Effects of the wet catching method on the detection of 
*Bursaphelenchus xylophilus* from trapped longhorn beetle vectors

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Summary – The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, causes pine wilt disease and is transmitted by *Monochamus* beetles. An efficient trapping system for these organisms is essential for their early detection in threatened regions. A wet catching method, using monoethylene glycol (MEG) in a collecting cup to preserve captured insects, was suitable for catching longhorn beetles. As a second step in developing this technique, we studied the influence of MEG on nematode detection. When *M. galloprovincialis* carrying PWN were submerged in MEG, nematodes died within 24 h. The preservative altered certain morphological features of nematodes immersed in solutions of 10, 30 or 60% MEG, impeding their morphological identification. However, molecular identification was possible as long as the MEG concentration was below 70%. At higher concentrations, a rinsing step with water before DNA extraction was enough to allow molecular detection. Wet trapping requires less frequent monitoring than dry trapping, thus reducing maintenance and related costs.

Keywords – invasive species, monitoring, *Monochamus galloprovincialis*, monoethylene glycol, pine wood nematode.

The pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 (Nematoda: Aphelenchoididae), is native to North America (Nickle *et al.*, 1981). It was introduced into Japan at the beginning of the 20th century via infested timber, and was subsequently spread to other Asian countries (*e.g.*, China, Taiwan and Korea) where it has had severe economic and ecological consequences by causing the pine wilt disease (PWD) in numerous pine forests (EPPO, 2013; Futai, 2013). When PWN infest a susceptible pine tree, the nematodes can disrupt physiological processes by destruction of cell structures, *e.g.*, through feeding on the epithelial cells around the resin canals and also through population build-up resulting in blocking of vascular tissues. Depending on several factors, mainly determined by host status and environmental conditions, a subsequent collapse of the plant’s upward water transport system results in wilting and death of the tree (Evans *et al.*, 2008). In 1999, PWN was found for the first time in Europe, in Portugal (Mota *et al.*, 1999), and subsequently caused large-scale mortality of *Pinus pinaster* Aiton trees. Despite intensive and expensive eradication measures in Portugal, the PWN spread to the centre of the country (Rodrigues *et al.*, 2015) and was detected several times in neighbouring Spain since 2008 (Abelleira *et al.*, 2011), demonstrating the prominent threat to other European countries. European pine stands are susceptible to the disease and the warm temperatures required for disease development occur in many European countries (Evans *et al.*, 1996, 2008). This has led to the PWN at-

PWN spreads via the trade of infested timber or wood-related products. Short-distance spread can also occur via its transportation by longhorn beetles of the genus Monochamus Dejean (Coleoptera: Cerambycidae) (Linit & Akbulut, 2008). Five Monochamus species are endemic to Europe: M. galloprovincialis (Ol.), M. sartor (F.), M. sutor (L.), M. saltuarius Gebler and M. urussovi (Fisher von Waldheim). The outbreaks of PWN in Portugal are associated only with the vector M. galloprovincialis (Souza et al., 2001). The insect transmits the nematodes at two stages: healthy trees can be inoculated during maturation feeding by PWN-infested adult beetles, whereas dying or dead trees can be inoculated during oviposition by PWN-infested female insects (Naves et al., 2015).

PWN is presumed to be absent in Belgium. From 2000 up to 2012, numerous wood samples were collected during phytosanitary controls of imported commodities and during national surveys in pine stands, public green areas and logging and wood processing facilities; PWN individuals were never detected (Berkvens et al., 2013). Two live Monochamus individuals, one adult and one larva, were intercepted in imported wood packaging in 2009 and 2010, respectively (Berkvens et al., 2013). Monochamus spp. appear to be very rare in Belgium. The Belgian collections contain three M. galloprovincialis, two M. sartor and four M. sutor, caught between 1870 and 1960. One M. urussovi and one M. alternatus Hope were intercepted in 2005 and 2004, respectively (A. Drumont, pers. comm.) During a mini-survey comparing two trap types, five adults of M. galloprovincialis (Fisher von Waldheim) were trapped in 2009 and one other M. galloprovincialis adult in 2014. The origin of these specimens is unknown (Berkvens et al., 2015). In addition to these Monochamus spp., other longhorn beetles were trapped during this mini-survey, of which Arhopalus rusticus, Spondylis buprestoides and Rhagium inquisitor were the most frequently captured (data unpubl.).

Two sets of tools are imperative in preventing the entry and establishment of a threatening invader: first, phytosanitary measures combining (inter)national regulations concerning trade with inspections of imported commodities that are possible carriers; secondly, the establishment of an early warning system, which prompts a rapid response against the possible establishment of an invader as it is known that the faster the response, the higher the likelihood of controlling the invader (Álvarez et al., 2015). PWD is assumed to be asymptomatic in pine trees in Belgium and other northern European countries based on an eco-climatic model developed by the European FP7 REPHRAME project (http://www.rephrame.eu). The goal of this study was to develop an efficient early warning system targeting the known PWN longhorn beetle vectors that could be examined for the presence of PWN. We assessed the ability of a wet catching strategy because previous monitoring by the Belgian NPPO (FASFC) indicated that dry catching methods must be inspected every 5-10 days due to the destruction of captured specimens by necrophagous insects such as the carrion beetles Nicrophorus vespilloides, N. humator and N. investigator (Coleoptera: Silphidae) (Berkvens et al., unpubl. obs.). We investigated a wet catching method using a preservative liquid, monoethylene glycol (MEG), which kills insects, including the necrophagous ones, thus preventing destruction of the captured insects and the nematodes that they might carry. We studied the effects of MEG on the detection and identification of PWN individuals, directly emerged in this liquid preservative or present inside emerged Monochamus beetles.

Materials and methods

Effect of MEG on survival, morphology and molecular detection of PWN

Nematodes of B. xylophilus were obtained from cultures using the fungus Botryotinia fuckeliana (de Bary) Whetzel (1945) (syn. Botrytis cinerea) grown on malt agar (Schröder et al., 2009). They consisted of juvenile stages and adult females and males but contained no dauer or any pre-dauer stages. Staining glasses were filled with either pure rainwater, pure MEG or a dilution of MEG with rainwater (10, 30 or 60% MEG); three staining glasses were used for each solution (15 staining glasses in total). We used the commercial antifreeze product Coolant TypeC/G11® (Jodima), containing 93-94% MEG, and refer to it as ‘pure MEG’. Antifreeze products containing MEG are easily acquired in most countries and often used in wet trapping of insects. Solutions of MEG were always made with rainwater to simulate the natural conditions where traps are hung outside and rain can enter the col-

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lection cups. More than 200 *B. xylophilus* individuals, a mixture of adult and juvenile stages, were handpicked and submerged in each staining glass and maintained in an incubator at 17°C. After 1, 3, 7, 14, 21 and 28 days, ten *B. xylophilus* adults from each of the three staining glasses (30 individuals per solution per collecting event) were inspected for survival using a stereo-microscope and scored for seven distinct morphological parameters described in the EPPO diagnostic protocol PM 7/4(3), using a microscope at 200 to 1000× magnification (EPPO, 2013). The visibility of each feature was scored on a scale from 1 to 5 for each nematode where 1 = not visible and 5 = clearly visible.

The individuals were inspected for the following general features of the *Bursaphelenchus* genus:
- Cephalic region high with six lips and offset by a constriction
- Lateral field with four lines
- Excretory pore at/or behind the median bulb

as well as for *B. xylophilus*-specific features:
- Female tail broadly sub-cylindrical with or without mucron
- Female vulval flap straight, not ending in a deep depression
- Male spicule length < 30 μm
- Male spicule with long and pointed rostrum; limbs of spicule with an angular curvature

The effect of MEG on the identification of *B. xylophilus* using molecular techniques was evaluated in a similar experimental set-up. The staining glasses were filled with either pure rainwater, a 30% MEG-70% rainwater solution or 100% MEG (three solutions, each in three staining glasses) and about 200 nematodes were submerged into each solution. After 1, 3, 7, 14, 21 and 28 days, seven *B. xylophilus* individuals were collected from each of the staining glasses (21 individuals per solution per collecting event). For each staining glass, DNA was extracted twice from one individual and once from a group of five individuals (Holterman *et al.*, 2006). A part of the 18S rRNA gene from the ribosomal DNA was then amplified using universal 18S rRNA primers (1813F and 2646R) (Holterman *et al.*, 2006). Successful amplifications were visualised using agarose gel electrophoresis followed by ethidium bromide staining. In addition, an experiment with a more gradual increase of MEG concentrations was performed. About ten handpicked nematodes were submerged in 20, 30, 40, 50, 60, 70, 80, 90 or 100% MEG solution, held in a staining glass for 28 days. One nematode of each dilution was processed for molecular detection as described above.

**Effect of MEG on the survival and detection of PWN inside Monochamus**

The method was further investigated with wild *M. galloprovincialis* vectors transporting PWN. This experiment was performed at the National Institute for Agricultural and Veterinary Research (INIAV, Oeiras, Portugal). Branches were collected in pine stands infested with PWD and brought to the laboratory where they were maintained in cages for natural emergence of adults of *M. galloprovincialis*. From a total of 76 *M. galloprovincialis* adults, half (38 individuals) were submerged separately in vials containing a 30% MEG dilution (made with rainwater), while the other half were submerged separately in vials containing a 70% MEG dilution. All adults were submerged within 1 day after emergence from the branches. It was presumed that some adults would be infested with PWN as they had been collected in pine stands, but the infestation status of the insects was unknown prior to submergence in MEG. After the insect vector died, the liquid (the MEG dilution) and the insect were examined for the presence of living or dead PWN. Insects were each dissected and visually inspected using a stereomicroscope (EPPO, 2013) and any retrieved PWN originating from the 30% and 70% dilutions were subjected to DNA extraction followed by PWN-primer-specific PCR using the protocol described by Inácio *et al.* (2015). Amplification was confirmed using electrophoresis of the final PCR product.

**Data analysis**

Data were analysed with R (R Development Core Team, 2014) using a generalised linear model (GLM) with link function and error distribution chosen depending on the nature of the data. The countable data were analysed with a Poisson or negative binomial regression (the latter in cases of overdispersion of the data). Data of each of the seven morphological features consisting of scores (categorical data) were analysed using an ordered logistic regression. If appropriate, the statistical analysis started with a saturated model and interactions and non-significant main factors were removed at a significance level (*P*) of 0.05.
Fig. 1. Changes in visibility (1 = not visible, 5 = clearly visible) over time of three morphological features of the genus *Bursaphelenchus* when submerged in rainwater (0% monoethylene glycol (MEG)) or different solutions of MEG (10, 30, 60, 100%). A: Cephalic region high and offset by a constriction with six lips; B: Lateral field with four lines; C: Excretory pore at/or behind median bulb.

**Results**

**EFFECT OF MEG ON SURVIVAL, MORPHOLOGY AND MOLECULAR DETECTION OF PWN**

While only five of the 30 *B. xylophilus* adults were dead after 24 h submersion in rainwater, all 30 PWN adults were dead when submerged in any of the solutions containing MEG (10, 30, 60 and 100%). Irrespective of the day, visibility was scored as 5 (clearly visible) in pure rainwater and in the dilutions with 10, 30 and 60% MEG for the features ‘Cephalic region high with six lips and offset by a constriction’ (Fig. 1A) and ‘Female tail broadly sub-cylindrical with or without mucron’ (Fig. 2A). These data could not be analysed statistically due to lack of variance. In a 100% MEG solution there was a clear decrease in scoring and thus visibility of both of these
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Fig. 2. Visibility scoring (1 = not visible, 5 = clearly visible) over time of four morphological features for females (A, B) or males (C, D) of *Bursaphelenchus xylophilus*, when submerged in 0, 10, 30, 60 or 100% monoethylene glycol (MEG) solution (mixed with rainwater). A: Female tail broadly sub-cylindrical with or without mucron; B: Female vulval flap straight, not ending in a deep depression; C: Male spicule with long and pointed rostrum; limbs of spicule with an angular curvature; D: Male spicule length < 30 μm.

features as the submergence period increased ($P = 0.001$ for both features; Figs 1A; 2A).

No interaction was found between the factors ‘solution’ and ‘submergence period’ for the morphological feature ‘Lateral field with four lines’ (Fig. 1B). In all solutions there was a decrease in visibility of this feature as the submergence period increased ($P \leq 0.001$), even in pure rainwater. There was a significant interaction between ‘solution’ and ‘submergence period’ for the features ‘Excretory pore at/or behind median bulb’ (Fig. 1C; $P = 0.048$), ‘Female vulval flap straight, not ending in a deep depression’ (Fig. 2B; $P = 0.003$) and ‘Male spicule
with long and pointed rostrum; limbs of spicule with an angular curvature' (Fig. 2C; \( P = 0.012 \)). The visibility of ‘Excretory pore at/or behind median bulb and ‘Female vulval flap straight, not ending in a deep depression’ decreased with increasing MEG concentration (both \( P \leq 0.001 \)) and with a longer submergence (both \( P \leq 0.001 \)). There were only a few males found in the different solutions (six or fewer per solution); the rest of the 30 individuals were females. The visibility of ‘Male spicule with long and pointed rostrum; limbs of spicule with an angular curvature’ seemed to decrease with an increase of the submergence period in the solutions containing MEG but this was not statistically significant (possibly due to the low number of males analysed). All 15 males were scored 5 for the feature ‘Male spicule length < 30 \( \mu \text{m} \)’ (Fig. 2D) no matter what the solution or the submergence period. No statistics could be performed on these data due to lack of variance.

Detection and identification of \( B. \) xylophilus using molecular techniques was possible for most nematodes that had been submerged in the preservative liquid MEG. We were able to amplify DNA of the 18S region of single and grouped PWN on all days, from day 1 to day 28 (Fig. 3), from all three solutions. In the cases of pure rainwater and the solution with 30% MEG, DNA extraction was successful with 25 \( \mu \text{l} \) lysis buffer mixed with 25 \( \mu \text{l} \) of either of the solutions containing the nematodes. However, an extra ‘rinsing’ step was needed for the 100% MEG solution, i.e., the nematodes had to be transferred from the 100% MEG solution into 25 \( \mu \text{l} \) pure water which was then mixed with 25 \( \mu \text{l} \) lysis buffer to start DNA extraction (Fig. 3, lanes marked with *). Without this additional rinsing, DNA from the nematodes in 100% MEG could not be detected, even when five individuals were pooled (Fig. 3). The additional experiments suggest that this extra rinsing step should be performed at MEG concentrations \( \geq 70\% \).

A clear amplicon could be visualised by electrophoresis at MEG concentrations \(< 70\% \), while bands were weak or invisible at MEG concentrations of 70% or higher (Fig. 4).

**EFFECT OF MEG ON THE SURVIVAL AND DETECTION OF PWN INSIDE *MONOCHAMUS***

All 76 emerged longhorn adults died within 4 h after being placed in either the 30% or the 70% MEG solution. Of these 76 longhorns, 23 were found to be transporting PWN (all as \( J_4 \) dauer juveniles); 13 of the insects had been immersed in the 30% MEG dilution and ten in the 70% MEG dilution. PWN were dead and retrieved from within the insect body of all infested beetles; the number
of PWN was fewer than ten per vector. In addition, for three insects immersed in the 30% MEG dilution one single dead PWN was also found in the liquid. A total of nine and six PWN samples retrieved from insects, from the 30% and 70% dilution, respectively, were analysed molecularly: eight out of nine and four out of six samples were positively identified as *B. xylophilus*. The case where the PCR had a negative result in the 30% MEG dilution was for a single PWN found in that insect. In addition, in three cases where one PWN was retrieved from the 30% MEG liquid, nematodes were each analysed molecularly but no DNA was amplified in the PCR runs.

**Discussion**

For countries not having *Monochamus* vectors, the most probable scenario for *B. xylophilus* to enter and establish is to have the nematode arriving together with invading *Monochamus* vectors. To assess rapidly the occurrence of this scenario, it is imperative that the likelihood of detecting or intercepting the invasive nematodes is maximised (Álvarez et al., 2015). Thus, the ability of utilising a wet catching strategy was assessed to maximise the coverage area of the trapping network. Our original interest for the wet catching method arose from the fact that the captured insects are preserved and protected for a longer period than with dry catching (at least up to 4 weeks based on our experience). The advantage is that the traps require fewer visits and with the same effort a much larger area can be monitored. Moreover, wet catching not only prevents destruction of the captured insects by predatory or necrophagous insects, it also prevents escape of captured insects; De Groot & Nott (2003) and Sweeney et al. (2006) observed that cross-vane traps with a wet collection cup containing a liquid, like soapy water or a mixture of propylene glycol and water, were more effective in retaining captured insects than cross-vane traps having a dry collection cup with twigs sprayed with the insecticide dichlorvos. Allison et al. (2014) determined that multi-funnel traps with a wet cup captured more Lamiinae and Cerambicinæ than multifunnel traps with a dry cup; treating the inner sides of the dry cup with Teflon improved the efficacy of capturing Cerambicinæ, but not of the Lamiinae. Álvarez et al. (2015) also found that treating the inner sides of dry collection cups with Fluon did not prevent escape of *M. galloprovincialis* adults.

Wet catching is frequently used in capturing longhorn beetles and other insects. However, it has not yet been applied for the monitoring of *Bursaphelenchus* sp. as the effects of preservative solutions, used in the collection cups, on the survival, detection and identification were previously unknown. We tested the effects of the preservative MEG and found that the PWN died within 4 h when submerged in any mixture containing different concentrations of MEG. This was observed for all nematodes, whether submerged as individuals or carried within the vector. In all cases where beetles were transporting PWN, nematodes were retrieved from the dead vector bodies. In only a few cases, nematodes were found also in the liquid, indicating they had been able to leave the beetle before dying. This only occurred at the low MEG concentrations of 30%, probably because the toxic effect on the nematodes was less than at high concentrations. Extraction of PWN from vectors, wood shavings or bark is based on the movement of the nematodes leaving the matrix into a liquid, usually water (e.g., Baermann funnel; EPPO, 2013), but here the liquid was MEG. More tests are needed to determine whether the migration of PWN from the insect vector into the liquid occurs more frequently at lower concentrations of MEG.

Morphological identification of PWN up to genus level appeared to be impossible when using MEG as several morphological features became unclear at higher MEG concentrations and with increasing submergence time. We evaluated adult and juvenile nematodes based on the EPPO diagnostic protocol PM 7/4(3) (EPPO, 2013) using cultured nematodes. In reality, if a PWN is detected in a *Monochamus* longhorn intercepted in a trap it will most likely be a dauer juvenile, as was the case in our test performed with the infested beetles. Occasionally, adult stages can be found in traps, e.g., when water is sitting at the bottom of the ‘dry’ traps dauer juveniles can leave the insect and moult into adults. Also, adult stages of other *Bursaphelenchus* spp. (e.g., *B. teratospicularis* and *B. hellenicus*) were found associated with insects in traps (Penas et al., 2006). We tried to obtain dauer stages by placing the cultures on *B. fucheliana* in unfavourable conditions (colder, drier environments) but failed to obtain this specialised dispersal juvenile form. Hence, we performed the tests using the other stages, allowing assessment of the influence of MEG over time on morphological characteristics of nematodes. Dauer juveniles are also recognised based on certain morphological features (dome-shaped head, a vestigial or no stylet, a degenerated pharynx, a poorly delimited median bulb, a subcylindrical tail with digitate terminus, body filled with stored lipids) (Penas et al., 2006), although these features are not useful for identification.
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to species level. We were able to conclude that a wet catching method using MEG would not be useful for morphological identification of nematodes. In cases where dauer stages are present, further development into adult stages required for species identification of *B. xylophilus* (EPPO, 2013) would definitely be impossible because the nematodes die rapidly in this liquid. In the rare cases where adult stages are present, their morphology is not sufficiently preserved in MEG to allow species identification. These findings are also useful for studies on other insect vectors of nematodes.

Alternatively, we showed that PWN captured in MEG can be identified using molecular techniques. Several methods have been developed to identify *B. xylophilus* using molecular methods (EPPO, 2013; Ínácio et al., 2015). These methods were developed with nematodes that were either reared or extracted from wood or vectors, but not with nematodes exposed to a preservative. Surprisingly, there were very few negative effects of MEG on the molecular identification of the PWN in our laboratory tests. Inhibition of DNA extraction and/or amplification was only observed when the nematodes had been submerged in high MEG concentrations of \( \geq 70\% \). This inhibition could nevertheless easily be reversed by rinsing the nematode in water prior to DNA extraction. We assume that other commercial antifreeze products containing MEG will most likely have similar effects as those found in our study for MEG using Coolant Type C/G11\(^*\), although this can vary if other chemicals are added. We note that commercial antifreeze products are much cheaper than pure MEG.

There was an irregular or lack of amplified DNA from single nematodes in two cases in the experiment with submerged cultured specimen (lanes 8 and 10; Fig. 3). A reason for these irregularities could be that it is almost impossible to verify whether a single nematode is successfully transferred to the Eppendorf tube during the preparation for the molecular analysis. In addition, it is not clear why in the last series of experiments the molecular identifications of some PWN retrieved from the vectors (three out of 15 samples) and from the liquid (30\% MEG, three single PWN) were negative for *B. xylophilus* as they were all rinsed thoroughly in water before DNA extraction. Perhaps insufficient amounts of DNA were extracted from these nematodes (two nematodes at most) or they were lost while handling. Another possibility is that other nematode species co-existed in the branches coming from the field and were accidentally picked up by the *M. galloprovincialis* adults and analysed in our experiments. As DNA extraction was followed by a PCR using PWN-specific primers and not by a universal primer, we cannot know if this was the case. The sensitivity of the molecular detection of *B. xylophilus* after submersion in MEG is relatively high; we assume that the detection level is 3-4 individuals, but certainly five nematodes, provided proper rinsing with water is performed when MEG solutions of 70\% are used and nematodes are not lost while handling. This estimate of the detection limit is based on the results of the tests with cultured specimens where five nematodes were always detected, but where samples with one specimen occasionally failed to be detected, and the experiments where nematodes were captured through beetles and where cases with two nematodes were negative.

Dawson et al. (1998), Kilpatrick (2002) and Yoder et al. (2006) suggested DESS, a DSMO-EDTA-saturated-NACl solution, as a preservative for nematodes and other invertebrates because not only is DNA preserved for an extended time period, morphological features also remain visible. This preservative is commonly used for storage and transportation of nematodes, but has not been reported for use in trapping. However, captured PWN dauer juveniles would probably die rapidly and not develop into adults, the stage necessary for morphological identification in EPPO (2013). Furthermore, Yoder et al. (2006) warn that DESS can cause mild skin irritations and facilitate transportation of other substances, including dangerous toxins, into the body. Although MEG can also cause mild skin irritation, it is easily available as a commercial antifreeze product (e.g., MEG in our study), which is not the case for DESS. However, we note that several other commercial anti-freeze brands based on MEG exist besides the coolant TypeC/G11\(^*\) (Jodima) used in this study. The chemical composition of other brands can differ slightly and certain chemical adjuvants could have negative effects on the DNA extraction or PCR reaction. It is necessary to evaluate other anti-freeze brands in respect to the molecular analysis and identification of PWN prior to their use in wet trapping.

It is our opinion that detection of PWN in this trapping system is easier and faster when the nematodes can be searched for in the vector (by dissection using a microscope or by direct molecular detection) rather than in the liquid. High MEG concentrations can be maintained in the collection cup by adding a sufficient volume in the cups at the time of setting up the traps, and/or by constructing the traps to prevent influx of rain (e.g., a larger top of the trap). The wet catching method targeting vec-
tors is not recommended in regions where Monochamus spp. are abundant. Processing numerous vectors captured in the wet catching traps individually to detect dead nematodes inside the insects leads to high personnel and reagent costs. In such conditions it is recommended to use dry catching, followed by PWN extraction of several beetles using a Baermann funnel technique where live nematodes exit the insects. Morphological identification of the PWN can then be performed using a microscope (EPPO, 2013) since nematode features will not be altered. The wet catching method is only recommended when the costs of maintaining traps with dry catching exceeds the costs for analysis of Monochamus individuals captured in the trapping network.

We demonstrated that wet catching using MEG as a preservative in the collection cup of the longhorn beetle trap is suitable for the detection of PWN. Captured Monochamus individuals should be examined for the presence of dead nematodes, which can then be identified as B. xylophilus using DNA-based methods because morphological identification is not possible. It should be noted that MEG concentration must be kept >30% for good preservation of the captured insects. This can be done by preventing entry of rain into the collection cup or by using sufficient MEG when setting-up the collection cup in the traps, depending on the local climatic conditions. With the wet catching method as an early warning system, less frequent visits of the traps to collect the trapped arthropods (i.e., every 3-4 weeks in our study) are needed, maximising the efficacy and coverage area of the trapping network with limited resources. It is recommended that the wet catching method is further tested in regions where both the vectors and the PWN occur.

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