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Pathway-analysis for pine wood nematode in bark from Portugal into the Netherlands

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Introduction

The pine wood nematode *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle (Bx) can infest *Pinus* spp. and various other coniferous species. Bx can cause tree death depending on tree species and environmental conditions (Mamiya, 1983; EPPO/CABI, 1997). Bx is believed to originate from North America and has been introduced in Asia (Japan, China, Taiwan and Korea) and Europe[1] (Portugal) (Mota et al., 1999; Evans et al., 2009). Bx is a regulated species in the EU and various measures apply to reduce the probability of spread from Portugal to other EU-member states and prevent new introductions from outside the EU. One of the possible pathways is import and trade of bark from areas where Bx is present. Therefore, one of the EU requirements is that bark originating from areas where Bx is present should be heat treated to achieve a minimum temperature of 56°C for at least 30 minutes (Commission implementing Decision 2012/535/EU).

The Netherlands imports pine-bark from Portugal (further referred to as "bark") and has tested bark lots from Portugal for presence of Bx since March 2009. Since mid-April 2012, bark lots are also tested for presence of other *Bursaphelenhus* spp. because their presence is an indicator that either the treatment of the bark has not sufficiently been effective or the bark has been re-infested after treatment by nematodes/*Bursaphelenchus spp*. which may originate from untreated bark. Also, EFSA PLH (2010) concluded that the composting process applied by Portugal does not ensure that all nematodes are killed in the bark. Since November 2012, bark that is traded from Portugal to the Netherlands is no longer composted but heat-treated by hot steam ("artificial heat-treatment") and this treatment has been shown to be 100% effective (Portugal, 2012b, 2012d). This new heat-treatment is applied at three locations (three companies). Despite this new heat- treatment, *Bursaphelenchus* spp. (other than Bx) and other nematode species are still found in samples of bark lots from Portugal (but not in all, data in Annex I). Most likely, *Bursaphelenchus* spp. recolonise the bark after the treatment.

The goal of the present analysis was to assess the probability of entry of Bx with import of bark from Portugal into the Netherlands. The probability of re-infestation of heat-treated bark was assessed and, in addition, the probabilities that Bx-infested bark would come into contact with a host plant and a plant becomes infested. Furthermore, the probability of entry of Bx with import of heat-treated bark was compared with the probability of entry of Bx through other pathways.

The present pathway-analysis applies to the three companies that has implemented the "artificial heat-treatment" since November 2012. According to recent information (received

[1] According to the available data until March 2013, Bx-infested trees have been found at three locations in Spain since 2008; eradication measures have been taken and the official pest status is "transient, actionable, under eradication" (EPPO, 2012).

12th April 2013), two more bark companies have been authorised by the Portuguese authorities. If these two companies apply a similar heat-treatment and work according to the same principles as the three companies visited, the risk associated with bark from these two new companies will be similar.

Methodology

In 2009, a Pest Risk Analysis (PRA) has been completed for the whole region of the European and Mediterranean Plant Protection Organization (EPPO) which include all EU-member states (Evans et al., 2009) and this PRA will be referred to where relevant.

The probabilities in the present pathway analysis were rated according to a 5-levels probability rating scale. The rating levels were "very low", "low", "medium", "high" and "very high" and were defined by probability intervals:

Rating level	Probability interval
Very high	0.33 – 1
High	0.1 - 0.33
Medium	0.01 – 0.1
Low	0.001 – 0.01
Very low	≤ 0.001

Evans et al. (2009) used the 5-levels rating scale, "very unlikely", "unlikely", "moderately likely", likely and "very likely", from the EPPO-scheme. These rating levels were not defined and have neither been defined in the entry section of the current EPPO-scheme (EPPO, 2011).

In the present analysis, the number of entries per year was assessed by multiplication of the probabilities (assessed per bark lot) of the different steps needed to enable entry (re-infestation of heat-treated bark, survival during transport, transfer to a host plant and infestation of a host plant) and the number of bark lots. This calculation was done using both extremes of the probability intervals assigned to each step. For "very low", $P \leq 0.001$, $P = 0.0001$ was used for the lowest extreme. A single probability or a narrower interval was used if the authors were more certain about the actual probability.

The probability of entry of Bx with bark from Portugal was compared with the probability of entry of Bx along other pathways in a more qualitative way.

The level of uncertainty of the ratings was indicated by "low", "medium" and "high". These ratings were also used by Evans et al. (2009) and still apply in the current EPPO-scheme (EPPO, 2011). In the present pathway-analysis, these "uncertainty ratings" were defined as follows (from Macleod et al., 2012: risk assessment method 2b):

- low uncertainty: "there is little doubt about the assessment and the risk rating. The assessor is confident."
- medium uncertainty: "there is some doubt about the assessment and the risk rating. The assessor has some confidence." [more specifically defined in the present analysis by: the actual risk level may be, although considered less likely, one level lower or higher but two rating levels difference between the actual risk and the rating level assigned is considered unlikely]
- high uncertainty: "there is considerable doubt about the assessment and the risk rating. The assessor has little confidence." [more specifically defined in the present analysis by: the actual risk level may be, although considered less likely, one or even two levels lower or higher than the rating level assigned]

Pathway-analysis

Probability of association before treatment

Origin of the bark
Surveys for Bx and other *Bursaphelenchus* spp. in conifer wood have been conducted in Portugal as part of the EU FP5 project PHRAME (Phrame, 2007). *Bursaphelenchus* spp. were found to occur widespread in low densities throughout Portugal. Various *Bursaphelenchus* spp. were identified. Bx was only found in a relatively small area but Bx has spread over a much larger area in Portugal since that time (Portugal, 2012a). *Pinus pinaster* is the most common pine species in central and northern Portugal (Euforgen, 2009) and bark of this pine species is processed and exported to the Netherlands for several purposes (see below: "Probability of transfer"). It is the main tree affected by Bx in Portugal (EFSA PLH, 2012) and many trees of this species have to be removed from areas in Portugal because of the presence of Bx but there are no figures on the percentage of bark that originates from infested trees and is processed by the three companies that have implemented the new heat-treatment. The companies may try to avoid bark of trees from infested areas. However, it is not forbidden to process bark from infested areas. There is also an uncertainty about the exact distribution of Bx in Portugal since there are practical limits to the number of trees that can be monitored and sampled.

Bx population densities in bark
Evans et al. (2009) have stated that "PWN [Bx] is likely to be associated with bark from PWN-infested trees [..and..] infested bark could contain high quantity of PWN.". This assessment was not supported with data. Data on numbers of Bx nematodes in isolated and untreated bark of *Pinus pinaster* have not been found. It has been reported and suggested that Bx is mainly present in the wood and only occasionally in the bark of mature pine trees (Bergdahl & Smeltzer, 1981; Myers, 1988; Ichihara et al., 2000). However, the isolated bark can also contain small parts of the sapwood which can harbour larger densities of Bx nematodes. Thus, the population densities of Bx in isolated bark will be greatly influenced by the quality of the debarking process and how much sap wood is still attached to the bark.

Bark is stored at the companies in large piles before they are processed. Data are available on the presence of Bx in bark piles that have been composted. The NPPO of Portugal found Bx in 5 out of approximately 6000 samples (0.08%) taken from 600 bark piles that had been composted in 2011 and 2012 (Portugal, 2012c; additional information from M.C. de Almeida Serra, DGAV, Portugal, March 2013). Bx has been intercepted and notified in isolated bark from Portugal 3 times (by Switzerland, Italy and Spain) before the artificial heat-treatment was implemented in November 2012. The Netherlands tested samples from about 240 lots of composted bark between February 2009 and November 2012 and never found Bx (in November 2012, the artificial heat treatment was implemented in Portugal). The low number of positive findings in Portugal and the lack of findings in the Netherlands indicate that Bx was usually not present or only present at low densities in the composted bark.

Biotic and abiotic conditions will affect the population dynamics of Bx in bark piles. Bx densities in untreated bark may be higher than in bark that has been composted because nematodes may have died during composting although it has been speculated that Bx may also increase during the composting process (EFSA PLH, 2010). Lethal temperatures can also be reached in bark piles during storage. Dwinell (1986) found that Bx population densities declined rapidly in wood chips at 45°C and higher and temperatures reached in a large part of a bark pile are probably above 45°C as illustrated by temperature registrations in bark piles before the 1st turning in the composting process (Portugal, 2012c). In those parts where temperatures are below about 45°C, Bx might reproduce. Dwinell (1986) found that after an initial increase at 35-40°C, Bx population densities declined in wood chips and were not much different from the initial densities after 21 days. At 25 and 30°C population densities declined. Halik & Bergdahl (1990) found an increase in population density of Bx in wood chips which had been frozen and subsequently inoculated with Bx and incubated at 30°C; between 2 and 12 weeks of incubation the population densities remained constant or decreased depending on the moisture content. It was speculated that as soon as fungal biomass becomes exhaustive nematode population development will slow down and densities decline. In conclusion, Bx densities will locally decrease (at temperatures above about 45°C) but might (temporarily) also increase in certain parts of a bark pile. It is assessed that Bx densities may not be much different (not different in order of magnitude) in bark piles than in the composted bark because also untreated bark is composted to some extent during storage.

Bursaphelenchus spp. other than Bx
Except Bx, many other *Bursaphelenchus* spp. can be associated with *Pinus* spp. (e.g. Braasch et al., 2002; Phrame, 2007; Carletti, 2008). *B. fungivorus*, known to be transmitted by bark beetles (Arias et al., 2005; Bx is transmitted by wood-boring beetles), was found in about 10% of bark lots of different origin sampled in Germany between 1996 and 2002 (Braasch et al., 2002). In the Netherlands, *B. fungivorus* was also the most frequently found *Bursaphelenchus* species in Portuguese bark (Annex I). Two other *Bursaphelenchus* spp. have also been found in Portuguese bark: *B. sexdentati* and *B. minutus* (Annex I). *B. sexdentati* has like *B. fungivorus* also been found associated with bark beetles (Vosilite, 1990; Braasch et al., 1999; Phrame, 2007) and has been considered a widespread species in the Mediterranean area and central Europe (Phrame, 2007). Few records have been found for *B. minutus* in literature (e.g. Carletti, 2008) but each of the *Bursaphelenchus* species mentioned have been found associated with pine trees (e.g. Phrame, 2007; Carletti, 2008). *B. fungivorus* and *B. minutus* have recently been reported as being associated with bark of *P. pinaster* in Portugal (Fonseca et al., 2012). Remarkably, *B. fungivorus* was not identified in 5587 wood samples during a survey on *Bursaphelenchus* spp. in Portugal (Phrame, 2007). It might be that *B. fungivorus* is mainly present in the bark (although it was found in wood samples in Spain (Phrame, 2007)) and less prevalent in wood of *P. pinaster* or that *B. fungivorus* rapidly multiplies on isolated bark.

<u>Conclusion</u>: the probability that Bx is present in untreated bark (i.e. arrives at least once a year at a company that process the bark) is rated as "very high" ($0.33 < P \leq 1$). If Bx is present in untreated bark, population densities are assessed to be low. The uncertainty of this assessment is medium because there are no data on the presence of Bx and their densities in untreated bark arriving at the three companies that have implemented the artificial heat treatment.

Probability of survival of the heat treatment
An artificial heat treatment (treatment by hot steam) has been implemented at three locations (three companies) in Portugal since the beginning of November 2012. Data from Portugal show that this heat treatment is 100% effective against *Bursaphelenchus* spp. (Portugal, 2012b, 2012d). The temperatures that are being achieved during the process are sufficient to kill the nematodes and experimental testing did not show any survival. Because interceptions of *Bursaphelenchus* spp. other than Bx continued after implementation of the artificial heat-treatment, the Portuguese authority has tested the bark directly after the heat-treatment since the first of February 2013. Each day, samples of the bark are taken directly after the treatment and tested for nematodes. Thus far, not a single nematode has been found (Portugal, 2013).

<u>Conclusion</u>: the probability that Bx survives the artificial heat treatment applied by three companies in Portugal is zero (low uncertainty).

Probability of re-infestation by Bx

Hygiene measures at the bark companies
After the heat-treatment, the bark is initially free of nematodes but may become re-infested[1]. Therefore, the three companies that heat treat the bark have implemented hygiene measures to reduce the probability of re-infestation with nematodes after the heat-treatment. They are RHP-certified and accordingly precautions are taken to avoid contact between treated and untreated bark and minimise spread by dust (RHP, 2012). For example, sprinklers are present

[1] Terminology: bark may become (re-)infested by Bx after the heat-treatment by contamination. Here, we use the term "re-infestation" both for bark that was already free of Bx before the heat-treatment as well as for bark that contained Bx before it was heat-treated.

at the companies to keep untreated bark wet to minimise spread by dust during dry periods and trucks delivering the untreated bark (raw material) may not enter the area where the treated bark is being stored ("treated area"). Also, shovels used in the "treated area" may not enter the "untreated area" and vice versa. A gutter is present to prevent water flux from the "untreated area" into the "treated area". At one company, the situation is different: the bark is fully screened both for removal of wood and fibres and separation in different size fractions before the heat treatment. After the heat-treatment, the bark is almost directly loaded into a container. The time between treatment and loading is less than three hours at this company. At the other two companies, the treated bark is stored under a shelter at least 25 m away from untreated bark for approximately 12 h and 2-3 days, respectively, before it is loaded.

Despite the hygiene measures, re-infestation with nematodes occurs including *Bursaphelenchus* spp. (Portugal, 2013; Europhyt interceptions records NPPO of the Netherlands; Annex I). The treated bark is probably rapidly recolonised by fungi which makes it a good substrate for fungivorous nematodes including *Bursaphelenchus* spp. Untreated and treated bark are kept separated at the companies in Portugal but nematodes from the untreated bark may reach the area where the treated bark is stored or loaded by wind dispersal of small particles (dust) or by particles attached to shoes or clothes which in practice seems difficult to avoid for 100%. Because of findings of *Bursaphelenchus* sp. (species not identified but other than Bx) in heat-treated bark, one company has changed the process. The company had found nematodes in 15 (including 14x *Bursaphelenchus* sp. other than Bx) out of 17 bark lots stored at the company and sampled more than 7 days after the heat-treatment (Portugal, 2013). The absence of nematodes in sample from two lots could be explained by the relative dryness of the bark. Because of these findings, the process was changed and bark is now screened before the heat-treatment and the bark is loaded into a container shortly after the heat-treatment. Loading takes 2-2.5 h after which the container is being closed. However, also this method may not prevent re-infestation with nematodes for 100% because re-infestation could take place during loading. The bark is exposed to open air (during a short time period) and nematodes may be present in dust dispersed by wind and machinery. Re-infestation may also take place in the container. Samples taken from dirt in a container (declared unsuitable for transport of the bark) were taken at one occasion and nematodes including *Bursaphelenchus* sp. were found (Portugal, 2013). However, even in clean-looking containers nematodes might be present. It is also possible that the bark becomes re-infested with nematodes including *Bursaphelenchus* spp. at arrival in the Netherlands. It is, however, much more likely that the findings of nematodes in samples taken in the Netherlands are due to re-infestation in Portugal or during transport because the probability to detect a nematode will be very low shortly after re-infestation. Shortly after re-infestation, nematodes are likely to be present at low densities and only locally in bark lots. In Portugal, nematodes have not been detected in bark sampled within one day after treatment (results from many samples). A pile of heat-treated bark was stored and sampled on 0, 6, 19, 42, 49 and 56 days after treatment; nematodes were only detected on day 19, 49 and 56 (Portugal, 2013).

Re-infestation by Bursaphelenchus spp other than Bx
Since the application of the artificial heat-treatment, *Bursaphelenchus* species (*B. fungivorus* and *B. minutus*) have still been found in Portuguese bark (Annex I; Portugal, 2013). *B. fungivorus* has been found in bark from each of the three companies before and after the implementation of the heat-treatment (including heat-treated bark from the company that loads the bark shortly after the heat treatment). These results and also the findings of Braasch (1999) suggest that *B. fungivorus* is commonly present at bark companies. *B. minutus* has been found three times in bark originating from one company since the implementation of the heat-treatment. The single finding of *B. minutus* in bark before the artificial heat-treatment was implemented (Annex I) also originated from this company. These findings suggest that this company also has a contamination source with *Bursaphelenchus minutus*.

B. fungivorus and *B. minutus* are probably associated with untreated bark arriving at the companies (see above: "Probability of association before treatment") and can come into contact with the treated bark despite the hygiene measures. These species may maintain themselves in (the vicinity of) the area where treated bark is processed and stored making it difficult to avoid re-infestation with these species. In Portugal, more than 10 *Bursaphelenchus* spp. have been found associated with *P. pinaster* (Penas et al., 2004, 2006; Phrame, 2007;

Fonseca et al., 2012). Thus far, only *B. fungivorus* and *B. minutus* have been found in heat-treated bark in the Netherlands (Annex I). These two *Bursaphelenchus* species might have certain ecological properties which increases their chance to be spread and to maintain themselves on bark companies but this hypothesis needs experimental testing. *B. fungivorus* has originally been described from rotting *Gardenia* buds infected with *Botrytis cinerea* in a glasshouse in the United Kingdom (Franklin & Hooper, 1962) and can probably maintain itself as long as a suitable substrate (e.g. organic material colonised by suitable fungi) is present. Isolated untreated bark (with pieces of sapwood) may contain nematode species of various genera (e.g. Phrame, 2007) and also nematode species other than *Bursaphelenchus* spp. found in bark after the heat-treatment might originate from untreated bark. The area where the heat-treated bark is processed and stored could be monitored for presence of nematodes and especially of *Bursaphelenchus* spp. to determine if the species composition is consistent over time or that new species are regularly introduced into the "treated area".

Re-infestation by Bx

The presence of *Bursaphelenchus* spp. other than Bx but also of any other fungivorous nematode (from other genera) in bark samples indicates that the bark lot is likely to be suitable for colonisation by Bx but does not indicate that Bx is present. Bx can be present in the bark lot. However, this is also the case when no *Bursaphelenchus* spp. are found in the samples because of detection limits of the testing procedure. Bark that has been heat-treated is probably rapidly re-invaded by fungi through air-borne spores or thermophilic fungi surviving the heat-treatment and may create a good food source for *Bursaphelenchus* spp. and other fungivorous nematode species. Heat-treated bark could, therefore, be a much better medium for growth of Bx populations than non-treated bark. There may initially be a surplus of fungal biomass to feed on and less competition with other organisms.

The main question is how likely bark can become re-infested with Bx at the place where the bark has been treated. Untreated and treated bark are kept separated at the companies in Portugal but as already discussed above nematodes from the untreated bark and the environment may reach the area where the treated bark is handled and stored. Re-infestation with Bx by *Monochamus* spp. is considered very unlikely because *Monochamus* spp. are very unlikely to oviposit on isolated bark because larvae of the beetle need wood to complete their development. It is, therefore, assumed that the probability of re-infestation by Bx is directly related to the prevalence of Bx at and in the direct vicinity of the place where the bark is handled and stored. Ecological differences between fungivorous nematodes[1] could play a role in the probability of re-infestation by a certain nematode species but there are currently no data that show that Bx would be a poorer coloniser of heat-treated bark than for example *B. fungivorus* and *B. minutus* which both have been found in heat-treated bark. Thus far, Bx seems to be present in low numbers in untreated bark (see above: "Probability of association before treatment") and Bx has not been found in heat-treated bark (Portugal, 2013; Annex I). The probability of re-infestation by Bx is, therefore, assessed as "very low". This probability may, however, vary over time as bark with higher Bx-infestation levels may arrive at the companies, e.g. when Bx would spread further in Portugal. Also, once the area where the heat-treated bark is handled, stored and/or loaded or the machinery (e.g. transport belts, screens, tractors etc) becomes contaminated with Bx, large volumes of bark may become re-infested by Bx until the infestation source has been removed. This risk of re-infestation can be mitigated by monitoring for possible presence of Bx, i.e. regular sampling and testing of the area where the treated bark is handled, stored and loaded including all machinery which comes into contact with the heat-treated bark (see below: "Additional measures to reduce the probability of entry of Bx with bark").

<u>Conclusions</u>:

- The probability that a bark lot (the volume of one container or truck load, see below: "Import volume") becomes re-infested by Bx is currently rated as "very low" (P≤0.001) because of the presumably low Bx-densities in untreated bark, the hygiene measures implemented by the bark companies in Portugal and the lack of positive findings of Bx in heat-treated bark thus far. The uncertainty of this assessment is medium. The

[1] Bx is mainly a fungivorous nematode as it feeds and reproduces on fungi; it can be phytoparasitic during part of its life cycle (Mamiya, 1983; Wingfield, 1987).

probability of re-infestation of one bark lot (see also below "Import volume) may be much lower than 0.001 but there are currently not sufficient data to assess the probability of re-infestation more accurately.

- The probability of re-infestation by Bx is assessed to increase with increasing densities of Bx in the untreated bark arriving at the bark-companies in Portugal. The probability of re-infestation by Bx is expected to increase strongly when Bx would enter and maintain itself (for some time) in the area where heat-treated bark is handled, stored and loaded.
- Re-infestation with nematodes including *Bursaphelenchus* spp. other than Bx of heat-treated bark is difficult to avoid at the companies that process the bark in Portugal.
- If no nematodes are found in samples of a bark lot, re-infestation with nematodes may not have happened, but neither can it be excluded because of detection limits.
- If nematode species other than Bx are found in samples of a bark lot, the probability that Bx is present is still assessed to be "very low" because of the presumed low prevalence at the bark companies (medium uncertainty).

Probability of survival during transport

The bark is loaded into a container and shipped to the Netherlands in most occasions. The time between loading of the bark and arrival in the Netherlands is between 1 and 3 weeks (usually 2 – 3 weeks) when transported by boat. The bark can also be transported by truck and transport by truck takes approximately 3-5 days (information obtained from the Portuguese bark companies and a Dutch company importing bark). Probably, more than 90% of the bark lots is currently transported by boat (estimate based on information from the NVWA, the Netherlands). There are no figures on temperatures that are reached within the containers or in the truck-loads but temperatures during transport may allow for reproduction of Bx (and other nematodes). At least, temperature allows for survival as shown by findings of *Bursaphelenchus* spp. in bark sampled at arrival in the Netherlands (Annex I) and Bx has been intercepted in bark from Portugal in other EU-countries. Evans et al. (2009) stated: "*B. xylophilus* will reproduce in the inner bark layer in live cambial and wood cells. It will also reproduce on any suitable fungi present on the bark." and rated the probability of survival as "very likely" with a "medium uncertainty". The interceptions of Bx and the findings of other *Bursaphelenchus* spp. at arrival show that Bx can survive transport. Dwinell (1987) found that Bx populations survived in wood chips during transport by boat from North America to Europe. Therefore, we assess the probability of survival as "very high" with a low uncertainty. Bx is also likely to reproduce in bark during transport when shipped. Nematodes including *Bursaphelenchus* spp. (other than Bx) have been found in bark lots from Portugal sampled at arrival while nematode populations, if present, will generally be below the detection threshold during the first days after treatment (see above: "Probability of re-infestation by Bx"). Reproduction during transport by truck will be limited because of the short transport time.

<u>Conclusion</u>: the probability of survival of Bx during transport is rated as "very high" ($0.33 < P \leq 1$) with a low uncertainty (the probability is assessed to be 1.0 for at least part of the population to survive). The probability that Bx populations will increase during transport is rated as "very high" with a medium uncertainty (there are currently no data that show that Bx-populations will increase in bark during transport and it is uncertain if the moisture content of the bark will always allow for reproduction).

Import volume

The import volume of bark of *Pinus* spp. from Portugal registered by the NPPO of the Netherlands in 2012 was:

- total 92,248 m^3
- 1153 lots: on average 80m^3 per lot (container/truck load)

The total import volume may have been higher because it is uncertain if all commodities have been registered. In addition, the import volume in 2012 may have been lower than in other years because the measures taken by the NPPO of the Netherlands (rejections of lots upon finding of *Bursaphelenchus* spp. irrespective of the species) may have limited import of the bark. Therefore, the total import volume without restrictions for *Bursaphelenchus* spp. other than Bx is estimated at about 150,000 m^3 per year (about 2000 lots).

Probability of transfer: bark comes into contact with a host plant

Bark from Portugal is especially used as growing substrate for orchids. One importer of Portuguese bark has indicated (March 2013) that it uses about 80% of the bark for the production of substrates for orchids and the other 20% in substrates (potting soil) for the hobby market (a few per cent of the bark), the tree nursery (shrubs and trees in containers, especially the larger sizes) and pot plant industry. Another importer has indicated (March 2013) that it uses about 70% of the bark for substrates for orchids and the other 30% for the tree nursery industry (substrate and mulch on pots) and the pot plant industry (substrate). There are also importers which use the bark mainly for the production of potting soil (not for orchids) and on average, it is assessed that approximately two thirds of the bark from Portugal are used for the production of orchids and one third is mixed through potting soil at a rate of 10 – 25% (v/v) (pers. comm. R. Keijzer, Horticoop, the Netherlands, February 2013). According to an inspector of the NPPO of the Netherlands, about 30 bark lots from Portugal are used in the chemical industry to filter air from odours every year. The bark is placed in special filters and replaced every 6-7 years when the old bark is probably discarded as waste. This application is not considered relevant for transfer of Bx. According to Dutch potting soil companies, Portuguese bark is not used as mulch (except for a limited extent on pots in the tree nursery industry). Bark from other origins (e.g. France, Spain and Germany) is sold as mulch for gardens, public green etc. The bark from Portugal has a very hard structure which makes it a very good growing medium for orchids. Furthermore, it is well separated from fibres and wood and is, therefore, more suited for use in growing substrates than bark from other origins. However, as people may use Portuguese bark as mulch in the future and Evans et al. (2009) discussed mulch as the possible way of transfer of Bx, the probability of transfer when the bark would be used as mulch will be discussed here as well.

Host plant species are defined here as species of *Abies*, *Picea*, *Pinus*, *Larix*, *Cedrus, Pseudotsuga* and *Tsuga,* the same species which are considered host plants in Commission implementing Decision 2012/525/EU. Evans et al. (2009) listed species of *Abies*, *Picea*, *Pinus*, *Larix*, *Cedrus* and *Pseudotsuga* as host plants of Bx and stated that the host plant status of species of *Juniperus*, *Chamaecyparis*, *Cryptomeria* and *Tsuga* was uncertain. In inoculation experiments, plants of *Tsuga heterophylla* and *T. mertensiana* had a low level of susceptibility or were resistant (Sutherland et al., 1991; Braasch, 1997). If more conifer species would be host plants of Bx than currently listed, the probability of transfer will increase but these would have a low level of susceptibility (see also EFSA PLH, 2012) which will decrease the probability of infestation (see below: "Probability of infestation of a host plant from infested bark"). Below the probability of transfer to a host plant is discussed for the known applications of Portuguese bark in growing media and its potential application as mulch.

Bark as growing medium for orchids

Bark used in growing media for orchids is very unlikely to lead to introduction of Bx because orchids are no host plants. The only possibility that Bx could transfer to a host plant would be when consumers discard the bark in their gardens. Bx might remain in the growing media for some time and feed on fungal biomass. Bx might still be present when orchids are removed and the growing medium is thrown away. This seems, however, rather theoretical and not considered relevant in the present pathway-analysis.

Bark in potting soil (including bark used to cover pots in the tree nursery)

Although about two thirds of the total volume of the bark are used as growing medium for orchids, bark from each of the estimated 2000 lots imported per year (except a limited number used for odour filters) can come into contact with a host plant because a lot is never entirely used for orchids. Certain fractions are (also) used for the production of potting soil. Below the probability that Bx will survive in potting soil and come into contact with a host plant is discussed.

Mamiya & Shoji (1989) could not recover Bx from soil 72 h after addition and concluded that Bx could not survive for extended time in soil. However, the survival rate and period will probably be different when bark pieces infested with Bx are mixed through potting soil. Bx survived (after an initial decrease) on wood pieces for at least 12 weeks in soil (Halik &

Bergdahl, 1992) and, therefore, it is considered likely that Bx can also survive for such periods on bark pieces in potting soil. If a host plant of Bx is planted in the potting soil, roots are very likely to come into contact with pieces of bark. Host plant species of Bx (species of *Abies*, *Picea*, *Pinus*, *Larix*, *Cedrus, Pseudotsuga* and *Tsuga*) are grown on about 1 – 10% of container fields at Dutch tree nurseries; on these fields other species are usually grown as well and an estimated 0.1 – 1.0 % of plants produced in containers are host plants with the percentage most likely close to 0.1% (Information from Naktuinbouw and consultants in the tree nursery industry, the Netherlands, March 2013). Assuming that about 50% or more of the potting soil with Portuguese bark is used in the tree nursery industry, an estimated 0.1% of this potting soil may be used for the production of host plants of Bx (based on the incidence of host plants in the tree nursery industry). Pot sizes used for *Pinus* spp. and *Picea* spp. the most common ones among the host plants produced in containers are average (Information from a consultant in the tree nursery industry, March 2013). Because of lack of information about the exact destinations of potting soil with Portuguese bark and because bark from one lot may have different destinations, we assess the probability that bark from one lot (one container) will come into contact with a host plant of Bx between 0.001 and 0.01 (0.1 – 1%) corresponding with the rating level: "low" (medium uncertainty). In case the bark is used a mulch on pots in stead of incorporation in the growing medium, the probability of transfer is assessed to be lower because only part of the bark can come into contact with the host plants: only those pieces directly adjacent to the stem base. In the present analysis, it is assumed that the bark is mainly used as potting soil component (if not used for orchids).

The probability that a host plant will come into contact with Portuguese bark in one year is, of course, much higher than the probability that bark from one lot will come into contact with a host plant. The total import volume of Portuguese bark is estimated to be about 150,000 m^3 of which 50,000 m^3 is used for potting soil (see above: "Import volume"). The volume of bark in potting soil varies from about 10 to 25%. Thus, about 200,000 – 500,000 m^3 potting soil contains Portuguese bark which is about 4 – 10 % of the total amount of potting soil produced every year in the Netherlands (the total production volume of potting soil is about 5 million m^3 of which about 1 million m^3 is being exported (Information from Stichting RHP, 1st March 2013).

Bark as mulch (in gardens, parks etc)
Bark is used as mulch in gardens and parks against weeds or as decoration and may also decrease soil evaporation. Bx may come into contact with superficial roots or the base of stems of host plants. Although no figures are available on the incidence of host plants in parks and gardens, the total number of host plants present in parks and gardens as compared to the total area on which bark can be applied as mulch is assessed to be very low. The area of host plants of Bx in forests in the Netherlands was about 194,000 ha in 2005 (Probos, 2009) but it is very unlikely that Portuguese bark is used as mulch in forests.

<u>Conclusion</u>: the probability of transfer, defined as the probability that bark from one lot comes into contact with a host plant is rated as "low" ($0.001 < P \leq 0.01$) with a medium uncertainty. The uncertainty is medium because it is uncertain to which extent potting soil with Portuguese bark is used for the production of host plants of Bx.

Probability of infestation of a host plant from infested bark
The only pathway by which Bx is known to infest trees naturally is through its vector *Monochamus* spp. (EPPO, 1997). Other possible pathways of natural transmission, e.g. by root contact is currently subject of an EU-project (Rephrame, 2013). Evans et al. (2009) concluded about the probability of infestation through infested bark the following: "The only significant international trade in bark is for use as mulch in agriculture, horticulture and gardening. This intended use increases the risk of PWN transfer to a suitable host but this risk (of non-vector transfer) itself is only theoretical." Evans et al. (2009) rated the question in the EPPO-scheme "How likely is the pest to be able to transfer from the pathway to a suitable host or habitat?" as "unlikely" with a high uncertainty. However, infestation of plants of various *Pinus* spp. have been found after addition of Bx to the growing medium with and without artificial wounding of roots (Kiyohara & Tokushige, 1971; Mamiya & Shoji, 1989; Halik & Bergdahl, 1987; 1992). Kiyohara & Tokushige (1971) found infestation after pine wood disks colonised by a fungus and

a *Bursaphelenchus* sp. (later indicated as Bx e.g. by Bedker (1987)) were buried around roots of a *Pinus* sp. without artificially wounding of the roots. Mamiya & Shoji (1989) only got infestation after artificially wounding of the roots (15,000 nematodes per plant). Halik & Bergdahl (1992) found infestation of wounded and unwounded *Pinus resinosa* seedlings after the stem base had been mulched with infested wood chips (1 out of 12 plants that had been deliberately wounded at the base and 1 out of 12 plants that had not been wounded became infested). It is unclear if presences of wounds are a prerequisite for infestation but their presence will probably increase the chances of infestation. In practice, roots of plants can have natural wounds depending on the conditions (e.g. wounds caused by other organisms or during potting of the plants) and two of the studies have shown that infestation can occur without deliberately wounding of roots or stem base. Thus, infestation of plants through infested growing medium and infested mulch is possible but it is uncertain how likely it will happen in practice. Below, the effect of Bx-density, host plant susceptibility and temperature on the probability of infestation is discussed.

Bursaphelenchus densities in bark and potting soil

The relationship between Bx-density in soil and the probability of infestation of plants is not known. The amount of wood chips mixed with soil in the study of Halik & Bergdahl (1992) was about 10% of the volume of the soil - wood chip mixture (pers. comm. S. Halik, 28th February 2013) which is similar or even lower than the bark volumes (10 – 25%) used in potting mixtures in the Netherlands. In two inoculation experiments of Halik & Bergdahl (1992) chips were mixed with soil, the chips contained 2,500 and 560 nematodes per g dry weight, respectively, and 150 g fresh weight was used as inoculum per potted seedling. The percentage of trees (*Pinus sylvestris* and *P. resinosa*) infested with Bx was on average 79.5 and 87.5% in the first experiment (2,500 nematodes per g dry weight) of potted seedlings placed at 20 and 30˚C, respectively. In the second experiment (560 nematodes per g dry weight and potted seedlings placed in soil in the field in June), the percentage infestation was on average 8%. In another study, Halik & Bergdahl (1987) observed wilting of 7 out of 12 seedlings treated with nematode-infested chips. Inoculum density was 50 nematodes per g dry weight of wood chips and 150 g fresh weight had been applied per seedling (being artificially wounded). The moisture content of the wood chips was 130%. Thus, about 3260 nematodes had been applied per potted seedling in a one litre pot. This study indicates that infestation of seedlings (that have been artificially wounded) can already be obtained with relative low nematode densities. Bx can probably maintain itself in bark in potting soil for some time as Halik & Bergdahl (1992) found that, after an initial decrease, Bx densities remained fairly constant in wood chips in soil during a 12-weeks study. In practice, Bx is unlikely to be evenly distributed in a bark lot after contamination (i.e. not each bark piece may be infested) which possibly decreases the probability of infestation as compared to the experimental studies with inoculated wood chips.

Host plant susceptibility

Species of the 7 genera recognised as host plants in the EU-regulation are produced in containers at a limited scale and of these host plants *Pinus* spp. (e.g. *P. mugo*, *P. nigra*, *P.strobus*) and *Picea* spp.. (especially *P. abies* "Conica") are among the most frequently grown host plant species in containers in potting soil (Information from Naktuinbouw and consultants in the tree nursery industry, the Netherlands, March 2013). The *Pinus* species are (highly) susceptible for Bx (Phrame, 2007). *Picea abies* had a low level of susceptibility and also the other host species (except *Larix* spp.) generally had low levels of susceptibility in inoculation experiments (Sutherland et al., 1991; Braasch, 1997; Phrame, 2007).

Temperature

Temperature will affect the probability of infestation. Mortality rates of Bx inoculated plants were higher at 30/25˚C day/night temperatures (8h night) than in another experiment, made under ambient summer-autumn temperatures in a shade house in Canada (Sutherland et al., 1991). At 15˚C, Bx was not detected or only found at relative low densities in *Pinus sylvestris*, *Larix decidua* and *Picea abies* as compared to ambient temperatures of 20 or 25˚C (Phrame, 2007). De Bilt, located in the central part of the Netherlands, has an average daily temperature of 17.9˚C during the warmest month of the year and the average temperature is below 15˚C during 9 months of the year (KNMI, long-term average 1981-2010; http://www.klimaatatlas.nl/tabel/stationsdata/klimtab_8110_260.pdf). Host plants of Bx are generally grown outdoors, i.e. not in tunnels or shade houses (Information from a consultant in the tree nursery industry, March, 2013).

Conclusion: Factors/evidence supporting infestation are (i) infestation of host plants have been shown after addition of Bx to soil, and (ii) the volume of bark in the potting soil (10 – 25% v/v) is sufficient to allow for infestation and (iii) (highly) susceptible *Pinus* spp. are produced in containers. Factors/evidence that do not support infestation are: (i) many host plant species produced in containers have a low level of susceptibility, (ii) presumably uneven distribution of Bx in a bark lot if contaminated, and (iii) the relatively cool summers in the Netherlands. The probability of infestation of a host plant if potted in soil containing bark from a Bx-infested lot is assessed "low" ($0.001<P<0.01$) with a medium uncertainty. The probability is rated as "low" mainly because many host plants have a low level of susceptibility and conditions in practice are expected to be generally less favourable for infestation than those in the experimental studies discussed above. The more plants are potted the higher the probability that at least one plant will become infested. If 100 plants are potted and the probability of infestation for one plant would be 0.001 or 0.01, the probability that at least one plant becomes infested is 0.1 and 0.6, respectively; if 1000 plants are potted these probabilities are 0.6 and 1.0 (example of calculation: P = 0.01 for one plant, then P(at least one out of 100 plants becomes infested) = $1 - 0.99^{100} \approx 0.6$). In practice, size of plant lots can vary largely, e.g. between tens to ten thousands at a few highly specialized companies (information from a consultant in the tree nursery industry). Assuming that in most cases at least 100 plants of the same species will be potted using the same potting mixture, the probability that at least one plant would become infested is rated "high" - "very high" ($P>0.1$). The uncertainty of this assessment is high:

- Exact figures are lacking on the production of the different host plant species in containers in the Netherlands and their relatively susceptibility to Bx (data on susceptibility are available for a limited number of host plant species only and differences in virulence between Bx-isolates may exist).
- It is uncertain at which frequency roots or stem bases will be invaded that have not been deliberately (or undeliberately) wounded.
- It is unknown to which extent the relatively low temperatures in the Netherlands will affect the probability of infestation.
- No information is available on Bx population densities that may occur in heat-treated bark in practice and how, after addition to soil, population densities relate to the probability of invading plants.

Note that for long-term establishment (although not part of this pathway-analysis), Bx will need to come into contact with an insect vector (EPPO, 1997). The only known vector of Bx is longhorn beetles of the genus *Monochamus* (EPPO, 1997). In the Netherlands, small numbers of adults of the vector *Monochamus* spp. have been detected in a small coastal area in the province of Noord-Holland but, thus far, not in other areas using pheromone traps (Heijerman et al., 2009, 2011; 2012 survey data from the NPPO of the Netherlands). Thus, it seems that natural spread of PWN cannot occur in the largest part of the Netherlands because of the absence of the vector. Therefore, infestation of a tree from infested potting mixture will probably not lead to spread of the PWN in the majority of cases and infestations may remain limited to the trees originally infested through contact with infested bark. Bx infestations will likely not lead to decline of its host plants because of the relatively cool summers in the Netherlands although this may change when average summer temperatures increases due to global warming (e.g. Sutherland et al., 1990; Sukovata et al., 2012). The absence of wilt symptoms under the current climatic conditions will also reduce the likelihood of natural spread of Bx: *Monochamus* spp. are attracted to dying or dead trees in which they oviposit and oviposition is a prerequisite for vector-transfer from infested to healthy trees (e.g. Wingfield, 1987; Phrame, 2007). Nevertheless, it should be noted that any finding of Bx on host plants in the Netherlands could have major consequences *inter alia* because of current EU measures (Commission implementing Decision 2012/525/EC). In case of the finding of one infested plant and even when there is sufficient evidence that the vector is not present in the area a clear cut zone of susceptible plants of 500 m around the infested tree should be created because of the presence of the vector in another area in the Netherlands; in addition various other measures should be applied (Commission implementing Decision 2012/525/EU).

Number of entries per year: combination of ratings

The number of entries per year calculated by combination of the individual risk ratings ranged from 0.02 down to 0.00002 (Table 1, Fig. 1). If the uncertainty for the different ratings were taken into account, the number of entries ranged from 2 down to 2 x 10^{-8}. The higher values in these range are, however, considered highly unlikely because the probability of re-infestation is currently assessed to be much lower than 0.001 and also the probability of transfer to a host plant that is (highly) susceptible is assessed to be at the lower range of its rating interval (see above: "Probability of transfer". Therefore, we assess the current probability of entry as "low" - "very low", i.e. less than 0.01 but more likely less than 0.002 and possibly much lower (Table 1). The probability of entry can, however, increase in the future for example when Bx densities would increase in untreated bark (see above: "Probability of re-infestation by Bx") or when bark would be more frequently used in growing media for host plants.

If one bark lot (one container) would be infested with Bx, the probability of infestation of a host plant, calculated as $P_{transfer}$ x $P_{infestation}$, is assessed to be "low" – "very low" (0.01 < P < 0.0001).

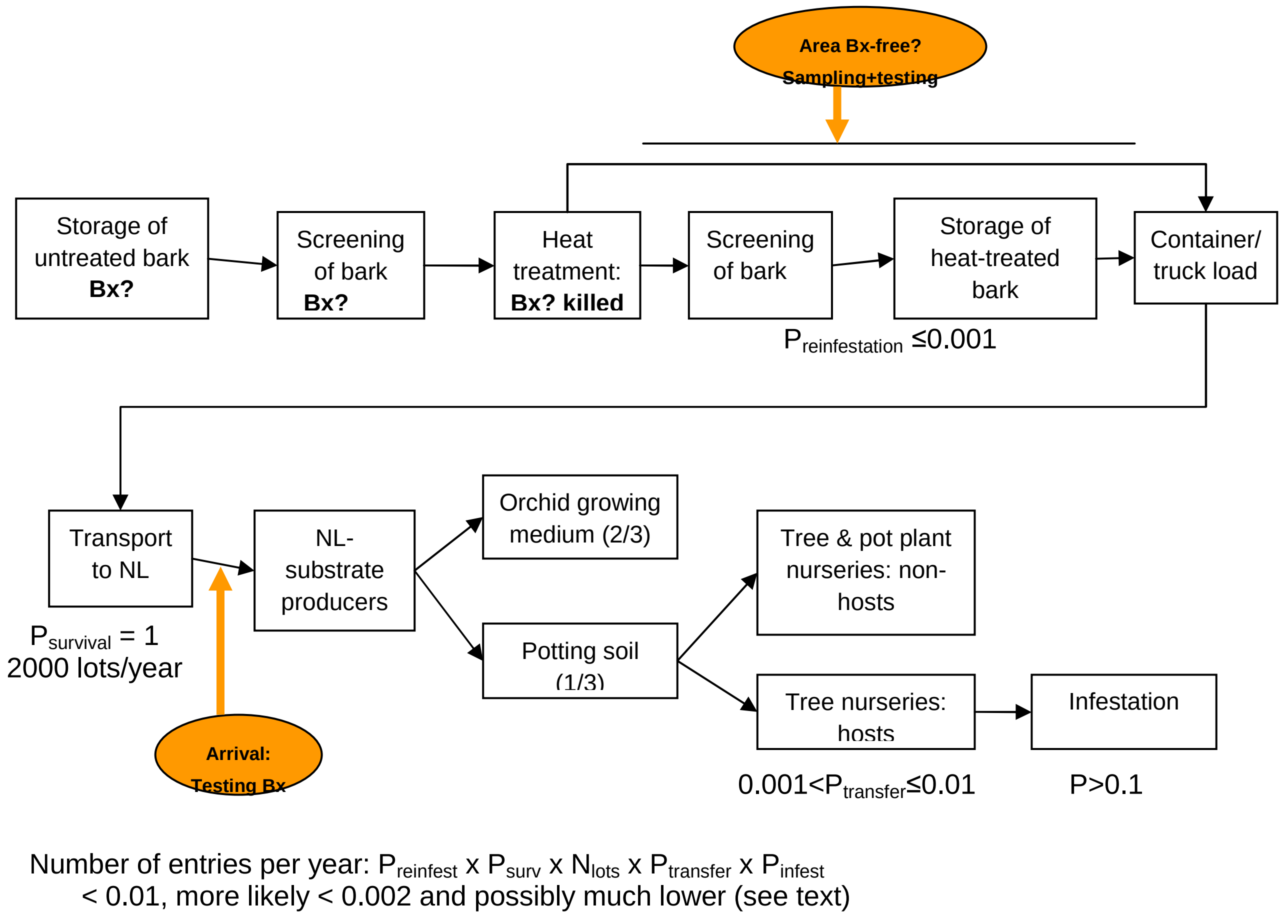

Fig. 1. Diagrammatic description of the pathway "importation of bark of *Pinus pinaster* from Portugal into the Netherlands" and the possibility that *Bursaphelenchus xylophilus* (Bx) is associated. Estimates are included of the import volume (number of lots per year), usage of the bark and probabilities per lot that Bx re-infests the bark after the heat-treatment, survives transport, transfers to a host plant and infests a host plant, respectively. Points in the process are indicated where bark samples could be taken to monitor for possible re-infestation by Bx after the heat-treatment.

Table 1. Assessment of the number of entries per year of *Bursaphelenchus xylophilus* with import of heat-treated bark from Portugal by combination of the probability (P) of re-infestation, the number of bark lots, the probability to survive transport, the probability of transfer and the probability to infest at least one plant (see text for explanation on probability intervals).

$P_{re\text{-}infestation}$	Number of lots	$P_{survival}$	$P_{transfer}$	$P_{infestation}$	Entry	
					Number of entries per year	Rating level
0.001	2000	1.0	0.01	1.0	0.02	Medium
				0.1	0.002	Low
			0.001	1.0	0.002	Low
				0.1	0.0002	Very low
0.0001	2000	1.0	0.01	1.0	0.002	Low
				0.1	0.0002	Very low
			0.001	1.0	0.0002	Very low
				0.1	0.00002	Very low

Additional measures to reduce the probability of entry of Bx with bark

The current probability of re-infestation with Bx after the heat-treatment is assessed "very low" with a medium uncertainty (see above). The probability of re-infestation is considered to be related to the infestation level of the untreated bark present at the same location (although separated from the treated bark) and hygiene measures taken by the bark companies. The probability of re-infestation is assessed to be lower when heat-treated bark is directly loaded into a container as compared to a situation where the bark after the heat-treatment is screened and stored for a (short) period before loading. However, even in the case where bark is loaded shortly after the heat-treatment, it may become re-infested (e.g. when the belt over which the bark is moved from the steam apparatus to the container becomes contaminated). Once the site where heat-treated bark is screened, stored and/or loaded ("treated area") becomes contaminated with Bx, many bark lots may become re-infested as it now appears to happen with *B. fungivorus* and *B. minutus*. Regular sampling and testing of the "treated area" can be used to detect possible infestation sources of Bx. Samples could be taken from dust and bark particles present on the screens used to separate the heat-treated bark in different size fractions, from the floor where the treated bark is stored, from the ground of the area where the treated bark is loaded and from walls of shelters, machinery, belts etc. in the "treated area". At the company where the bark is loaded almost immediately after the heat-treatment, the belt over which the bark is transported into the container and the direct vicinity of the belt could be sampled in a regular way (Fig. 1). In case Bx is found, measures should be taken to avoid any re-infestation of treated bark or trading of bark that may have become re-infested by Bx. Untreated bark is screened and handled on a daily basis with the risk of spread of Bx containing particles. Daily sampling of the "treated area" may not be practical but sampling on a weekly basis could be reasonable both from the point of view of the presumably very low probability of re-infestation and practical aspects.

Sampling of the bark shortly after treatment is considered useful for monitoring the efficacy of the heat treatment but not for monitoring possible re-infestation. During the first days after treatment re-infestation may already occur but nematode densities will probably be too low to enable detection with high confidence (see also above: "Probability of re-infestation"). Sampling and testing for Bx of treated bark at arrival in the Netherlands will be more effective because nematode populations are expected to increase during transport (see above "Probability of survival during transport") (Fig. 1). However, testing (including incubation of samples before the extraction of the nematodes) takes 3 weeks and if bark lots are not allowed to be moved or used before test results are available this can have major logistic consequences for the potting soil companies using the bark to produce growing media. The testing procedure may be shortened significantly if the samples are not incubated before extraction of nematodes. During transport the bark may already have been "incubated" to a certain extent, i.e. conditions may be favourable for reproduction of Bx (transport time by truck will be too short but transport by boat usually takes 2 – 3 weeks). The sensitivity of a testing procedure for *Bursaphelenchus* spp. with and without incubation after sampling could be compared to determine if the procedure could be shortened. In addition, the temperature at different places in the container could be monitored to assess how favourable temperatures are for reproduction of Bx during transport.

Probability of entry, including infestation of plants, through other pathways
Evans et al. (2009) concluded the following about pathways of Bx:
"In order of priority, the probability of entry of PWN [Bx] and its vectors is:
1) untreated coniferous wood packaging materials (but the implementation of ISPM No 15 reduces this risk to an acceptable level),
2) wood,
3) plants for planting,
4) particle and waste wood,
5) cut branches,
6) isolated bark.

Nevertheless the risk of PWN [Bx] entry, but not necessarily establishment, with those commodities is substantial." Thus, isolated bark was considered the least likely pathway for entry of Bx in absence of any regulatory measures.

The current EU-regulation has strict measures for each of these pathways to prevent introduction from infested areas into the rest of the EU (Commission implementing Decision 2012/525/EU):

- susceptible wood in the form of WPM have been treated according to ISPM No. 15. (pathway 1).
- susceptible wood and bark have been treated to achieve a minimum temperature of 56°C for at least 30 minutes (pathways 2, 4, and 6).
- susceptible plants have been grown at a place of production where no PWN or its symptoms have been observed since the beginning of the last complete growing cycle and plants have been grown under complete physical protection ensuring that the vector cannot reach the plants (pathways 3 and 5).

In addition, the following is required for wood and bark that has been treated: "it is moved outside the flight season of the vector or, except in the case of wood free from any bark, with a protective covering ensuring that infestation with PWN or the vector cannot occur" (Commission implementing Decision 2012/525/EU). This requirement should prevent re-infestation by the vector but cannot prevent re-infestation of isolated bark due to contamination as discussed above: "Probability of re-infestation by Bx". Except from the possibility of contamination of isolated bark after heat treatment, these regulatory measures are in principle 100% effective against introduction of Bx but in practice these measures may not reduce the probability of spread by trade to zero for several reasons, such as lack of knowledge about the distribution of Bx, no 100% implementation (fraud) etc. Since 2010, Bx has been notified thirteen times by EU member states and Switzerland on commodities (wood and bark) originating from Portugal (Europhyt, last access 19th March 2013). Three of these notifications concerned bark, the other 10 wood or woody materials. In addition, Bx has been notified six times in WPM originating from Morocco but the WPM had probably been constructed from wood originating in Portugal. The Netherlands intercepted Bx once on WPM from Portugal (in 2012). This interception was not among the 13 notifications listed in the Europhyt database (notifications from 2010 and later).

Except for isolated bark (see above "Import volume"), trade data for the other 5 commodities (pathways) mentioned above are lacking. For the Netherlands, wood packaging materials (WPM) (pathway 1) and isolated bark (pathway 6) seem to be the most important pathways for Bx from Portugal because trade of wood, plants for planting of conifers, particle and waste wood and cut branches from Portugal into the Netherlands may be absent or very limited (uncertainty because of lack of trade data).

Untreated WPM from Portugal is considered a higher risk than isolated bark because of the possible presence of *Monochamus* spp in the WPM together with Bx which makes transfer to and infestation of a plant more likely than with isolated bark. This does not apply for wood sizes that are too small to carry the vector. Such wood poses a lower risk than isolated bark because of the very low or negligible probability of transfer from WPM to a host plant without a vector. WPM should be treated according to international standards (ISPM No. 15) which will kill all *Bursaphelenchus* nematodes present in the wood. Despite this requirement, the NPPO of the Netherlands intercepted Bx on WPM from PT in 2012. This interception was traced back by the Portuguese authorities and it appeared to be a case of fraud (pers. comm. M.C. de Almeida Serra, Portugal, 19^{th} February 2013). Most likely pallets had been composed from wood that had been marked and untreated wood (without the ISPM15 mark). Although, mitigation measures have been taken to prevent such incidents from happening, it is no guarantee that such fraud could not happen again. There are approximately 280 companies that process wood in Portugal and the probability of non-compliance with EU- regulation may be higher than bark becomes re-infested with Bx after heat-treatment. Also note that WPM from PT is not regularly tested because there is no official system which registers import from Portugal (or other EU-countries).

Except in Portugal, Bx is also present in North America and Eastern Asia and may also be introduced from those continents into the EU including the Netherlands. For example, EU-member states notified Bx 5 times on WPM or other kinds of wood from North America since 2010 (Europhyt, last accessed 19^{th} March 2013).

Summary and conclusions

The current probability of entry of Bx (including transfer and infestation of a host plant) through import of isolated bark from the three locations in Portugal where the bark is steamed and exposed to at least 56°C during 30 min or more and hygiene measures are taken is assessed to be "low" - "very low" (i.e. less than 0.01 and more likely less than 0.002 per year and possibly much lower). This low probability is mainly due to the very low probability assessed for re-infestation of treated bark by Bx. The artificial heat treatment of bark implemented since autumn 2012 has been shown to be 100% effective but there is uncertainty about the probability of re-infestation by Bx. Isolated bark is inter alia used for the production of potting soil. If a host plant is planted in potting soil containing Bx-infested bark, infestation of the plant is considered realistic. If one bark lot (one container) would be infested with Bx the probability of infestation of a plant is, however, assessed to be "low" – "very low" ($0.01 < P < 0.0001$), mainly because of the low probability (medium uncertainty) that this bark will come into contact with a host plant.

Re-infestation of treated bark by other nematode species including *Bursaphelenchus* spp. other than Bx is difficult to avoid and when *Bursaphelenchus* spp. other than Bx are found in heat-treated bark at arrival in the Netherlands, the probability that Bx is present is still assessed as "very low". The probability of re-infestation by Bx, and thereby the probability of entry may, however, increase in the future for example when Bx would spread further in Portugal and untreated bark may contain higher densities of Bx. Higher Bx-densities in untreated bark are assessed to lead to an increased probability of re-infestation of treated bark which is stored and loaded at the same production place. Heat-treated bark probably offers a good medium for Bx and once the area where the treated bark is processed (e.g. screened), stored and loaded becomes contaminated with Bx, several lots may become re-infested. Therefore, and because of the uncertainty about the probability of re-infestation with Bx an additional requirement (additional to the current EU-requirement of heating the bark) could be that heat-treated bark should be processed, stored and loaded at a site (part of the production place) that is free of Bx as confirmed by regular sampling and testing. Samples could be taken on a weekly basis from dust and bark particles on the screens used to separate the heat-treated bark in different size fractions, from the floor where the treated bark is stored, from the ground of the area where the treated bark is loaded and from walls of shelters, machinery, belts etc. in the "treated area".

Sampling and testing shortly after the treatment is not considered effective to determine possible re-infestation by Bx. Sampling and testing of the bark shortly after the heat treatment could be done at a lower frequency to test the efficacy of the heating process (additionally to the temperature monitoring in the hot steam apparatus). In addition, bark could be sampled for presence of Bx upon arrival in the Netherlands (at a random basis). Re-infestation of heat-treated bark by nematodes in general, including *Bursaphelenchus* spp. other than Bx, is difficult to avoid in practice. Direct loading of the bark after the heat treatment into a container is assessed to reduce the probability of re-infestation with Bx as compared to the situation were bark is screened after the heat treatment and stored at the site before being loaded.

Despite the large volumes of bark from Portugal being shipped to the Netherlands, the probability of entry of Bx into the Netherlands by import of isolated bark which has been heat-treated and where hygienic measures have been taken to minimise contact with untreated bark, etc. is currently not assessed to be higher (it may even be lower) than the probability of entry along other pathways taking into account the current EU-regulation. The uncertainty of this assessment is medium because figures are lacking on import volumes of commodities other than bark and on the implementation level of the current EU-regulation for the commodities.

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Annex I: Test results of Portuguese bark sampled at arrival in the Netherlands
1st January 2012 – 8th April 2013 (data from the NPPO of the Netherlands)

Date[2]	Results[1]	
	Nematodes	***Bursaphelenchus* sp.**
2012		
5-Jan	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
16-Jan	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
26-Jan	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
26-Jan	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
9-Feb	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
9-Feb	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
9-Feb	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
17-Feb	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
17-Feb	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
9-March	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
13-March	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
16-March	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
19-March	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
27-March	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
16-April	yes	no *B. xylophilus*, not tested for other *Bursaphelenchus* spp.
17-April[3]	yes	*B. sexdentati*
23-April	yes	no
7-May	yes	no
10-May	yes	no
22-May	yes	*B. fungivorus*
24-May	yes	no
31-May	yes	no
4-Jun	yes	no
14-Jun	yes	no
15-Jun	no	no
15-Jun	no	no
18-Jun	yes	no
22-Jun	yes	*B. fungivorus*
22-Jun	yes	*B. fungivorus*
2-Jul	yes	no
2-Jul	yes	*B. fungivorus*
18-Jul	yes	no
31-Jul	yes	*B. fungivorus*
31-Jul	yes	*B. fungivorus*
3-Aug	yes	*B. fungivorus*
13-Aug	yes	no
14-Aug	yes	no
21-Aug	no	no
5-Sep	yes	*B. minutus*
21-Sep	yes	*B. fungivorus*
21-Sep	yes	*B. fungivorus*
21-Sep	yes	*B. fungivorus*
8-Oct	no	no
8-Oct	yes	*B. fungivorus*
8-Oct	yes	no
8-Oct	no	no
8-Oct	no	no
11-Oct	no	no
11-Oct	yes	*B. fungivorus*
23-Oct	no	no
24-Oct	yes	no
29-Oct	no	no

Date[2]	Results[1]	
	Nematodes	***Bursaphelenchus* sp.**
1-Nov	yes	no
1-Nov	no	no
1-Nov	no	no
1-Nov	yes	*B. fungivorus*
1-Nov	no	no
13-Nov*[4]	no	no
13-Nov*[4]	yes	*B. fungivorus*
19-Nov	yes	*B. fungivorus*
19-Nov	no	no
19-Nov	yes	*B. fungivorus*
20-Nov	yes	*B. fungivorus*
23-Nov	yes	*B. fungivorus*
23-Nov*[4]	yes	*B. fungivorus*
30-Nov	yes	*B. fungivorus*
7-Dec	no	no
10-Dec	yes	*B. minutus*
13-Dec	no	no
13-Dec	no	no
14-Dec	yes	*B. fungivorus*
14-Dec	no	no
21-Dec	yes	*B. fungivorus*
21-Dec	no	no
31-Dec	no	no
2013		
8-Jan	yes	*B. fungivorus*
8-Jan	yes	*B. fungivorus*
11-Jan	yes	*B. fungivorus*
14-Jan	no	no
18-Jan	yes	no
25-Jan	no	no
4-Feb	yes	*B. minutus*
8-Feb	no	no
8-Feb	yes	no
11-Feb	yes	no
14-Feb	no	no
14-Feb	no	no
21-Feb	yes	*B. minutus*
27-Feb	no	no
6-March	yes	*B. fungivorus*
11-March	no	no
18-March	no	no

[1] Short description of the analysis: A sample is incubated at 25˚C for two weeks. After incubation a subsample of 500 ml is submerged in water for 3 days. The water suspension is poured through a course sieve onto a cotton wool filter. The cotton wool is placed in a shallow layer of water for one night to allow nematodes to move into the water. Next morning the nematode suspension is collected. All nematodes in the suspension are counted at a magnification of 20-40x with a stereo microscope. From the suspension 20 *Bursaphelenchus*-like specimens (if present) are transferred onto a glass slide and identified morphologically to species level at a magnification of 1000x. If *B. xylophilus* will be detected, 20 specimens will be additionally identified using a real-time PCR test.

[2] Date the sample was received by the National Reference Laboratory; the sampling date is usually 1-3 days before this date.

[3] Samples dated 17th April 2012 and later were tested for *B. xylophilus* and other *Bursaphelenchus* spp.

[4] The artificial heat-treatment (steaming of bark) has been applied since the beginning of November and transport time is between 1 to 3 weeks. Thus, samples from 7th December and later have most likely been treated by hot steam. For samples received in November it is indicated by an asterisk (*) if they originated from steamed bark as far as known.