



# Euphresco

## Final Report

For more information and guidance on completion and submission of the report contact the Euphresco Call Secretariat ([bgiovani@euphresco.net](mailto:bgiovani@euphresco.net)).

Project Title (Acronym)
Focusing on <i>Monochamus</i> spp., insect vectors of <i>Bursaphelenchus xylophilus</i> (MONOCHAMUS)

**Project Duration:**

Start date:	01/11/13
End date:	31/10/16



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## 2 Executive Summary

### 2.1 Project Summary

#### **MONOCHAMUS – vectors of pine wood nematodes (PWN) causing pine wood death (PWD)**

The main objectives of the project were:

- To optimize the monitoring methods using attractant traps.
- To map the incidence for PWN and possible vectors
- To evaluate phenology and biology of relevant vectors and their PWN relationship
- To develop molecular identification tests for *Monochamus* spp.

Participating research institutions included 7 research organisations from 5 countries; in four of these countries, the vectors were known to be present, in one country the PWN was known to be present and in one country no vector nor the nematode was known to be established.

#### **Suggesting a trans-national monitoring strategy: Optimal monitoring trap type, lure, locality, hight, servicing interval, wet/dry, vector or PWN monitoring**

In Slovenian experiments organized between 2007 and 2010 *Monochamus galloprovincialis* had showed a clear preference for the lure Gallowit®. In 2011–2012 Galloprotect 2D® attracted significantly more cerambycids than ethanol+α-pinene, and *M. galloprovincialis* was by far the most numerous species. However, results during this project 2014-16 from Belgium, The Netherlands and Denmark showed Galloprotect Pack to be the most efficient lure agent. Recommended trap types are: Crosstrap® from Econex®, Multifunnel (Lindgren funnel®) from Contech®, BC as they are solid state traps.

The consortium recommends monitoring at a combination of trade-related risk locations and pine stands in old or natural reserves. Regarding monitoring the risk locations – e.g. import harbours - adjacent pine stands could be a more favourable place to set up the traps than putting them direct on the risk site itself.

We recommend putting traps at a hight of about 2 m as this could reduce some of the non-target catches of bark beetle predators (*Rhizophagus* spp.) and makes serviceing easier.

Recommended trapping period is when you expect the vectors to have their flight activity. For Central- and Northern Europe that would be mid-June until end-August. Service intervals for the traps could be reduced if conserving agent – mono-ethylene glycol (MEG) - is used in the traps. This however has some implications for identification of possible *Bursaphelenchus* species inside the *Monochamus* specimens. This is because morphological characters useful for identification wanish in MEG.

#### **Reducing non-target catches (predators/saproxylic insects).**

It is recommended to try to improve a bit the design of the traps used at present.

Escape possibilities for the smaller non-target beetles should be possible. On the other hand, this should not be an obstacle to the present monitoring method, since it could be argued that the practical effect on the predator population could be considered negligible

### **PWD – visibility of infections**

In northern Europe, PWN may be transmitted to healthy, susceptible trees but the infestation will not necessarily lead to PWD and tree death. As susceptible tree species are readily available in northern Europe, this will not be a limiting factor to the spread of the disease. Development of the disease seems to require high temperatures, so this is unlikely to occur under the climate conditions found at present in northern European countries.

Once a tree has become infested, the nematode population may persist for several years. If the trees experience significant stress in the future due to water stress, pollution or presence of other pathogens, and a moderate temperature increase occurs, PWD may be able to develop in certain locations.

### **Molecular identification of *Monochamus* spp.**

Molecular-based identification is of great importance when the sample is not suitable for morphology (such as larvae or damaged specimens). European *Monochamus* species are supposed to be phylogenetically related, the most ancestral seems to be *M. saltuarius*. Sequence obtained from *M. grandis* (GenBank) groups closely with European *Monochamus* species, thus suggesting close relationship with *M. sutor*, *M. urusovi*, *M. galloprovincialis* and *M. sartor*. One gene region, part of cytochrome oxidase I gene (COI), was successfully sequenced in almost all analyzed specimens. The other gene region - 28S ribosomal rRNA gene - was successfully sequenced only for five specimens of *M. galloprovincialis*. In the GenBank, only three sequences were available from *Monochamus* genus. Phylogenetic comparison of Slovenian specimens revealed groupings with *M. galloprovincialis* (KC692744). Identifications of collected *Monochamus* specimens based on selected molecular markers (COI and 28S rDNA) gave evidence for the presence of *Monochamus* species in Slovenia/Europe.

### **Incidence mapping – important tool for focus on internal trade.**

PWN has not been discovered in any new country during the project. *Monochamus galloprovincialis* was discovered as established in Denmark for the first time and as result of the efforts in developing a monitoring system. A complete European incidence map for *Monochamus* still has to be drawn.

## 3 Report

### **3.1 Objectives and tasks of the project**

The goal of the MONOCHAMUS project was to optimize monitoring strategy and improve the background for pest management. In short, the objectives of the MONOCHAMUS project were:

- To optimize the monitoring methods using attractant traps and other methods
- To map the incidence of PWN and possible vectors,
- To evaluate the phenology of relevant vectors and their PWN relationship,
- To develop molecular identification tests based on molecular markers of selected specimens of *Monochamus*.



### **3.2 Materials & methods as well as results and discussion will be presented in the following sections per deliverable and WP/task**

#### **WP1: Monitoring methods and strategy**

Any insect that is able to transfer nematodes, particularly members of the genus *Bursaphelenchus*, can be considered a potential vector of the PWN. However, experience until now shows that longhorn beetles of the genus *Monochamus* are 100-1000 times more efficient vectors than other insects living on pine in transmitting PWN (Hugh Evans, pers. comm.) The efficacy of attractants available had shown to be very different depending on where monitoring occurs (Jurc *et al.* 2013a; Misser 2013). In co-operation with the national plant health authorities, the consortium partners (UC, BF-Forestry, SFI, ILVO, NVWA, INIAV) collaborated to develop the best possible survey program using attractants, visual inspections and other suitable methods.

#### **Task 1.1. Comparison of results of the previous monitoring methods (Methods 1-6 of *Monochamus* spp. used in Slovenia in the 2007-2015 period) (Slovenia)**

The PWN has not been recorded in Slovenia. However, the introduction of the species might present a large threat to Slovenian pine forests.

The Department of Forestry and Renewable Forest Resources at the Biotechnical Faculty (University of Ljubljana) had been monitoring sawyer beetles in conifer forest stands with cross vane funnel traps and different attractants since 2007. Until 2015, sample collection was carried out at 19 locations with *Pinus halepensis*, *Pinus nigra*, *Pinus sylvestris*, *Picea abies* and *Abies alba* as the dominant tree species. The traps were baited with a combination of primary attractants (ethanol +  $\alpha$ -pinene), attractants with kairomonal components (Pheroprax® and Gallowit®) and three different dispensers which include the aggregation pheromone of the pine sawyer beetle (*Monochamus galloprovincialis*), 2-undecyloxy-1-ethanol (Galloprotect 2D®, Gallohost® and Galloprotect Pack®). The total catch of stag beetles on 16 locations was 1328 specimens belonging to four species: *Monochamus galloprovincialis* (655 specimens), *Monochamus saltuarius* (29 specimens), *Monochamus sartor* (345 specimens) and *Monochamus sutor* (265 specimens). The average annual catch per trap was 5.85 specimens. An above average catch was recorded in the locations of Trnovo (22.41 specimens), Dekani (9.38 specimens), Snežnik (6.54 specimens) and Brdo pri Kranju (5.93 specimens). Dispensers containing 2 undecyloxy-1-ethanol (Galloprotect 2D®, Galloprotect Pack® in Gallohost®) proved to be more effective (average annual catch per trap 14.47, 13.50 and 8.00 specimens) than the other tested attractants.

The catch was also affected by the position of the trap, forest management practices and weather conditions. On the Brdo pri Kranju location, the number of captured *M. galloprovincialis* significantly increased in 2015, one year after a catastrophic ice storm. More frequent sampling on the same location also showed that individual

sawyer beetles were captured in the traps as late as the first half of October, if the weather conditions were favourable. On several locations, detailed identification of the additional, non-target catch was carried out. On the Brdo, Dekani and Kastelec locations, the additional catch from 45 Coleoptera families was established between 2007 and 2012. The additional catch 2 of predatory beetles from the *Cleridae* family, especially *Thanasimus formicarius* and *Thanasimus femoralis*, is unwanted and may significantly exceed the catch of the target *Monochamus* species. (Pavlin *et al.*, 2016).

## **Task 1.2 Case study: comparing six monitoring methods previously used in Slovenia, in a new *Pinus* stand during 2015**

### **Comparison of methods 1-3.**

Target and non-target beetles in semiochemical-baited cross vane funnel traps used in monitoring *Bursaphelenchus xylophilus* (PWN) vectors in pine stands. (Slovenia)

In 2007–2012 scientists assessed dendrobiotic insects at three locations in stands of *Pinus nigra*, *Pinus halepensis* and *Pinus sylvestris* in Slovenia. The samples were collected from May to November using four (three in 2007) cross vane funnel traps per location with wet collecting cups and attractants. In 2007 we used ethanol+ $\alpha$ -pinene, and from 2008 to 2010, ethanol+ $\alpha$ -pinene, Pheroprax® (ipsdienol, cis-verbenol, 2- methylbut-3-en-2-ol) and Gallowit® (ipsdienol CAS 14434-41-4, ipsenol CAS 60894-96-4, DMWK CAS 115-18-4, cis-verbenol CAS 18881-04-4,  $\alpha$ -pinene CAS 80-56-8, ethanol CAS 64-17-5) were used with traps 1.5m above the ground. In 2011–2012 ethanol+ $\alpha$ -pinene and Galloprotect 2D® (Galloprotect F: an aggregation pheromone [2-undecyloxy-1-ethanol] and Galloprotect A: kairomonal substances [ipsenol and 2- methyl-3-buten-1-ol]) were used with traps in the lower canopy. 31,228 individuals from 45 beetle families were collected. Curculinidae (Scolytinae, 23,325) were the most numerous, and the target family Cerambycidae was represented with 1945 specimens from 28 taxa and 25 species. In 2007, *Spondylis buprestoides* was by far the most abundant species. In 2008–2010 ethanol+  $\alpha$ -pinene more effectively attracted *S. buprestoides* and *A. rusticus*, whereas *Monochamus galloprovincialis* showed a clear preference for Gallowit®. In 2011–2012 Galloprotect 2D® attracted significantly more cerambycids than ethanol+ $\alpha$ -pinene, and *M. galloprovincialis* was by far the most numerous species. In addition to cerambycids, other saproxylic beetle species and also scolytine predators (mostly Cleridae, Histeridae, Trogossitidae) were found in the traps, highlighting the need to consider the potential negative impacts of the long-term monitoring of PWN vectors on species with important functional trophic traits in forests. (Jurc & Meterc, 2013; Jurc, 2014; Jurc *et al.*, 2016)

### **Methods 4-5.**

In the year 2014, scientists in Slovenia tested the impact of two attractants, Galloprotect 2D® and Galloprotect Pack®, on the catch of *Monochamus* species. In



the locations Snežnik and Trnovo (Slovenia) they chose plots with a radius of 70 meters, in forest stands of Norway spruce (*Picea abies*) and silver fir (*Abies alba*) and with three levels of cutting (100%, 50% and 0%). Cross vane funnel traps (Witasek) with wet collecting cups were placed in the middle of each plot. In total, 276 specimens of sawyer beetles were collected. Three different species were identified: *Monochamus sartor* (127 specimens), *M. sutor* (104 specimens) and *M. galloprovincialis* (45 specimens). All three species were 3 times more frequent on the plots with Norway spruce. On two plots with Norway spruce, the catch on Galloprotect Pack® was significantly higher. However, on one plot with silver fir, significantly high number of catches were obtained with Galloprotect 2D®. The highest number of catches was established on plots with 100% cutting. The additional catch of predatory coleopteran species from Cleridae and Troggositidae families was also detected (Pavlin *et al.* 2015).

### Method 6.

In 2015, scientists in Slovenia tested a new type of trap for monitoring of *Monochamus* spp. We set the Crosstrap® traps (Econex, Spain) at two locations, Rožnik in Ljubljana and Domžale. Crosstrap® traps were used, coated with Teflon which allows easier catch of target species and are equipped with special collecting containers that allow catches of live *Monochamus* beetles (dry-catch). Live beetles collected with Crosstrap traps were more suitable for the molecular analysis.

At both locations they set 4 traps, two equipped with Galloprotect Pack®, one with Galloprotect 2D® and one with a combination of ethanol and  $\alpha$ -pinene. Traps were monthly re-filled with fresh attractants. Traps were placed from mid-June 2015 until mid November 2015, and the catches were collected 2–3 times per week. Morphological identification of the collected specimens was done in the Laboratory for ecological research BF-Forestry (Partner 2). After morphological identification, all caught *Monochamus* beetles were stored in 99% ethanol and delivered to Laboratory of forest protection SFI (Partner 3), to perform molecular analysis.

Altogether, 26 beetles from *Monochamus* genus were caught. Morphological identifications revealed that all belong to the species of *M. galloprovincialis*. Most of those beetles (22) were caught in traps with Galloprotect Pack, 4 beetles were found in traps with Galloprotect 2D, while no target beetle was caught in traps with ethanol+ $\alpha$ -pinene.

The main purpose to use dry-catch type of traps was achieved, as all caught target beetles were found alive. Successful molecular identification further confirmed the adequacy of the traps and the above-described method. The biggest disadvantage of method 6 is the need for regular monitoring and emptying of the traps. On the other hand, the advantage is that most of the specimens are still alive when the catches are collected from the trap. Therefore, all non-target organisms (especially beetle families Cleridae and Monotomidae, important natural enemies of bark beetles, were caught in high numbers) can be released back in the nature.

The use of Crosstrap® traps was in 2016 already introduced in two Survey programmes (non-European *Monochamus* spp., PWN), under the authority of the Administration for food safety, veterinary and plant protection.

### Task 1.3 Evaluating new monitoring methods and strategies (Belgium and the Netherlands)

#### Belgium – monitoring traps:

##### Materials and methods

In collaboration with Dutch colleagues of the Nederlandse Voedsel- en Warenautoriteit (NVWA), a small scale trial comparing two trapping systems for cerambycid beetles was performed. Method A consisted of a Teflon-coated cross-vane trap (Crosstrap® (Econex, Murcia, Spain)), combined with the lure package Galloprotect Pack® (SEDQ S, Barcelona, Spain). Method B consisted of a panel trap (WitaPrall IntPT-Wet Trap® (Witasek, Feldkirchen in Kärnten, Austria)), and the lure package Galloprotect 2D® (SEDQ S, Barcelona, Spain). Both lure packages are commercially available from SEDQ for *Monochamus* spp. trapping and contain 2-undecyloxy-1-ethanol, ipsenol and 2-methyl-3-buten-1-ol components. The Galloprotect Pack® contains two additional dispensers, which emit a kairomonal attractant ( $\alpha$ -pinene). Both trapping systems were deployed about 100 m apart in similar pine structures in the pine forests Withoefseheide (Kalmthout), Elsakker (Hoogstraten), Eindepoel (Wortel), Kolisbos (Neerpelt) and Kloosterbos (Wachtebeke). This study was performed from May 2014 to 0September 2014, the contents of the traps were collected every 3-4 weeks and the lures were renewed every 7 weeks.

##### Results

The Cerambycidae captured in the traps are shown in Table 1.

**Table 1. Number of adult Cerambycidae caught with Method A (cross-vane Crosstraps and Galloprotect Pack) or Method B (intercept trap and Galloprotect2D) traps at five locations between 21/05/2014 and 03/09/2014.**

Community	Pine forest	Trap type	Cerambycid spp.	Date			
				17/06	08/07	06/08	03/09
Hoogstraten	Eindepoel	Type A	<i>Arhopalus rusticus</i>	0	3	13	1
			<i>Spondylis buprestoides</i>	5	44	43	9
			<i>Strangalia maculata</i>	0	0	0	1
			<i>Leptura rubra</i>	0	0	1	0
			<i>Rhagium inquisitor</i>	9	0	0	0
			<i>Clytus arietis</i>	1	0	0	0
		Type B	<i>Rhagium inquisitor</i>	4	0	0	0
			<i>Pyrrhidium sanguineum</i>	1	0	0	0
			<i>Rhagium bifasciata</i>	1	0	0	0
Hoogstraten	Elsakker	Type A	<i>Arhopalus rusticus</i>	0	2	4	1
			<i>Spondylis buprestoides</i>	9	23	21	12



Kalmthout	Withoefseheide	Type B	<i>Rhagium inquisitor</i>	4	0	0	0
			<i>Spondylis buprestoides</i>	1	0	1	1
			<i>Rhagium inquisitor</i>	6	0	0	0
		Type A	<i>Arhopalus rusticus</i>	0	0	7	0
			<i>Spondylis buprestoides</i>	0	0	1	0
			<i>Rhagium inquisitor</i>	10	0	0	0
		Type B	<i>Rhagium inquisitor</i>	9	0	0	0
			Type A	<i>Arhopalus rusticus</i>	0	2	3
		<i>Spondylis buprestoides</i>		66	30	74	39
		<i>Leptura rubra</i>		1	0	0	0
Neerpelt	Kolisbos	Type B	<i>Rhagium inquisitor</i>	2	0	0	0
			<i>Rhagium inquisitor</i>	9	1	0	0
		Type A	<i>Arhopalus rusticus</i>	0	1	3	0
			<i>Leptura rubra</i>	0	1	0	0
		Type B	<i>Rhagium inquisitor</i>	19	0	0	0
			<i>Strangalia maculata</i>	0	0	1	0

With the exception of Kloosterbos and Withoefseheide, where the trap types had a similar Cerambycid-trapping efficiency, the results of the other 3 pine stands clearly illustrate the higher efficiency of trapping cerambycids with trap Method A compared to Method B.

### Discussion

The relatively higher numbers of Cerambycids captured in the Method A traps tend to support our choice for using this trapping system (i.e. Crosstraps and Galloprotect Pack lures). Although no *Monochamus* spp. were captured in any trap, we refer to the Dutch field experiments evaluating both same trapping types.

### The Netherlands

The Netherlands conducted surveys for *Monochamus* species since 2010 (Heijerman *et al.*, 2011ab, 2013; NVWA 2010, 2011, 2012, 2013). During the project period from 2014 until 2016 they followed up their previous surveys (2010 – 2013) with an integrated evaluation of the survey strategy and results, including description of survey methodology, overall survey results in relation to methodological (survey grid, traps and lures, etc.) and biological aspects and risk factors and consequences for potential PWN introduction and detection. Two types of surveys were conducted:

1. Monitoring natural stands of pine for the presence of *Monochamus* species across the Netherlands; this monitoring programme largely had an experimental component, i.e. directing researching questions of concern such as the use of different trapping methods and monitoring the Dutch – Belgian border (2014), monitoring for the presence of *Monochamus* spp. in stands of different pine species (2015) and the presence of *Monochamus* species in natural stands and surrounding ports of entry (2016).
2. Monitoring ports of entry (an annual selection of 30 import and distribution sites of stones firewood and wasted wood) with wood and or wood packaging material



(WPM) originating from various countries in the world, largely China) for the presence of longhorn beetles, i.c. *Monochamus* spp.

### Materials and methods

During the surveys in the Netherlands the NVWA used and compared 2 types of flight interception traps: WitaPrall IntPT-Wet Trap® (Witasek, Feldkirchen in Kärnten, Austria) (WBB hereafter) and Crosstrap® (Econex, Murcia, Spain), a teflon-coated cross-vane trap (CV hereafter) (see figure 1).



Figure 1 - Trapping types used during the surveys 2015 and 2016 in the Netherlands. WitaPrall Bark Beetle Trap (left), Econex Cross-vane Trap (right) (© Th. Heijerman, (Heijerman & Noordijk, 2016))

In all surveys and traps a mixture of alcohol (ethanol), water, glycerine and acetic acid was used in the ratio of 4:3:2:1 (so-called EWGA mix). Except for a preservative effect this mixture has an attractant effect on wood-boring beetles as well.

The monitoring period was chosen as expected for native species of longhorn beetles in natural stands (from end of July until end of September) and at ports of entry (from half of June until end of September). The trapping liquid EWGA was collected and replaced at every collection date: in natural stands every 2 weeks, at ports of entry every 3 weeks.

As lures the Galloprotect 2D® (without  $\alpha$ -pinene) and Galloprotect Pack® (Galloprotect 2D incl.  $\alpha$ -pinene) were tested. Both lure packages were bought from SEDQ S (Barcelona, Spain) for trapping *Monochamus* spp. and contain 2-undecyloxy-1-ethanol, 2-methyl-3-buten-1-ol and ipsenol as components. The Galloprotect Pack® contains two additional dispensers which emit a kairomonal attractant ( $\alpha$ -pinene).



Figure 2 - Trapping types used during the experiment in the Netherlands. WitaPrall Bark Beetle Trap (left), Econex Cross-vane Trap (right) (© Th. Heijerman, (Heijerman & Noordijk, 2016))

## Results

In 2014 research was conducted in the pine forest at Bergen-Schoorl where a natural population *Monochamus galloprovincialis* occurs. Two types of flight interception traps combined with different types of lures (figure 1): a) WitaPrall Bark Beetle Trap (WBB-Trap) with Galloprotect 2D (no  $\alpha$ -pinene) + EWGA mix and b) Econex Cross-vane Trap combined with Galloprotect Pack (Galloprotect 2D incl.  $\alpha$ -pinene) + EWGA mix. Three pairs of both traps were placed 10-15 m apart.

In addition, in 2014 a survey was conducted in 10 natural stands of *Pinus sylvestris* near the Dutch – Belgian border. The reason for this survey was the finding of *Monochamus galloprovincialis* (Olivier, 1795) (5 individuals in 5 locations) and *M. sartor* (Fabricius, 1787) (1 individual) in Belgium, just across the Dutch border (Berkvens *et al.* 2013). At each of 6 locations 3 WBB-traps were deployed (n= 18), at 4 locations a set of 1 CV trap and 2 WBB trap were placed by (CV n=4; WBB-trap n = 8). Galloprotect Pack® (incl.  $\alpha$ -pinene) was used in CV traps Galloprotect 2D (no  $\alpha$ -pinene) was used in the WBB traps;

In 2015 6 stands of black pine (*Pinus nigra*) were monitored in the province of Noord-Holland using CV traps (n=18). At each location also 1 WBB trap was used (n=6), 5-10 m apart from the most nearby CV-trap (see figure 3) and the catches were compared.

In 2016 surveys were conducted at 2 locations: 1) pine stands in a dune forest at Bergen- Schoorl where there is a population of *Monochamus galloprovincialis* of a considerable size and 2) in Nuenen in the pine stands in the surroundings of a risk



location were imported stones with WPM are stacked and distributed and where in 2015 a single specimen of *M. galloprovincialis* was trapped. Only CV-traps were used, with Gallprotect Pack® (incl.  $\alpha$ -pinene) as lures and EWGA mix for wet catching and preservation. In Bergen we explored the area of spread of *M. galloprovincialis*, in Nuenen (NB) we monitored several *Pinus* stands for the presence of *M. galloprovincialis*.

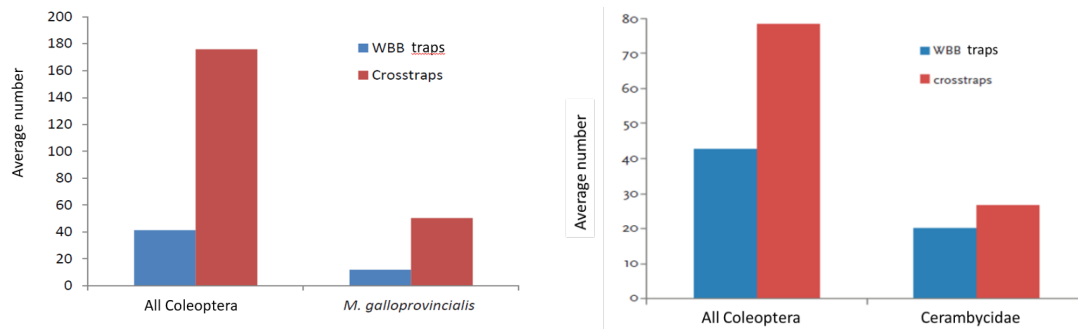


Figure 3 - Comparison of WBB traps and CV traps: in *Pinus sylvestris* stands with *Monochamus galloprovincialis* (left) and in *Pinus nigra* stands without *Monochamus galloprovincialis* (right)

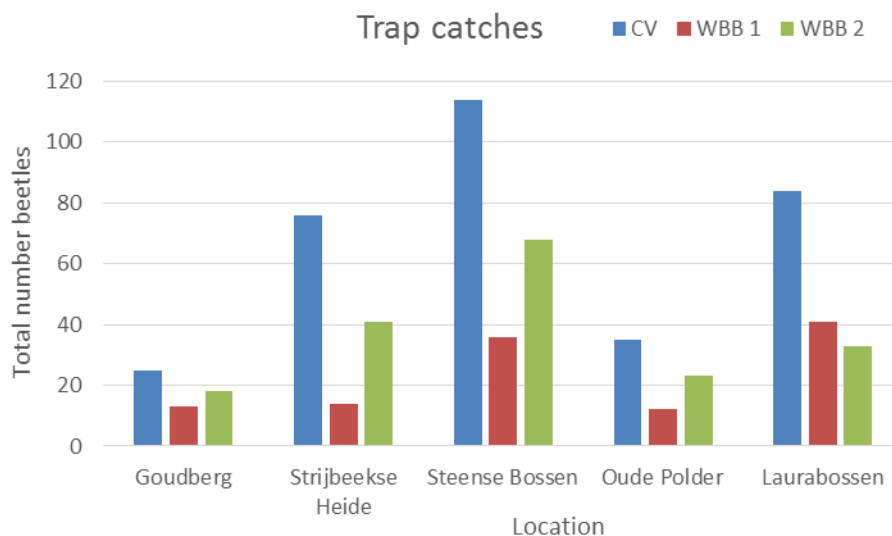


Figure 4 - Comparison of trap catches (total Coleoptera, no *Monochamus* found) using WBB traps and CV traps at 5 locations in 2014 in *Pinus sylvestris* stands near the Dutch Belgian border

The cross-vane (CV) traps captured significantly more Coleoptera, Cerambycidae and *M. galloprovincialis* than the WBB traps; in 2014 on average more than four times as much (mixed model with fall pair as random factor and fall type as fixed factor,  $F = 31.556$ ,  $p = 0.005$ ) (figure 3). The same difference can be seen when we compare all trapped beetles, here too the number of average trapped beetles per trap is significantly higher in the CV traps than in WBB traps: on average more than 4 times as much (mixed model with trap pair as random factor and fall type as a fixed factor,  $F = 55.944$ ,  $p = 0.002$ ) (figure 3). The same pattern can also be found for the Scolytinae, where at each and every instance more specimens were caught in CV trap (33 versus 15, 66 versus 11, 104 versus 11) (Heijerman *et al.* 2015).

If we compare 4 CV traps in natural stands with the WBB traps that were exposed in the same area, then there is also a clear difference too (see figure 4). It should be noted, however, that a real comparison is not possible because the 3 traps per location were more widespread in various kinds of sub-areas. Nevertheless, the CV-traps in all cases caught more beetles than each of the 2 WBB traps in the same area. This also applied to the longhorn beetles (Goudberg 9 versus 0 and 0, Strijbeekse Heide 19 versus 0 and 6, Laurabossen 19 versus 2 and 0). The number of Scolytinae showed no clear pattern: at two locations there were more individuals in the CV-traps, at two locations one of the WBB traps trapped most of the specimens and at one location a CV-trap and a WBB-trap had both the same Scolytinae (Heijerman *et al.* 2015).

In 2015 a similar pattern was found in stands of *Pinus nigra*: no specimens of *Monochamus* were found, but CV-traps captured significantly more beetle individuals than the WBB-traps; on average about 1.8 times as much (mixed model with trap pair as random factor and trap type as fixed factor,  $F = 8,104$ ,  $p = 0.036$ ). CV-traps also caught on average 1.3 times as many Cerambycidae, but the differences were not significant ( $F = 0.810$ ,  $p = 0.409$ ) (Figure 3, right). Comparison between both years is difficult because WBB traps did not have  $\alpha$ -pinene as a lure and trap catches in 2014 were highly biased because of the high numbers of *Monochamus*. (Heijerman & Noordijk, 2016).

In 2016, using CV-traps in combination with GalloprotectPack in natural *Pinus* stands, 3481 Coleoptera were trapped: 27 species of Curculionidae ( $n=1338$ , 1219 of which belonged to Scolytinae & Platypodinae) and 5 species of Cerambycidae (951, 898 of which was *Spondylus buprestoides*, 49 *Arhopalus rusticus*, 2 *Rhagium inquisitor*, 1 *Pogonocherus decorates* and 1 ♀ *Monochamus galloprovincialis*). This is the first specimen of *Monochamus* collected in a natural *Pinus* stand outside the Bergen-Schoorl area. Whether this specimen has a relation with the import risk-location nearby is yet unknown. This isolated capture of a single specimen is comparable to the single Captures of *Monochamus* species in Belgium during 3-year survey (2013-2015) (see table 1).

## Discussion

*Monochamus* species were captured in Hoogstraten (Belgium) on the border with the Netherlands. The Dutch NPPO monitored for *Monochamus* spp. throughout the Netherlands from 2009-2012, including within the border region where *Monochamus* spp. were captured. However, different attractants (no  $\alpha$ -pinene) and a different trap (WBB) type were used back then. However, an additional survey by the Dutch NPPO in the border region including old (WBB + 2D) traps and new (CV + 2D+  $\alpha$ -pinene) did not result in any captures of *Monochamus* in 2014. Using the CV trap + pheromone and  $\alpha$ -pinene did result in the capture of 1 *Monochamus galloprovincialis* at the site of import in 2015 and 1 *M. galloprovincialis* in a natural *Pinus* stand in the direct surroundings in 2016. Some of the catches (*M. galloprovincialis*) correspond to sparse, endemic populations:

**Conclusion:** Comparative research in both Belgium and the Netherlands showed that the Galloprotect Pack® combined with the Crosstrap is effective for monitoring purposes of *Monochamus* spp. However, from the comparative study it was not possible to determine whether the CV-traps worked better due to the added attractant  $\alpha$ -pinene or due to the properties of the trap. The use of  $\alpha$ -pinene (as in Galloprotect Pack®) greatly increased total beetle and cerambycid catches, adding *Monochamus* pheromone resulted in higher catches of this species and is recommended when monitoring for *Monochamus* species. It also could provide a greater attraction to other *Monochamus* species compared to traps where Galloprotect 2D is used (without  $\alpha$ -pinene). The results have been published in detail as Heijerman *et al.* (2015), Heijerman & Noordijk (2016).

## **Task 1.4 Recommendations for a trans-national monitoring strategy (Belgium)**

### ***Suggestions for a trapping network in Europe***

- Trap type

Combined use of Teflon-coated cross-vane traps (Crosstrap®, Econex, Muria, Spain) and Galloprotect Pack® (SEDQ, Barcelona, Spain) attractants appear to be the best system based on our own comparative experiments performed throughout the project and the experiences by European colleagues (e.g. Alvarez *et al.* 2014).

- Wet catching

In the course of the project, “wet catching” using mono-ethylene glycol (MEG) was successfully used throughout the 3-year field survey to trap *Monochamus* spp. Laboratory experiments showed that DNA extraction of PWN (Holterman *et al.* 2006) followed by PCR is still possible from nematodes submerged in the MEG for up to 28 days. Experiments conducted with Portuguese colleagues revealed that most PWN inside the vector *M. galloprovincialis* were rapidly killed when vector and nematode were submerged in concentrations of MEG (30 and 70%). In addition, most PWN remained in the insect, few PWN were retrieved in the MEG solvent and only in the 30% solution. These results are promising and suggest the use of the wet catching approach to monitor both vectors and PWN having the great advantage that for a certain amount of (personnel) resources a much larger area can be surveyed with wet catching compared to dry catching. An extra rinsing step of the collected nematodes before extraction of their DNA is needed when PCR will be performed. In addition, to keep the MEG concentration in the collection cups higher than 30% one can either add a sufficiently large volume of 100% MEG in the collection cups when setting them up, or modify the traps to prevent infiltration of rain.

- Locations

- Individuals were caught both at risk locations and in pine stands.



- Regarding the risk locations (and based on the information we have from FASFC), samples were captured in risk locations related to European trade (not points-of-entry) and not in the traps deployed in the harbours (points-of-entry) where intercontinental trade arrives.
- The pine stands generally occurred in areas (i) with a high concentration of industry and enterprises, and intensive European road transport passing through and (ii) having the largest and highest densities of pine stands in Belgium.
- We thus recommend sampling a combination of both European and intercontinental trade-related risk locations (the latter in respect to invasive non-European spp.) and pine stands in natural reserves. Regarding the risk locations, adjacent pine stands could be more favourable to set the traps up in than on the risk site itself.
- Random changes in the monitoring network in the pine stands could be advisable due to:
  - Possible long distance dispersal, e.g. 5 km (Torres-Vila *et al.*, 2015), 8.3 km (Gallego *et al.*, 2012) and 13.6 km (Mas *et al.*, 2013) ;
  - Small area coverage of attractants of traps (diameter of ca. 100-125 m reported by Jactel (2013), Sanchez-Husillos *et al.* (2015) and Extebeste *et al.* (2015));
  - A general small surface area of most pine stands and substantial scattering throughout Belgium;
  - Monochamus* individuals never found at a same location in successive years during the monitoring survey of the project.
- A focus should remain on monitoring pine stands, however, monitoring of spruce stands could also be important as was done, e.g. in Sweden (Magnusson *et al.* 2007). *Monochamus sutor* and *Monochamus urussovii* can reproduce and feed on spruce (Schroeder 2012).
- The natural dune reserve “de Duinbossen” in De Haan is an ecosystem similar to the coastal dune reserve in Schoorl en Bergen (the Netherlands) where a *M. galloprovincialis* has established for some years (Heijerman *et al.* 2009, 2011a & b, 2013). For this reason, it is advisable to monitor this reserve.



## **WP2: Incidence mapping**

An assessment of indigenous and exotic *Monochamus* spp. as well as information on the incidence of PWN and other potential vectors was not yet available at project's start. Where *Monochamus* spp. was not yet established, it was examined which other longhorn beetles and wood boring insects. During the project scientists:

- 2.1 Examined the presence of *Monochamus* spp. and other potential PWN vectors in Europe including alleged *Monochamus*-free regions in Portugal, Belgium Netherlands and Denmark
- 2.2 Determined the incidence of PWN in different European areas
- 2.3 Mapped the incidence of *Monochamus* spp. and PWN in all partner countries

### **Task 2.1 Monitoring for *Monochamus* spp. and other potential PWN vectors in Belgium, Denmark, Slovenia, and The Netherlands**

#### **Task 2.1a Analysis of cerambycids and other xylophagous beetles, potential vectors of PWN.**

- a) Surveys for *Monochamus* species in natural stands (Belgium, Netherlands, Denmark, Slovenia)
- b) Surveys for *Monochamus* species at locations of imports with wood packaging material (WPM) (Belgium, Netherlands, Slovenia)
- c) Phytosanitary status of indigenous and exotic *Monochamus* spp. in Belgium. Denmark, The Netherlands and Portugal.
- d) Identification of non-*Monochamus* longhorn beetles in Belgian and Dutch pine stands and their potential as vector of PWN.

This part/task is more or less completely integrated into and covered by the text in the following sections.

### **Task 2.1b Monitoring for *Monochamus* spp. and other potential PWN vectors in Belgium, Netherlands and Denmark**

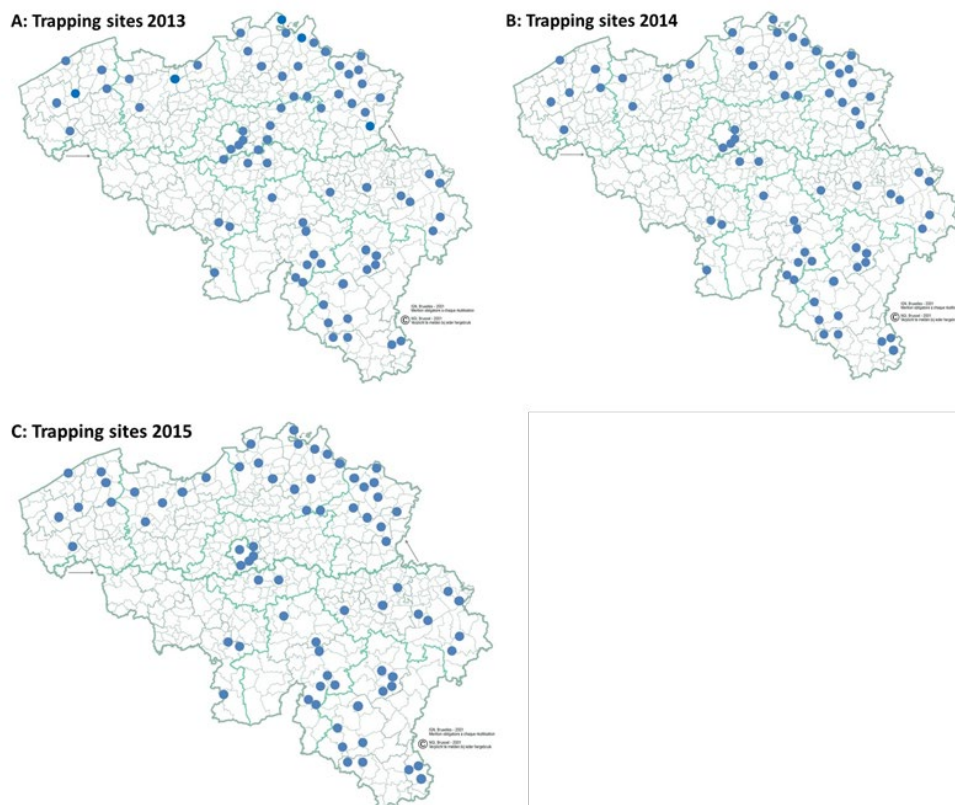
#### Belgium

##### Material and methods

The following wet catching trapping system was used throughout Belgium during 2013, 2014 and 2015:

- Traps: Crosstrap® (Econex: Murcia, Spain): Teflon coated cross-vane traps.
- Attractants: Galloprotect Pack® (SEDQ: Barcelona, Spain): a multi-component lure containing the *Monochamus* aggregation pheromone (monochamol) and the kairomones (Ipsenol + 2-methyl-3-buten-1-ol, and  $\alpha$ -pinene).
- Preservation/capturing liquid: Antifreeze liquid based on monoethylene-glycol. Approximately 5 cm polyethylene glycol (or 250 ml) was added to the collection cup at the base of the Crosstrap.





**Figure 5.** *Monochamus* trapping network established in Belgium during 2013 (A), 2014 (B), 2015 (C); with respectively 79, 80 and 82 total trapping locations, each having one trap (i.e. Crosstrap+ Galloprotect Pack® attractants)

In 2013 a total of 79 traps were established in pine stands containing *P. sylvestris* or *P. nigra* from the beginning of July until the beginning of October (5A). In 2014 and 2015, a total of 80 and 82 traps, respectively, were monitored in Belgium (5B and C). In both years traps were deployed from the end of April to the beginning of October. Captures were collected approximately every 2 to 4 weeks and the lures of the traps were replaced approximately every 6-8 weeks. Simultaneously throughout the three years an additional 10 traps aimed at imported *Monochamus* species (European and non-European) were established in locations near companies importing foreign goods and collected every two weeks, the latter was done by the Belgian NPPO, the Federal Agency for the Safety of the Food Chain (FASFC). FASFC employed the 'dry collecting' method in order to allow identification of nematodes (if any) vectored by the beetles, therefore the cup contained pieces of bark impregnated with a contact insecticide in order to allow the collection and identification of the nematodes potentially associated with the beetles.

## Results

A total of 7 adult *Monochamus* specimens were collected by ILVO and FASFC in Belgium, comprising of two species, *M. sartor* and *M. galloprovincialis* (1 and 6 specimens, respectively) (Table 2). In Elsaker (Hoogstraten) both species were found in the same trap.

**Table 2.** Captures of *Monochamus* species in Belgium during 3-year survey (2013-2015).

Species	Location	Province	Date
<i>M. galloprovincialis</i>	Bezoensbeek (Zuttendaal)	Limburg	14-08-2013
<i>M. sartor</i>	Elsakker (Hoogstraten)	Antwerp	19-08-2013
<i>M. galloprovincialis</i>	Kolisbos (Neerpelt)	Limburg	19-08-2013
<i>M. galloprovincialis</i>	Kloosterbos (Wachtebeke)	East-Flanders	16-09-2013
<i>M. galloprovincialis</i>	Elsakker (Hoogstraten)	Antwerp	16-09-2013
<i>M. galloprovincialis</i>	Bulskampveld (Beernem)	West-Flanders	17-09-2013
<i>M. galloprovincialis</i>	Hoge Vijverbos (Arendonk)	Antwerp	06-08-2014

### Discussion

*Monochamus* species were captured in Hoogstraten (Belgium) on the border with the Netherlands. The Dutch NPPO monitored for *Monochamus* spp. throughout the Netherlands from 2009-2012, including within the border region where *Monochamus* spp. were captured. However, different attractants and a different trap type were used. This confirms that the Galloprotect Pack® combined with the Crosstrap is effective for monitoring purposes of *Monochamus* spp.

### Netherlands

Inspections in natural stands of pine in 2014 2015 and 2016 mainly had a research component. These results are discussed under Task 1.3 Evaluating of new monitoring methods and strategies. At the ports of entry in the Netherlands (commercial importers where wood and other products such as stoneware with wood packaging material are gathered and distributed) annual surveys have been performed since 2009. In 2014 we used a WBB-trap (WitaPrall IntPT-Wet Trap® (Witasek, Feldkirchen in Kärnten, Austria)) and Galloprotect 2D® as a lure. In 2015 and 2016, based on the research performed in 2014 the NVWA at each port of entry this combination of trap and lure was replaced by the teflon-coated cross-vane trap (Crosstrap® (Econex, Murcia, Spain)), combined with the lure package Galloprotect Pack®. Samples was collected every 3 weeks using EWGA liquid collection and preservation mix between half of June and early October.

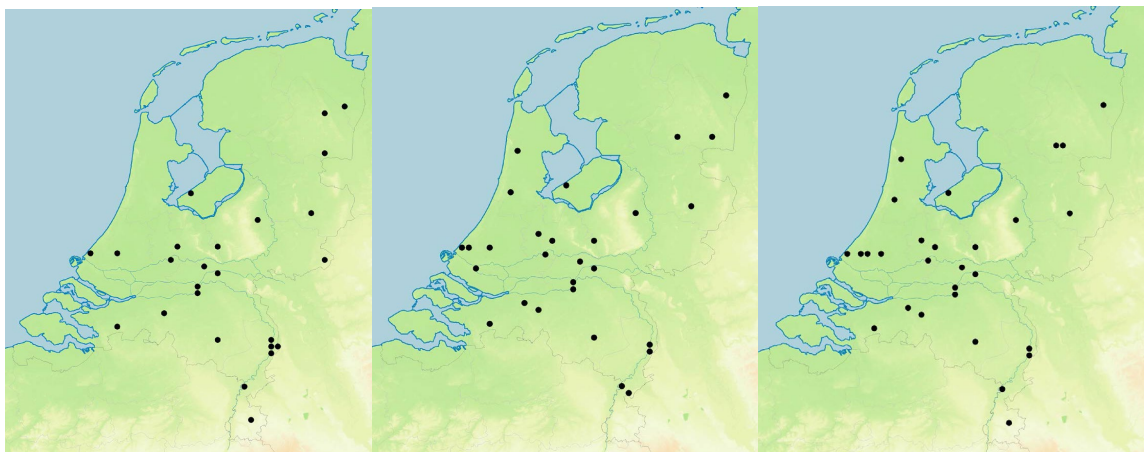


Figure 6 – distribution maps survey locations WPM Netherlands 2014 (left), 2015 (middle), 2016 (right).

Table 3 - Overview of longhorn beetle species trapped at ports of entry 2014-2016; \*\* are species not know to occur in the Netherlands.

<i>Cerambycidae</i>	2014	2015	2016
<i>Spondylis buprestoides</i> (Linnaeus, 1758)	540	396	400
<i>Arhopalus rusticus</i> (Linnaeus, 1758)	10	5	3
<i>Phymatodes testaceus</i> (Linnaeus, 1758)	26	119	17
<i>Acanthocinus griseus</i> (Fabricius, 1792) **		1	
<i>Monochamus galloprovincialis</i> (Olivier, 1795) **		1	
<i>Trichoferus cf campestris</i> (Faldermann, 1835)**	1	1	1
Other species ( n=5, 5, 3 )	6	7	3
<b>Total</b>	<b>583</b>	<b>530</b>	<b>424</b>

During the project period, 424-583 specimens of longhorn beetles were collected: predominantly native species such as *Spondylus buprestoides*. Also 3 exotic longhorn species were found at ports of entry: 3x *Trichoferus cf. campestris* at 3 locations, 1x *Acanthosinus griseus*, and 1x *Monochamus galloprovincialis*. *Trichoferus campestris* is originally an Asian species, which has been imported to other countries by means of wood packaging material (Grebennikov *et al.* 2010). It develops, in addition to dead wood, in all kinds of deciduous trees, including healthy trees; the species is often seen as an undesirable type of pest. *Acanthosinus griseus* originates in Eastern Europe. The finding of *Monochamus galloprovincialis* in 2015 was the first specimen trapped at a port of entry in the Netherlands and outside the native population in Bergen – Schoorl.

### Conclusion

During the project period 2014-2016 no wood-boring species of quarantine importance have been trapped using different traps and lures. At sites where imported wood and or goods with WPM from other countries is stored and distributed the prevalence of exotic species, however, is significant: exotic species of phytosanitary importance such as those belonging to Cerambycidae, Curculionidae (Scolytinae), Buprestidae and Bostrichidae are recorded in low numbers, but on a regular basis. Some are pest species of live, damaged or dying trees which are native elsewhere in Europe (e.g. *Ancathosinus griseus*) or already have established in other areas of Europe (e.g. *Trichoferus campestris*), others are living on dead trees or (processed) wood. Therefore, it is necessary to monitor those sites where wood or wooden products or WPM is present originating from other countries, using the best traps and lures, either tailor-made for certain taxa or as multiplex traps combining characteristics of different taxa.

### **Denmark**

Until 2014 monitoring of *Monochamus* spp. took place as a visual examination of imported wooden material. PWN monitoring took place by testing samples from wooden material. In 2012, the inspection at one harbour resulted in a collection of live specimens of 22 species of long horn beetles found on wood, imported to Denmark from Latvia and France. Among these *Monochamus sutor*, *M. sartor* and *M.*



*galloprovincialis* – all species that – together with seven other long horn beetle species found does not belong to the native fauna of Denmark (Misser, 2013).

**2014:** Monitoring experiments were initiated in 2014 using commercially available trap type WitaTrap® 'Segmenttrichterfalle 5+1'. The monitoring period was chosen as expected for native species of longhorn beetles. Efforts included 7 traps placed in harbours or suitable places in vicinity (pine stands) of harbours on Zealand (Hundested and Køge) and Jutland (Randers). This was a dual approach: detect emerging longhorn beetles via import and possible populations established near the harbours due to earlier importation (also attracted to the traps). Traps were equipped with Galloprotect Pack® lures. There was however a problem with placing attractants. Attractants were by error put in center of funnels (based on experience with bark beetle drainpipe traps), but this clogged the funnel traps. Traps in Danish monitoring campaign were set up in accordance with the expected optimal emergence of longhorn beetles – that is from early August.

**Results:** No *Monochamus* specimen was caught in the traps; and neither other longhorns. The only 'relevant catch' was several scolytids of the species *Ips sexdentatus*. This was in a trap placed among pine trees west of Hundested harbor. The imported wooden material at the harbor was a mixture of broadleaved and conifers.

At the harbor of Randers neither longhorns nor scolytids were caught in the traps. That has actually been the case before when using older types of lures and even in situations when live *Monochamus* specimens were seen in vicinity of traps. This could be due to host compound lures (kairomones) from pines being specific for *Monochamus* reared from pines, whereas the *Monochamus* found in harbors developed on spruce.

The design and duration of the WitaTrap 'Segmenttrichterfalle' worked out to be too poor for the windy conditions of the harbors. It simply fell apart too easy and had to be repaired.

As in 2014, the 2015 monitoring experiments were carried out in co-operation between the plant health institution and the University of Copenhagen. 24 traps were used. Trap type was either Multifunnel® (Contech) or Crosstrap® (Econex, Murcia, Spain), combined with the lure package Galloprotect Pack® (SEDQ S, Barcelona, Spain). Traps were used earlier than previous year, that is end June/early July. The traps were serviced several times until they were taken down in October. The lures were placed on the outside of the traps. Experiments were carried out on the effect of position of the traps: either low - 2 meters - or high 8 meters – above the ground. Localities in 2015 were on Zealand: Harbor of Hundested and the old pine forest of Asserbo on the north coast of Zealand. The last place was where the position experiment was carried out. In Jutland and Funen 20 traps were in use at harbors and in old pine forests.

Results: All traps caught several beetles. At the harbor of Hundested the species caught reflects a mixture of beetle species living on conifers as well as broadleaves. In the old pine forest of Asserbo the catch consisted of beetle species mainly living on conifers or even specific on pine. *Spondylus buprestoides* appeared as the most abundant species. No *Monochamus* species was caught at this place. The high/low traps showed that the low positioned traps have a higher number of catches. Except that for beneficial insects such as *Thanasimus formicarius* and *Rhizophagus* spp. Here the low trap caught less *Rhizophagus* spp. than the high positioned trap. The first record of *Monochamus galloprovincialis* in Denmark appeared in a monitoring trap in the most western part of Jutland (Oksbøl).

In 2016, about 60 traps in use in DK - mainly Crosstrap and MultiFunnel. HPR serviced 11 traps on Zealand and Bornholm. Galloprotect Pack® lures were used in all traps. Where the one *M. galloprovincialis* was found in 2015, the trap density was increased. Besides this the target monitoring areas were import harbours, forests with long continuation of pine and forests close to infantry military areas from where staff and material had been brought back from Balkan in the 1990'ies.

Results: In the same area in southwest Jutland 76 specimens of *M. galloprovincialis* were caught in 2016. The only other trap catch of this genus was one specimen of *M. sartor* in the harbor of Randers.

8 species of long horn beetles were caught in the traps, about 20 species of bark beetles.

Comparison Crosstrap and MultiFunnel-traps showed about same catch capacity. Monthly catches showed highest catches in July-August.

#### Discussion:

Before this project, *Spondylis buprestoides* was considered quite rare in Denmark. In one trap 284 specimens were caught! The trap catch of such a species and other pine beetle species makes it legitimate to conclude that this monitoring system works. If any specimen of *Monochamus* had been present in the area influenced by the scent plume from the traps, where CrossTrap or MultiFunnel traps with Galloprotect Pack® were used, it was expected to catch them – as it happened in Western Jutland. This result is – by the way - slightly in contradiction with the result from Belgium where no catches were observed in the same region two years in a row.

Non-target catches of beneficial insects such as *Thamasimus* spp. and *Rhizophagus* spp. are of course regrettable, and when it is in high numbers as in this case even more regrettable. However, the high numbers tell us how abundant these species are in the area. The following question could be asked: If a permanent and comprehensive system of monitoring traps across Europe was established, would it have a negative impact on natural enemies of our forest insect pests? To answer this question, someone has to remember that the percentage of all insects in the air



caught with monitoring traps is like taking a small water sample in the ocean! The high number tells how abundant these species are in the environment. A pheromone trap will only influence the area or sector above which the air will distribute the pheromone plume. It is not to be compared to a vacuum cleaner sucking up every insect in a certain radius.

Nevertheless by elaboration on the trap design, improvements could surely be achieved on the escape possibilities for the smaller sized predatory beetles – *Rhizophagus* spp. and *Thanasimus* spp. – as it has been the case for in the history of drainpipe type barkbeetle traps.

### **Task 2.1c Phytosanitary status of indigenous and exotic *Monochamus* spp. in partner countries (Portugal, Belgium)**

#### **PORTUGAL**

##### Materials and methods

Owing to the lack of detailed knowledge on the distribution of *Monochamus galloprovincialis* (Olivier 1795) in Portugal, we conducted an exhausted detailed bibliographic review on international and "grey" literature on its distribution and associated hosts. Furthermore, we incorporated the unpublished field data gathered over the years by the forest entomology team of the INIAV Institute (Instituto Nacional de Investigação Agrária e Veterinária, Oeiras, Portugal), in order to produce a mapping on the distribution and hosts of this insect in Portugal.

Field surveys were mostly directed to areas where *P. pinaster* is the dominant tree species (ICNF, 2010), recording the locations in the UTM 10x10 km coordinates grid (Universal Transverse Mercator coordinate system). The surveyed areas were chosen based on the current distribution of the zones affected by the pine wilt disease, and on the proximity with Spain (buffer zone). The distribution of *M. galloprovincialis* was assessed by the presence of adult insects or larval instars inside decaying or dead trees. Besides *P. pinaster*, other conifers were also sampled, namely *Pinus halepensis* Mill., *Pinus pinea* L., *Pinus sylvestris* L., *Pinus radiata* Don, *Cupressus lusitanica* Mill., *Larix decidua* Mill. and *Chamaecyparis lawsoniana* (A. Murraybis) Parl.

##### Results

The pine sawyer *M. galloprovincialis* was found in 90 out of 96 surveyed UTM grids (94%), being widely distributed in the Portuguese continental territory (Fig. 7). The cerambycid was found associated mainly with maritime pine but also with other hosts, namely *P. sylvestris* in Viana do Castelo (UTM NG21) and *Pinus halepensis* in Cascais (UTM MC68). The pine sawyer was not detected in any of the other surveyed conifers.

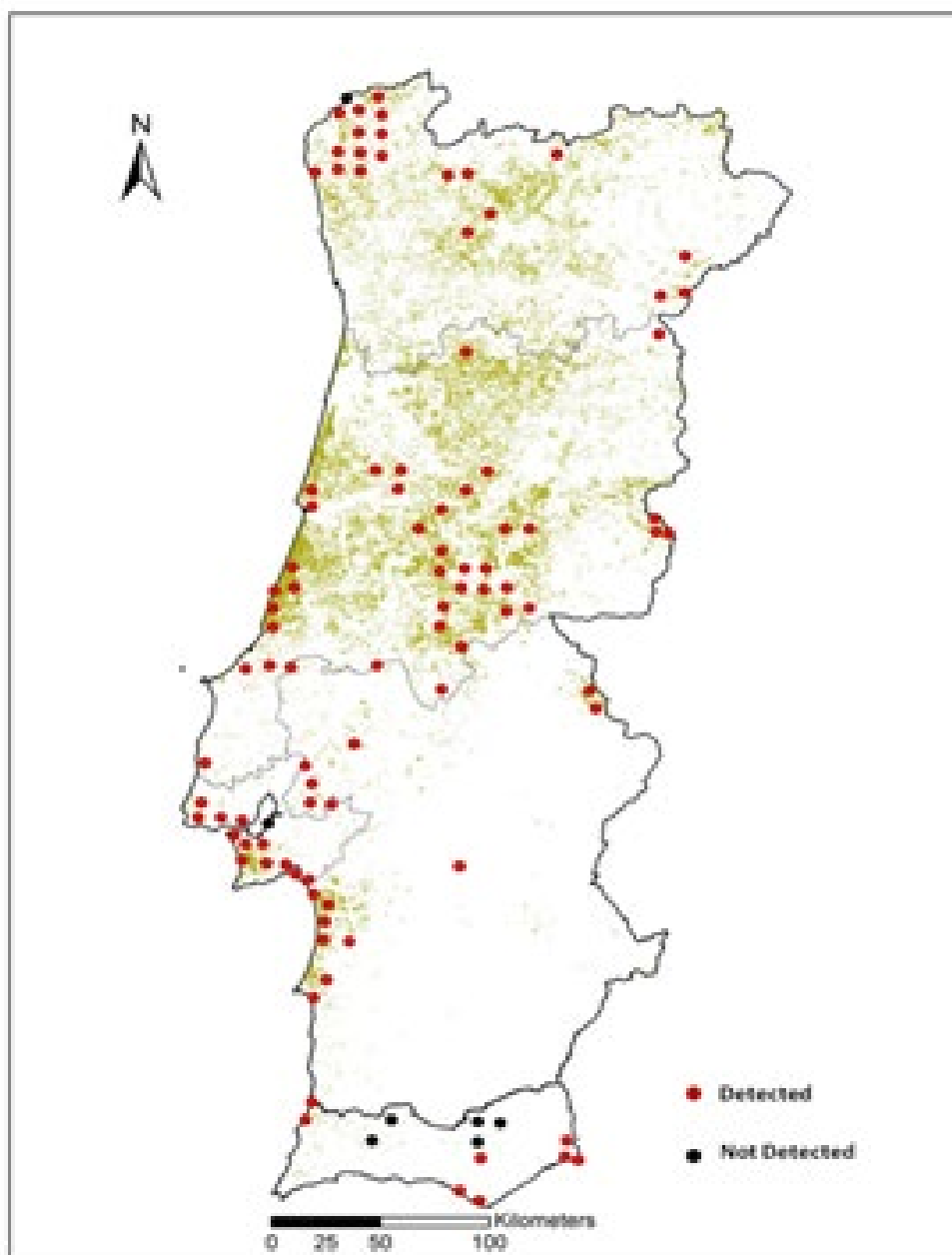


Figure 7. Distribution of *Monochamus galloprovincialis* in continental Portugal (Map created with ESRI® ArcMap™ 10.0).

### Discussion

A detailed knowledge on the distribution and hosts of the vector beetle is important to understand the establishment and incidence of wilt disease in the field. Our results confirm *M. galloprovincialis* as the sole member of its genus in Portugal, and also reflect its adaptability and ecological plasticity, as this insect was found to be widely distributed on the Portuguese territory where pine forests are abundant, independently of edapho-climatic differences between locations. Historically, in Portugal there is also the record of a second *Monochamus* species, *Monochamus sutor* Linnaeus, which was firstly reported in the end of the XIX<sup>th</sup> century from the Leiria region. Nevertheless, subsequent reviews of the insect specimens found them

to be erroneously identified, and should be assigned to *M. galloprovincialis*. This species was only referred in 1978, when it was collected from burned *P. pinaster* trees near Arganil, central Portugal.

As for the hosts, the association of *M. galloprovincialis* with maritime, scots and aleppo pines is not surprising, as these are common hosts of this beetle throughout other locations in southern Europe. Our results also confirm pines as the primary hosts of this cerambycid.

## **Belgium:**

### Materials and methods

The trapping network described in Task 2.1 for Belgium was used

### Results

As discussed a total of 7 adult *Monochamus* specimens were collected in Belgium, comprising of two species, *M. sartor* and *M. galloprovincialis* (1 and 6 specimens, respectively) (see Table 2). Harbours and import-risk locations were equally surveyed by FASFC, but no exotic *Monochamus* spp. were captured in these traps nor in the traps set up in the pine stands throughout Belgium.

### Discussion

The limited number of *Monochamus* spp. individuals found during the 3-year survey, which were never found at the same location in successive years, suggests 2 possibilities:

1. Some of the catches (*M. galloprovincialis*) correspond to sparse, endemic populations:

- *Monochamus galloprovincialis* occurs in Belgium (endemic);
- The species is very sparsely distributed at very low population densities, possibly occurring only in the Flemish part of Belgium;
- In addition, the fact that individuals were never found at the same location in successive years could imply that *M. galloprovincialis* is highly dispersive. David *et al.* (2014) report a mean distance of 16 km flown during a lifetime.

## 2. *Monochamus* spp. were imported via trade into Belgium (non-established).

- *Monochamus sartor* is assumed native to Eastern and Central Europe and in mountainous areas regions in Western Europe (*Fauna Europaea*; IUCN red list), suggesting that the individual caught in Elsakker in 2013 was imported. However, it is present in all countries surrounding Belgium, except The Netherlands;
- The pine stands in which the *Monochamus* individuals were collected are situated in regions having a high concentration of industry and enterprises, and intensive European road transport passing through them;
- A DNA microsatellite analysis by Haran and Roux-Morabito (2014) at INRA resulted in linking 3 of the *M. galloprovincialis* individuals (other individuals could not be analyzed) to *M. galloprovincialis* populations from Central (Germany, Austria) and Eastern Europe (Poland and the Balkan region);
- *Monochamus* spp. individuals were never found at a same location in successive years, which could imply there are no established populations (in release-recapture experiments by Etxebeste *et al.* (2013, 2015), Sanchez-Husillos *et al.* (2015) and Torres-Vila *et al.* (2015) most released individuals remained close by 50 – 150m). However, Jactel (2013) caught a maximum of 10% of the beetles they released, irrespective of the distance from the traps to the release point.

## **Analysis of cerambycids and other xylophagous beetles, potential vectors of PWN in Slovenia**

After discovery of the pine wood nematode causing death of *Pinus pinaster* trees in Portugal in 1999, *Bursaphelenchus xylophilus*, the pine wood nematode (PWN), has begun to spread and *Monochamus galloprovincialis* has been confirmed as the vector of *B. xylophilus* in Portugal (Mota *et al.* 1999, Sousa *et al.* 2001). There is no doubt that *B. xylophilus* is a significant threat to the pine forests of Europe. *B. xylophilus* is an EPPO A2 pest. Details about its biology, distribution and economic importance can be found in EPPO/CABI (1997).

Its natural way of transmission from tree to tree is by transfer through activity of the adult stages of wood-inhabiting longhorn beetles of the genus *Monochamus* (Coleoptera: Cerambycidae) (OEPP/EPPO, 2011). Some of woodborers are also associated with different *Bursaphelenchus* spp. Technically, therefore, any insect able to carry nematodes, especially if belonging to the genus *Bursaphelenchus*, could be considered a potential vector of PWN. In our case, we also found some of the species of woodborers that are possible vectors of PWN.

If other vectors are proved in Europe, the suppression or mitigation of pine wilt disease could be designed, which would have greater probability of success than has been with suppression of *Monochamus* spp. vectors only.

We analyzed the Cerambycidae and Curculionidae entomofauna, collected in traps in pine forests at three different locations (Kastelec, Dekani and Brdo pri Kranju) during 2007 and 2012 (Jurc *et al.* 2012). The Kastelec location lies on limestone parent rock



with monoculture of *Pinus nigra*, the Dekani location embraces a stand of *Pinus halepensis* on flysch (both in the Sub-mediterranean ecological region), whereas the Brdo pri Kranju location encompasses a stand of *Pinus sylvestris* on brown soil (Pre-alpine ecological region). According to the literature, we summarize the potential vectors PWN of the order Coleoptera, which were found at our research plots.

In Slovenia, the species from the genus *Bursaphelenchus* were isolated only from the wood. *B. mucronatus* has been detected in samples of the decaying wood of red pine (*Pinus sylvestris*) from the surrounding of Brnik and Kidričevo, species *B. hofmanni* was isolated from the trees of Austrian pine (*Pinus nigra*) in karst areas near Sežana (Urek and Širca, 2005; Urek *et al.*, 2007).

In the frame of the CRP project, again, *B. mucronatus* was isolated in samples of wood in location Brnik. Analysis of nematodes from the sample collected at the site Golovec near Ljubljana revealed the presence of the species *B. pinaster* Baujard (Nematoda: Parasitaphelenchidae), which has not yet been detected on the territory of Slovenia (Širca *et al.*, 2013).

### **Monitoring of longhorn beetles and other xylophagous beetles in the Port of Koper, possible entry points of PWN in Slovenia**

In the Port of Koper a collaboration was initiated with the phytosanitary inspection services. Three black cross vane funnel traps (Witasek) were placed on two different positions within the port. The first trap, baited by the combination of  $\alpha$ -pinene + ethanol, and the second trap, baited by Galloprotect Pack® were placed near a wood mill. The third trap, baited by Galloprotect 2D® was placed inside the wood terminal. The collection of the catch was monthly, from the beginning of March until the end of November. All captured Arthropodes were recorded with the determination to the species level for the class of Coleoptera only. In 2014, 397 specimen of Beetles (Coleoptera) from 30 different families, 82 genera and 90 determined species were caught. We determined a few alien beetle species (*Stelidota geminata* (Say, 1825), *Xylotrechus stebbingi* Gahan, 1906, *Harmonia axyridis* (Pallas, 1773) and study of their significance for Slovenia is under way. Taxonomic work (of the catch from year 2015) is still going-on and determined material is stored in LVG-BF-GOZD-entomology collection.

Partial results were presented during the EUPHRESCO II – *Monochamus* workshop meeting in Slovenia, 31<sup>st</sup> May – 3<sup>rd</sup> June 2015 in Ljubljana, Pavlin / Borkovič / Jurc: Report on monitoring of long-horn, and other xylophagous beetles in the Port of Koper (2014), and also on 6<sup>th</sup> Forest protection workshop meeting, 16<sup>th</sup> of June 2015, Kostanjevica na Krki, Slovenia.

### **Monitoring of longhorn beetles and other xylophagous beetles in Belgium**

#### **Belgium:**

#### Materials and methods

The trapping network described in Task 1.2 for Belgium was used.



## Results

The longhorns, other than *Monochamus* spp. captured in the traps during the 3-year survey are reported in Table 3.

Table 3. Total number of non-*Monochamus* longhorn beetles captured in Belgium during 3-year survey (2013, 2014 and 2015) in 79, 80 and 82 traps (Crosstrap and Galloptotect Pack), respectively.

Longhorn species	2013	2014	2015	<b>total</b>
<i>Acanthocinus griseus</i>	1	0	0	<b>1</b>
<i>Anaglyptus mysticus</i>	0	5	3	<b>8</b>
<i>Arhopalus rusticus</i>	577	656	529	<b>1762</b>
<i>Asemum striatum</i>	0	7	8	<b>15</b>
<i>Brachyleptura maculicornis</i>	0	1	0	<b>1</b>
<i>Clytus arietus</i>	0	15	9	<b>24</b>
<i>Cortodera humeralis</i>	0	1	0	<b>1</b>
<i>Hylotrupes bajulus</i>	0	3	2	<b>5</b>
<i>Leiopus femoratus</i>	0	0	1	<b>1</b>
<i>Leiopus nebulosus</i>	0	0	1	<b>1</b>
<i>Leipus linnei</i>	0	1	2	<b>3</b>
<i>Leptura quadrifasciata</i>	4	15	11	<b>30</b>
<i>Molorchus minor</i>	0	1	0	<b>1</b>
<i>Oxymirus cursor</i>	1	1	0	<b>2</b>
<i>Phymatodes testaceus</i>	0	0	1	<b>1</b>
<i>Pogonecherus hispidulus</i>	2	0	0	<b>2</b>
<i>Pogonecherus hispidus</i>	1	2	5	<b>8</b>
<i>Prionus coriarius</i>	0	2	1	<b>3</b>
<i>Pyrrhidium sanguineum</i>	0	1	0	<b>1</b>
<i>Rhagium bifasciatum</i>	2	65	132	<b>199</b>
<i>Rhagium inquisitor</i>	30	2080	2949	<b>5059</b>
<i>Rhagium mordax</i>	3	40	10	<b>53</b>
<i>Rutpela maculata</i>	3	39	12	<b>54</b>
<i>Spondylis buprestoides</i>	1580	4851	4976	<b>11407</b>
<i>Stenurella melanura</i>	3	6	4	<b>13</b>
<i>Stictoleptura rubra</i>	14	10	9	<b>33</b>
<i>Tetropium castaneum</i>	3	5	1	<b>9</b>

Discussion will follow under task 4.3.

## Task 2.2 Incidences of PWN in Europe (including a more detailed study for Belgium, Denmark, Slovenia and The Netherlands)

### Introduction

While establishing an early warning detection system for PWN in the field, the work performed by different partners on the incidences of PWN in Europe focused on 2 aspects: a) establishing the incidences of PWN in Europe (all partners) and b) sorting out the effect of wet-catching, in particular the effect of Mono-ethylene glycol (MEG), on the survival, the morphological and molecular identification of *Bursaphelenchus xylophilus* (Belgium).

### **a) Establishing the incidences of PWN in Europe (all partners)**

#### Materials and methods

**Portugal:** The trapping network in Portugal for the capture of *M. galloprovincialis* uses multifunnel traps with a kairomone and pheromone lure, Galloprotect 2D-Plus, in responsibility of the ICNF (Portuguese Governmental Agency for Nature and Forests). It involved the setting-up of 6,520 traps, monitored from 2012 to 2014. The captured insects were received in the Nematology lab of INIAV and processed for the extraction of nematodes, according with the protocol EPPO PM 7/4(3). When the dauer juveniles were not able to moult to the adult form, preventing a reliable morphological identification, specimens were sent for additional molecular analysis.

**Belgium:** The trapping network described in Task 1.2 for Belgium was used. All 7 *Monochamus* adults captured during this field monitoring were inspected for the presence (= transportation) of nematodes in accordance with the EPPO protocol PM 7/4 (EPPO, 2013). Nematodes were extracted from the Cerambycids with the Baermann funnel technique in a mistifier for 24-48 hours. The obtained suspension was then examined for the presence of nematodes using a stereomicroscope. Isolated nematodes were identified based on their morphological features.

**Netherlands:** The trapping network described in Task 1.2 was used for surveys in natural stands and at ports of entry. In the period prior to 2014, many specimens of the naturally occurring population in Bergen-Schoorl had been examined already for PWN, without any findings. During this survey project, no *Monochamus* was caught except from the already known population in Bergen-Schoorl; from this population a sample (20%) of the adults trapped was examined for the presence of nematodes using the mistifier extraction technique described above.

**Denmark:** PWN survey according to EU regulation was carried out by the plant health inspectors.

### Results

**Portugal:** From 2012 to 2014 a total of 1,121,744 insects were captured on the traps. Of them, 90.1% were scolytids and only 0.2% were *M. galloprovincialis*. In average,

the PWN was detected in 29% of the beetles caught. In this work additional data was used from insects caught and sent directly to INIAV by forest associations and owners, namely in 2011 (with 1,053 beetles) and less in subsequent years (Table 5).

Table 5 – *Bursaphelenchus xylophilus* incidence (%) in *Monochamus galloprovincialis* analysed during 2011 to 2014, in Portugal (by district); n= number of insects. Numbers in red represent increase of infection ratio between successive years and in green represent the decrease.

Districts	2011		2012		2013		2014	
	n	%	n	%	n	%	n	%
Aveiro	10	30	20	10				
Braga	4	50						
Bragança	11	9						
Castelo Branco	103	12	46	4	11	0		
Coimbra	409	20	15	27	1,057	27	91	16
Faro	2	0						
Guarda	1	0						
Leiria	253	17	299	12	1,073	18	486	15
Porto	1	0						
Santarém	26	27	1	0	2	0		
Setúbal	177	23	20	35	77	18		
Viana do Castelo	4	0						
Viseu	19	11						
Madeira Island	31	29	252	36				

**Belgium:** None of the 7 *Monochamus* individuals captured during the 3-year survey in Belgium were transporting *B. xylophilus*. In addition, no other *Bursaphelenchus* spp. were found on/in the 7 longhorn beetles.

**Netherlands:** From 2014-2016, no PWN and or other *Bursaphelenchus* species could be extracted from the only *Monochamus galloprovincialis* population present in Bergen-Schoorl.

**Denmark:** According to EU-regulation sampling and testing of woodsamples and *Monochamus* for PWN has been carried out. No PWN has so far been found.

## Discussion

**Portugal:** The trapping effort of ICNF in Portugal was not identical in all surveyed areas, and data for some years were not available for all districts. So, the results must be taken with some caution. However, this work confirms the importance of the insect vector in the Pine Wilt Disease spread and how a good control of the insect populations is a key factor for the success of the PWD management, since both the number of caught *M. galloprovincialis* and the PWN infection ration decrease along the years and had the lowest values in the last year. Exception to Madeira Island where the disease shows particularly aggressive behavior and control measures applied revealed to be insufficient. These results were first presented by Inácio *et al.* (2015)

**Belgium:** *Bursaphelenchus* spp. were never captured or observed during the study in Belgium. Two studies describe the diversity of nematode species occurring in Belgium, i.e. Bert *et al.* (2013) and Steel *et al.* (2014); they do not report any *Bursaphelenchus* spp. found in Belgium. In addition, FASFC (i.e. the Belgian NPPO) has never intercepted any commodities introduced into Belgium which were infested with *B. xylophilus* (bark, wood, transport pallets, etc.) (Berkvens *et al.* 2013). Samples were brought to the diagnostic lab of ILVO for investigation: the sample was incubated for 14 days at room temperature, then submerged in water overnight, after which the nematodes are extracted from the water using a zonal centrifuge. The obtained nematode suspension is then investigated using a stereomicroscope for the presence of nematodes. Sometimes saprophytic nematodes (mostly *Aphelenchoides* spp., but also *Monaphelenchus* spp. and Rhabditidae) are found in these wood commodities. This is rather suspicious for the wood samples that are supposed to have been treated with heat or fumigation (ISPM 15). However, it is possible that contamination with these nematodes occurred after the treatment.

**Netherlands:** during the project period 2014-2016 as well as in the preceding period, Pinewood Nematode (*Bursaphelenchus xylophilus*) was never detected in specimens from the single natural occurring population of *Monochamus galloprovincialis* in Bergen-Schoorl. Also specimens of other *Monochamus* species that were intercepted during import inspections, no PWN has been extracted.

## **b) Wet catching: the effect of mono-ethylene glycol on identification of *Bursaphelenchus xylophilus* (Belgium)**

### Introduction

Mono-ethylene glycol (MEG) is used as a preservation solvent for insects in the Belgian survey as the trap captures can then be collected less frequently (a wider range can subsequently be monitored). However, the effect of this solvent on the survival and morphological and molecular identification of *B. xylophilus* is unknown. The latter is important when establishing an early warning detection system for PWN in the field.

### Material and methods

Staining glasses were filled with either rain water, pure MEG or a dilution of MEG with rain water (10, 30 or 60%); 3 staining glasses were used per liquid solution. More than 200 *B. xylophilus* adults cultured on *Botrytis* were submerged in each staining glass and maintained in an incubator at 17°C. After 1, 3, 7, 14, 21 and 28 days a total of 10 *B. xylophilus* individuals from each of the 3 staining glasses (30 individuals per solution) were inspected for survival and scored for 7 distinct morphological parameters described in the EPPO diagnostic protocol PM 7/4(3) (EPPO, 2013). The individuals were inspected for the features of the *Bursaphelenchus* genus:

- Cephalic region high and offset by a constriction with 6 lips
- Lateral field with 4 lines
- Excretory pore at/or behind median bulb



and for *B. xylophilus*-specific features:

- Female tail broadly sub-cylindrical with or without mucro
- Female vulval flap straight, not ending in a deep depression
- Male spicule length < 30µm
- Male spicule with long and pointed rostrum; limbs of spicule with an angular curvature

The features were scored on a scale from 1 to 5 for each nematode; 5 indicating clearly visible and 1 not visible. All individuals were then discarded. The scores were analysed using an ordered logistic regression.

We then investigated the feasibility of identifying *B. xylophilus* adults submerged in the MEG solvent via molecular techniques. On the same days as the morphological observations, 7 individuals were taken out of each of the 3 staining glasses (21 individuals per solution) from the 'pure rainwater', '30% MEG' and '100% MEG' solutions. DNA was extracted from the submerged individuals (Holterman *et al.* 2006). From each staining glass, DNA was extracted twice from 1 separate nematode individual and once from a group of 5 nematode individuals. DNA of the 18S region was then amplified using universal 18S rRNA primers (1813F and 2646R) as described in Holterman *et al.* (2006). Successful amplifications were visualized via electrophoresis.

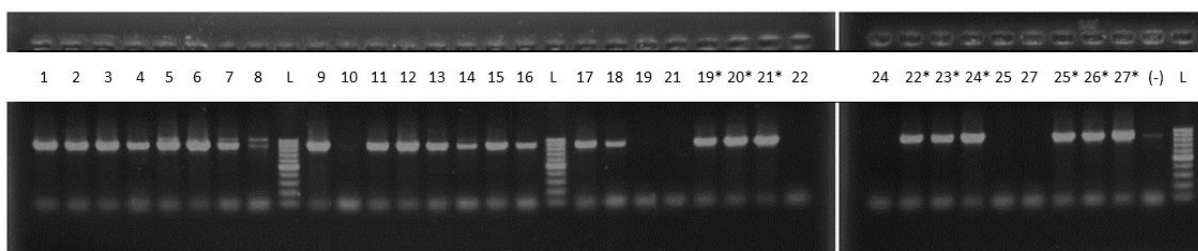
The detection and identification of PWN in wet catching with MEG was then further investigated with wild *M. galloprovincialis* vectors transporting PWN. This experiment was performed by Maria Lurdes Inácio at the National Institute for Agricultural and Veterinary Research (INIAV, Oeiras, Portugal). In total, 76 *M. galloprovincialis* adults were reared in the laboratory from artificially infested logs. Half of the emerged adults (38 individuals) were each submerged separately in vials containing a 30% MEG dilution, while the other half (38 individuals) were submerged separately in vials containing a 70% MEG dilution. After mortality of the insect vector, both the solvent and the dissected insect vector were visually inspected for the presence of PWNs using a stereomicroscope. Of the retrieved PWN, 18 were subjected to DNA extraction and PWN-primer-specific PCR analysis using the protocol described by Inácio *et al.* (2014) who used the primers developed by Takeuchi *et al.* (2005) (15 of these nematodes were retrieved from within the insect vector and 3 from within the solvent). Amplification was checked via electrophoresis of the final PCR product.

## Results

While only 5 of the 30 *B. xylophilus* adults were dead after 24h when submerged in rainwater, all 30 adults were dead when submerged in a MEG solution (10, 30, 60 and 100%). Except for the feature "spicule <30µm", the MEG concentration and/or submersion time had a significant effect on the visibility of the morphological features of the nematodes. While the use of MEG as a wet catching approach in our traps had

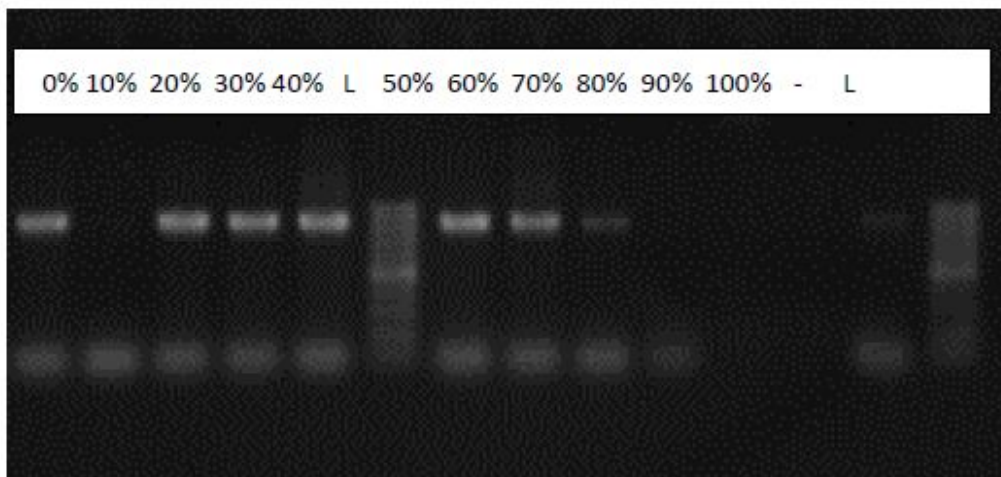
a negative effect on some of the morphological features of the PWN it did not prevent the detection/identification of *B. xylophilus* via molecular techniques.

On all days, from the first to the 28<sup>th</sup> day, we were able to amplify the 18S region of the nematodes from all three solutions (0%, 30% and 100% MEG). In the cases of pure rainwater and a solution with 30% MEG, the DNA extraction could start with 25µl lysis buffer mixed with 25µl of the solutions containing the nematodes. An extra step was needed for the 100% MEG solution, *i.e.* the nematodes had to be transferred from the 100% MEG solution into 25µl pure water which was then mixed with 25µl lysis buffer. DNA extraction was then performed with this mixture. Figure 8 illustrates the electrophoresis performed for the nematodes submerged for 3 days in the solutions.



**Figure 8.** Electrophoresis of 18S DNA region (Holterman et al., 2006) of *B. xylophilus* adults submerged in one of three solutions (pure water, 30% MEG or 100% MEG) for 3 days.

Lanes 1-9: nematodes submerged in pure water for 3 days; lanes 10-18: nematodes submerged in 30% MEG for 3 days; lanes 19-18: nematodes submerged in 100% MEG for 3 days; an alternative DNA extraction was performed for the individuals related to the lanes marked with \*, these nematodes were transferred from the 100% MEG solution into 25µl pure water before performing DNA extraction



**Figure 9.** Electrophoresis of 18S DNA region (Holterman et al., 2006) of single *B. xylophilus* adults submerged at different MEG concentrations (0 to 100%) for 1 day; mixtures of the solutions were made with pure water.

The MEG generally had no influence on the quality of the DNA of *B. xylophilus*, however, in high concentrations it probably inhibits certain reactions in the PCR. This effect occurred at a concentration of 70% MEG or higher (Figure 9).

All of the 76 longhorn adults submerged in the MEG solutions died within 4 hours after being placed in the MEG solutions. Of these 76 beetles, 23 were transporting PWN: 13 of the insects submerged in the 30% MEG dilution and 10 in the 70% MEG

dilution. Of all 23 beetles transporting PWN, nematodes were detected in and retrieved from all insect bodies, while only for 3 insects, in the 30% MEG dilution, were nematodes also found in the solvent.

A total of 9 and 6 PWN samples of the 30% and 70% dilution, respectively, were analysed molecularly; 8 out of 9 and 4 out of 6 samples were positively identified as *B. xylophilus*. A negative PCR result in the 30% MEG dilution occurred for a sole PWN found in that insect which may have been a result of an insufficient amount of extracted DNA. The 2 negative identifications in the 70% MEG dilution are in accordance with previous results of where we found that an extra 'rinsing' step was necessary for adequate DNA extraction in higher MEG concentrations.

The PWN retrieved from the 30% MEG liquids were also subjected to molecular analysis, however, no DNA was amplified in the PCR runs. This contradicts the results of previous experiments and it is unclear why there was no amplification of the DNA.

### Discussion

MEG can be used as wet catching solvent since the solvent did not inhibit molecular identification of the PWN, albeit an extra rinsing step was necessary at high concentrations of MEG ( $\geq 70\%$ ). When PWN were present in the vector, (nearly) all individuals were found within the insect host, indicating that they died inside their host due to the MEG. Further research is necessary to determine if and to what extent PWN leave the host at MEG concentrations below 70%. The first results in this WP are promising for the use of wet catching with MEG. A higher MEG concentration can be created in the collection cup during field use by adding more MEG in the cups when collecting the samples (a layer of about 5 cm pure MEG was used throughout this project) or by modifying the traps to prevent infiltration of rain (e.g. larger top of the trap). In addition, an extra rinsing step before DNA extraction from potential PWN's is recommended to obtain a positive identification with PCR. It is recommended that the wet-catching approach is further tested in other regions/countries.

### **Task 2.3 Natural distribution of native *Monochamus* spp. throughout Europe (all partners)**

#### Materials and methods

A study of the natural distribution of *Monochamus* spp. was performed based on information already present at the different partners, added with information from literature. Figure 10 illustrates the natural distribution of the native European *Monochamus* spp. according to the *Fauna Europaea* (2010).

#### Results

This distribution is as follows according to the *Catalogue of Palaearctic Coleoptera* (2013):



- ***Monochamus galloprovincialis***: Azerbaijan, Albania, Armenia, Austria, Bosnia Herzegovina, Bulgaria, Belarus, Croatia, Czech Republic, Estonia, Finland, France, Germany, Georgia, Greece, Hungary, Italy, Latvia, Lithuania, Macedonia, Moldavia, The Netherlands, Poland, Portugal, Romania, Slovakia, Sweden, Switzerland, Ukraine, Russia (Central European Territory), Russia (North European Territory), Russia (West Siberia), Russia (East Siberia), Russia (Far East), Serbia and Montenegro, Algeria, Morocco, Tunisia, Kazakhstan, Mongolia, China (Northeast Territory), Turkey
- ***M. saltuarius***: Austria, Bosnia Herzegovina, Belarus, Croatia, Czech Republic, Germany, Hungary, Italy, Latvia, Poland, Romania, Slovakia, Slovenia, Ukraine, Russia (Central European Territory), Russia (North European Territory), Russia (West Siberia), Russia (East Siberia), Russia (Far East), Kazakhstan, Japan, Mongolia, North Korea, South Korea, China (Provinces: Heilongjiang, Jilin, Jiangxi, Nei Mongol, Shaanxi, Shanghai, Shanxi, Xinjiang, Zhejiang)
- ***M. sartor***: Austria, Bosnia Herzegovina, Bulgaria, Belarus, Croatia, Czech Republic, Estonia, France, Great Britain, Germany, Hungary, Italy, Latvia, Lichtenstein, Lithuania, Poland, Romania, Slovakia, Slovenia, Switzerland, Ukraine, Serbia and Montenegro
- ***M. sutor***: Albania, Austria, Bosnia Herzegovina, Bulgaria, Belarus, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Great Britain, Germany, Georgia, Greece, Hungary, Italy, Latvia, Lichtenstein, Lithuania, The Netherlands, Norway, Russia (Central European Territory), Russia (North European Territory), Russia (South European Territory), Russia (West Siberia), Poland, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Ukraine, Serbia and Montenegro, Kazakhstan
- ***M. urussovii***: Belarus, Czech Republic, Estonia, Finland, Latvia, Lithuania, Norway, Poland, Sweden, Russia (Central European Territory), Russia (North European Territory), Russia (South European Territory), Russia (East Siberia), Russia (Far East), Russia (West Siberia), Ukraine, Kazakhstan, Mongolia, North Korea, Japan, South Korea, China (Northwest Territory), China (Provinces: Ningxia, Nei Mongol, Hebei, Heilongjiang, Henan, Jilin, Shaanxi, Xinjiang)



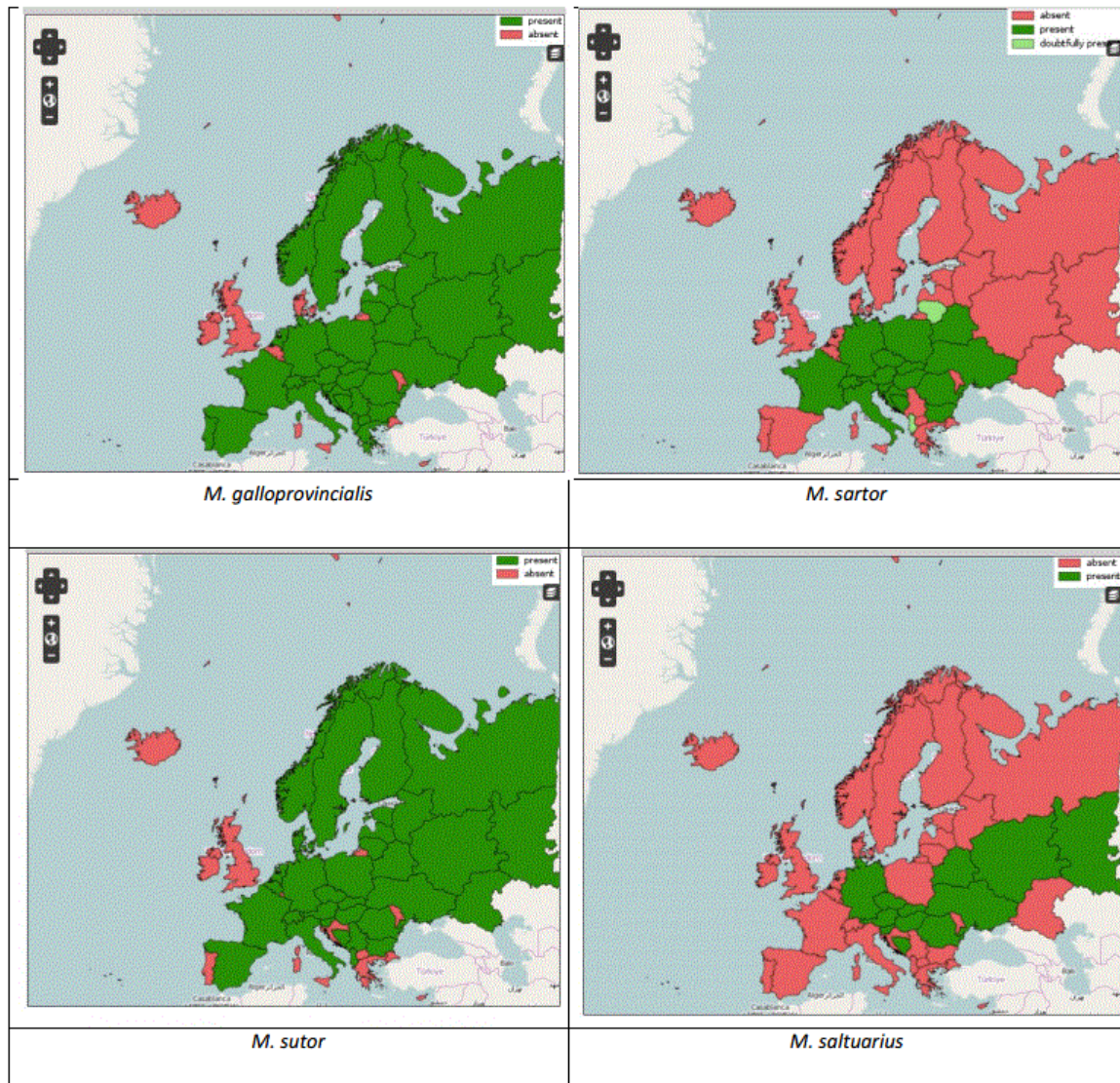


Figure 10. Distribution of European *Monochamus* spp. (source *Fauna Europaea*; [www.fauna-eu.org](http://www.fauna-eu.org))

The host plant distribution for the native *Monochamus* spp. in Europe is described in the European Atlas of Forest Tree Species (San-Miguel-Ayán et al. 2016, and can freely be downloaded at <http://forest.jrc.ec.europa.eu/european-atlas-of-forest-tree-species/atlas-download-page/> after logging in with a registered account). In this atlas distributional maps can be found for the following tree species in Europe:

- Abies alba* - Silver fir
- Abies* spp. - Circum-Mediterranean firs
- Larix decidua* - European larch
- Picea abies* - Norway spruce
- Picea omorika* - Serbian spruce
- Picea sitchensis* - Sitka Spruce
- Pinus cembra* - Arolla pine
- Pinus halepensis* and *Pinus brutia* - Aleppo and Turkish pine
- Pinus mugo* - Dwarf mountain pine
- Pinus nigra* - Black pine

*Pinus pinaster* - Maritime pine

*Pinus pinea* - Stone pine

*Pinus sylvestris* - Scots pine

### Discussion

Even in the Fauna Europea there were some obvious errors – e.g. *M. sutor* is still not an indigenous species of Denmark, even though there has been multiple interceptions.

The study was not concluded during the MONOCHAMUS project. The consortium took actions to gather information that is more detailed on the distribution of *Monochamus* spp. in the EU-countries. However, we discovered that another group had already begun this work. Instead of doing parallel work we took contact with the researchers involved in this to suggest co-operation on the task. The co-ordinator have made agreement with Jean-Claude Gregoire, Université Libre de Bruxelles, Belgium to continue and finish the work. Prof. Gregoire was participant of ISEFOR project, FP7 [https://cordis.europa.eu/result/rcn/163906\\_en.html](https://cordis.europa.eu/result/rcn/163906_en.html)

### WP3: Life cycle studies

#### **Task 3.1. Case phenological study of Coleoptera species, captured in traps for controlling PWN vector *Monochamus* spp. at the Brdo estate complex location (Slovenia)**

In 2011 and 2012, the entomofauna was collected using black cross vane funnel traps (Witasek PflanzenSchutz) at five different locations in three different ecological regions: Sub-mediterranean, Dinaric and Pre-alpine in forests with 5 dominant conifer species: *Pinus nigra*, *P. halepensis*, *P. sylvestris*, *Abies alba* and *Picea abies*. The targets were PWN vector species (*Monochamus* spp.), but additional catch in form of other Coleoptera species was also collected and preserved. The catch was collected monthly during the growing season (May-October). The preliminary results show that the majority of *Monochamus galloprovincialis* individuals were collected in traps in June (2011) and July (2012).

In 2013, new case phenological study started in the Brdo estate complex near Kranj (Slovenia, Pre-alpine region). The forests of Brdo, which had been proclaimed forests of special purpose, are managed by the State Protocol Services of the Republic of Slovenia in accordance with the valid forest managing plan. The forest stand of 60-65-year old *Pinus sylvestris* on brown soil, developed on silicate and carbonate sediment rock, was selected. A set of 3 traps, baited by  $\alpha$ -pinene + ethanol, GalloProtect 2D and control (without attractants) was lifted to the lower canopy of trees; the distance between the traps was approx. 50 m. In order to capture the early flight of insects, the traps were mounted on 4th March 2013. The samples were collected every 14 days. The study was repeated in 2014. The selected site was located on a plot of Intensive Monitoring Programme in Slovenia (Level II) under the competence of the Slovenian Forestry Institute. The plot provided meteorological and phenological data as well as measurements of air pollution, acidification of soil, nitrogen enrichment and damage of crowns.

### Results

In the 2013-2016 period the sampling of insects in the frame of the monitoring of sawyer beetles (*Monochamus* spp.) at the location Brdo near Kranj (Slovenia) was undertaken.

Witasek cross vane funnel traps were elevated to the lower part of the tree crowns and equipped with different dispensers: Galloprotect 2D®, ethanol +  $\alpha$ -pinene and empty control trap. The traps were activated each year on the first Wednesday of March and emptied at the regular two-week intervals till the second week in December (in total 20 times). The selected sampling location in the forest stand of red pine (*Pinus sylvestris*) was a part of the plot for intensive monitoring of Forest Ecosystems (IMP-SI, level II), carried out by Slovenian Forestry Institute. The results of sawyer beetles monitoring for the years 2013 and 2014 were presented at EUPHRESKO II - *Monochamus* meeting in Slovenia, Ljubljana, 31<sup>st</sup> May – 3<sup>rd</sup> June 2015. These results are summarised in figure 11.



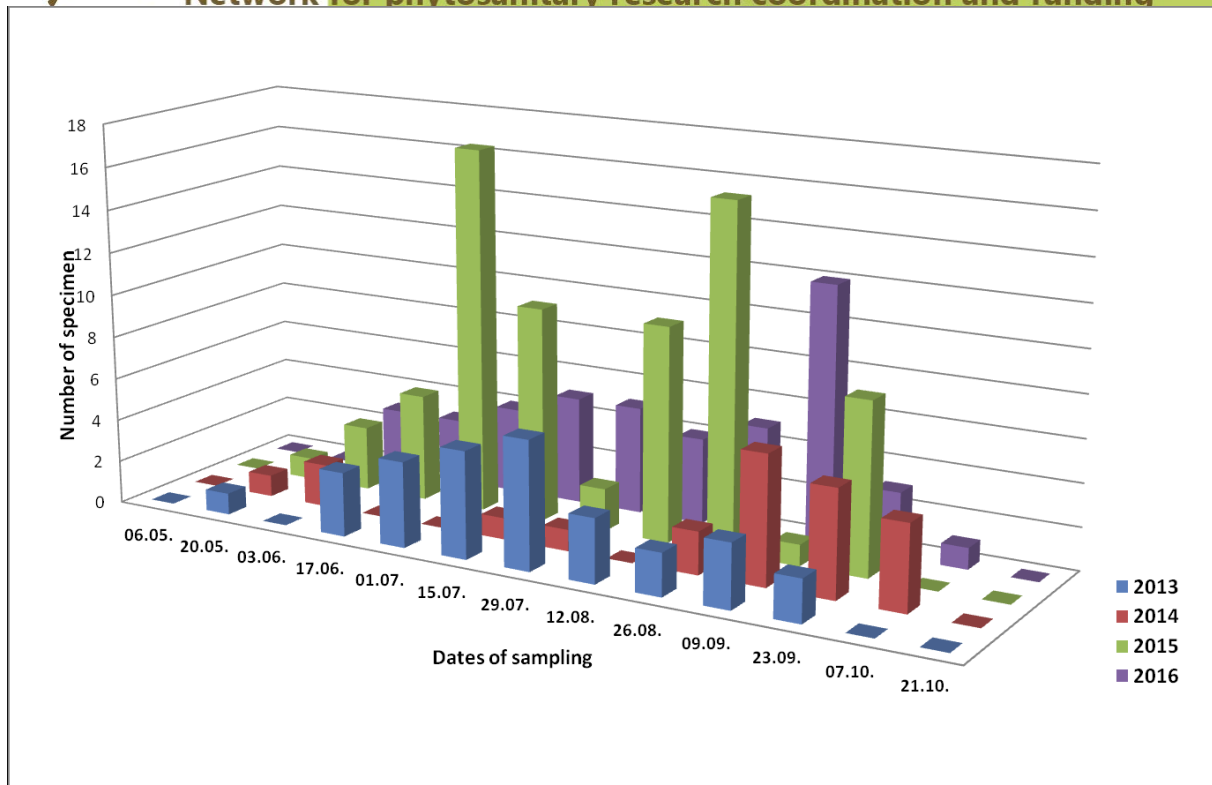


Figure 11. Phenology of *Monochamus* spp. in Slovenia based on trap catches 2013-16.

The most frequent trapped species was *M. galloprovincialis*. The dynamic of the catch varied each year significantly. Sawyer beetles were caught from the second half of May till the first half of October. In 2013 the largest catch was recorded in July and August. The research plot was damaged by the disastrous ice storm in February 2014. Sanitary cut of the damaged trees lasted till the end of September. The mass-trapping of the sawyer beetles did not start until the autumn. In 2015 the number of caught sawyer beetles increased highly, with two peaks of the catch at the beginning of July and at the end of August. In 2016 the catch was more homogeneously distributed, with a peak at the beginning of September. In 2016, the total catch of sawyer beetles on the research plot was higher than the average annual catch before the ice storm.

### Task 3.2. Assessment of the dispersal capacity of *M. galloprovincialis* (Portugal, Belgium)

#### Portugal

##### Materials and methods

A mark-release recapture study was made with immature (105 insect with less than 15 days of age) and mature (110 insects over 16 days of age) nematode-free *M. galloprovincialis*, at Herdade da Comporta in 2011, inside an adult *Pinus pinaster* stand, in Portugal. The marked insects were released in the center of a 8x8 grid of Multifunnel traps, distanced by 200 meters, and lured with Galloprotect 2D-plus (bark beetle and *Monochamus* specific pheromones and  $\alpha$ -pinene as components).



Another study was carried out to assess in more detail the flight behavior of mature adult *M. galloprovincialis* in natural environment and involved the release of marked beetles (30 females and 43 males), in the center of a 55 meters side square plot (0,3 ha) inside a healthy, seven-year old, maritime pine forest at Companhia das Lezírias. All trees in the plot were surveyed and the insects recaptured were placed in the same tree where they were found.

## Results

The first main result of the 2011 assay was that Galloprotect 2D-plus is only efficient for mature insects when searching for weakened hosts to mate and lay their eggs (since the younger recapture beetle had 16 days of age, and 14 days after being released), which reflected on the time between the release and the recapture that was longer for immature beetles ( $34,2 \pm 10,8$  days) than for laboratory matured beetles ( $32,9 \pm 15,4$  day), but the average distance was identical:  $462,6 \pm 231,0$  m for immature beetles and  $392,8 \pm 284,8$ m for beetles released after maturation stage. The average recapture rate was 24,7% and higher for immature released marked beetles, 33,3% against 16,4% of mature beetles. One immature beetle was accidentally recaptured on an identical trap placed around 2.500m from the release site, in the center of the traps grid.

In the second assay, that took place at Companhia das Lezírias, over a third of the released insects (36.2%) were recaptured in the same tree where they had been placed, and 21 days later, the males had flown, on average, 4,97 m (the longest flight distance was 13,9m) while the females had flown 4,3 m (up to a maximum of 14,1 m) (Bonifácio, 2009) (Figure 12).

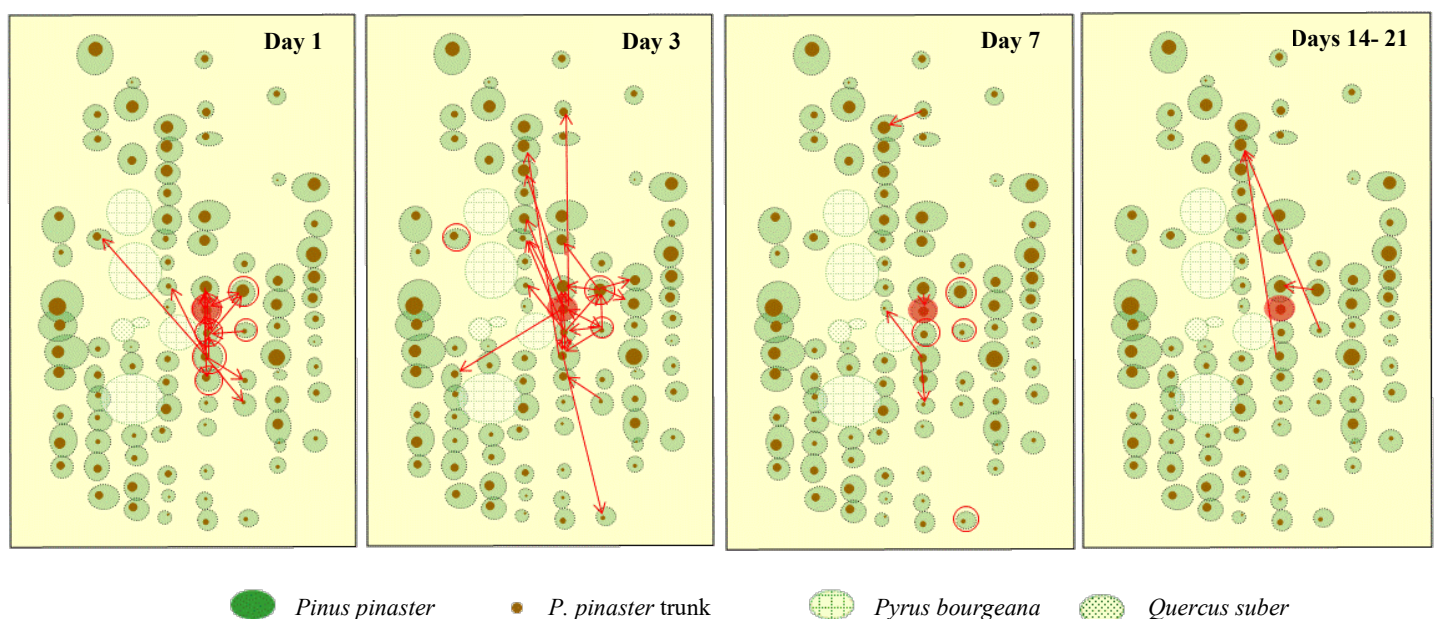


Figure 12. Movement behavior of *Monochamus galloprovincialis* adults released after maturation feeding, at the center (red mark) of a plot, in a maritime pine stand (*Pinus pinaster*). Red arrows represent the flights between

the tree where the beetle was placed and the tree where it was captured. Red circles represent recaptures that occurred on the same tree where the insect had been placed (no movement).

The flight distance travelled by *M. galloprovincialis* was mainly dependent on the size of the pine tree where the flight had started, and the final choice of the tree, depended on the dimensions of the pines surrounding the starting point. The insect chose the tree with the largest canopy.

### Discussion

In Spain, mark-recapture trails found that lifetime adult dispersal was 107–122 m on average, with a maximum dispersal of 464 m (Torres-Vila *et al.* 2015). Similar observations were made in Norway and Sweden with *M. sutor*, which flew an average of 157 m from clear-cut areas in search of branches to feed (Økland *et al.* 2010).

Similar studies conducted in Japan, with *M. alternatus*, also found that the insects perform short distance flights after sexual maturity, staying for several days on the same tree, and average distances traveled were similar 12,3 m for females and 10,6 m for males (Shibata, 1986; Togashi, 1990a).

This low mobility shown by the insect vector, when food sources are available, is apparently the main reason for the clustered mortality pattern associated to PWN infection in Portugal (Bonifácio, 2009), as described in Japan for *M. alternatus* (Shibata, 1986). The marked insects left the study plot and some were recaptured on traps placed in an adult pine stand located more than 250 meters away from the point of dispersal.

Laboratory trials, with insects placed in a flight mill, showed that *Monochamus* have the physiological capacity to perform long flights (up to 10 km) if, hypothetically, they need it. During their entire life span in laboratory conditions ( $99,5 \pm 4$  days since emergence) *M. galloprovincialis* can flight, on average, 15,6 km (males) and 16,3 km (females), but the mean distance covered with a single flight, was around 2 km, for both sexes (David *et al.*, 2014).

Natural dispersion of *Monochamus* results from the sum of short distance flights inside the stands, and longer flights, mainly when they need to fly over areas without suitable hosts. In Japan, the annual natural dispersion of the disease, resulting exclusively from the flight of *M. alternatus*, was estimated to be of 6 km, on average, up to a maximum of 15 km (Togashi & Shigesada, 2006).

The PWD natural expansion, due to the dispersal of the infected insect vectors, occurs in a limited spatial scale, within the original stand, or to neighboring pine forests. The long-distance dispersal is mainly promoted by human activity, through the transport of infected wood. This was the major cause of the disease spread in Japan (Kawai *et al.*, 2006) and also in Portugal, when new outbreaks were detected in late 2007, at municipalities of Arganil and Lousã, located more than 140 km from the northern limit of the area initially affected, at the Setúbal Peninsula.

## Belgium

Several studies have investigated the flight activity of *M. galloprovincialis* reporting distances of 7 km (Hernandez *et al.* (2011) referred to in Torres-Vila *et al.* (2015)), 8.3 km (Gallego *et al.* (2012) referred to in Etxebeste *et al.* (2015)), 13.6 km (Mas *et al.* (2013) referred to in Etxebeste *et al.* (2015)) and 16.3 km (David *et al.*, 2014). However, these are more exceptional observations resulting from experiments in fragmented woodland ecosystems to stimulate long-distance dispersal among forest patches or resulting from experiments using flight mills with tethered adults (e.g. David *et al.* (2014)) in which the insects are driven to their maximum flight capacity. On the other hand, several field studies have also studied the mean lifetime adult dispersal. Torres-Vila *et al.* (2015) and Bonifacio (2009) (referred to in Etxebeste *et al.* (2015)) report a lifetime dispersal of 107-122 m (females-males) and 237 m for *M. galloprovincialis*, respectively. Etxebeste *et al.* (2013) observed that 50 % of the *M. galloprovincialis* adults used in their mark-recapture field experiments did not disperse beyond 40 m when released in a central point in different pine stands. Sanchez-Husillos *et al.* (2015) studied the effective sampling area of the trapping system and its radius  $r$  and estimated 95% of the occurring *M. galloprovincialis* individuals would be removed at a trapping density of 0.82 traps/ha or, in other words,  $r = 197$  m (including a trapping attraction radius between 31-100m for these types of traps (Jactel, 2013; Etxebeste *et al.*, 2015; Torres-Vila *et al.*, 2015)).



#### **WP4: Evaluation of forestry practises and of phenology and biology of relevant vectors and their PWN relationship**

##### **Task 4.1. Case Study: the impact of forest management practice on the populations of *Monochamus* species and other xylophagous beetles, potential vectors of PWN (Slovenia).**

On the locations Snežnik and Trnovo (Slovenia) plots were chosen with a radius of 70 meters, in the forest stands of Norway spruce (*Picea abies*) and silver fir (*Abies alba*) and with three levels of cutting (100%, 50% and 0%). Cross vane funnel traps (Witasek) with two attractants, Galloprotect 2D® and Galloprotect Pack®, and wet collecting cups were put in the middle of each plot. In total 276 specimens of sawyer beetles were collected. Three different species were identified: *Monochamus sartor* (127 specimens), *M. sutor* (104 specimens) and *M. galloprovincialis* (45 specimens). All three species were more frequent on the plots with Norway spruce. On two plots with Norway spruce, the catch on Galloprotect Pack® was significantly higher. However, on one plot with silver fir, the significantly higher catch was detected on Galloprotect 2D®. The highest catch was established on the plots with 100% cutting (Appendix 1, 'Annex 3', p.6).

##### **Task 4.2 Title: Analysis of differences in expression of disease caused by PWN across Europe (Denmark)**

PWN is widespread in North America and occurs in Canada up to 60° latitude. However, the disease PWD is uncommon in North America where many trees are resistant or do not occur in zones warmer than the 20°C mean July air temperature isotherm (Rutherford & Webster 1987). The disease is rarely expressed north of 40° latitude in North America, even in susceptible species (Lawson & Sathyapala 2008). In areas where this pest has been introduced, the economic losses are the result of transmission of PWN to healthy susceptible tree species by its vector during maturation feeding. Once inside the tree the nematodes feed on essential tissues eventually resulting in death of the tree (Naves *et al.* 2007). Nematodes are also transmitted by ovipositing female *Monochamus* to dead or dying trees where they mainly feed on wood inhabiting fungi (Wingfield & Blanchette 1983).

##### **Susceptible host trees**

A list of susceptible tree species is given in Evans *et al.* (1996). They state that *Pinus sylvestris*, *P. luchuenis*, *P. densiflora* and *P. nigra* are the only species reported to have succumbed to PWD as mature trees in the field. More recently severe losses in *P. pinaster* have been reported from Portugal (Mota *et al.* 2009). *P. sylvestris*, which is very susceptible to PWN, is the main species of *Pinus* in Europe, mainly in the northern, eastern and central regions, extending into the Mediterranean region (Evans *et al.* 1996, EUFORGEN 2009a). This species does not occur naturally in areas warmer than the 20°C July isotherm except at higher elevations (Rutherford &



Webster 1987). Other susceptible conifer species, e.g. *P. nigra* and *Picea abies*, are common in Europe (Köble and Seufert 2001, EUFORGEN 2009b, 2009c) and could become infested with PWN if it spreads further.

### **Climate conditions correlated with PWD**

Environmental factors determine whether a susceptible tree is affected and dies. High temperature seems to be most important, maybe in combination with water stress as described in a number of papers. The following observations have been made on the distribution of PWD in relation to macroclimatic conditions: development of PWD is predicted to be severe in areas with summer temperatures above 25°C and annual rainfall less than 600 mm (Braasch & Enzian 2004). Trees stressed by low rainfall and mean temperatures above 25°C for 55 days have shown the greatest damage (Takeshita *et al.* 1975, referred in Rutherford & Webster 1987). According to other sources the expression of disease coincides with the 25.2 °C isotherm in July or August (Yokoberi 1986, referred in Evans *et al.* 2008); in Japan it is confined to areas warmer than the 20°C August mean air temperature isotherm and in North America the 20°C July mean air temperature isotherm, or where the July or August mean air temperatures exceed 20°C over several weeks (Rutherford & Webster 1987).

Soliman *et al.* (2012) concluded, based on climate modelling for Europe, that if uncontrolled, PWD would spread from Portugal to all of Spain, the southern part of France and parts or all of Italy by 2030, depending on the model applied. They used a temperature threshold value of 20°C (average summer temperature in July and August) to identify areas suitable for the development of disease. Evans *et al.* (2008) performed model simulations that showed that in the UK (where the soil water content is high and conditions were less favorable to PWN growth) trees would survive an infestation for longer than the two years of modeling. The model results suggest that seasonal drought and/or high temperature represent a stress situation which is made more serious with a PWN infestation.

Several studies using small *P. sylvestris* inoculated with PWN under controlled climate conditions show varying results regarding the lower temperature threshold for tree mortality. Sikora & Malek (1991) found no tree death at 18°C and below when observed after 8 weeks. Melakeberhan *et al.* (1992) reported 22% mortality at 15°C after 310 DD (day degrees) above 10°C as opposed to almost 75% tree mortality at 20 and 25°C after 270 and 240 DD, respectively. Braasch (2000) found very little tree mortality at 15°C, but at 20°C and above tree mortality was higher, approaching 100% at 30°C. Several studies have dealt with the population development of PWN at low temperatures: Futai (1980) studied the rate of development of PWN grown on a fungus culture in relation to temperature and determined the developmental zero point to be 10.0°C. Development at 25 and 30°C required approximately 60 DD (day-degrees) above 10°C. Melakeberhan *et al.* (1992) found that the PWN population increase 215 DD days after inoculation was low and very similar at 15, 20 and 25°C in symptomless trees. In dead trees the population increase at 20 and 25°C was twice as high as it was at 15°C, all after 215 DD. Population increase of PWN was

very slow at 15°C compared with 20 and 25°C (Daub 2007). However Tomminen (1993) found that at 14°C PWN reproduction on a fungus culture was poor, reaching a maximum of 10 times after 20 days, in comparison with 400-500 times reproduction after 5 days at 34°C. Zhao *et al.* (2007) found that low temperature is important for the development of dispersal stages of PWN, and suggest that this relationship is important in synchronizing the emergence of suitable stages in relation to its vector. In conclusion it could be suggested that temperature dependant asynchronisation between vector and PWN could be the explanation why PWN causes pinewilt above 20° C July and August isotherm.

### **Other stress factors for trees**

It has been suggested that the threshold temperature for expression of PWD may be lower under conditions of water stress (Magnusson 1986). Mamiya (pers. com. in Magnusson 1986) considers water stress to be even more important than high temperatures. It has been suggested that low light intensity (shade) may speed up the appearance of disease symptoms as a result of a reduction in photosynthetic activity (Kaneko 1989).

Other factors may make trees more susceptible to PWD, e.g. environmental pollution, presence of other pathogens, poor site conditions, high tree density, severe winter climate etc. (OEPP/EPPO 1989, PHRAME 2007). In addition, fungi may play an important role in pine wilt, e.g. certain wood inhabiting fungi that serve as a food source for the nematodes (Wingfield 1987, Maehara & Futai 1996, 2000). The amount of mycorrhizal fungi on pine roots differs somewhat among lightly and heavily damaged pine stands (Ugawa *et al.* 2009). It has been suggested that mycorrhiza may mitigate the effect of stress due to drought, and thus the development of pine wilt (Akema & Futai 2005).

### **PWN migration and dispersal by vectors**

The dispersal of PWN to new trees may occur more slowly in cooler climates due to several factors and the development time of the vector may be prolonged up to 2 years. In addition, at the time of emergence of adult vector beetles the nematode population in the tree may be very low and thus the number carried by the beetle to a healthy tree is low (Zinno *et al.* 1987, referred in Rutherford *et al.* 1990). Iwahori & Futai (1995) found that speed of migration of PWN within a tree was greatly reduced at temperatures below 15°C: very little movement occurred at 5 and 10°C. Jikumaru & Togashi (2000) found that nematode transmission by vectors was delayed and the number of nematodes was lower at low temperatures. Furthermore, they found that the longevity of the vector *Monochamus alternatus* was lower at 16°C compared to 20 and 25°C. In Portugal development of the vector *M. galloprovincialis* takes approximately one year (Naves *et al.* 2008). Day degree estimates suggest that development of the vector may be prolonged to two years or more under cooler climates (Naves & Sousa 2008). All these factors may affect the incidence and speed of development of the disease in areas with cool climate.

## Virulence and persistence of PWN

The virulence of isolates of PWN from different locations varies. This variation has been related to many factors (differences in reproductive potential, behavior, in the amounts and types of phytotoxins, cell wall degrading enzymes, and surface coat proteins that can affect the ability of PWN to evade host immune responses etc.) (references in Futai 2013). There seems to be differences in the biology of different strains of PWN, which may affect their relative pathogenicity (Rutherford *et al.* 1990). Halik & Bergdahl (1994) inoculated 20 year old *Pinus sylvestris* in a cool climate (northeastern USA) with PWN. Nematodes could be found in 20% of the trees after 6 years, but a gradual decline was observed in the PWN population in the majority of inoculated trees. At that time half of the inoculated trees from which PWN had been extracted were still alive and healthy. They also found that PWN persisted for up to 2 years after a tree had died. This study indicates that in cool climates the development of disease is slow but eventually occurs, maybe with a lower prevalence. The long persistence of PWN in dead trees in cool climates is a factor that must be taken into account if PWN spreads to northern Europe.

## Conclusion

Based on the available literature it seems that the PWN will be able to survive and reproduce in northern temperate regions of Northern Europe. However, once the PWN has become introduced, many of the above stated factors in combination may result in a slower dispersal in cooler than it does in warmer areas, i.e. where the July mean temperature is above 20 to 25°C. More specifically: low temperatures mean slower buildup of PWN populations in the trees, longer development time for the vector, decreased transmission of PWN by vectors, slower speed of movement of PWN within the host tree and suboptimal synchronization between vector and nematode life cycles.

In northern Europe, PWN may be transmitted to healthy, susceptible trees but the infestation will not necessarily lead to PWD and tree death. As susceptible tree species are readily available in northern Europe, this will not be a limiting factor to the spread of the disease. Development of the disease seems to require high temperatures so this is unlikely to occur under the climate conditions found at present in most northern European countries.

For example, in Denmark the July and August mean temperatures are below 16°C, and annual precipitation is 700 mm. These conditions are not considered to be conducive for development of disease in trees in Denmark even though susceptible trees are common. In addition, the insects that have been described as vectors of the PWN are not present in Denmark (IIPC 2011) and a few other northern European countries (U.K. and Ireland) (Evans *et al.* 1996).

Climate change will probably not lead to the temperature increase which seems to be necessary for the development of disease within the near future. On the other hand, once a tree has become infested, the nematode population may persist for several years. If the trees experience significant stress due to water stress, pollution or

presence of other pathogens, and a moderate temperature increase occurs, PWD may be able to develop in certain locations.

#### **Task 4.3. Evaluation of non-target insects, captured in cross vane funnel traps baited by attractants based on kairomonal components for monitoring of *Monochamus* populations (Portugal and Belgium)**

##### Materials and methods

Trapping *M. galloprovincialis* was a main strategy in the management of Herdade da Comporta pine forest affected by the Pine Wilt Disease, from 2009 to 2014. Every year a grid of traps (mainly multifunnel traps) was placed inside the most affected areas and along the borders of the pine stands, from May to October, with generalist lures (bark beetles and Galloprotect 2D-plus). The traps were visited periodically (from weekly to monthly) to collect the trapped insects and renew the lures (every six weeks).

Belgium & the Netherlands: The trapping network described in Task 1.2 was used, as was the field experiment evaluating 2 trapping setups (type A and B) in Belgium resp. Netherlands described in Task 1.3.

##### Results

###### Portugal

During a six-year monitoring period the traps captured 15,000 *M. galloprovincialis* adults. However, they represented less than one percent of the bark and wood boring insects caught (over 2,200,000 insects), where the bark beetle *Orthotomicus erosus* (Wollaston) was massively captured and represented 98% of total captured insects. Considering other wood-boring species several cerambycids were also caught in significant numbers, during these six years trapping: 4,500 *Spondylis buprestoides* (L.), 2,650 *Acanthocinus griseus* F., 900 *Ahropalus syriacus* (Reitter) and fewer (less than 10) *Ahropalus ferus* Mulsant, *Pogonocherus perroudi* Mulsant, *Rhagium inquisitor* L. and *Trichoferus* spp. Some buprestids, namely *Chrysobothris solieri* Gory & Laporte, *Calcophora mariana* (L.), and *Anthaxia* spp. and weevils (Curculionidae) such as *Pissodes castaneus* (De Geer.), were also caught every year but less than 1 beetle per trap, in average. More important were over the Coleoptera predators, about 2,000 specimens mainly from Cleridae (i.e. *Thanasimus formicarius* (L.) and *Opilo domesticus* (Sturm)), Trogositidae (i.e. *Temnochila coerulea* (Olivier)), but also Colididae and Histeridae.

###### Belgium

The non-*Monochamus* longhorn species captured in the Belgian traps are reported in Table 1 and Table 3. Non-longhorn arthropods captured during the field experiment described in task 1.3 were also identified morphologically to either the (super)family or (sub)order taxonomic level (see table 4).



Table 4. Total number of non-longhorn arthropod individuals caught in Belgian pine stands during 2014 per taxonomic group and trap type system (A = cross-vane trap + Galloprotect Pack attractants, or B = panel trap and Galloprotect 2D attractants, each system consisted of 5 traps set up at Eindepoel, Elsakker, Withoefseheide, Kolisbos and Kloosterbos)

Taxonomic group	Type A	Type B
<i>Acariformes</i>	11	0
<i>Aphididae</i>	24	3
<i>Apoidea</i>	3	30
<i>Araneae</i>	19	34
<i>Caelifera</i>	0	1
<i>Cantharidae</i>	1	3
<i>Carabidae</i>	9	39
<i>Cleridae</i>	688	605
<i>Coccinellidae</i>	0	1
<i>Collembola</i>	10	1
<i>Culicomorpha</i>	2	8
<i>Curculionidae</i> *	8	15
<i>Cynipoidea</i>	12	0
<i>Dermaptera</i>	0	10
<i>Diplopoda</i>	1	0
<i>Diptera</i> *	15	10125
<i>Ectobiinae</i>	12	13
<i>Elateridae</i>	67	27
<i>Endopterygota</i>	4	3
<i>Formicidae</i>	52	3
<i>Heteroptera</i>	16	14
<i>Histeridae</i>	0	1
<i>Ichneumonidae</i>	1	13
<i>Isopoda</i>	13	36
<i>Ixodoidea</i>	2	0
<i>Lepidoptera</i>	3	502
<i>Mecoptera</i>	0	4
<i>Monotomidae</i>	1607	1221
<i>Sciaridae</i>	1	2
<i>Scolytinae</i>	142	263
<i>Silphidae</i>	198	116
<i>Staphylinidae</i>	5	357
<i>Vespidae</i>	0	5
<i>Vespoidea</i> *	1	43

Curculionidae\* = all Curculionidae individuals exclusive the Scolytinae individuals; Vespoidea\* = all Vespoidea individuals exclusive the Formicidae and Vespidae individuals; Diptera\* = all Diptera individuals except the Culicomorpha and Sciaridae individuals

In general, more longhorns were captured in the type A trapping system than in type B ( $p=0.024$ , negative binomial regression). Extremely high numbers of Diptera and Lepidoptera were found in the type B trapping system compared to the type A

trapping system ( $p \leq 0.001$  for both groups, negative binomial regression). In addition, more Staphylinidae individuals were captured in the type B traps than the type A traps ( $p \leq 0.001$ , negative binomial regression). On the other hand, more Formicidae individuals were captured in the type A system ( $p=0.016$ , negative binomial regression). No significant differences between both trapping types were found for the other arthropod species.

## Discussion

### Portugal

The removal from the pine stands of the caught *M. galloprovincialis* had a clear impact on the PWD spread control (along with other measures, such as the dead pines eradication in the winter months). Since the detection of the first infected pines in Portugal in 1999, enormous efforts have been made to control the disease, namely by cutting and destroying the infested trees before the exit of the insect vector and by capturing the *M. galloprovincialis* insect vector during its flight period.

Many traps were developed to catch conifer bark and wood-boring beetles. They can be divided into different types: sticky traps (Ikeda *et al.*, 1980; Fatzinger, 1985), transparent flight interception (Billings & Cameron, 1984), silhouette or barrier traps (i.e. multifunnel) (Lindgren, 1983; Chénier & Philogène, 1989b; Nakamura *et al.*, 1999; Groot & Nott, 2001; McIntosh *et al.*, 2001). Comprehensive comparison studies were made to establish the most effective for *Monochamus* beetles (Pajares *et al.*, 2004; Bonifácio *et al.*, 2012; Álvarez *et al.*, 2015).

Host selection by bark and wood borers is based on chemical odors and its acceptance regulated by tasting, all depending on the volatile terpene content of the wood (Hanks, 1999; Faccoli *et al.*, 2005; Ginzl & Hanks, 2003; Allison *et al.*, 2004).

As in other parts of the world, initially in Portugal only kairomone lures from the maritime pine tree host (ethanol, turpentine and  $\alpha$ -pinene) (Chénier & Philogène, 1989a; Dyer & Seabrook, 1978; Zhang *et al.*, 1993; Bonifácio *et al.*, 2012), and bark-beetles pheromones (Ipsenol, Ipsdienol and Methyl-butenol) (Pajares *et al.*, 2004; Bonifácio, 2009) were used. Only later a specific pheromone was identified as 2-undecyloxy-1-ethanol (Ibeas *et al.*, 2008; Pajares *et al.*, 2010), and its inclusion increased significantly the numbers of *Monochamus* caught. However, the use of pine volatiles combined with ethanol, and bark beetles pheromones attract many other insects that colonize pine trees (Borden *et al.*, 1982; Schroeder & Lindelow, 1989; Byers, 1992; Bonifácio *et al.*, 2012). The impact of non-target predator species was previously reported as a major problem of the *M. galloprovincialis* trapping techniques that needed to be studied in order to be minimized (Pajares *et al.*, 2004; Bonifácio 2009).

### **Task 4.4. Analysis of the mass of dead wood in forests as habitats of longhorn beetles and other potential vectors PWN (Belgium)**

Dead wood of conifers represents potential habitat for longhorn beetles. Model of potential habitat for longhorn beetles will be prepared on the basis of dead biomass

data from sample plots, which follows the rules of the United Nations Economic Commission for Europe (UNECE), Convention on Long-range Transboundary Air Pollution and International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests). The data from Level I and Level II sample plots will be supplemented with data from timber database which is in the domain of Slovenia Forest Service. Model validation will be performed on the basis of data from catches in traps that will be set on sample plots.

### Materials and methods

In 2013, one *M. galloprovincialis* adult was captured in a pine stand in Wachtebeke (BE) during the national Belgian survey. We performed a more comprehensive monitoring of this pine forest in 2014 due to the presence of several trees exhibiting evidence of cerambycid activity (Figure 13) in proximity to the trap.



**Figure 13.** Symptoms of cerambycid beetles, exit holes in dying pine trees, in Kloosterbos (Wachtebeke) in the autumn of 2013.

On 20/03/2014 a total of 16 damaged trees were felled in Kloosterbos (Wachtebeke). Each trunk from about 0.5 m to 4 m from the ground, was cut into smaller segments of about 60-80 cm. The segments having visual damage were collected and transported to ILVO for further research.

A total of 20 trunk segments were cleaved open in search of Cerambycid larvae. The larvae were placed individually in Eppendorf tubes and then stored at -20°C. Morphological identification of larvae of the Cerambycid species *A. rusticus* and *S. buprestoides* is straightforward using a stereomicroscope based on the position of the urogomphi (identification was confirmed via molecular analysis and sequencing of a small set of larvae; DNA was extracted and the CO1 region was amplified using the LCO1490 and HCO2198 primers and the protocol designed for Coleoptera during the Q-bol project<sup>1</sup>. The other larvae were examined morphologically using larval-identification keys of *Monochamus* spp. and *Rhagium* spp. However, due to the more challenging task of identifying these larvae, further identifications were performed

<sup>1</sup> QBol project available at [http://www.q-bank.eu/arthropods/LocalFiles/Protocols%20Arthropods\\_2.pdf](http://www.q-bank.eu/arthropods/LocalFiles/Protocols%20Arthropods_2.pdf)



using molecular techniques (using the same protocol designed by the Q-bol project, see above for *A. rusticus*).

Simultaneously, an experiment was conducted in which 30 trunk segments were placed individually under a cylindrical netting cage and maintained in a glasshouse at ambient temperatures. The segments were placed on growing-mats which were moistened twice a week to prevent desiccation. Each trunk segment was inspected periodically from 31/03/2014 to 08/08/2014 for naturally emerging arthropods.

### Results

ILVO collected 500 Cerambycid larvae from the 20 trunk segments that were dissected. A total of 462 larvae were morphological or molecular identified as *A. rusticus* (92,4%), *R. inquisitor* (3.4%) or *S. rubra* (2.4%). The insects emerging naturally from the trunk segments are shown in Table 5. No *Monochamus* spp. emerged from the segments, although *Monochamus* spp. was captured in the pine stand the previous year, however, several other Cerambycidae and their natural enemies (parasitoids and predators) emerged from the trunks.

**Table 5. Number of adult insects (unless specified) emerged from 30 naturally damaged caged pine trunk-segments. Numbers are shown for each species per month.**

Species	April	May	June	July
<i>Rhagium inquisitor</i>	2			
<i>Arhopalus rusticus</i>		10	174	75
<i>Stictoleptura rubra</i>			1	
<i>Sirex noctilio</i> (Hymenoptera: Siricidae)				10
<i>Odontocolon dentipes</i> (Hymenoptera: Ichneumonidae)	41			
<i>Rhyssa persuasoria</i> (Hymenoptera: Ichneumonidae)		1	1	
Pyrochroidae larva (Coleoptera)	1			

### Discussion

The Cerambycid diversity collected from the trunks is similar to the dominant diversity captured in the traps, except for the high numbers of *S. buprestoides*. The latter could be explained by the fact that segments from 0.5 to 4m above the ground were collected. Although no *Monochamus* individual was found in the damaged trunks, it is not clear whether *M. galloprovincialis* is endemic in low populations to Belgium or whether the individuals captured were related to possible import. The results of this experiment are thus inconclusive for the exploitation of dead wood by *Monochamus* spp.



## WP5 Molecular Identification

### **Task 5.1. Molecular identification of *Monochamus* specimens based on molecular markers (Slovenia)**

Molecular analyses of *Monochamus* specimens, trapped in Slovenia in 2014, 2015 and 2016 were performed. Nucleotide sequences were deposited in the International Nucleotide Sequence Database. Relevant literature was checked and phylogenetic trees were calculated based on sequences, obtained during this research and from publicly available databases. A diagnostic protocol for molecular identification of species from *Monochamus* genus was developed. The insight into genetic variability of four species from the genus *Monochamus*, which have been reported from Slovenia, was performed partially. Namely, specimens trapped in 2015 for this study (identified by morphological and molecular markers) were all *M. galloprovincialis*. Six wet-trapped specimens (years 2013, 2014, 2015 and 2016) representing species *M. sutor*, *M. sartor* and *M. saltuarius* were thus added to this study.

The protocol of extracting DNA from live trapped beetles proved to be successful and was also partially successful with specimens which were wet-trapped. Wet-trapped specimens can have contaminations and the DNA could be degraded. One gene region was successfully sequenced at almost all analyzed specimens - part of cytochrome oxidase I gene (COI). Based on phylogeny, this region is informative enough to enable identification of European *Monochamus* species, nevertheless the sole identification based on one gene region and without morphological examinations should be performed with great caution, especially since sequences of *M. sutor* for gene region COI are grouping inside *M. galloprovincialis* clade. Numerous ambiguous sites were recognized in COI sequences obtained during this study.

When these sites were excluded from the further analyses, the dataset still gave groupings related to species level. European *Monochamus* species are supposed to be phylogenetically related, the most ancestral seems to be *M. saltuarius*. Sequence obtained from *M. grandis* (source GenBank) groups closely with European *Monochamus* species, thus suggesting close relationship with *M. sutor*, *M. urusovi*, *M. galloprovincialis* and *M. sartor*. Interestingly, this specimen is according to GenBank caught in Japan, Tokyo. There is a limited number of *Monochamus* related sequences deposited in publicly accessible databases (eg. GenBank) and little research is conducted on the phylogenetic relationships inside *Monochamus* genus, with little emphasis on the broader worldwide insight. Nevertheless, we believe that COI sequence information can be successfully implemented for diagnostic purposes, especially in combination with morphological identification and can serve as a diagnostic tool to distinguish European from non-European *Monochamus* species. Identifications based on molecular (genetic) analyzes are of great importance when the incoming material is not suitable for morphology (eg. larvae, damaged specimens, ...).



The other gene region - 28S - was successfully sequenced only for five specimens of *M. galloprovincialis*. In the GenBank, only three (3) sequences were available from *Monochamus* genus. Phylogenetic comparison of Slovenian specimens revealed groupings with *M. galloprovincialis* (KC692744) and can as such also be an additional confirmation for identification. At this moment, due to the problems we had with this region (unsuccessful sequencing) and lack of reference sequences in GenBank, this gene region (28S) is less appropriate for molecular based identification. Identifications of collected *Monochamus* specimens based on selected molecular markers (COI and 28S rDNA) represent additional confirmation of morphological determinations and are as such an important evidence for the presence of *Monochamus* species in Slovenia.

A molecular test for the identification of the four known *Monochamus* species in Slovenia were delivered and put into practice.



Specimens of *Monochamus*: from left to right - *Monochamus galloprovincialis* Olivier, 1795; *Monochamus sartor* Fabricius, 1787; *Monochamus saltuarius* Gebler, 1830; *Monochamus sutor* Linnaeus, 1758 (photo's: Maja Jurc)

### **3.3 Main conclusions**

The project has fulfilled most of its objectives with respect to optimizing monitoring methods, mapping the incidence of PWN and vectors, studying the pest's phenology and life cycle in different part of Europe and developing molecular tests for its identification:

- During the project testing of different types of lures and traps at the end resulted in a combination that could be recommended for use in all countries and that had proven to efficient (discovered presence of Monchamus in a country where its presence had not been recorded before)
- The national plant health responsible authorities have been involved in this development. This means that the dissemination of results has already taken place
- There could still be improvements. E.g. the trap design could be further improved to enhance escape of beneficial and other non-target insects. However, this could be considered more an ethical than a practical problem.
- Incidence mapping is an on-going process. It has been commenced during this project but not finished.

### **3.4 Research articles, other publications and dissemination activities linked to the project**

#### **Belgium:**

Boone C., Berkvens N., Sweeney J., Silk P., Hughes C., Webster R., Stephen F., Maclauchlan L., Bentz B., Casteels H., Grégoire J.-C. (2013) Detection of exotic *Monochamus* spp. in Belgium: testing the tools in the areas of origin. Oral presentation at International joint IUFRO-REPHRAME-EUPHRESKO conference: Pine Wilt Disease 2013, Braunschweig, Germany (12-15 October 2013)

Berkvens N., Casteels H., Damme N., Bighiu A., Witters J., Grégoire J.-C., Boone C., Michelante D., Viaene N. (2013) *Bursaphelenchus xylophilus* does not occur in Belgium, but what about its vectors, the *Monochamus* spp.? Poster presentation at International joint IUFRO-REPHRAME-EUPHRESKO conference: Pine Wilt Disease 2013, Braunschweig, Germany (12-15 October 2013)

Berkvens N., Viaene N., Boone C., Grégoire J.-C., Michelante D. and Casteels H. (2014) Vector required: the case of the quarantine pine wilt nematode *Bursaphelenchus xylophilus* and the *Monochamus* longhorn beetles in Belgium. Oral presentation at *Aliens on the Horizon Congress* (BELSPO, 12 March 2014, Brussels)

Berkvens N., Viaene N., Waeyenberge L., de Sutter N., Boone C., Grégoire J.-C., Michelante D., Casteels H. (2015) Vector required; the case of *Bursaphelenchus xylophilus* and *Monochamus* spp. for Belgium. Oral presentation at International Symposium on Crop Protection (Ghent, May 2015)

#### **Netherlands:**

Heijerman, Th. & Noordijk, J. 2014. *Monochamus*-inventarisatie nabij risicolocaties. – Rapport EIS2014-04. EIS Kenniscentrum Insecten, Leiden.

Heijerman, Th., Noordijk J., Keijl, G.O. & Smit, J.T. 2015. *Monochamus*-monitoring 2014, met een vergelijking van twee vangstmethoden. – Rapport EIS2015-02. EIS Kenniscentrum Insecten, Leiden.

Heijerman, Th. & Noordijk, J. 2016. *Monochamus*-monitoring 2015: inventarisatie van zwarte den-opstanden in Noord-Holland. – Rapport EIS2016-02. EIS Kenniscentrum Insecten, Leiden.

#### **Slovenia :**

Pavlin, R., Meterc, G., Borkovič, D., Hauptman, T., Jurc, M., 2016. Pregled monitoringa žagovinarjev (*Monochamus* spp., Cerambycidae), vektorjev borove ogoričice (*Bursaphelenchus xylophilus*) v Sloveniji (2007-2015) = Review of the monitoring of sawyer beetles (*Monochamus* spp., Cerambycidae), vectors of the pine wood nematode (*Bursaphelenchus xylophilus*) in Slovenia. (2007-2015). V: JURC, Maja (ur.). Invazivne tujerodne vrste v gozdovih ter njihov vpliv na trajnostno rabo



gozdnih virov: zbornik prispevkov posvetovanja z mednarodno udeležbo = Invasive alien species in forests and their impact on the sustainable use of forest resources: lectures presented at the conference with international participation. Ljubljana: Biotehniška fakulteta, Oddelek za gozdarstvo in obnovljive gozdne vire, 2016, str. 59-69. [COBISS.SI-ID 4362918].

Jurc, M., Hauptman, T., Pavlin, R., Borkovič, D., 2016. Target and non-target beetles in semiochemical-baited cross vane funnel traps used in monitoring *Bursaphelenchus xylophilus* (PWN) vectors in pine stands. *Phytoparasitica*, ISSN 0334-2123, 2016, vol. 44, iss. 2, str. 151-164, ilustr. <http://dx.doi.org/10.1007/s12600-016-0515-4>, doi: 10.1007/s12600-016-0515-4. [COBISS.SI-ID 4367014],

Hauptman, T., Jurc, M., Pavlin, R., 2016. Žagovinarji (*Monochamus* spp.) - vektorji borove ogorčice (*Bursaphelenchus xylophilus*): predavanje na delavnici Karantenski škodljivi organizmi za gozdne in druge lesnate rastline: delavnica za fitosanitarne in gozdarske inšpektorje, 28 September 2016, Ljubljana, Gozdarski inštitut Slovenije. <http://www.zdravgozd.si/dogodki.aspx?iddogodek=13>. [COBISS.SI-ID 4522662]

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Pavlin, R., Borkovič, D., Hrvatin, M., Jurc, M., 2015. Ulov hroščev (Coleoptera) v pasteh za monitoring žagovinarjev (*Monochamus* spp.) v Luki Koper s povdarkom na tujerodnih vrstah. Predavanje na 6. seminarju in delavnici iz varstva gozdov, Kostanjevica na Krki, 16. junij 2015. <http://www.zdravgozd.si/dat/dogodki/90.pdf>.

Pavlin, R., Meterc, G., Jurc, M., 2015. Primerjava učinkovitosti atraktantov Galloprotect 2D in Galloprotect pack za spremljanje žagovinarjev (*Monochamus* spp., Cerambycidae), vektorjev borove ogorčice (*Bursaphelenchus xylophilus*). V: TRDAN, Stanislav (ur.). Zbornik predavanj in referatov 12. slovenskega posvetovanja o varstvu rastlin z mednarodno udeležbo, Ptuj, 3.-4. marec 2015 = Lectures and papers presented at the 12th Slovenian Conference on Plant Protection with International Participation, Ptuj, March 3-4 2015. Ljubljana: Društvo za varstvo rastlin Slovenije: = Plant Protection Society of Slovenia, 2015, str. 242-247.

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