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Pinewood nematode presence and survival in commercial pallets of different ages

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Abstract
The pinewood nematode (PWN) *Bursaphelenchus xylophilus* is an important forestry quarantine organism in the world and can be disseminated through the international trade of infested wood packaging materials. Although heat treatment (HT) and kiln-drying (KD) are used to disinfest the wood and reduce its moisture, doubt remains if treated wood can be re-infested and support viable populations of the nematode. In this study, the risk associated with disseminating the PWN with kiln-dry pallet timber was evaluated by sampling commercial pallets of different ages and by inoculating *B. xylophilus* into treated wood to assess the nematode's survival. A total of 229 timber samples (boards and blocks) were randomly sampled, finding no *Bursaphelenchus* species. Nematodes of the families *Aphelenchidae, Aphelenchoididae, Diplogasteridae, Rhabditidae* and *Tylenchidae* were found in 45% of the samples, being absent from timber with less than 6 months but present with increasing frequency in older pallets. Fungi were encountered frequently, with the genus *Trichoderma* dominant. In the second trial, artificial inoculations of *B. xylophilus* were made in pallet timber of different age and assessed after 7, 14, 28, 56 and 84 days. The PWN was recovered in the first sampling, but infestation rates and nematode numbers decreased until disappearing in the subsequent samplings. The results confirm that ISPM 15 treatments effectively sanitize wood, and older, drier wood does not support re-infestation with *B. xylophilus*, while other saprophitic nematodes and fungi colonise the treated timber. Future research should evaluate the risk associated with KD timber subjected to rewetting and its ability to support viable populations of the PWN.

1 Introduction

Wood packaging (pallets, crates, and dunnage) are indispensable commodities in today's globalised modern societies, and it is estimated that over 1.5 billion pallets are produced every year, consuming approximately 60 million cubic meters of timber (TIMCON 2014). Pallets are the most abundant form of wood packaging, and in the United States alone, they account for nearly 80% of all packaging used by small and large exporters, of which 90% are made of solid wood (Molina-Murillo et al. 2005).

Historically, the intensive worldwide trade involving untreated and often green wood pallets and other wood packaging material (WPM) has been associated with the dispersal and introduction of various exotic insects and pathogens (e.g., Allen and Humble 2002; Haack 2006; Zahid et al. 2008; Haack et al. 2014; Lovett et al. 2016), including bark and wood-boring insects *Scolytinae*, *Cerambycidae* and *Buprestidae* (Haack 2006; Haack and Petrice 2009; Benker 2012; Wu et al. 2017), and also fungi and nematodes (Braasch et al. 2001; Tomiczek et al. 2003; Gu et al. 2006).

To significantly reduce the risk of introduction and spread of pests and pathogens, wood packaging material must comply with an international standard for phytosanitary measures - "Guidelines for Regulating Wood Packaging Material in International Trade", or ISPM 15 (IPPC 2009; Anonymous 2009). The three currently approved treatments are heat treatment, dielectric (microwave) heating and fumigation, following specific guidelines, and which have proven

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effective against many insects and pathogens (Eyre et al. 2018).

One of the most important forest quarantine organisms is the pinewood nematode (PWN) *Bursaphelenchus xylophilus*, the causal agent of pine wilt disease and one of the most serious forest threats worldwide (Kishi 1995; Evans et al. 1996; Futai 2013). This nematode can survive in processed timber for several months (Sousa et al. 2011a, b), and can be potentially transported over long distances with international trade of goods and merchandise, being regularly found in assorted untreated wood packaging (e.g., Tomminen 1991; Dwinell 1997, 2004; Tomiczek et al. 2003; Gu et al. 2006; Eyre et al. 2018). Heat treatment [HT, submitting the wood to a minimum core temperature of 56 °C for at least 30 min (Dwinell 1990, 1997; Smith 1992)] is a conventional option to sanitize the wood. However, *B. xylophilus* can colonise HT-timber if kept in direct contact with nematode-infested timber, although infestation can be prevented by kiln-drying (KD) the wood and lowering its moisture content to around 18–22%, which hampers nematode transfer (Sousa et al. 2011).

Despite its low moisture content, even kiln-dried timber may be vulnerable to fungal colonisation if it becomes wet during transport, storage, or use (Melencion and Morrell 2007), and reports of nematodes and fungi in kiln-dried, heat-treated ISPM 15 stamped timber (Payne et al. 1998; Zahid et al. 2008) create doubt whether treated timber can support viable populations of the PWN, although inadequate treatment of wood or fraudulent use of the ISPM 15 stamp can also explain the presence of nematodes and fungi (Eyre et al. 2018). Considering this knowledge gap, the objective of this study was to evaluate the risk associated with disseminating the PWN with in service commercial pallet timber sanitized according to international standards (mandatory ISPM 15 HT and recommended KD treatment), with two experiments: the sampling of commercial pallets of different age for the presence of the PWN, other nematodes and fungi (Experiment 1), and the artificial inoculation of *B. xylophilus* into commercial pallets with ISPM 15 stamp to assess its colonisation and survival in timber of different age and type (Experiment 2).

2 Materials and methods

2.1 Selection of in-service commercial pallet timber (Experiment 1 and 2)

Commercial pallets were supplied by CHEP®, a leading international company in pallet and container pooling services with a pool of approximately 300 million pallets worldwide. In-service maritime pine timber (*Pinus pinaster*) pooled pallets were used, having a longevity from 6 months to 10 years or more. Commercial pallets of various ages complying with ISPM 15 standard (HT) and presenting the appropriate stamp were randomly selected at the CHEP facility at Castanheira do Ribatejo (Portugal), where they had been sent to repair. Pallet wood from the Portuguese pallet circuit was deliberately sampled in order to increase the risk of detecting potential PWN re-infestation of the pallet wood, as the nematode has been present in Portugal since 1999.

Four age classes were defined for the in-service pallets: less than six months old since manufacturing, 6–12 months, > 12–36 months and over 36 months. Pallets were divided into boards ($1000 \times 100 \times 25$ mm^3) and blocks ($160 \times 95 \times 95$ mm^3) and sent, separated by wood batches (wood type and age class), to the INIAV laboratories in Oeiras during April 2013.

Upon arrival, the timber was individually labelled and analysed for its moisture content (MC) by oven-drying wood samples of ≈ 100 g at 95 °C for 24 h and subtracting the dry weight from the initial weight and dividing by the dry weight, multiplied by 100 (Glass and Zelinka 2010).

2.1.1 Nematode sampling and identification (Experiment 1.1)

The number of replicates of boards and blocks analysed for the presence of nematodes is presented in Table 1. To maximise the detection of nematodes, two separate samples of timber were randomly selected from each replicate, peeling off manually the external outer wood layer (1–2 mm) and sawing the two sections into small wood cubes of approximately 1 cm^3, in order to obtain a combined sample of ≈ 100 g of wood per replicate. The sawing equipment was carefully disinfected (cleansed with alcohol 70% and water) between samplings to avoid contaminations.

Table 1 Number (Nb) of samples and moisture content (MC, mean ± SD) of timber from in-service boards and blocks of commercial pallets with different ages analysed for the presence of nematodes

Age (months)	Timber type	No of replicates	Initial MC (%)[1]
0–6	Board	30	30.2 ± 15.1a
	Block	26	24.7 ± 4.8ab
> 6–12	Board	30	22.7 ± 7.1b
	Block	27	25.7 ± 2.5ab
> 12–36	Board	29	19.4 ± 7.9b
	Block	30	31.4 ± 14.3a
> 36	Board	30	21.2 ± 5.8b
	Block	27	25.2 ± 6.2ab

Means within the column followed by the same letter do not differ after Kruskal–Wallis analysis of variance test, $p \leq 0.05$

Live nematodes were extracted from the wood samples using the modified tray method (Penas et al. 2002), where the wood cubes were immersed in water for 48 h. The water was then passed through a 400 mesh (38 µm) sieve and the residues washed with water into small Petri dishes.

Extracted nematodes were observed using an Olympus BX-51 bright field light microscope (Hamburg, Germany), and morphological identification was based on the main diagnostic characteristics of species from the *xylophilus* group: number of lateral incisures, shape of the spicules, presence of a vulval flap and female tail shape (Ryss et al. 2005; Braasch et al. 2009a, b; Braasch and Schönfeld 2015). Additional molecular analyses were performed to detect *B. xylophilus* when morphologically similar nematodes were present in the wood (Inácio et al. 2015). Nematodes other than *Bursaphelenchus* were classified into genus based on their morphological characteristics.

2.1.2 Fungal sampling and identification (Experiment 1.2)

Fungal isolation was made simultaneously with nematodes sampling (see Sect. 2.1.1; Table 1). Two sections of wood (fragments with 1 cm^2) were taken from each replicate and surface-sterilized by dipping for 1 min in sodium hypochlorite solution (1%) and rinsed with sterilized distilled water. Samples were further divided and plated into 9 cm diameter Petri dishes with two different media (three small subsamples/plate): malt extract agar (Difco MEA, USA) amended with streptomycin (Sigma-Aldrich, USA) (500 mg/L), and MEA added with cycloheximide (Sigma-Aldrich, USA) (500 mg/L), a semi-selective antibiotic for *Ophiostoma* species and related anamorphs (Harrington 1981).

Cultures were incubated at 25 ± 1 °C in the dark. Axenic cultures of each fungus were obtained and grouped according to their macroscopic characteristics, and submitted to DNA extraction and sequencing. The genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, California, USA) and the universal primers ITS3/ITS4 (White et al. 1990) were used to amplify a large portion of the 5.8S rDNA and the adjacent ITS2 region. The amplicons were sequenced at STABVida Sequencing Laboratory (Lisbon, Portugal). Sequence alignment and phylogenetic analyses were conducted using MEGA version 6 software (Tamura et al. 2013), using the maximum likelihood (ML) and neighbour-joining (NJ) algorithm with the Jukes-Cantor model.

2.2 Artificial inoculation of the pinewood nematode into in-service commercial pallet timber (Experiment 2)

Commercial pallets with ISPM 15 stamp were randomly selected as in the previous experiment. Four age classes were defined (less than 6 months since manufacturing, 6–12 months, > 12–36 months and over 36 months), and wood MC was determined by the oven-dry method (Glass and Zelinka 2010). Only pallets with MC above 20% were selected for the experiment, with the objective of maximizing the establishment of *B. xylophilus* in wood with higher moisture content (Sousa et al. 2011).

The pallets were divided into boards and blocks, selecting 10 replicates per treatment (timber type and age class), corresponding to 80 replicates in total. All the wood was sampled for the presence of nematodes prior to inoculations, as described in Experiment 1.

Five holes (1 cm diameter and 3–4 cm long) were obliquely drilled on each board and four holes on blocks for the inoculation of the PWN, and a suspension of *B. xylophilus*, with ≈3000 nematodes/mL, was injected with a syringe into the holes (0.5 mL on each hole), resulting in an estimate of ≥7500 *B. xylophilus* inoculated into each board and ≥6000 individuals per block. An additional group of ten boards was obtained from freshly felled healthy maritime pine trees and equally inoculated to serve as the control treatment. All inoculated timber, totalling 90 replicates, was maintained at 22 °C and 60–70% RH throughout the experiment.

Nematode extraction was done as previously described. The number of nematodes was determined by placing a 1 mL aliquot inside a counting slide (Chalex Corporation, Grasonville, MD, USA) and nematodes were counted under the microscope. To estimate the total number of PWN in the wood, this counting was multiplied by the volume of each extracted suspension, considering a constant weight (100 g) of wood samples. Nematode sampling was done prior to inoculation and subsequently at days 7, 14, 28, 56 and 84, with a total of 540 samples analysed. The moisture content of the timber was also determined on each occasion.

2.3 Statistical analysis

A nonparametric Kruskal–Wallis analysis of variance test was used to compare moisture content (MC) of timber from boards and blocks of commercial pallets with different ages. A parametric analysis of variance test (ANOVA) was used to compare MC between boards and blocks, timber with different ages, nematode and fungi presence in regard to timber type and age, and mean number of *B. xylophilus* in timber with and without other nematode species. The Fisher Least Significant Difference test (LSD) was used to compare means within each significant factor in the ANOVA.

3 Results

3.1 Nematode sampling and identification (Experiment 1.1)

A total of 229 timber samples were analysed to assess the presence of nematodes, including boards and blocks (Table 1).

Nematodes were absent from timber with up to 6 months following manufacturing. In older timber (age class over 6 months), nematodes were found and, in general, their frequency increased with age (Fig. 1).

Live nematodes were found in 45% of the timber samples, with comparable frequencies between boards (49%) and blocks (42%). *Bursaphelenchus xylophilus*, or other species of the *Bursaphelenchus* genus, were never found. Detected nematodes included members of the families *Aphelenchoididae, Diplogastridae, Rhabditidae* and *Tylenchidae*. Populations were generally abundant, sometimes reaching hundreds of individuals per sample and with the presence of eggs and juveniles, indicating breeding populations were present in the timber. The genus with the highest frequency was *Rhabditis* (found in 37% of the timber samples), followed by *Parasitorhabditis* (32%), *Aphelenchoides* (13%), *Laimaphelenchus* (8%), *Diplogaster* (2%), *Tylenchus* (2%) and *Aphelenchus* (1%). These genera are mostly fungal feeders and saprophagous. Specimens of the predacious genus *Mononchus* were also observed but in very low numbers (< 2 nematodes/ 100 g wood sample in 1% of the sampled wood).

The moisture content of the wood did not influence nematode presence ($F = 0.053$; $df = 1$; $p < 0.819$). In general, blocks had significantly higher moisture content (MC) than boards ($F = 7.429$; $df = 1$; $p < 0.001$; Table 1), although mean values were below 30% for both timber types (26.9% ± 8.8 for blocks and 23.4% ± 10.4 for boards). Moisture content was similar for timber with different ages ($F = 2.271$; $df = 3$; $p < 0.001$).

Other organisms such as protozoans were abundant in the timber samples, although they were not quantified nor identified.

3.2 Fungal sampling and identification (Experiment 1.2)

Fungi were ubiquitous and very abundant in the timber, regardless of type or age (Fig. 2).

Only sporulating fungi were obtained, and mould fungi of the genus *Trichoderma* were prevallent, being found in 97% of the timber samples. The ITS2 noncoding region was successfully amplified from the representative isolates, and the PCR amplification generated only one fragment of approximately 0.36 kb. The sequences showed a similarity of 99% with the sequences available at NCBI, allowing the identification of two species, *Trichoderma atroviride* (the most abundant) and *Trichoderma longibrachiatum*.

Species of *Penicillium* were present in 12% of the samples and were more frequent in boards and in younger wood with higher MC ($F = 4.6258$; $df = 1$; $p < 0.033$). Members of the order *Mucorales* (genus *Mucor*) were detected in 33% of the boards and blocks, regardless of their ages or MC ($F = 0.4540$; $df = 1$; $p < 0.5012$).

Fungi of the genus *Ophiostoma*, or related blue-stain fungi, were not isolated from any of the samples.

3.3 Artificial inoculation of pinewood nematode into in-service commercial pallet timber (Experiment 2)

The pinewood nematode was absent from the in-service timber prior to inoculation (Table 2), although other nematodes

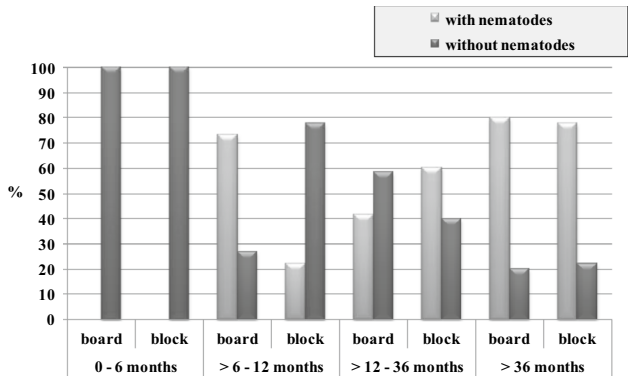

Fig. 1 Frequency of nematode infestation (in %) on boards and blocks of commercial pallets with different ages

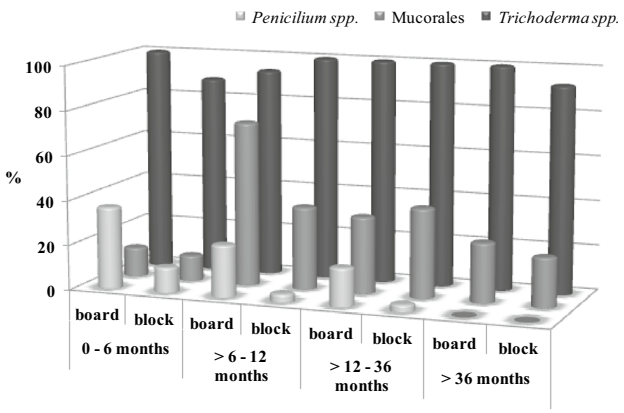

Fig. 2 Frequency of fungi isolation (in %) on boards and blocks of commercial pallets with different ages

Table 2 Infestation rate (in %) and number of *B. xylophilus* (mean ± SD) recovered from in-service boards and blocks from commercial pallets and from fresh boards inoculated with the PWN

Age	Timber	Sampling (days)											
		0		7		14		28		56		84	
		PWN infested (%)	PWN/g	PWN infested (%)	PWN/g	PWN infested (%)	PWN/g	PWN infested (%)	PWN/g	PWN infested (%)	PWN/g	PWN infested (%)	PWN/g
0–6	Board	0	–	40	0.11 ± 0.21	0	–	0	–	0	–	0	–
	Block	0	–	20	0.12 ± 0.17	20	0.18 ± 0.24	10	0.01 ± 0.01	0	–	0	–
> 6–12	Board	0	–	50	0.02 ± 0.02	10	0.01 ± 0.01	0	–	0	–	0	–
	Block	0	–	30	0.04 ± 0.12	30	0.13 ± 0.17	0	–	0	–	0	–
> 12–36	Board	0	–	30	0.02 ± 0.04	30	0.02 ± 0.02	0	–	0	–	0	–
	Block	0	–	30	0.15 ± 0.23	30	0.11 ± 0.09	0	–	0	–	0	–
> 36	Board	0	–	10	0.01 ± 0.02	0	–	0	–	0	–	0	–
	Block	0	–	40	0.17 ± 0.32	40	0.07 ± 0.06	0	–	0	–	0	–
Control	Board	0	–	100	76.3 ± 14.6	100	82.2 ± 24.4	100	94.2 ± 34.6	100	130.3 ± 37.2	100	191.8 ± 44.6

N = 10 replicates for each treatment (boards and blocks of different ages)

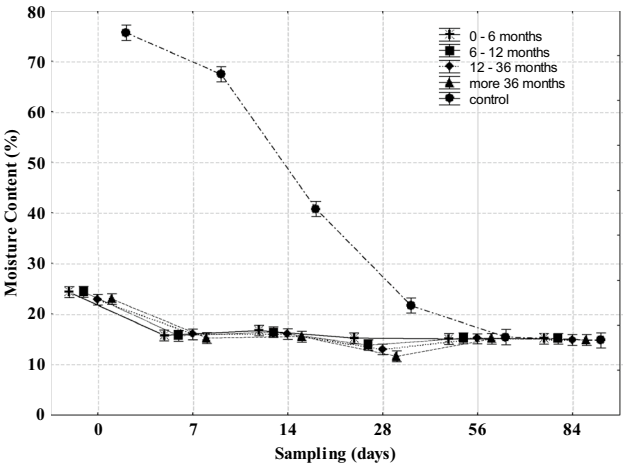

Fig. 3 Changes in moisture content (mean ± SE) of in-service timber from commercial pallets with different ages and from fresh control timber inoculated with *B. xylophilus*

(the same genera found in the previous experiment) were detected in 36% of the replicates.

Bursaphelenchus xylophilus was detected in timber of all ages in the first sampling (at day 7) after the artificial inoculation, although at very low frequencies of a few dozen individuals maximum. Nematodes were found with significantly higher abundance in blocks than in boards ($F = 4.366$; $df = 1$; $p < 0.040$).

Infestation rates and mean nematode numbers generally decreased in the subsequent samplings at days 14 and 28, and no *B. xylophilus* were recovered in the last two observations at days 56 and 84 (Table 2). No eggs nor juveniles of *B. xylophilus* were detected throughout the experiment, suggesting that reproduction was rare or inexistent.

Bursaphelenchus xylophilus was more frequent in timber free of other nematodes, with 67% of the *xylophilus*-positive samples found in timber without other nematodes. The mean number of *B. xylophilus* was also slightly higher in nematode-free timber, although the differences were not statistically significant ($F = 2.1014$; $df = 1$; $p < 0.152$). Nonetheless, the general pattern of collapse and complete disappearance of *B. xylophilus* populations from the wood was similar for timber with and without other nematode species.

The *B. xylophilus* inoculated into the fresh pine boards (control treatment) showed a different pattern, with the PWN successfully colonising and establishing breeding populations with a clear tendency to increase populations over time (Table 2).

Moisture content of the in-service timber was 24 ± 2% at the inoculation time, and rapidly decreased and stabilized to values around 15% in the subsequent samplings (Fig. 3). There were no differences in MC of blocks and boards ($F = 0.182$; $df = 1$; $p < 0.005$) or of different age-timber ($F = 2.344$; $df = 3$; $p < 0.072$). Moisture content of the

control samples (fresh timber) was initially above 70% but also decreased to values around 15 ± 1% in the last sampling dates (Fig. 3).

4 Discussion

The pinewood nematode *B. xylophilus* was not found in commercial pallets of various age (Experiment 1), which probably reflects previous HT sterilization according to ISPM 15 standards. In fact, the complete absence of nematodes (of any species) from the recently treated timber (with less than 6 months) suggests that heat treatment is effective in eliminating nematodes from the wood, with low moisture content and presence of competitive saprophitic organisms possibly preventing subsequent re-infestation.

Bursaphelenchus xylophilus did not persist in the wood of commercial pallets when artificially inoculated in large numbers (Experiment 2), and the inoculated population could not be detected within 4 weeks and did not reproduce. The failure to establish viable populations may result from the absence of favourable food resources (blue-stain fungi) in the timber, and/or by the wood's low moisture content which was initially between 20 and 30% but rapidly decreased to around 15–16%, near the expected equilibrium moisture content (Simpson 1998). Moisture content is one of the most important parameters regulating *B. xylophilus* populations, with high (Halik and Bergdahl 1990) and low values (Hisai et al. 2006; Sousa et al. 2011a, b, b; Hopf-Biziks et al. 2017) conditioning the establishment and development of the nematode's populations. In contrast, the PWN successfully colonised the fresh pine boards when artificially inoculated, which can be explained by their higher initial MC, which supports the nematode's establishment. Even in the fresh-timber, the MC decreased to values around the equilibrium moisture content, but the already established nematodes were able to subsist in this dried wood for some weeks, as already described by Sousa et al. (2011a, b).

The presence of other nematode species in the timber appeared to negatively affect the establishment and development of *B. xylophilus*, although the populations rapidly collapsed and prevented further analysis. It is possible that timber with other nematodes was more decayed and/or had lower availability of adequate fungal food resources than nematode-free timber, thus hampering the establishment of the newly arrived *B. xylophilus*, although further studies are required to elucidate this.

Fungi were abundant in the commercial pallets, and even in timber treated in the previous six months, suggesting that heat treatment either did not eliminate all fungi from the wood [as conventional HT may not guarantee 100% mortality of the specifically targeted fungi (Ramsfield et al. 2010)], or/and that fungi were able to rapidly colonise the treated timber, as suggested by Uzunovic et al. (2008). Nevertheless, pathogenic species of known phytosanitary risk were absent in the current survey, and the fungal communities were dominated by mould genera ubiquitous on plant and wood materials, namely oligotrophic species adapted to grow in environments with low nutrient levels. Some species of *Trichoderma* can be found on decaying wood (Gams and Bissett 1998) and wood chips (Halik and Bergdahl 1990; Hopf-Biziks et al. 2017). The most abundant fungi in the present sampling, *T. atroviridae*, is a highly competitive organism that can be used as a biocontrol agent against wood decay fungi (Schubert et al. 2008). Members of the order *Mucorales* and species of *Penicillium* are cosmopolitan fungi found on almost all substrata and generally considered saprobes associated with decaying materials without causing structural damage to wood (Kubátová 2000; Seifert and Frisvad 2000; Webster and Weber 2007). Various species of *Penicillium* are xerophilic and able to survive in dry environments, such as the desiccated timber of pallets (Webster and Weber 2007), and this genus has been found in wood packaging material displaying the ISPM 15 stamp (Zahid et al. 2008).

The prevailing fungal community found in the pallets, dominated by species of *Trichoderma*, was not suitable for the survival and development of *B. xylophilus* or other species of the *Bursaphelenchus* genus, and may explain their absence from the samplings. In fact, many species of *Trichoderma* and *Penicillium* are considered neutral or unsuitable for PWN development (Kobayashi et al. 1974, 1975; Fukushige 1991; Maehara and Futai 2000, 2008; Maehara et al. 2005, 2006; Sriwati et al. 2007), with some *Trichoderma* thought to be control agents of the PWN (Maehara 2008; Yang et al. 2010, 2012). Hopf-Biziks et al. (2017) found the PWN could not survive when reared on *T. atroviridae*, while Davies and Spiegel (2011) suggested a nematicidal activity by this fungus. The ability of *Trichoderma* spp. to be competitive inhibitors, produce antibiotics and behave as mycoparasites has been recognised for decades (e.g., Weindling 1932; Dennis and Webster 1971; Cutler et al. 1986; Ghisalberti and Sivasithamparam 1991; Harman 2000; Howell 2003; Harman et al. 2004), and is also indirectly supported by the results of the present study.

Fungi of the genera *Ceratocystis, Ophiostoma, Pestalotiopsis* and *Botrytis cinerea* are suitable food sources for the PWN (Fukushige 1991; Maehara and Futai 1996, 2000; Sriwati et al. 2007) but were absent from the surveyed timber. Failure to detect *Ophiostoma* and related blue-stain fungi may be explained by their preference for wood with higher moisture content and greener condition (Gibbs 1993), by loss of viability of fungal propagules at humidity below 95% (Dowding 1969) and temperatures above 30 °C (Payne et al. 1998), and/or by competition with *Trichoderma* spp., which interfere antagonistically by competing for nutrients and by

using mechanisms of antibiosis and mycoparasitism (Rossman 1996; Jaklitsch 2009).

Pallet timber was colonised by a community of opportunistic saprophytes and fungal feeder nematode species with low ecological requirements that became more abundant and diverse in older pallets. Nematodes are regularly found in wood packaging materials, and include fungal feeders, saprobic and predatory species (Tomiczek et al. 2003; Gu et al. 2006). Zahid et al. (2008) found *Aphelenchus* spp. nematodes while sampling ISPM 15-treated wood packaging material, whereas Meissner et al. (2014) found live fungivorous nematodes of the genera *Aphelenchoides, Aphelenchus* and *Filenchus* in a similar sampling, although with much lower infestation rates (3% against 45% in the present study). These authors sampled older (more than 5 year-old) and drier timber that had been stored and isolated for several years, while the present sample was conducted on timber recovered from the international pallet trade, where it had recently been in contact with other WPM and varied substrata (fruit, soil, plants, etc) that could have served as multiple sources for nematode infestation.

5 Conclusion

Commercial pallets of variable ages with ISPM 15 stamp harboured a diversified community of organisms, including fungi, protozoan and nematodes, dominated by non-quarantine/non-pathogenic species. The pathogenic *B. xylophilus* was not detected in wood of commercial pallets and was unable to establish viable populations when artificially inoculated into boards and blocks. The present studies suggest a low likelihood of KD/HT-treated wood being infested with *B. xylophilus* through the international pallet trade, due to the PWN's requirement of wetter wood for colonisation and establishment of breeding populations, and the inadequacy of available food resources in the treated timber (dominance of unsuitable *Trichoderma* and related species, and lack of suitable blue stain fungi). With this information, the wood packaging industry can quantify the risks associated with old pallets being infested with *B. xylophilus* that could eventually represent a threat to new pallets in circulation. Pallets are frequently backhauled, repaired and reused over extended periods of time to increase their longevity and reduce costs (Bengtsson and Logie 2015). PWN-infested components could circulate throughout the world for extended periods of time and be in contact with ISPM-15's treated timber components, although the present results suggest that contamination is unlikely.

Moisture content is a key factor in the risk associated with old pallets in circulation, and future research should evaluate the capacity of HT and KD timber to re-absorb and hold moisture when exposed for extended periods to rain or submersed in water, and the ability of this rewetted material to support viable populations of the PWN and mycoflora which support its development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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