Technical assistance on the sampling statistics to be applied pursuant to Commission Implementing Decision 2012/535/EU on emergency measures to prevent the spread of *Bursaphelenchus xylophilus* (the pine wood nematode) within the European Union [1]

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SUMMARY

The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is listed in annex 2 part A of the council directive EC/2000/29 as a harmful organism “whose introduction into, and spread within, all member states shall be banned, if they are present on certain plants or plant products”. The European Commission implementing decision 2012/535/EC obliges the member states to “carry out annual surveys for the presence of PWN in areas where it is not known to occur and adopt contingency plans to be prepared for findings of the presence of PWN” by the end of 2013.

When a Member State detects the presence of PWN in its territory, it is required to establish immediately a demarcated area. All trees in this area which are dead, in poor health or situated in storm- or fire-affected areas shall be identified and felled. All felled trees shall be subject to sampling and testing for the presence of PWN, according to certain statistical sampling schemes.

The eradication scheme, applied to the entire demarcated area requires, that all felled susceptible plants in which PWN has not already been found to be present shall be sampled and tested for the presence of PWN, according to a sampling scheme able to confirm with 99% reliability that the level of presence of PWN in those susceptible plants is below 0.1%.

The containment scheme, applied to the buffer zone of the demarcated area requires that all felled susceptible plants shall be sampled and tested for the presence of PWN, according to a sampling scheme able to confirm with 99% reliability that the level of presence of PWN in those susceptible plants is below 0.02%.

This report clarifies the sampling strategies to be applied for the sampling of susceptible plants in weak conditions within the area under concern, for gathering material from the plant, for detecting

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PWN in the material and for determining the number of samples, for a range of total numbers of susceptible plants ranging from 1 to 1 000 000. Summary tables are provided in the appendix.

KEY WORDS

Pine wood nematode, *Bursaphelenchus xylophilus,* pest freedom, risk based sampling, detection method, sample size

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Commission Implementing Decision 2012/535/EU on emergency measures to prevent the spread within the European Union of the pine wood nematode (PWN) requires Member States that detect the presence of PWN in their territory to establish a demarcated area, consisting of an infested zone and a buffer zone. In the buffer zone, all trees which are dead, in poor health or situated in storm- or fire-affected areas shall be identified and felled. All felled trees shall be subject to sampling and testing for the presence of PWN, according to certain statistical sampling schemes:

- According to point 7 of Annex I, which concerns demarcated areas subject to eradication measures, felled susceptible plants in which PWN has not already been found to be present shall be sampled and tested for the presence of PWN, according to a sampling scheme able to confirm with 99% reliability that the level of presence of PWN in those susceptible plants is below 0.1%.

- According to point 3(b) of Annex II, which concerns demarcated areas subject to containment measures, felled susceptible plants, other than plants completely destroyed by forest fires, shall be sampled and tested for the presence of PWN, according to a sampling scheme able to confirm with 99% reliability that the level of presence of PWN in those susceptible plants is below 0.02%.

A harmonised implementation of the above provisions by the Member States and auditing by the Commission of that implementation requires a common understanding of the numbers of felled plants to be sampled and tested.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

Therefore, in the context of Article 31 of Regulation (EC) No 178/2002, EFSA was requested to provide scientific assistance to the Commission in the form of an analysis of the numbers of felled plants in the buffer zone of demarcated areas that are to be sampled and tested for PWN, pursuant to the provisions of point 7 of Annex I and point 3(b) of Annex II of Decision 2012/535/EU. The analysis should clarify which statistical method should be applied and should provide the mathematical formula to be applied in each case for determining the number of felled trees to be sampled and tested, for a range of total numbers of felled trees from 1 to 1 000 000. Summary tables should also be provided, allowing Member States to implement the provisions without recourse to the statistical equation and without consulting a statistician.

ASSESSMENT

1. Introduction

The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is listed in annex 2 part A of the council directive EC/2000/29 as a harmful organism "whose introduction into, and spread within, all member states shall be banned, if they are present on certain plants or plant products". PWN causes the Pine Wilt Disease (PWD) in several *Pinus L.* species. Other susceptible plants are *Abies* Mill., *Picea* A. Dietr., *Larix* Mill., *Cedrus* Trew., *Pseudotsuga* Carr. and *Tsuga* Carr. The Commission implementing decision 2012/535/EC also states all wood of conifers (*Coniferalis*), with the exception of sawn wood and logs of *Taxus* L. and *Thuja* L., are classified as "susceptible wood", and all bark of conifers (*Coniferales*) are classified as "susceptible bark".

The life of PWN and transmission between trees is closely related to its vectors of *Monochamus* species (Cerambycidae). Schröder et al. (2009) mention that *M. saltuarius* and *M. galloprovincialis* are known vectors, and *M. sutor, M. sator* as well as *M. urussovi* are possible vectors of the PWN, which are present in Europe.

Two principle pathways of transmission exist (Schröder et al. 2009, Evans et al. 1996): The "oviposition pathway" and the "maturation feeding pathway".

Pine sawyers beetles (*Monochamus* spp.) are attracted to physiologically weakened or physically damaged (incl. affected by wind, snow or forest fires) or freshly cut trees (with bark), and colonise these, hereby transmiting bluestain fungi also. Depending on the species of the beetle oviposition can take place in small branches in the crown or throughout the entire length of the trunk. For example, *M. galloprovincialis*, which prefers thin bark mainly, oviposits in the crown, while other species are less precise in their choice of oviposition sites.

During oviposition infested beetles transmit PWN, which first feed on fungi in the wood and later become attracted to the pupal chamber. The fourth dispersal juvenile stage of PWN (J_{IV}) enters into the bodies of the eclosed beetles before the beetles bore out from the breeding material, e.g. trees and logs. To complete development the beetles fly to healthy trees and feed on young shoots, bark or needles, this is called maturation feeding. Over the entire life cycle they feed on young shoots, bark and needles, so called nutrition feeding. During maturation feeding PWN (J_{IV}) leave the infested beetles and enter the tree via the feeding wounds. They multiply and colonise the whole tree except needles, cones and seeds (Schröder et al. 2009). Depending on tree species and climatic conditions the multiplication of nematodes inside the host can result in PWD, which makes the tree attractive to beetles for oviposition.

Under favourable climatic conditions (e.g. high temperatures, low humidity) the PWN can kill a pine tree within a few months, as reported for *Pinus pinaster* in Portugal (Mota et al. 1999) and *Pinus sylvestris* in the United States (Malek, Appleby, 1984). Usually the tree will die during the same vegetation period or the coming season (Mamiya 1984). Schröder et al. (2009) explicate that several factors influence the distribution of PWN throughout the tree. In dying trees the PWN can be found from the roots to the crown and is able to survive for up to three years after the death of the tree (Malek, Appleby, 1984). But the infestation can also be very localised or irregular without any symptoms of PWD for many years (latent infections on asymptomatic carrier trees) (Halik and Bergdahl 1994, Futai and Takeuchi 2008).

Currently all of Portugal is considered as demarcated area for PWN and there is an obvious risk of spread to other European countries. Therefore the European Commission obliges the member states with their implementing decision 2012/535/EC to "carry out annual surveys for the presence of PWN in areas where it is not known to occur and adopt contingency plans to be prepared for findings of the presence of PWN" by the end of 2013.

Where the annual survey shows the presence of PWN in a susceptible plant or other evidence is given for that, the member state should without delay demarcate the area of concern, so called "demarcated area".

Within the demarcated area the zone where the PWN is found to be present is called the "infested zone". In a zone with a minimum radius of 500m (under some circumstances reduced to 100m) around each infested plant all susceptible plants shall be felled, removed and disposed. This zone is called "clear-cut zone". Finally the demarcated area consists of a "buffer zone" of a width of at least 20km (under some circumstances not less than 6km) around the infested zone.

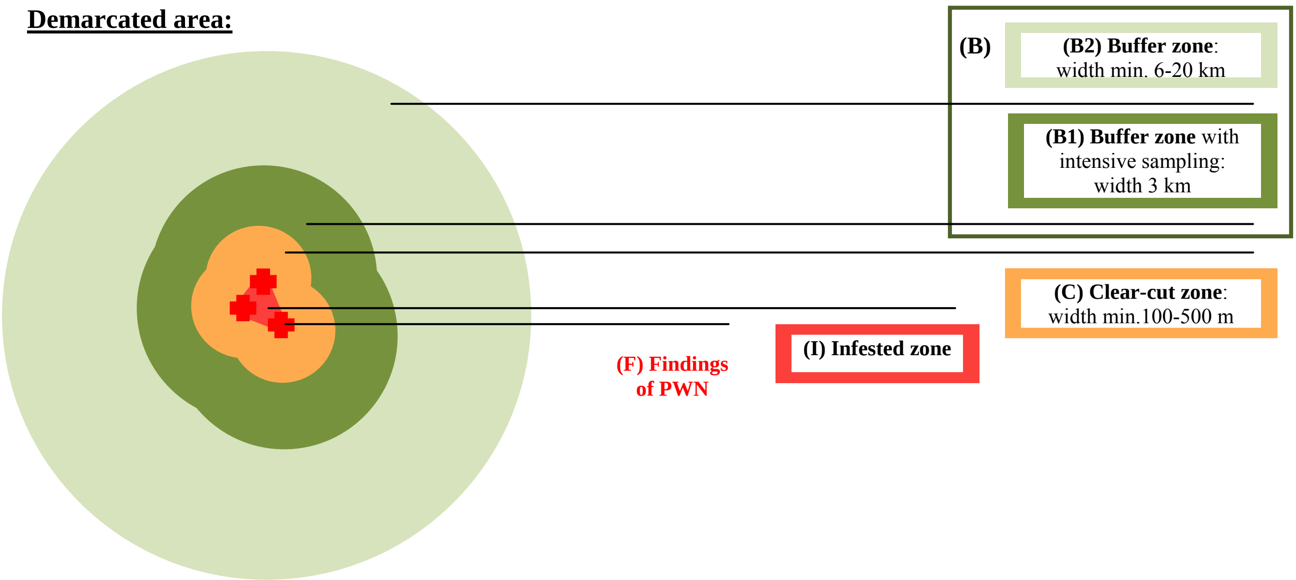

Figure 1: Structure of the demarcated area

In an eradication scheme all susceptible plants within the demarcated area fulfilling one of the following conditions:

(1) PWN has been found to be present in the plant
(2) the susceptible plant is already dead
(3) the susceptible plant is in poor health condition
(4) or the susceptible plant is situated in fire- or storm-affected areas

shall be felled, removed or disposed. Felled susceptible plants in which PWN has not already been found to be present (conditions 2-4) "shall be sampled and tested for the presence of PWN, according to a sampling scheme able to confirm with 99% reliability that the level of presence of PWN in those susceptible plants is below 0.1%." (2012/535/EC, annex I, point 7)

Point 6 explains the characteristics of surveys within the demarcated area: *"Member States shall perform annual surveys of the susceptible plants and the vector in the demarcated areas by inspecting, sampling and testing those plants and the vector for the presence of PWN. Those surveys shall give particular attention to susceptible plants which are dead, in poor health or situated in fire- or storm-affected areas. Those surveys shall also include systematic sampling of healthy-looking susceptible plants. The intensity of the surveys 3 000 m around each susceptible plant in which PWN has been found shall be at least four times higher than from 3 000 m thereof to the outer limit of the buffer zone."* (2012/535/EC, annex I, point 6)

In a containment scheme all susceptible plants within the buffer zone fulfilling one of the following conditions:

(1) the susceptible plant is already dead
(2) the susceptible plant is in poor health condition
(3) or the susceptible plant is situated in fire- or storm-affected areas

shall be felled, removed or disposed. Felled susceptible plants not completely destroyed by forest fires "shall be sampled and tested for the presence of PWN, according to a sampling scheme able to confirm with 99% reliability that the level of presence of PWN in those susceptible plants is below 0.02%." (2012/535/EC, annex II, point 3(b))

Regarding the two schemes mentioned above following numbers are defined:

(F) Findings:

F Number of susceptible plants PWN has been found to be present (Findings)

(W) Susceptible plants in weak conditions:

(1) the susceptible plant is already dead
(2) the susceptible plant is in poor health condition
(3) or the susceptible plant is situated in fire- or storm-affected areas, but not completely destroyed by forest fires

W_I Number of susceptible plants in weak conditions in which PWN has not already been found to be present in the infested zone (not including F)

W_C Number of susceptible plants in weak conditions in the clear-cut zone (not including F and W_I)

W_{B1} Number of susceptible plants in weak conditions in the buffer zone with intensive sampling (not including F, W_I and W_C)

W_B Number of susceptible plants in weak conditions in the complete buffer zone (including W_{B1}, but not F, W_I and W_C)

(N) All susceptible plants:

(1) the susceptible plant is already dead
(2) the susceptible plant is in poor health condition
(3) or the susceptible plant is situated in fire- or storm-affected areas, but not completely destroyed by forest fires

N_I Number of all susceptible plants in the infested zone (including F and W_I)

N_C Number of all susceptible plants in the clear-cut zone (including W_C, but not N_I)

N_{B1} Number of all susceptible plants in the buffer zone with intensive sampling (including W_{B1}, but not including N_I and N_C)

N_B Number of all susceptible plants in the complete buffer zone (including W_B and N_{B1}, but not N_I and N_C)

Thus:

$F_{Tot} = F$ is the number of susceptible plants PWN has been found to be present in the whole demarcated area

$\mathbf{W_{Tot} = W_I+W_C+W_B}$ is the number of susceptible plants in weak conditions in the whole demarcated area

$\mathbf{N_{Tot} = N_I+N_C+N_B}$ is the number of all susceptible plants in the whole demarcated area

For the purpose of the report it is assumed that these numbers are known or estimated by a valid procedure.

1.1. Purpose of the report

The purpose of this technical report is to analyse the numbers of felled susceptible plants in different parts of the demarcated area which should be sampled and tested for PWN to fulfil the requirements of the eradication and containment scheme of the annexes of the implementing decision 2012/535/EC (annex I point 7, annex II point 3(b)). The appropriate statistical method is described in chapter 3.3 by formulas and the results are exemplified by tables in the appendix with typical numbers of felled trees between 1 and 1 million. Special emphasis is given to the fact the results are applicable without further calculations.

To complete the task chapter 3.1 discusses good practise of sampling trees, while chapter 3.2 focuses on the good practise to sample material from a susceptible plant and detect PWN in the sample.

1.2. Scope of the report

The report is restricted to the sampling of felled susceptible plants in weak conditions. This implies

- that the total number of felled susceptible plants is known and can be used in the calculation of the sample size. For planning of felling and sampling a valid estimator of the total number of susceptible plants in weak conditions can be used, when it is guaranteed that the final number of samples will not be less than the number of samples necessary for the total number of finally felled susceptible plants.

- that the procedure to collect and analyse is done with a reliable method, even when the susceptible plants in weak conditions were not felled due to exceptions described in 2012/535/EC, annex I point 3.

2. Methodology used in the assessment

The report uses and describes standard procedures for sampling trees, detecting PWN and determine sample sizes, as given in the scientific literature. No specific literature search was performed for this report. Instead the results of intensive literature searches on PWN from former mandates of the Plant Health Panel of EFSA were used, including the “Scientific Opinion on the phytosanitary risk associated with some coniferous species and genera for the spread of pine wood nematode” (EFSA Journal 2012; 10(1):2553); the “Scientific Opinion on a composting method proposed by Portugal as a heat treatment to eliminate pine wood nematode from the bark of pine trees” (EFSA Journal 2010; 8(9):1717); the scientific opinion on “Mortality verification of pinewood nematode from high temperature treatment of shavings” (EFSA Journal 2009: 1055)

3. Assessment results

3.1. Sampling procedures for trees

The estimation of the prevalence of infestation by PWN in a population of susceptible plants is made by random sampling, where samples are collected from a fraction of all susceptible plants. The susceptible plants are the sample units of the survey. The number of plants which need to be sampled is statistically determined to reach a specific level of reliability. This method will be explained later in chapter 3.3.

Prevalences of infestations by PWN in Europe were reported in Portugal. Penas et al. (2004) reported an infestation rate of 38.6% of symptomatic trees in the demarcated zone. Sousa et al. (2011) showed that between 13 and 77% of *Pinus pinaster* showing symptoms were infested on the Troia peninsula depending on the year of sampling. Regarding all felled trees in the demarcated zone the infestation rate can be calculated as about only 0.1-0.5%.

The detection of a susceptible plant infested by PWN will therefore be a rare event. It is recommended to increase the prevalence by focussing on susceptible plants with a higher risk (risk-based sampling).

- Under warm climatic condition (like in Asia and southern or continental Europe) nematodes transmitted by beetles to healthy trees may induce development of PWD symptoms (miscoloration, partial die-back or death) in susceptible plants. In a month or more, after being transmitted, the PWN population will increase to reach about 1000 individuals per g wood (Kuroda et al. 1988). This level of infection can be easily detected.

- Trees may be weakened for reasons other than PWN-infection: Wind-thrown trees, trees weakened by forest fires or attacked by pathogens and insects like bark beetles also become attractive to *Monochamus* spp. for breeding. Bark beetles carry various species of "blue-stain fungi" which may colonise the wood. Many species of "blue-stain fungi are excellent hosts for PWN, which typically reach high numbers in blue-stained wood. If this material is oviposit by Monochamus carrying PWN, the nematodes will develop rapidly to easily detectable levels.

Dead susceptible plants, plants showing PWD symptoms or other poor health conditions, along with susceptible plants weakened by wind or forest fires constitute a subpopulation with reasonably higher risk of infestations by PWN.

Detached wood (logging waste) is also highly attractive for Monochamus spp as a breeding substrate. Similar to weakened trees fungal contaminants can serve as hosts for PWN development. In an area where the beetles carry PWN the nematodes will be transmitted in connection with oviposition of the beetles, and then readily reach high levels in wood. The inclusion of detached wooden material might be further considered in the sampling scheme. (EPPO 2011)

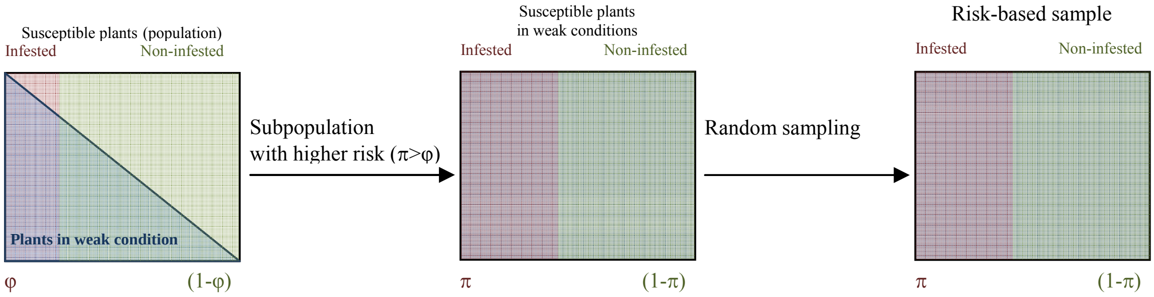

Figure 2: Increasing of the rate of infestation by risk-based, random sampling

3.2. Detection of *Bursaphelenchus xylophilus* (the pine wood nematode)

Combination samples from susceptible plants should be taken from several subsamples from the whole tree, i.e. crown and trunk. This is of particular importance in *P. pinaster* because the vector *M. galloprovincialis* preferably feeds and oviposits on thin bark which is typical of the crown (PHRAME 2007). Since PWN J_{IV} enter through the feeding wounds made by the beetle the transmission of nematodes will occur in this part of the tree. Studies on the axial distribution of nematodes in field grown *P. pinaster* have demonstrated that in early development of pine wilt disease PWN is most prevalent in the branches of the crown and in the upper trunk compared to other parts (Lopez et al. 2011). Samples taken from dying and recently dead maritime pine should focus in particular on branches in the crown and the upper part of the trunk.

Also the transmission biology could generate localised infestations of trees. At least for the North American species *Monochamus carolinensis* reports on the rates of transmission of PWN J_{IV} ("dauer larva") to branches of *P. sylvestris* ranged between 0-314 nematodes per feeding experiment. (Linit 1990). If this is true also of *P. pinaster* the nematode infection may be highly localised in the crown.

To increase probability of detection samples should be taken from wood showing signs of *Monochamus* activity like feeding wounds, larval galleries and grub holes (Schröder et al. 2009). Samples should be taken from areas with "blue-stain fungi" (normally best seen in wood discs). Also in this situation PWN is expected to be present in high numbers as a result of feeding on these fungi.

An appropriate sample size would be 100-300 g of wood shavings. Depending on the method of sampling the bark may be removed before incubation and nematode extraction to reduce the development of saprophytic nematodes, which could complicate the analyses.

3.2.1. Which method should be used to detect PWN?

Wood samples should reach an incubation temperature of approximately 25 °C for at least 14 days to allow any nematodes present to breed and maximise the likelihood of detection. Live nematodes can be extracted from infested wood using the Baermann funnel technique involving immersion of small pieces of wood (not larger than 1 cm in width using a cutting method that does not generate heat) or wood shavings in tap water for 48 hrs. Nematodes migrate from the chopped wood through a filter (like Ederol, Munktell-2001, disposable sheets or Kleenex) into the water, and can be collected from the closed bottom of the funnel (Hooper 1986a). Chemical filter paper and kitchen paper both have too dense a structure for successful nematode migration. Other methods involving immersion of wood in water like "the Whitehead tray" or a mistifier (Hooper 1986ab) are also suitable.

In Portugal PWN is extracted from wood pieces by a modification of the Whitehead tray. Wood shavings are wrapped in a cloth and placed in a tray with water at room temperature. After 48 hours the water is sieved through a 400 mesh (38 µm) and nematodes are identified by an experienced nematologist or by molecular techniques (Sousa et al. 2011, with clarification). Molecular tests can be used for identification of PWN, but should be confirmed by morphological identification. When only juveniles are present, they should be cultured on *B. cinerea* to produce adult specimens for identification (Schröder et al., 2009).

Guidance on methods for identifying PWN is found in the EPPO Standard PM7 (4) Diagnostic Protocol for *Bursaphelenchus xylophilus.* (EPPO 2009, Schröder et al. 2009).

The methods above are standard methods, and at present there are only few alternative techniques available.

Novel molecular techniques are under development and may prove to be an important complement to ocular analysis in the future.

3.3. Sample size calculations

In this chapter statistical methods are described to determine the minimal sample size to detect infestations in large and restricted populations, as well as the main part of infested susceptible plants. These calculations assume that all susceptible plants with weak conditions were selected by random or using risk-based sampling strategies with a perfect method of PWN detection in the plant.

The last subchapter 3.3.4 describes necessary adjustments when the detection method is less accurate in the sense of a non perfect sensitivity of the detection method:

Sensitivity = Likelihood of detection, when material is infested by PWN

3.3.1. Minimal sample size to confirm pest freedom in large populations

A population is large, when the number of susceptible plants is much higher than the total sample size. Under these circumstances the random selection of an individual plant can be assumed to be independent from the remaining sample. The probability to observe no infested plant (k=0) in a sample of K plants can be therefore calculated easily by

$$\text{P(No infestations in the sample of K plants)} = P(k=0) = (1-\pi)^K$$

with π the rate of infested plants in the population
$1-\pi$ the rate of not infested plants in the population.

To test that the infestation rate π is below a given threshold π_0, the hypothesis H

$$H_0: \pi \geq \pi_0 \quad \text{versus} \quad H: \pi < \pi_0$$

with π_0 the threshold of the infestation rate in the population

should be looked at. No observed infested plants in the sample (k=0) will confirm the hypothesis H, when the false-positive error (type I error) of the decision "rejecting the null hypothesis H_0" is below the significance level α, a small probability:

$$\text{P(false-positive error)} = P(k=0|\pi \geq \pi_0) = (1-\pi)^K < (1-\pi_0)^K \leq \alpha$$

with α the significance level of the statistical test
$1-\alpha$ the reliability of the hypothesis H, in case of confirmation.

Solved for K gives the minimal sample size needed:

$$K \geq \frac{\ln(\alpha)}{\ln(1-\pi)}$$

to confirm with no observed infested plants (k=0) the hypothesis, that the infestation rate in the population is below the given threshold π_0 with reliability 1-α.

Using the result for the questions of interests in the eradication and containment scheme:

Table 1: Minimal sample size to confirm pest freedom in large populations according the eradication and containment scheme of the implementation decision 2012/535/EC

Parameter of the scheme	Test and minimal sample size
Eradication scheme: Threshold for infestation rate: 0.1% Reliability: $1-\alpha = 99\%$	**Infestation below threshold**, if no observed infested plants in the sample Minimal sample size needed: $K \geq 4603$ out of $W=W_I+W_C+W_B$
Containment scheme: Threshold for infestation rate: 0.02% Reliability: $1-\alpha = 99\%$	**Infestation below threshold**, if no observed infested plants in the sample Minimal sample size needed: $K \geq 23\,024$ out of $W=W_B$

These minimal sample sizes are independent from the size of the tested population of susceptible plants in weak conditions, when the population size is much larger than the sample size.

If the sample size increases the minimal number, the observation of infested plants may be possible, even if the hypothesis is still confirmed. This case will be no more considered here, because the finding of additional infested plants will change the demarcated area and hence the whole setting of the test.

To summarise, the test is confirming the pest freedom of the area of concern with a threshold of π_0 (precision of the sampling) and a reliability of $1-\alpha$ of the confirmation

An additional condition of the eradication scheme is that the sample density d_1 in the inner zone ($W_I + W_C + W_{B1}$) is at least four times higher than the density d_2 in the outer buffer zone ($W_B - W_{B1}$). This is another strategy of risk-based sampling, assuming that additional infestations will be more likely close to the infested area than further away. To calculate the proportion r of the samples to be taken in the inner zone following equations have to be fulfilled:

Density in the inner zone: $d_1 = (K \cdot r) / (W_I+W_C+W_{B1}) \geq 4 \cdot d_2$

density in the outer buffer zone: $d_2 = (K \cdot (1-r)) / (W_B - W_{B1})$

$$r / (1-r) \geq 4 \cdot (W_I+W_C+W_{B1}) / (W_B - W_{B1}) = 4\,\rho$$

with $\rho = (W_I+W_C+W_{B1}) / (W_B - W_{B1})$

It follows the proportion of sample in inner zone:

$$r \geq (4 \cdot \rho) / (1+4 \cdot \rho)$$

Table 2: Minimal sample size to confirm pest freedom in large populations according the eradication scheme with stratified sampling of the implementation decision 2012/535/EC

Parameter of the scheme	Test and minimal sample size
Eradication scheme: Threshold for infestation rate: 0.1% Reliability: $1-\alpha = 99\%$ Sampling densitiy in the inner zone $W_1 = W_I + W_C + W_{B1}$ is at least 4 times the density in the outer buffer zone $W_2 = W_B - W_{B1}$	**Infestation below threshold**, if no observed infested plants in the sample Minimal sample size needed: $K_1 \geq 4603 \cdot r$ out of $W_1 = W_I + W_C + W_{B1}$ $K_2 \geq 4603 \cdot (1-r)$ out of $W_2 = W_B - W_{B1}$ with $r \geq (4 \cdot \rho) / (1 + 4 \cdot \rho)$ $\rho = W_1 / W_2$

This risk based sampling will increase the precision of the test to show pest freedom

3.3.2. Minimal sample size to confirm pest freedom in restricted populations

In restricted populations the assumption of independent samples is not valid and the calculation of the false-positive error is more complicated. But the principle of the statistical test remains the same.

The probability to observe no infested plants (k=0) in a sample of K out of a population of W susceptible plants with $W \cdot \pi$ infested plants follow a hypergeometric distribution:

$$\text{P(No infestations in the sample of K plants)} = P(k=0) = \text{HyperGeometric}(k=0 | K, W \cdot \pi, W)$$

$$= \frac{\binom{K}{0} \cdot \binom{W-K}{W \cdot \pi}}{\binom{W}{W \cdot \pi}} = \frac{(W-K)! \cdot (W - W\pi)!}{W! \cdot (W - W\pi - K)!}$$

with
- π the rate of infested plants in the population
- K the sample size
- W the population size
- $W \cdot \pi$ the number of infested plants in the population (integer), with more or equal non-sampled plants than infested: $(W-K) \geq W \cdot \pi$

The test does again limit the false-positive error of the decision:

$$\text{P(false-positive error)} = P(k=0 | \pi \geq \pi_0) = \text{HyperGeometric}(k=0 | K, W \cdot \pi, W) < \text{HyperGeometric}(k=0 | K, \lfloor W \cdot \pi_0 \rfloor, W) \leq \alpha$$

with
- α the significance level of the statistical test
- $1-\alpha$ the reliability level of the hypothesis H, in case of confirmation
- $\lfloor W \cdot \pi_0 \rfloor$ the floor function of $W \cdot \pi_0$ (largest integer not greater than $W \cdot \pi_0$)

The minimal sample size will be calculated by solving the equation above. To get an explicit solution the hypergeometric distribution can be approximated (Cannon 2001) using:

$$\frac{n!}{(n-m)!} = n \cdot (n-1) \cdot \ldots (n-m+1) = \left(n - \tfrac{1}{2}(m-1)\right)^m$$

to

$$\text{HyperGeometric}(k=0|K, \lfloor W \cdot \pi_0 \rfloor, W) \cong \left(1 - \frac{K}{W - \frac{1}{2}\left(\lfloor W \cdot \pi_0 \rfloor - 1\right)}\right)^{\lfloor W \cdot \pi_0 \rfloor}$$

The condition for the sample size is than

$$\left(1 - \frac{K}{W - \frac{1}{2}\left(\lfloor W \cdot \pi_0 \rfloor - 1\right)}\right)^{\lfloor W \cdot \pi_0 \rfloor} \le \alpha$$

with the explicit solution:

$$K \ge \left(1 - \alpha^{1/\lfloor W \cdot \pi_0 \rfloor}\right) \cdot \left(W - \tfrac{1}{2}\left(\lfloor W \cdot \pi_0 \rfloor - 1\right)\right)$$

if at least one infestation is expected, when the infestation rate is equal to the threshold: $\lfloor W\pi_0 \rfloor > 0$. Otherwise total sampling (K=W) is needed.

The resulting sample size is calculated for the different schemes and a collection of population sizes in the appendix A of this report. The approximation within the range of the appendix is almost exact.

If the sample size increases the minimal number, the observation of infested plants may be possible, even if the hypothesis is still confirmed. This case will no more be considered here, because the finding of additional infested plants will change the demarcated area and hence the whole setting of the test.

Table 3: Minimal sample size to confirm pest freedom in restricted populations according the eradication and containment scheme of the implementation decision 2012/535/EC

Parameter of the scheme	Test and minimal sample size
Eradication scheme: Threshold for infestation rate: π_0=0.1% Reliability: 1–α = 99% Population size: $W=W_I+W_C+W_B$ Sampling densitiy in the inner zone $W_1= W_I+W_C+W_{B1}$ is at least 4 times the density in the outer buffer zone $W_2= W_B-W_{B1}$	**Infestation below threshold**, if <u>no observed infested plants in the sample</u> Minimal sample size needed: $K \geq \left(1-\alpha^{1/\lfloor W\cdot\pi_0\rfloor}\right)\cdot\left(W-\frac{1}{2}\left(\lfloor W\cdot\pi_0\rfloor-1\right)\right)$ $K_1 = K\cdot r$ out of $W_1= W_I+W_C+W_{B1}$ $K_2 = K\cdot(1-r)$ out of $W_2= W_B-W_{B1}$ with $r \geq (4\cdot\rho) / (1+4\cdot\rho)$ $\rho = W_1 / W_2$
Containment scheme: Threshold for infestation rate: π_0=0.02% Reliability: 1–α = 99% Population size: $W=W_B$	**Infestation below threshold**, if <u>no observed infested plants in the sample</u> Minimal sample size needed: $K \geq \left(1-\alpha^{1/\lfloor W\cdot\pi_0\rfloor}\right)\cdot\left(W-\frac{1}{2}\left(\lfloor W\cdot\pi_0\rfloor-1\right)\right)$ out of $W=W_B$

3.3.3. Minimal sample size to detect infested trees

The task of the last two subchapters was to confirm that no additional findings of infested plants exist in the demarcated area. Only the impossibility to confirm the total absence with a sample, that is only a part of the whole population of susceptible plants in weak conditions, makes it necessary to introduce a threshold for the existing rate of infestation and be able to plan the sampling design with the given precision.

The situation is different, when it is assumed that additional infested plants are existing and the task of the survey is to identify these plants. In this case the proportion τ of infested plants, which should be detected with a given reliability, must be defined.

Now we interpret π_0 as an upper estimator of the existing infestation rate in the population of size W. This means that $\lfloor W\cdot\pi_0\rfloor$ susceptible plants are unknown and out of these $\lfloor \tau\cdot W\cdot\pi_0\rfloor$ should be identified with a probability higher than 1-α.

This model follows again a hypergeometric distribution with following condition:

$$1-\alpha \leq P\left(k \geq \lfloor \tau\cdot W\cdot\pi_0\rfloor\right) = \sum_{k=\lfloor \tau\cdot W\cdot\pi_0\rfloor}^{\lfloor W\cdot\pi_0\rfloor} \text{HyperGeometric}\left(k \mid K, \lfloor \tau\cdot W\cdot\pi_0\rfloor, W\right) = \sum_{k=\lfloor W\cdot\pi_0\rfloor}^{\lfloor W\cdot\pi_0\rfloor} \frac{\binom{K}{k}\cdot\binom{W-K}{W\pi-k}}{\binom{W}{W\cdot\pi}}$$

The likelihood is null, when the sample size K is below the number of infestations to find $\lfloor \tau\cdot W\cdot\pi_0\rfloor$, and is one (complete safety), when the sample size is total K=W. The designated sample size here is the minimal number K, which fulfils the condition above. The computation is again not simple and can be done case by case.

3.3.4. Adjustments needed for imperfect detection

Of course the detection of PWN in the sample will not be perfect by any test system. Therefore following situation has to be considered:

	Actual infestation	
Detection result	Infested	Non-infested
Positive (detected)	True positive (A)	False positive (B)
Negative (not detected)	False negative (D)	True negative (C)

Because positive results, i.e. the detection of nematodes, will be tested and confirmed by additional examinations, it can be assumed that there are no false positive findings (B=0). This includes that the sample material was extracted, stored and analysed correctly by avoiding all kind of cross-contaminations.

But some infestations will not be recognised by the detection method. The probability that an infestation is not detected, although the material is infested, is called sensitivity of the test:

$$\sigma = A / (A+D)$$

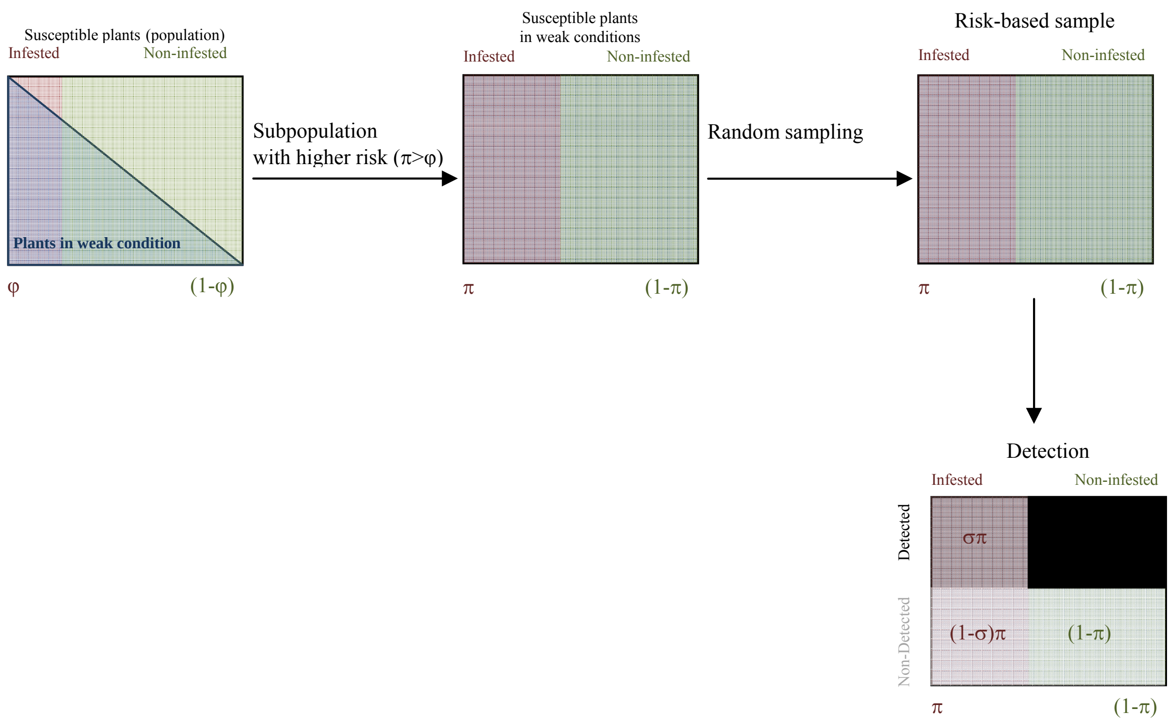

Figure 3: Correcting the threshold by the sensitivity of detection σ

Indications on the actual sensitivity of the detection method can be obtained from multiple testing of infested susceptible plants.

Changing the viewpoint from "infestation" to "detected infestation" imputes additional uncertainty into the sampling scheme. The easiest way to adjust the sample size is to adjust the threshold from

$$\pi < \pi_0 \quad \text{to} \quad \sigma \cdot \pi < \sigma \cdot \pi_0$$

giving

$$K \geq \min\left(W,\ \left(1-\alpha^{1/\lfloor W\cdot\sigma\cdot\pi_0\rfloor}\right)\cdot\left(W-\tfrac{1}{2}\left(\lfloor W\cdot\sigma\cdot\pi_0\rfloor-1\right)\right)\right) \qquad \text{if } \lfloor W\pi_0\rfloor \geq \tfrac{\ln(\alpha)}{\ln(1-\sigma)} > 0$$

Nevertheless Cannon (2001) showed that

$$\left(1-\frac{K\cdot\sigma}{W-\tfrac{1}{2}\left(\lfloor W\cdot\sigma\cdot\pi_0\rfloor-1\right)}\right)^{\lfloor W\cdot\pi_0\rfloor} \leq \alpha$$

with the explicit solution:

$$K \geq \min\left(W,\ \tfrac{1}{\sigma}\cdot\left(1-\alpha^{1/\lfloor W\cdot\pi_0\rfloor}\right)\cdot\left(W-\tfrac{1}{2}\left(\lfloor W\cdot\sigma\cdot\pi_0\rfloor-1\right)\right)\right) \qquad \text{if } \lfloor W\pi_0\rfloor \geq \tfrac{\ln(\alpha)}{\ln(1-\sigma)} > 0$$

is a better approximation for the minimal sample size.

Table 4: Minimal sample size to confirm pest freedom in restricted populations adapted to imperfect detection methods (sensitivity less 100%) according the eradication and containment scheme of the implementation decision 2012/535/EC

Parameter of the scheme	Test and minimal sample size
Eradication scheme: Sensitivity of the detection: σ Threshold for infestation rate: π_0=0.1% Reliability: $1-\alpha = 99\%$ Population size: $W=W_I+W_C+W_B$ Sampling densitiy in the inner zone $W_1= W_I+W_C+W_{B1}$ is at least 4 times the density in the outer buffer zone $W_2= W_B-W_{B1}$	**Infestation below threshold**, if <u>no observed infested plants in the sample</u> Minimal sample size needed: $K \geq \tfrac{1}{\sigma}\cdot\left(1-\alpha^{1/\lfloor W\cdot\pi_0\rfloor}\right)\cdot\left(W-\tfrac{1}{2}\left(\lfloor W\cdot\sigma\cdot\pi_0\rfloor-1\right)\right)$ $K_1 = K\cdot r$ out of $W_1= W_I+W_C+W_{B1}$ $K_2 = K\cdot(1-r)$ out of $W_2= W_B-W_{B1}$ with $r \geq (4\cdot\rho) / (1+4\cdot\rho)$ $\rho = W_1 / W_2$
Containment scheme: Sensitivity of the detection: σ Threshold for infestation rate: π_0=0.02% Reliability: $1-\alpha = 99\%$ Population size: $W=W_B$	**Infestation below threshold**, if <u>no observed infested plants in the sample</u> Minimal sample size needed: $K \geq \tfrac{1}{\sigma}\left(1-\alpha^{1/\lfloor W\cdot\pi_0\rfloor}\right)\cdot\left(W-\tfrac{1}{2}\left(\lfloor W\cdot\sigma\cdot\pi_0\rfloor-1\right)\right)$ out of $W=W_B$

RECOMMENDATIONS

The task of sampling is mainly to confirm that no more infestations exist in the demarcated area, except the already identified susceptible plants, PWN has been found to be present:

- Random sampling of susceptible plants should be used to select plants for taking potentially infested material.

- The focus on susceptible plants in weak conditions, this means already dead plants, plants in poor health conditions and plants affected by forest fire or storm, is an appropriate strategy for risk based sampling. Another risk based approach is to sample within 3 km around the exiting findings with a higher density than outside this zone. Depending on the conditions of forestry detached wood (e.g. logging waste) should also included into the sampling scheme.

- Per tree about 100-300 g of wood shavings should be taken from several locations of the whole tree, including crown and trunk. Wood showing signs of infestations (poor health), *Monochamus* activitiy (e.g. feeding wounds, larval galleries or grub holes) or areas with “blue-stain” fungi should be preferred for taking samples.

- Sample material should be incubated and PWN detected using standard methods, like Baerman funnel techniques, Whitehead trays or similar. The diagnostic protocol is described in the EPPO Standard PM7(4)

- The minimal sample size to confirm freedom of PWN with perfect detection can be calculated using the hypergeometric distribution or appropriate approximations for a given threshold of the infestation rate (precision of the sampling) and a given reliability. Sample sizes for different numbers of susceptible plants are given in the appendix A.

- When the sensitivity of the detection method is below 100% identification of infested material, the minimal sample size to confirm freedom of PWN can be calculated using the adapted approximation for a given threshold of the infestation rate (precision of the sampling) and a given reliability. Sample sizes for different numbers of susceptible plants are given in the appendix C.

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APPENDICES

A. SAMPLE SIZES NEEDED TO CONFIRM FREEDOM OF PWN WITH PERFECT DETECTION

Table 5: Sample sizes to confirm pest freedom in restricted populations for intervals of populations sizes (approximate formula see chapter 3.3.2)

Eradication scheme:
Threshold of infestation rate: π_0= 0.1%
Reliability: 1−α=99%
Sample size rounded up to next 10, maximum per interval

Population size: $W=W_I+W_C+W_B$ from	to	Sample size: K=
1	999	999[1]
1000	1999	1980[1]
2000	2999	2700[1]
3000	3999	3140[1]
4000	4999	3420
5000	5999	3610
6000	6999	3750
7000	7999	3860
8000	8999	3940
9000	9999	4010
10000	10999	4060
11000	11999	4110
12000	12999	4150
13000	13999	4180
14000	14999	4210
15000	15999	4230
16000	16999	4250
17000	17999	4270
18000	18999	4290
19000	19999	4310
20000	20999	4320
21000	21999	4330
22000	22999	4350
23000	23999	4360
24000	24999	4370
25000	25999	4380
26000	27999	4390
28000	28999	4400
29000	30999	4410
31000	31999	4420
32000	33999	4430
34000	35999	4440
36000	38999	4450
39000	41999	4460
42000	44999	4470
45000	47999	4480
48000	52999	4490
53000	57999	4500
58000	63999	4510
64000	71999	4520
72000	81999	4530
82000	94999	4540
95000	112999	4550
113000	138999	4560
139000	181999	4570
182000	261999	4580
262000	465999	4590
466000	999999	4600
1000000	infinity	4603

[1]= If K greater than W, total sampling of the population

Containment scheme:
Threshold of infestation rate: π_0= 0.02%
Reliability: 1−α=99%
Sample size rounded up to next 50, maximum per interval

Population size: $W=W_B$ from	to	Sample size: K=
1	4999	4999[1]
5000	9999	9900[1]
10000	14999	13500[1]
15000	19999	15700[1]
20000	24999	17100
25000	29999	18100
30000	34999	18800
35000	39999	19300
40000	44999	19700
45000	49999	20050
50000	54999	20300
55000	59999	20550
60000	64999	20750
65000	69999	20900
70000	74999	21050
75000	79999	21150
80000	84999	21300
85000	89999	21400
90000	94999	21450
95000	99999	21550
100000	104999	21600
105000	109999	21700
110000	114999	21750
115000	119999	21800
120000	124999	21850
125000	129999	21900
130000	134999	21950
135000	144999	22000
145000	149999	22050
150000	159999	22100
160000	169999	22150
170000	179999	22200
180000	189999	22250
190000	204999	22300
205000	219999	22350
220000	239999	22400
240000	259999	22450
260000	284999	22500
285000	314999	22550
315000	349999	22600
350000	399999	22650
400000	459999	22700
460000	544999	22750
545000	669999	22800
670000	859999	22850
860000	999999	22900
1000000	infinity	23024

B. Proportion of Sampling in the Inner Zone for Risk-Based Sampling

Table 6: Proportion of samples in the inner zone (formula see chapter 3.3.1)

Relation of susceptible plants in the inner zone compared to the outer buffer zone		Proportion of samples to be taken in the inner zone with 4-times higher density
$(W_I+W_C+W_{B1}) : (W_B-W_{B1}) =$	ρ =	r =
1:50 000	0.00002	0.01%
1:20 000	0.00005	0.02%
1:10 000	0.0001	0.04%
1:5000	0.0002	0.08%
1:2000	0.0005	0.20%
1:1000	0.001	0.40%
1:500	0.002	0.79%
1:200	0.005	1.96%
1:100	0.01	3.85%
1:50	0.02	7.41%
1:20	0.05	16.67%
1:10	0.1	28.57%
1:5	0.2	44.44%
1:2	0.5	66.67%
1:1	1	80.00%
2:1	2	88.89%
5:1	5	95.24%
10:1	10	97.56%
20:1	20	98.77%
50:1	50	99.50%
100:1	100	99.75%
200:1	200	99.88%
500:1	500	99.95%
1000:1	1000	99.98%
2000:1	2000	99.99%
5000:1	5000	100.00%
10 000:1	10000	100.00%
20 000:1	20000	100.00%
50 000:1	50000	100.00%

C. Sample Sizes Needed to Confirm Freedom of PWN with Imperfect Detection

Table 7: Sample sizes to confirm pest freedom in restricted populations with imperfect detection for intervals of populations sizes (approximate formula see chapter 3.3.3)

Eradication scheme:
Threshold of infestation rate: π_0= 0.1%
Reliability: 1–α=99%
Sample size rounded up to next 10,
calculations were done for the upper limit of the interval

Population size: $W=W_I+W_C+W_B$		Sample size: K = Sensitivity: σ =			
from	to	100%	80%	60%	40%
1	999	999[1]	–[2]	–[2]	–[2]
1000	1999	1980[1]	–[2]	–[2]	–[2]
2000	2999	2700[1]	–[2]	–[2]	–[2]
3000	3999	3140[1]	3930[1]	–[2]	–[2]
4000	4999	3420	4280[1]	–[2]	–[2]
5000	5999	3610	4520	–[2]	–[2]
6000	6999	3750	4690	6250[1]	–[2]
7000	7999	3860	4820	6430	–[2]
8000	8999	3940	4930	6570	–[2]
9000	9999	4010	5010	6680	–[2]
10000	10999	4060	5080	6770	10150[1]
11000	11999	4110	5130	6840	10260
12000	12999	4150	5180	6910	10360
13000	13999	4180	5220	6960	10440
14000	14999	4210	5260	7010	10510
15000	15999	4230	5290	7050	10580
16000	16999	4250	5320	7090	10630
17000	17999	4270	5340	7120	10680
18000	18999	4290	5360	7150	10730
19000	19999	4310	5380	7180	10760
20000	20999	4320	5400	7200	10800
21000	21999	4330	5420	7220	10830
22000	22999	4350	5430	7240	10860
23000	23999	4360	5450	7260	10890
24000	24999	4370	5460	7280	10910
25000	25999	4380	5470	7290	10940
26000	27999	4390	5490	7320	10980
28000	28999	4400	5500	7330	11000
29000	30999	4410	5520	7360	11030
31000	31999	4420	5520	7370	11050
32000	33999	4430	5540	7380	11070
34000	35999	4440	5550	7400	11100
36000	38999	4450	5570	7420	11130
39000	41999	4460	5580	7440	11160
42000	44999	4470	5590	7460	11180
45000	47999	4480	5600	7470	11200
48000	52999	4490	5620	7490	11230
53000	57999	4500	5630	7510	11260
58000	63999	4510	5640	7520	11280
64000	71999	4520	5650	7540	11310
72000	81999	4530	5670	7560	11330
82000	94999	4540	5680	7570	11360
95000	112999	4550	5690	7590	11380
113000	138999	4560	5710	7610	11410
139000	181999	4570	5720	7620	11430
182000	261999	4580	5730	7640	11460
262000	465999	4590	5740	7660	11480
466000	1000000	4600	5750	7670	11500

Containment scheme:
Threshold of infestation rate: π_0= 0.02%
Reliability: 1–α=99%
Sample size rounded up to next 50,
calculations were done for the upper limit of the interval

Population size: $W=W_B$		Sample size: K = Sensitivity: σ =			
from	to	100%	80%	60%	40%
1	4999	4999[1]	–[2]	–[2]	–[2]
5000	9999	9900[1]	–[2]	–[2]	–[2]
10000	14999	13500[1]	–[2]	–[2]	–[2]
15000	19999	15700[1]	19650[1]	–[2]	–[2]
20000	24999	17100	21400[1]	–[2]	–[2]
25000	29999	18100	22600	–[2]	–[2]
30000	34999	18800	23450	31300[1]	–[2]
35000	39999	19300	24150	32150	–[2]
40000	44999	19700	24650	32850	–[2]
45000	49999	20050	25050	33400	–[2]
50000	54999	20300	25400	33850	50750[1]
55000	59999	20550	25700	34250	51350
60000	64999	20750	25900	34550	51800
65000	69999	20900	26100	34800	52200
70000	74999	21050	26300	35050	52600
75000	79999	21150	26450	35250	52900
80000	84999	21300	26600	35450	53150
85000	89999	21400	26700	35600	53400
90000	94999	21450	26850	35750	53650
95000	99999	21550	26950	35900	53850
100000	104999	21600	27000	36000	54000
105000	109999	21700	27100	36100	54150
110000	114999	21750	27150	36200	54300
115000	119999	21800	27250	36300	54450
120000	124999	21850	27300	36400	54600
125000	129999	21900	27350	36450	54700
130000	134999	21950	27400	36550	54800
135000	144999	22000	27500	36650	55000
145000	149999	22050	27550	36750	55100
150000	159999	22100	27650	36850	55250
160000	169999	22150	27700	36950	55400
170000	179999	22200	27750	37000	55500
180000	189999	22250	27800	37100	55600
190000	204999	22300	27900	37200	55750
205000	219999	22350	27950	37250	55900
220000	239999	22400	28000	37350	56000
240000	259999	22450	28100	37450	56150
260000	284999	22500	28150	37500	56250
285000	314999	22550	28200	37600	56400
315000	349999	22600	28250	37700	56500
350000	399999	22650	28350	37750	56650
400000	459999	22700	28400	37850	56750
460000	544999	22750	28450	37950	56900
545000	669999	22800	28550	38050	57050
670000	859999	22850	28600	38100	57150
860000	1000000	22900	28600	38150	57200

[1]= If K greater than W, total sampling of the population
[2]= Number of expected, infested plants $\lfloor W\pi_0 \rfloor \geq \ln(\alpha)/\ln(1-\sigma)$

ABBREVIATIONS

EPPO	European and Mediterranean Plant Protection Organisation
PWN	Pine wood nematode
PWD	Pine wilt disease