Final Report

Project title
Xylella fastidiosa and its insect vectors

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Contents

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2. Short project report

2.1. Short executive summary

Xylella fastidiosa is a bacterial pathogen transmitted by insect vectors. In the Americas, the primary vectors are Cicadellidae, subfamily Cicadellinae, i.e. glassy-winged and blue-green sharpshooters whereas in Europe the common meadow spittlebug or froghopper *Philaenus spumarius* has been identified as the primary vector beside two other spittlebugs, *Neophilaenus campestris* and *Philaenus italosignus*. *P. spumarius* is abundant in Europe, wide-ranging geographically and present in a variety of habitats as well as being highly polyphagous. However, it is important to recognise that although *Philaenus spumarius* is the most important vector identified at this time, any xylem feeding insect could potentially act as a vector.

Although routine surveillance for *X. fastidiosa* is carried out on plant material it is also possible to detect the bacterium within the foregut of insects. Recent studies have indicated that, in conjunction with plant surveys, testing vectors for *Xylella* could be an important tool for monitoring for the bacteria within the wider environment. The main activities of this project focussed on:

- Surveys of potential vector species and association with plant hosts within different habitats including agroecosystems such as vineyards and olive groves collecting data on abundance and host plant preferences.
- Evaluation of sampling and trapping methods for vectors surveillance. Sweep netting is the most effective method and is therefore recommended. However yellow sticky-traps or pan-traps as passive trapping methods also proved to be suitable for monitoring for the presence of spittlebugs.
- Development and evaluation of molecular tests to identify vectors alongside well-established DNA barcoding techniques. CO1 sequences are readily available within GenBank and BOLD for several *Philaenus* and other species. A specific real-time PCR test was developed for the identification of *Philaenus spumarius*.
- Development and evaluation of molecular methods (PCR, Real-time PCR and LAMP) for the detection of *X. fastidiosa* in vectors. CTAB is the most suitable extraction method for the obtention of high concentration of *X. fastidiosa* genomic DNA from insects. Initial results indicate that although real-time PCR is more sensitive than LAMP, the LAMP test provides advantages as a useful tool for screening vectors in the field.
- A CTAB extraction protocol is the most suitable for the obtention of high concentration of Xf-genomic DNA from insects. Comparative analysis was also undertaken between different molecular techniques (PCR, Real-time PCR and LAMP). Initial results indicate that although qPCR is more sensitive than LAMP that the LAMP assay is a useful tool for screening vectors.

2.2. Project aims

While the vectors of *X. fastidiosa* are relatively well studied in South and North Americas, knowledge on the European vectors needs to be improved. Therefore, this project aimed to improve our understanding of the biology of the main vectors of *X. fastidiosa* in a range of differing habitats and environments in Europe through surveying for xylem feeding Auchenorrhyncha as potential vectors and determining associations, if any, with plant hosts. Sampling and trapping methods were also evaluated to determine which would be most appropriate for surveillance purposes.

CO1 barcoding is a well-established diagnostic method for the identification of arthropods and can be used to confirm identity in conjunction with morphological techniques, particularly when identifying immature specimens. Evaluation of current DNA barcoding techniques was carried

out alongside development of species-specific tests for the identification of the most important vectors.
Finally, comparison of three molecular methods for detection of *X. fastidiosa* in insect-vectors was investigated to determine their potential usefulness in monitoring for the presence of the bacterium in spy-insects.

2.3. Description of the main activities

The project was organised into three main activities:

- Surveying vectors, including evaluation of vector sampling and trapping techniques
- Molecular identification of vectors
- Detection of *X. fastidiosa* in vectors

2.3.1. Vector surveys including evaluation of sampling and trapping techniques

National surveys were conducted in the partner countries in order to gather data on the presence of xylem feeding insects within natural habitats and agroecosystems such as vineyards and olive groves.

2.3.1.1. Austria

Survey for potential *Xylella fastidiosa* vector species on grapevine

Surveys for potential *X. fastidiosa* vectors were conducted in 29 vineyards in Lower Austria and 3 vineyards in Burgenland in 2019 (Figure 1).

Yellow sticky traps (Rebell® giallo) and the beating tray method were applied from the beginning of July until the beginning of October. Five yellow sticky traps per site were placed in the grapevine canopy. Yellow sticky traps were replaced every two weeks. In addition to the yellow sticky traps, insects were collected by beating every two weeks. One beating sample consisted of 10 strokes per grapevine with four replications per site.

Survey for potential *X. fastidiosa* vector species in an olive orchard

The olive orchard is located in Mörbisch, Burgenland and consisted of two fields with 13 different cultivars of *Olea europaea*.

The number of spittle masses per $1m^2$ in the herbaceous vegetation in the olive orchard was recorded by visual inspection on the 25th of April and the 8th of May 2019. In total 30 m^2 quadrats were evaluated on each date. The transects were randomly chosen. From June to September 2019, sampling for adult *X. fastidiosa* vectors in the olive groves was carried out by sweep-net: six sampling spots were randomly chosen and ten sweeps per spot were carried out. All Auchenorrhyncha species were collected from the net by an aspirator for later identification in the laboratory.

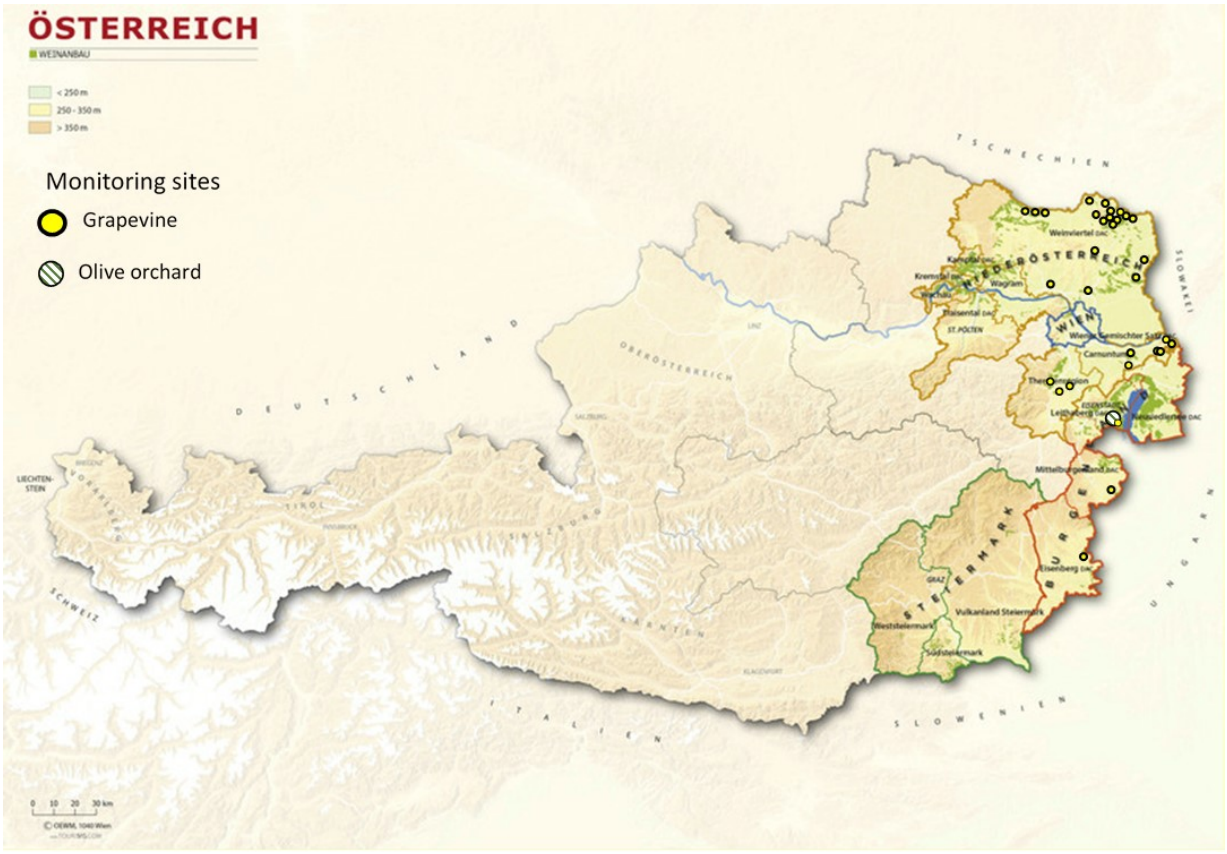

Figure 1. Monitoring sites in Austria surveyed for potential *X. fastidiosa* vector species, in 2019 in wine-growing areas and including one olive orchard.

2.3.1.2. Portugal

Regular monitoring of insect vectors of *X. fastidiosa* was carried out in two olive groves, one located in the south of Portugal (Alentejo region) and the other in the north (Trás-os-Montes region), in 2018 and 2019, from middle April to the end of October.
Three monitoring techniques were tested:
1) Yellow sticky traps (25 x 20 cm) hung in the branches of randomly chosen olive trees and changed periodically. Two traps per tree were suspended at an approximated height of 1.50 m from the soil, in eight trees, and were replaced by new ones after a period of about 3 weeks. In the second year, an additional yellow sticky trap was added per tree, hung at about 0.5 m height, in the same tree or in the nearest tree, depending on the presence of weeds, in order to target insects closer to the ground. Six groups of three traps each were installed per grove: two low traps and one high trap (respectively at 0.5 and 1.5 m height), replaced in 4 sampling dates.
2**)** Beating and sweep-net sampling in the trees' canopy was used only in the first year. It was abandoned because it was very inefficient in catching the target insects, with the additional problem of collecting a large quantity of plant debris, being especially harmful during flowering.
3) Sweep-net sampling in the natural vegetation (weeds and bushes) in three places per grove per date (weekly in 2018 and less frequently in 2019), with 30 sweeps performed in each place. During the summer, the ground cover weeds were restricted to small areas, owing to drought, being found mainly around the trunk of the drip-irrigated olive tree. So, sampling by sweep-net had to be adapted and restricted to those few weeds and with fewer number of sweeps.
Additionally, in spring, white spittlebug foam was observed on the axils of live herbaceous weeds indicating the presence of potential vectors' nymphs. They were collected, together with

the host plants. In the laboratory, the spittlebug nymphs were separated from the spittlebug foam and identified.

2.3.1.3. Germany

Monitoring of xylem-sap feeding Auchenorrhyncha as potential vectors of *Xylella fastidiosa* in vineyards

Routine sampling was carried out by sweep-net in ten vineyards of the wine-growing regions of Palatinate and Mosel to determine which xylem-sap feeding species are present in substantial density in vineyards. In some plots, sticky traps were exposed in addition to sweep netting. Furthermore, the suitability of coloured pan-traps in comparison to sweep-netting and sticky traps for monitoring *P. spumarius* was evaluated in two habitats, a vineyard and a meadow.

Sweep-netting: Ten to 30 sampling spots were randomly chosen per vineyard according to the plot size, and five sweeps per spot - each sweep a back and forth movement of the net - were carried out fortnightly along a distance of five meters on the ground vegetation between the rows and the canopy of the vines, respectively. Spittlebugs, froghoppers and sharpshooters were selectively collected from the net by an aspirator and transported to the lab for final identification.

Sticky-traps: Ten to 29 sticky traps (Aeroxon, Germany; 10.5 cm x 25.5 cm) were exposed per plot. They were installed at the bottom edge of the canopy and replaced every two weeks except for the comparison of trapping methods, when they were replaced every week. Species like *P. spumarius* that are easily to identify were counted directly on the traps. Other specimens were removed from the glue with a drop of benzine and identified by morphological traits.

Pan-traps: Since yellow pan-traps (Temmen, Germany; 30 cm diameter) are highly attractive for insects, they were exposed for only three days per week, filled with 1 L of tap water with a drop of detergent. Caught insects were sieved from the liquid, all Auchenorrhyncha were separated and stored in ethanol until identification.

2.3.1.4. The Netherlands

Commissioned by the Dutch National Plant Protection Organization in 2018 the European Invertebrate Surveys Foundation (EIS) performed a study on xylem feeding Auchenorrhyncha as potential vectors for *X. fastidiosa* around tree nurseries and greenhouse horticulture. This study was based on a two-fold approach: a) collecting information available in literature, databases and collections, as well b) sampling in the field around tree nurseries and greenhouse horticulture. The sampling strategy was aimed to determine (i) which xylem feeding Auchenorrhyncha species are present around tree nurseries and greenhouse horticulture, (ii) what their phenology is, and (iii) how they can be sampled efficiently (Noordijk *et al.*, 2019[1]).

Year round sampling was carried out 10 times at 3-4 week intervals from February until November along 2 transects at 4 locations: two sites in regions where there are a high number of tree nurseries that breed potential host plants of *X. fastidiosa* outdoors (Boskoop, Haaren) and 2 sites in regions with greenhouse horticulture where potential host plants are grown indoors (De Lier, Made). In addition, a single survey was carried out in 8 regions distributed over the country with tree nurseries and greenhouse horticulture, serving as back-ground control of species occurring in the open field in the Netherlands. Xylem feeding Auchenorrhyncha species found during the study were not tested for *X. fastidiosa*.

[1] Noordijk, J., C.F.M. den Bieman, M.C. de Haas & E. Colijn (2019). Xyleemzuigende cicaden, potentiële vectoren van *Xylella fastidiosa*, rondom boomkwekerijen en glastuinbouw. Rapport EIS Kenniscentrum Insecten en andere ongewervelden, Leiden. EIS2019-06, 54 pp. Available at https://www.eis-nederland.nl/rapporten. (in Dutch with English summary).

2.3.1.5. United Kingdom - Scotland

In 2018, numbers of Aphrophoridae insects (*Philaenus*, *Neophilaenus* and *Aphrophora* species) were recorded every two weeks over a period of five calendar months (May-September) at two different sites in Scotland: a grassy meadow and the understorey of broadleaved woodland. In 2019 the monitoring was expanded to include to two further sites: an arable habitat (grass field margin) and a second woodland habitat with tree species such as *Salix* and *Corylus* spp. The number of spittles were recorded from thirty quadrants along a 100 m transect. Adult insects were sampled from herbaceous plants using a sweep net; 4 sweeps a total of 30 times over the 100 m transect. A selection of trees and hedges were also sampled for adult spittlebugs at one timepoint in August 2018 and 2019. More non-systematic surveys were carried out across Scotland from varying habitats to investigate presence and basic prevalence of vectors in the wider environment.

Data on host plant preferences of *P. spumarius* was collected via a citizen science project in 2018. The 'spittlebughunt' hashtag was originally used in 2017 by the International Plant Sentinel Network (https://plantsentinel.org/news/1493/) in collaboration with Fera Science Ltd., Royal Botanic Gardens (Kew), Botanic Gardens Conservation International (BGCI) and Defra to collect information from their gardens in the United Kingdom and in 2018 SASA utilized a similar method to gather further information more specific for Scotland (http://www.sasa.gov.uk/sites/default/files/spittlebughunt%20poster.pdf). Individuals were encouraged to tweet images of 'cuckoo spits' using the hashtag 'spittlebughunt' including information on the host plant and location within the tweet. Several volunteers also collected nymphs along with a sample of the host plant. Identification of the nymphs and plant material was carried out using a combination of morphological identification techniques and molecular barcoding.

2.3.1.6. Spain - Balearic Islands

In November 2017, EFSA launched a project for the collection of data and information in the Balearic Islands on the potential vectors of *X. fastidiosa* and their biology. The study focussed on three main tasks i) improving understanding of biology, ecology, life cycle and abundance of vectors and potential vectors in agroecosystems through macrocosm field observations and ii) microcosm observations and finally iii) transmission studies under controlled conditions.

For the long-term study of macrocosm, nine organic farms (three olive, three vineyards and three almond farms) were selected for surveys in Majorca. The islands of Minorca, Ibiza and Formentera were sampled twice a year, in summer and autumn. Insects were collected biweekly in each plot in Majorca by using a sweeping net for adults and a wood frame of 0.25 m^2 for nymphs. In the other islands of the Balearic archipelago, only adults were collected since nymphs were not present at the time of the sampling. For Task 2, 50 cages containing one male and one female of *P. spumarius*, one plant per cage and substrate for oviposition (straw) were placed at field conditions. The plants species were the following *Rosmarinus officinalis, Mentha sativa, Ocimum basilicum, Pistacia lentiscus* and *Lavandula officinalis*. Insects were placed inside the cages from September to November 2018. Analysis of oviposited eggs was conducted in February 2019. For Task 3 (only vector competence studies), field collected insects were caged with *X. fastidiosa* free *Medicago sativa* plants for 96 h. After that period insects were analysed by real-time PCR for the detection of *X. fastidiosa*. Samples taken from plants were analysed 15, 30, 45 and 60 day post inoculation.

2.3.2. Molecular Identification of vectors

2.3.2.1. DNA Barcoding

CO1 barcoding is a well-established diagnostic method for the identification of arthropods and in conjunction with morphological techniques can be used to confirm identity particularly in

regards to identifying immature specimens. To establish how complete existing records are for vectors within online databases such as NCBI and BOLD, several xylem feeders were sequenced for comparison as follows. Genomic DNA from vectors was isolated using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's instructions. The 658 bp Folmer region of the COI gene was amplified by PCR and sequenced following the EPPO protocol for DNA barcoding of arthropods (EPPO, 2016[2]). CO1 sequences from both Genbank and this project were used to construct a Neighbour-Joining Tree using Geneious.

2.3.2.2. Development of tests for molecular identification of confirmed vectors

Prior to method development, the ITS2 spacer and partial sections of rRNA 28S and 5.8S genes were amplified and sequenced for vectors of interest.
Briefly, genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's instructions. The ITS2 region was amplified using the primers CAS5p8sFcm (5'-CGAACATCGACAAGTCGAACGCACA-3') and CAS28sB1d (5'-TTGTTTTCCTCCGCTTATTAATATGCTTAA-3') from Ji *et al.* (2003)[3]. PCR reactions were carried out using 10 µL of Bio-X-act mastermix (Bioline), 0.2 µM of each primer and water and 2 uL of DNA were added to a total volume of 20 µL. The amplification reaction was as follows: 95°C for 5 min, 35 cycles of 95°C for 30 secs, 64°C for 30 secs and 72°C for 30 secs, with a final elongation at 72°C for 10 min. All reactions were visualized on 1 % (w/v) agarose gel. The PCR product was then purified and sequenced. Sequences were analysed with Geneious. Chromatograms from DNA sequences were assembled for each individual and then aligned with the ClustalW algorithm and visually inspected. The ITS2 sequences were then used to develop specific tests for the identification of vectors.
Real-time PCR was carried out using a real-time PCR system (Life Technologies) using 96 well plates. A reaction mixture was prepared containing 0.25 µM of each of the forward and reverse primers, 0.125 µM of probe and 1 µL of 18S Endogenous control mix (Applied Biosystems). Water and 30 ng of DNA template were added to a total volume of 20 µL. Thermal cycles in the real-time PCR consisted of an initial denaturation at 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Three technical replicates were prepared for each reaction and the average threshold cycle (Ct) value was calculated.

2.3.3. Detection of *X. fastidiosa* in vectors

2.3.3.1. Comparative efficiency analysis of PCR, real-time PCR and LAMP tests in detecting *X. fastidiosa* in insect-vectors using three different DNA-based extraction methods

Insects-vectors (with a special regard to *P. spumarius* and *N. campestris*) were collected using a sweeping net from the *X. fastidiosa*-infected olive canopy and ground vegetation of 'De Donno orchard' (Gallipoli, Lecce province, Italy) in September 2018. The captured insects were conserved in 95% ethanol (*in-situ*) and brought to the laboratory for species identification. After identification, one hundred specimens for each of *P. spumarius* and *N. campestris* were singly separated and stored in a 1.5 mL microcentrifuge tube containing ethanol (100 %) and stored at -20 °C for further molecular analyses.

- Insects were washed with de-ionized water to remove any ethanol residue and three different extraction methods of total nucleic acids (TNA) were used:Extraction method

[2] PM 7/129 DNA barcoding as an identification tool for a number of regulated pests https://onlinelibrary.wiley.com/doi/epdf/10.1111/epp.12344
[3] Ji *et al.* (2003). Evolutionary conservation and versatility of a new set of primers for amplifying the ribosomal internal transcribed spacer regions in insects and other invertebrates. https://doi.org/10.1046/j.1471-8286.2003.00519.x

1 (EM1): The entire insect was enclosed in 200 µL of extraction buffer containing 1X TE (Tris-EDTA), 0.5% TRITON-X100, and boiled for 5 min at 94°C and afterward chilled on ice for 5 min. 25 µL of TNA from the EM1 were stored at -20°C for further molecular analysis.

- Extraction method 2 (EM2): The head of single specimen was removed as described by Bextine *et al*. (2004)[4] and treated as described in the EM1 for TNAs extraction. Afterward, 25 µL were collected for subsequent molecular analysis, whereas the remaining 175 µL of extract was kept aside for successive usage in EM3.
- Extraction method 3 (EM3): The insects-organs of each insect, i.e. head and body, treated in EM1 and EM2 were recuperated and entirely ground/crushed in this step using a plastic pestle in the presence of 500 µL of CTAB (2% Hexadecyl trimethyl-ammonium bromide, 0.1 M Tris-HCl pH 8, 20 mM EDTA and 1.4 M NaCl) following the protocol of Hendson *et al*. (2001)[5], De Souza *et al*. (2003)[6] and Rodrigues *et al*. (2003)[7]. Extract aliquots were incubated at 65°C and chloroform treated. TNA were then isolated by precipitating the supernatant with 0.6 volume of cold 2-Propanol (Murray and Thompson, 1980)[8]. Prior TNA precipitation, TNA extracts of each specimen coming from EM1 and EM2 (175 µL from each EM) were added to those extracted in this phase, in order to recover the entire amount of bacterial genomic DNA present in each specimen.

The TNAs extracts obtained for each insect from the three EM were used for the detection of *X. fastidiosa*, using PCR, real-time PCR and LAMP.

Conventional PCR has been performed using the primers RST31/33 (Minsavage *et al*., 1994)[9], widely used for detecting *X. fastidiosa* in quarantine programs (EPPO, 2019)[10]. PCR reactions were performed in a 1X amplification buffer in a final volume of 25 µL containing 2.5 µL of TNA, 5 mM of dNTPs, 5 µM of each primer and 1.25 U of DreamTaq™ DNA polymerase (Thermofisher, Italy). The amplification reaction was conducted as follow: 94°C for 5 min, 35 cycles of 94°C for 30 secs, 55°C for 30 secs and 72°C for 45 secs, a final elongation at 72°C for 7 min. All reactions were visualized on 1.2 % (w/v) agarose gel. Samples were considered positive when the DNA band of the expected size (733 bp) was clearly visualized after electrophoresis.

Real-time PCR was performed as described by Harper *et al*. (2010, erratum 2013)[11] in 20 µL reaction volumes containing 10 µL of the SsoAdvanced TM Universal Probes Supermix (BioRad, Milan, Italy), 0.6 µL of 10 µM Xf -forward (XF-F) and reverse (XF-R) primers 0.2 µL of labelled XF-P probe (10 µM), and 2.5 µL of TNA. Thermocycling conditions were as follows:

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[4] Bextine *et al.* (2004). Evaluation of Methods for Extracting Xylella fastidiosa DNA from the Glassy-Winged Sharpshooter. https://doi.org/10.1093/jee/97.3.757

[5] Henderson *et al.* (2001). Genetic Diversity of Pierce's Disease Strains and Other Pathotypes of Xylella fastidiosa. doi: 10.1128/AEM.67.2.895-903.2001

[6] de Souza *et al.* (2003). Analysis of Gene Expression in Two Growth States of Xylella fastidiosa and Its Relationship with Pathogenicity. https://doi: 10.1094/MPMI.2003.16.10.867.

[7] Rodrigues *et al.* (2003). Detection and Diversity Assessment of Xylella fastidiosa in Field-Collected Plant and Insect Samples by Using 16S rRNA and gyrB sequences. DOI: 10.1128/AEM.69.7.4249–4255.2003

[8] Murray and Thompson (1980). Detection of *Xylella fastidisoa* in olive groves by molecular and serological methods. http://www.sipav.org/main/jpp/index.php/jpp/article/view/3096

[9] Minsavage *et al.* (1994). Development of a Polymerase Chain Reaction Protocol for Detection of Xylella fastidiosa in Plant Tissue. DOI: 10.1094/phyto-84-456

[10] EPPO PM 7/024(4) *Xylella fastidiosa* https://onlinelibrary.wiley.com/doi/epdf/10.1111/epp.12575

[11] Harper *et al.* (2010). Development of LAMP and real-time PCR methods for the rapid detection of Xylella fastidiosa for quarantine and field applications. https://apsjournals.apsnet.org/doi/10.1094/PHYTO-06-10-0168

95°C for 6 min, followed by 40 cycles of 94°C for 10 secs and 62°C for 40 secs. A cycle threshold (Ct) value below 35 was scored as a positive result.

Loop-mediated isothermal amplification (LAMP) assays were carried out using Enbiotech's LAMP system® (Yaseen *et al*., 2017)[12]. Reactions have been carried out in a final volume of 25 µL and conducted at 65°C for 30 min according to the manufacturers' instructions.

2.4. Main results (knowledge, tools, etc.)

2.4.1. Vector surveys including evaluation of sampling and trapping techniques

Austria

Results for potential *X. fastidiosa* vector species in an olive orchard in Mörbisch, Burgenland, AT.

The total number of spittle masses was recorded on two dates (Figure 2). On the 25th of April only *Neophilaenus campestris* nymphs were recorded compared to the 8th of May, where nymphs of both species *N. campestris* and *P. spumarius* were recorded during the survey. Within each spittle the number of nymphs observed ranged between one to a maximum of three. The total number of spittle observed tripled in the two weeks between each timepoint.

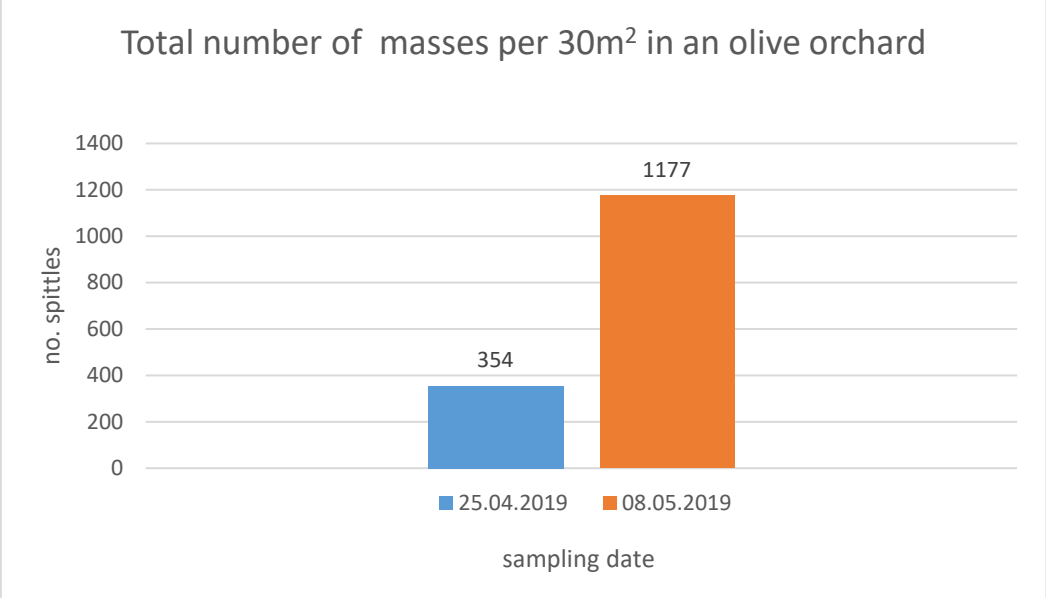

Figure 2. Number of spittle masses/30m^2 in an olive orchard in Mörbisch, Burgenland, AT in 2019.

The surveys carried out later in the year for adults show that *N. campestris* is the most common species in this olive orchard. Beside *N. campestris*, two other species of this genus were recorded: *Neophilaenus lineatus* and *Neophilaenus minor* but in much lower numbers with only one individual of each recorded (Table 1).

[12] Yaseen *et al.* (2015). On-site detection of Xylella fastidiosa in host plants and in "spy insects" using the real-time loop-mediated isothermal amplification method. https://doi.org/10.14601/Phytopathol_Mediterr-15250

Table 1. Xylem-sap feeding Auchenorryncha species collected in an olive orchard in Mörbisch, Burgenland by sweeping net in 2019.

Aphrophoridae	No. of individuals
Neophilaenus campestris	51
Philaenus spumarius	4
Neophilaenus lineatus	1
Neophilaenus minor	1

The number of Aphrophoridae individuals dropped from June to August presumably because of high temperatures and drought in 2019 (Figure 3). During the summer months (June–September), there was almost a complete absence of *Philaneus spumarius* (but also other Auchenorrhyncha species) on ground vegetation. More adults were recorded at the beginning of September and several mating individuals were observed in the olive orchard at this time. It appears that in September they returned for mating.

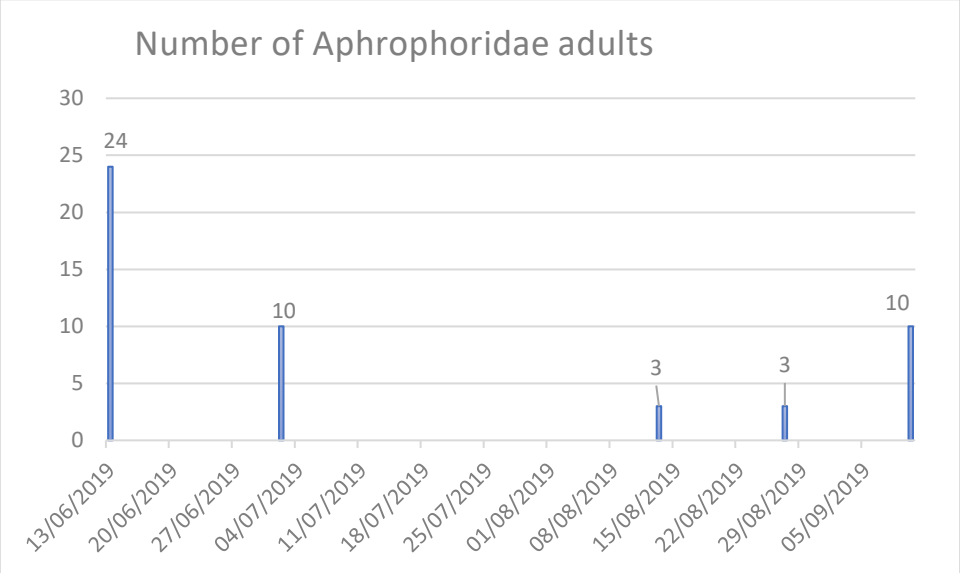

Figure 3. Number of adult Aphrophoridae (*Philaenus* sp., *Neophilaenus* sp.) in an olive orchard, Mörbisch, Burgenland, AT from June to September 2019.

Results for potential *X. fastidiosa* vector species on grapevine
In 2019, 4 potential X. fastidiosa vector species were recorded in the canopy of grapevines. Only *N. campestris* and *P. spumarius* were present in high numbers on grapevines. *C. viridis* and *A. alni* were recorded rarely and only at a few monitoring sites (Table 2).

Table 2. Total numbers of Xylem-sap feeding Auchenorryncha recorded in the canopy of grapevines in Lower Austria and Burgenland 2019.

Aphrophoridae	Cicadellidae	No. individuals
Neophilaenus campestris		52
Philaenus spumarius		46
Aphrophora alni		1
	Cicadella viridis	2

Only 9 individuals of Aphrophoridae species in total were detected during the three months survey on three sites in Burgenland. On 14 monitoring sites only one vector species was recorded, on 9 monitoring sites two and only on one monitoring site three different potential vector species were recorded. On 7 monitoring sites no vector species were recorded (Seefeld, Haugsdorf, Großkrut, Zwingendorf, Drasenhofen, Jedenspeigen, Edelstal and Eisenberg) (Figure 4). See tables for a breakdown of all potential vector numbers from different wine growing regions and sites. Differences with regard to the abundance of xylem-sap feeding Auchenorryncha between the monitoring sites were found. It is assumed, that factors like green cover, application of plant protection products and the surrounding vegetation influenced the occurrence and abundance of Auchenorryncha.

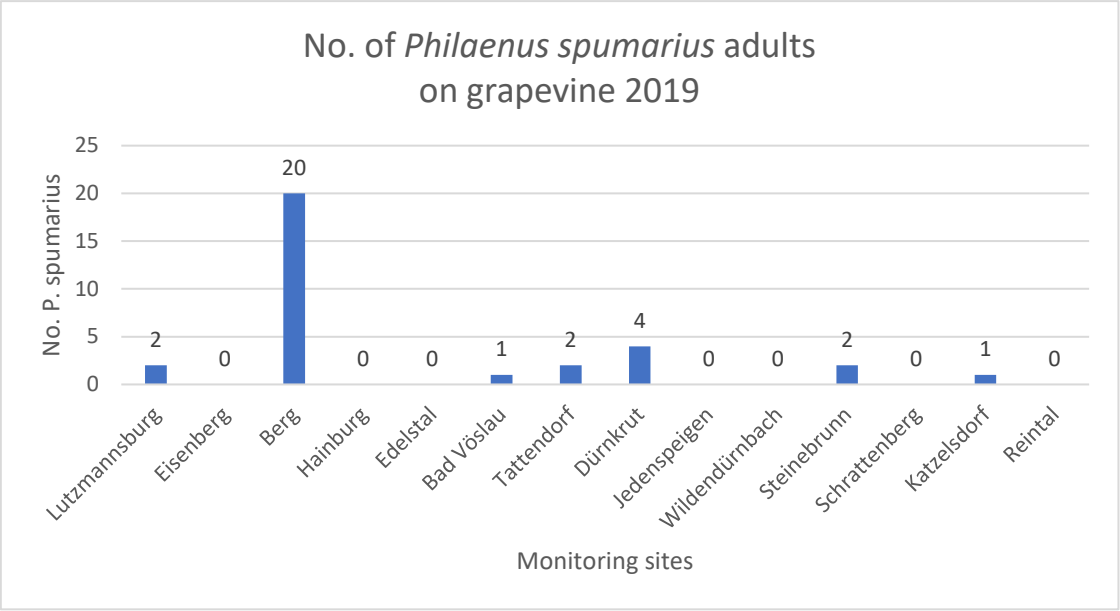

Figure 4. Number of *Philaenus spumarius* adults in grapevine canopies

Phenological observations: Nymphs of *P. spumarius* were observed on grapevine leaves on few monitoring sites (Berg) by the end of May 2019. Adult *P. spumarius* was observed in the grapevine canopy in June and July whereas *N. campestris* was recorded later in the summer from mid-August until October.

Table 1. Xylem-sap feeding Auchenorrhyncha collected in the canopy of grapevines in Burgenland. Methods used: beating tray and yellow sticky traps.

Site	*Philaenus spumarius*	*Aphrophora alni*	*Neophilaenus campestris*
Mörbisch	5	0	0
Lutzmannsburg	2	1	1
Eisenberg	0	0	0
Total	**7**	**1**	**1**

Table 2. Xylem-sap feeding Auchenorrhyncha detected in the canopy of grapevines in Carnuntum and Thermenregion, Lower Austria, in 2019. Method used: yellow sticky traps.

Wine-growing region	Monitoring site	*Neophilaenus campestris*	*Philaenus spumarius*
Carnuntum	Berg	3	20
Carnuntum	Göttlesbrunn	1	1
Carnuntum	Sommerein	3	0
Carnuntum	Prellenkirchen	0	1
Carnuntum	Hainburg	1	0
Carnuntum	Edelstal	0	0
Thermenregion	Tattendorf	4	2
Thermenregion	Bad Vöslau	2	1
Thermenregion	Günselsdorf	4	1
	Total	**18**	**26**

Table 3. Xylem-sap feeding Auchenorrhyncha detected in the canopy of grapevines in Weinviertel, in Lower Austria, in 2019. Method used: yellow sticky traps.

Monitoring site	*Cicadella viridis*	*Neophilaenus campestris*	*Philaenus spumarius*
Wolkersdorf	0	12	5
Dürnkrut	0	4	4
Poysdorf	0	4	0
Falkenstein	1	2	0
Niederrußbach	0	2	0
Mitterretzbach	0	2	0
Schrattenberg	0	3	0
Reintal	0	1	0
Walterskirchen	0	1	1
Herrnbaumgarten	0	1	0
Mistelbach	0	1	0
Wildendürnbach	1	0	0

Steinebrunn	0	0	2
Katzelsdorf	0	0	1
Total	**2**	**33**	**13**

Portugal

Insect vectors detected

Caught adult insects were identified by their morphological characteristics with taxonomic keys, namely Biederman & Niedringhaus (2009)[13] and Wilson *et al*. (2015)[14]. The nymphs found inside spittlebug foam were identified by molecular tools.

The insects caught belonging to the group of *X. fastidiosa* vectors were all Aphrophoridae: *Philaenus spumarius*, *Philaenus tesselatus* and *Neophilaenus campestris*. The *P. tesselatus* specimens were found only in the southern olive grove. Another Aphrophoridae genus caught was *Lepyronia* sp. and whilst this is likely to be *Leypronia coleoptrata* the identification was not confirmed to species level.

The nymphs collected from the foam masses were *P. spumarius*.

Vector adults were already detected in April. By the end of October, adults were still in the field. Nymphs were found only in early spring.

Regarding *X. fastidiosa* vectors, in other surveys conducted in Portugal, the presence of *Cicadella viridis*, *Aphrophora* sp., *Cercopis vulnerate* was also detected. Individuals of *Euscelis sp.* have also been found.

Overall efficacy of the monitoring methods

The yellow sticky traps operating for about 3 weeks periods had a much lower efficiency in catching insect vectors than a single sweep-net sample.

Comparing traps installed at the weeds' level with those placed higher in the canopy, a higher number of insects was caught in the lower traps.

Traps have the advantage of being easier to be deployed in the field by less trained technicians (in relation to sweeping) and require fewer visits to the field which reduces the cost of the monitoring. Placing traps near the ground next to the weeds optimizes the monitoring, but it should be periodically complemented with sweep-net sampling on weeds.

Beating and sweep-net sampling in the trees' canopy proved not to be a good option. Visual monitoring for nymph foam masses during spring is useful for identifying which habitats or locations vectors and their preferred host plants are found, and examination of spittle can also provide further information on the nymphal stage the insects are at.

Germany

Xylem-sap feeding species in vineyards

There were 12 different species of xylem-sap feeders detected in German vineyards, five species of Aphrophoridae with *Philaenus spumarius* and *Neophilaenus campestris* as the most common taxa, three species of Cercopidae with *Cercopis vulnerata* as the predominant one, and four species of Cicadellinae (Table 4). While the green leafhopper *Cicadella viridis* was the most common sharpshooter, the introduced Rhododendron leafhopper *Graphocephala fennahi* was also caught in vineyards.

[13] Biedermann, R. and Niedringhaus, R. (2009). The plant and leafhoppers of Germany. Identification keys to all species.WAB-Frund; Scheessel.

[14] Wilson, M., Stewart, A., Biedermann, R., Nickel, H., Niedringhaus, R. (2015). The planthoppers and leafhoppers of Britain and Ireland. WAB-Frund; Scheessel.

Table 4. Xylem-sap feeding Auchenorrhyncha detected in the ground cover and/or canopy of vineyards.

Aphrophoridae	Cercopidae	Cicadellidae: Cicadellinae
Aphrophora alni	*Cercopis sanguinolenta*	*Cicadella viridis*
Lepyronia coleoptrata	*Cercopis vulnerata*	*Evacanthus acuminatus*
Neophilaenus campestris	*Haematoloma dorsata*	*Evacanthus interruptus*
Neophilaenus lineatus		*Graphocephala fennahi*
Philaenus spumarius		

Philaenus spumarius accounted for 97 % of the total sticky trap catches among vineyards (n = 1161, Figure 5), followed by *N. campestris* (0.95 %). The dominance structure of xylem-sap feeder species was slightly more balanced in the sweep-net collections, but *P. spumarius* and *N. campestris* still made up for 92 % of all specimens (n = 3046), followed by *C. viridis* (2.7 %) , *A. alni* (2.3 %), and *C. vulnerata* (1.2 %).

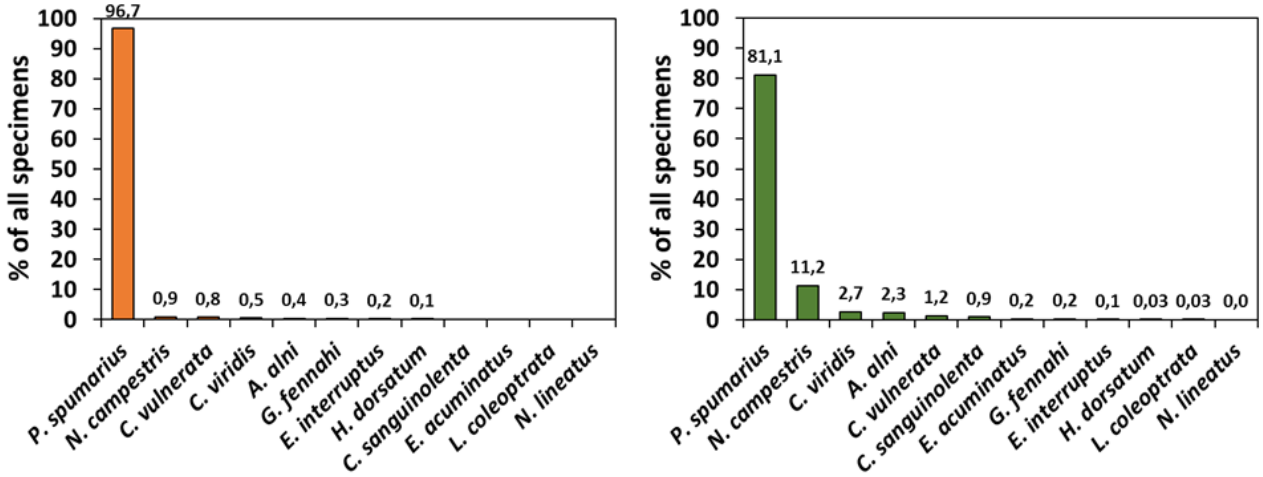

Figure 5. Dominance patterns of xylem-sap feeding Auchenorrhyncha in vineyards collected with a) sticky-traps and b) sweep-net.

Philaenus spumarius catches on the ground and canopy levels of vineyards

To compare sampling methods and the distribution of spittlebugs between the ground cover and the grapevine canopy, regular sampling of *P. spumarius* was carried out in vineyards by sweep net and sticky traps. The comparison was carried out on one plot in the Palatinate wine-growing area in 2018 and 2019, and two different plots in the Mosel area. Since the green cover was completely removed in one plot in the Mosel area in 2018 due to drought conditions in the fall, an alternative plot had to be chosen for 2019.

In the Palatinate plot, the first captures were achieved in both years by sweep-netting on the ground cover, while sticky trap catches and captures by sweep net in the canopy started later in the season (Figure 6). The numbers of *P. spumarius* per trap were compared with those achieved with the five sweeps carried out at the same spot. However, it should be noted that the sticky trap data represent the cumulative captures over 14 days compared to the sweep net collections as single events. The big difference between the sweep-net captures of the two years cannot be attributed directly to weather conditions; however, the ground cover was completely removed in the summer of 2018 due to drought conditions but retained in 2019. In

addition, the data correspond with the general trend of Auchenorrhyncha captures of the two years observed in other projects, too. The average number of adult specimens collected from the ground cover by sweep-net was higher compared to the canopy in both years (except for one week in 2019), but the difference was significant only in 2019.

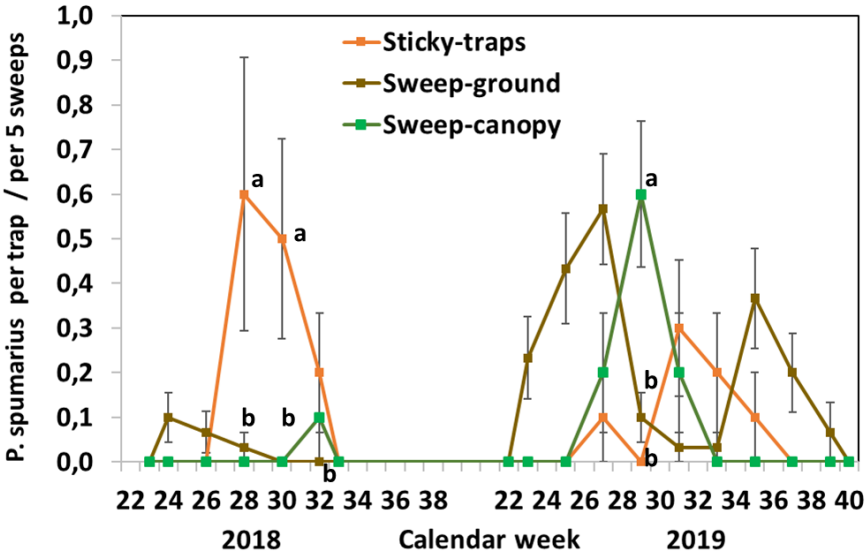

Figure 6. Numbers of *P. spumarius* (mean ± standard error) captured by sticky-traps or sweep-netting of the groundcover and the grapevine canopy in the Palatinate plot. Significant differences (ANOVA with Tukey's post hoc test, α=0.05) between trapping methods at each date are indicated by different letters.

The density of adult *P. spumarius* in the two experimental plots sampled in 2018 and 2019, in the Mosel wine-growing area was higher than the values from the Palatinate region. There was no significant difference between sweep net captures from ground and canopy in the mature vineyard in 2018, while on the plot examined in 2019 captures by both sweep net on the ground and sticky traps were higher than those achieved by sampling the still undersized canopy of the two-year-old vines. Sticky trap and sweep net data for the ground vegetation revealed comparable results in June and July. However, *P. spumarius* captures increased in the ground cover from August on, whereas the numbers of spittlebugs caught by sticky traps remained on a low level. Remigration to the ground level of the spittlebugs for egg laying could be an explanation for this observation.

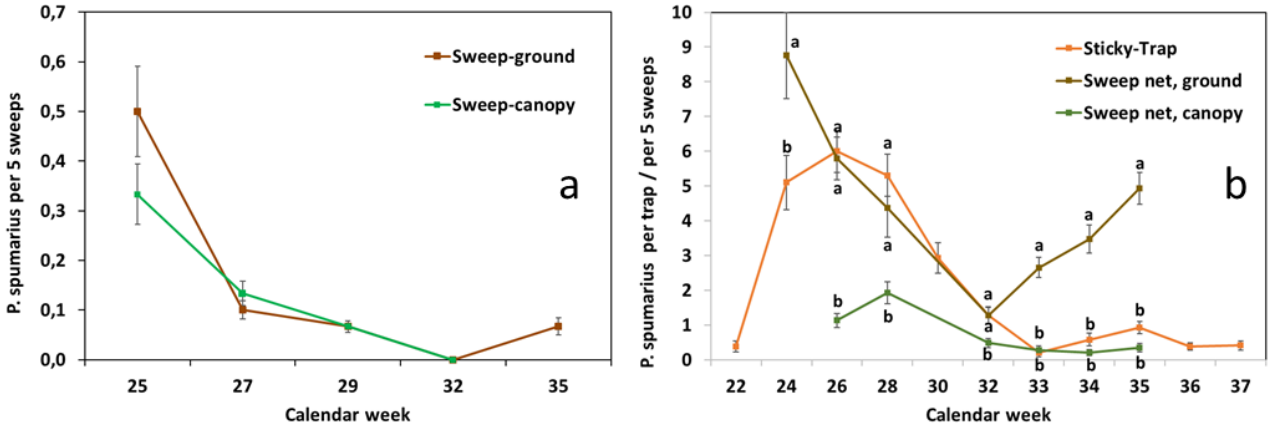

Figure 7. Numbers of *P. spumarius* (mean ± standard error) captured by sticky-traps or sweep-netting of the groundcover and the grapevine canopy in vineyards of the Mosel region. a) in an eight-year-old vineyard in 2018 and b) in a two-year-old vineyard in 2019. Significant differences (ANOVA with Tukey's post hoc test, α=0.05) between trapping methods at each date are indicated by different letters.

Efficiency of different trap types for the monitoring of *P. spumarius*

To compare the efficiency of another trap type - yellow pan-traps - with sticky-traps and sweep-netting, additional experiments were carried-out in the vineyard examined in 2019 in the Mosel area (15 randomly chosen spots) and on a meadow (a transect with 10 spots) as a habitat without vertical structuring. At each spot, one pan-trap and one sticky trap were installed (vineyard: lower edge of the canopy, approximately 50 cm from the ground; meadow: approximately 30 cm above ground), and 5 sweeps were carried out on the ground. Since pan-traps need to be checked frequently, they were installed just for three days. Sweep-netting and the replacement of sticky- and pan-traps were carried out at the same dates.

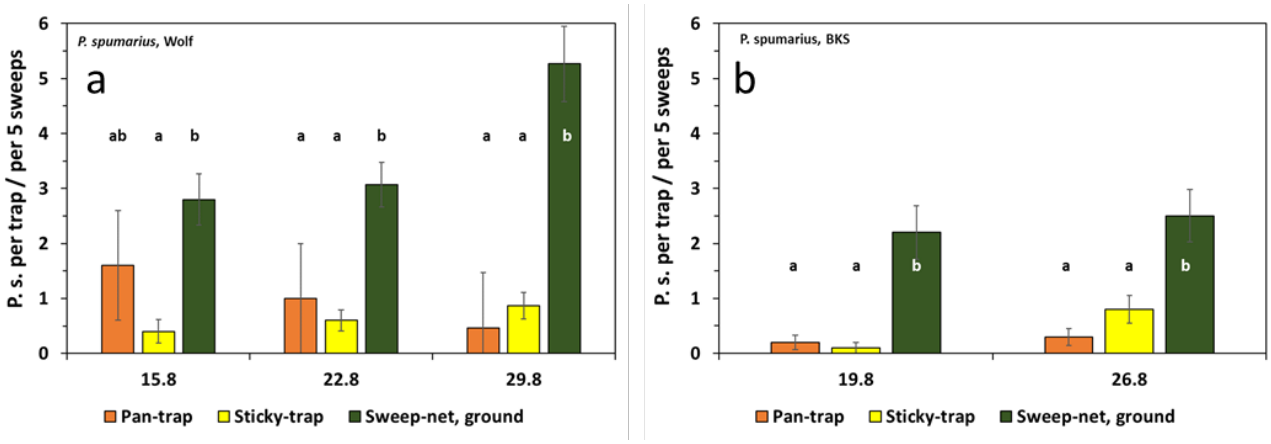

Figure 8. Comparison of different capturing methods for *P. spumarius* (mean ± standard error) at the Mosel region in 2019 in a) a vineyard (n=15; randomly chosen spots) and b) a meadow (n=10; spots along a transect). Significant differences (ANOVA with Tukey's post hoc test, α=0.05) between trapping methods at each date are indicated by different letters.

A high density of *P. spumarius* was observed in both habitats (Figure 8). Sweep-netting resulted in significantly higher captures compared to the two passive sampling methods. Numbers of spittlebugs caught by sticky-traps and pan-traps were not significantly different.

The Netherlands

From the Netherlands, 420 Auchenorrhyncha species are known. During our 1 year survey, 98 cicada species were caught in total, eight of which are xylem feeding species (*Cercopis vulnerata* Rossi, 1807 – Cercopidae; *Aphrophora alni* (Fallén, 1805), *A. pectoralis* Matsumura, 1903, *A. salicina* (Goeze, 1778) and *Philaenus spumarius* (Linnaeus, 1758) (Aphrophoridae); *Cicadella viridis* (Linnaeus, 1758) and *Graphocephala fennahi* Young, 1977 (Cicadellidae). Additionally, nine other xylem feeding species are known from the Netherlands: *Haematoloma dorsatum* (Ahrens, 1812), *Aphrophora corticea* Germar, 1821, *A. major* Uher, 1896 (= *A. alpina* Melichar, 1900), *Lepyronia coleoptrata* (Linnaeus, 1758), *Neophilaenus campestris* (Fallén, 1805), *N. lineatus* (Linnaeus, 1758), *N. minor* (Kirschbaum, 1868), *Errhomenus brachypterus* Fieber, 1866, *Evacanthus acuminatus* (Fabricius, 1794) and E. *interruptus* (Linnaeus, 1758). Three others might be added to the fauna in the future (*Neophilaenus albipennis* (Fabricius, 1798), *N. exclamationis* (Thunberg, 1784) and *Cicadella lasiocarpae* Ossiannilsson, 1981). The biology, distribution and phenology of all xylem feeders was worked out as well. This was also done for six further species that are not xylem feeding but are nonetheless mentioned in literature as potential vectors for *Xylella*. *Aphrophora alni* and *Philaenus spumarius* are the most important potential vectors for *Xylella* in the Netherlands: these species are present during the summer in relatively large numbers on both trees and herbaceous plants respectively at almost all locations. Furthermore, *Aphrophora pectoralis* and *Aphrophora salicina* (on *Salix*) and *Graphocephala fennahi* (on *Rhododendron*) are potential vectors that occur widely. Two species might become noteworthy potential vectors in future: *Cicadella viridis* (very common and widely present on grasses) and *Neophilaenus campestris* (uncommon, present in certain parts and habitats only on *Agrostis*).

The peak of adult abundance of the most important potential vectors for *Xylella*, and thus the ideal moment for sampling, is in the period June - September. If all xylem feeding cicadas need to be caught, additional sampling in May would be necessary. Cicadas can be sampled by beating woody vegetation (in particular *Salix*) and sweeping grassy and herbaceous vegetation. By specifically sampling their preferred host plants, many species of cicadas can be caught relatively rapidly. However, grassy and herbaceous vegetation needs to be sampled for several (3-4) hours to collect sufficient individuals (dozens) of especially *Philaenus spumarius*. This is due to the fact that, in the areas monitored, road verges and ditches are relatively often mown, and population densities are low.

For further details see Noordijk J, Bieman CFM den, Haas MC de, Colijn EO (2019). Xyleemzuigende cicaden, potentiële vectoren van *Xylella fastidiosa*, rondom boomkwekerijen en glastuinbouw. Rapport EIS Kenniscentrum Insecten en andere ongewervelden, Leiden. EIS2019-06, 54 pp. Available at https://www.eis-nederland.nl/rapporten.

United Kingdom - Scotland

Plant Hosts

Records of ‘spittle’ and host plants were received from both the public and volunteers however 90% of samples were contributed by plant health professionals based at SASA or within the Agriculture and Rural Economy directorate of the Scottish Government (Table 4).

Table 4. Response to Citizen Science Survey

Response	No. of respondents	No. of locations	No. of records
Twitter	32	32	94
Email	13	11	27
Nymph & host plant sample	38	32	219
Total	83	75	340

The spittlebug nymphs and associated plant material were identified to species and genus taxonomical level, respectively. The survey carried out in 2018 identified 93 plant genera ranged across 39 botanical families and it was possible to differentiate which of those records were from urban *i.e.* gardens and those which were observed from natural habitats and parks (Table 5).

Table 5. Number of host plants for *Philaenus spumarius*

Urban and suburban Habitat *i.e.* garden		Natural Environment including parks & waste ground		Total	
No of families	31	No of families	26	No of families	39
No of genera	57	No of genera	57	No of genera	93

Overall, the botanical families presenting the highest number of plants hosts recorded with *P. spumarius* nymphs were Lamiaceae (10%), Rosaceae (10%), Onagraceae (9%) and Poaceae (9%). The most common garden host recorded was lavender (*Lavandula* spp.) and within the natural environment including areas such as waste ground and parks, Poaecea species were the most common. Adult *Philaenus spumarius* were also collected from trees and hedges such as birch (*Betula* sp.), hawthorn (*Crataegus* sp.) and willow (*Salix* spp.) in August. However more methodical sampling of trees is required to fully understand prevalence in tree canopies.

Phenology and Abundance

The number of *P. spumarius* nymphs steadily increased from early May peaking in mid-June with the first adults emerging in July (Figure 9). It is unknown if the drop in adult numbers observed in mid-August was due to insect migration or poor sampling conditions caused by heavy rain.

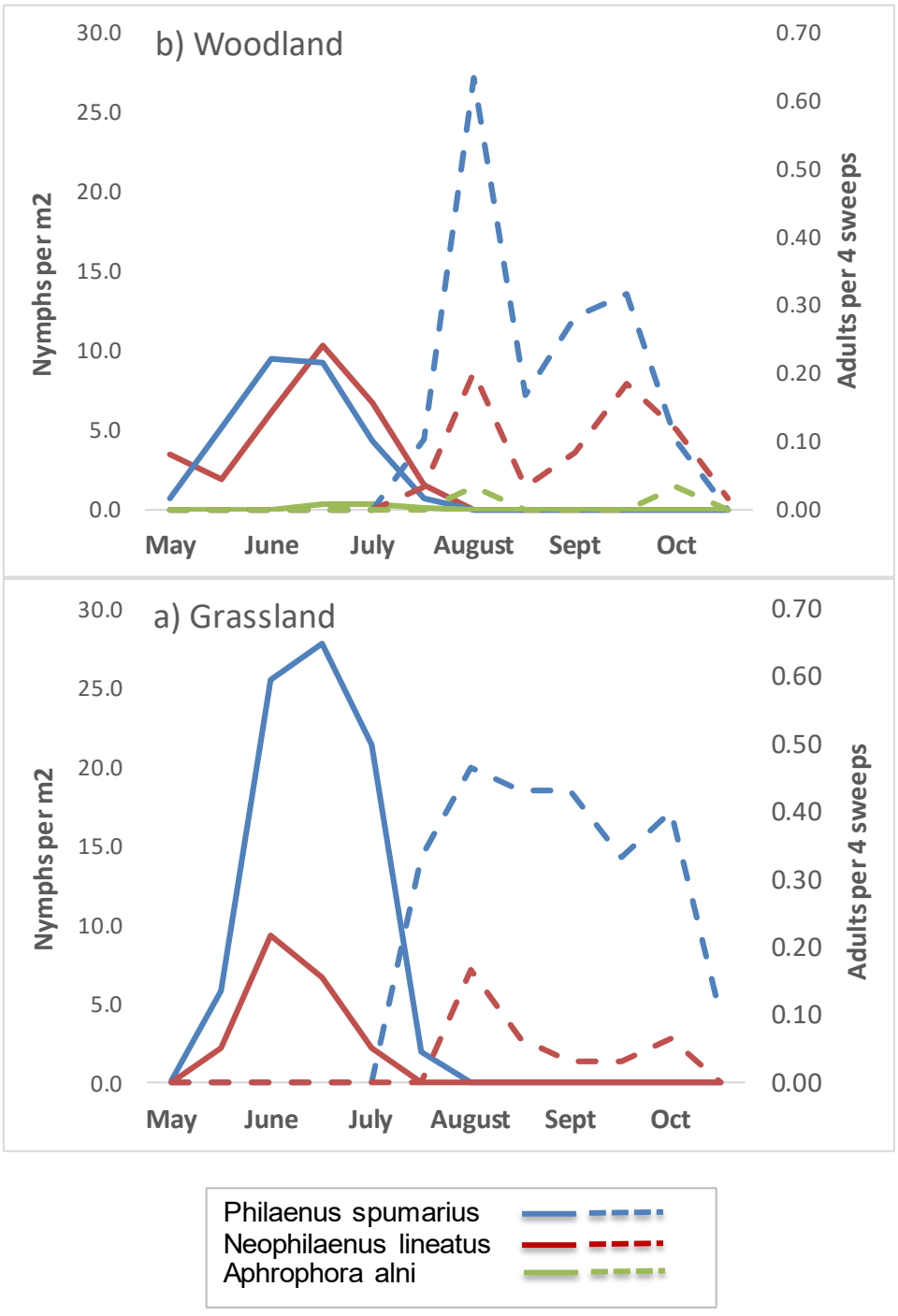

Figure 9. Phenology of Aphrophoridae. The average numbers of Aphrophoridae from different habitats in Edinburgh, Scotland in 2019 for nymphs and adult insects. a) Grassland and Field margin b) Herbaceous understorey of woodland.

In total six different species of xylem-sap feeders were collected in Scotland (Figure 10). Prevalence of these six vectors differs between each habitat, however it is clear that *Philaenus spumarius* is abundant, wide-spread and present in numerous habitats. It should be noted that *Cicadella viridis* and *Neophilaenus lineatus* are also common although not as abundant.

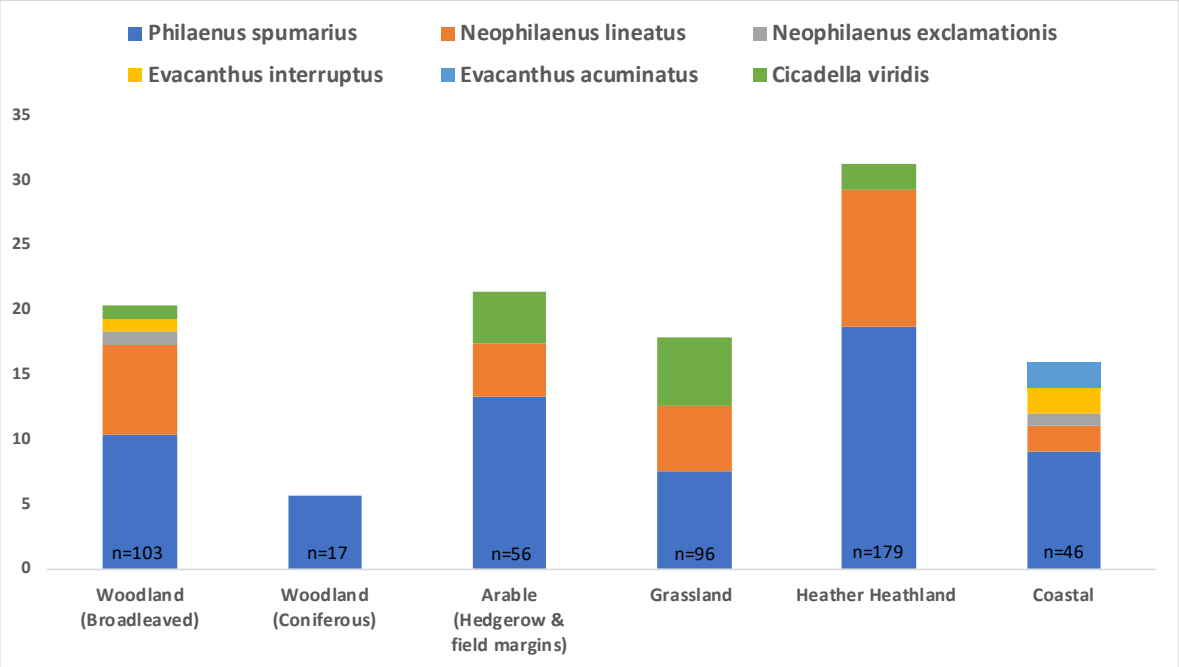

Figure 10. Potential *Xylella* vectors present in Scotland, 2018-2019. Average numbers of adult vectors collected from a 100 m transect at two timepoints (July and August) in six different habitats.

Possible differences in abundance were observed between habitats. Initial results indicate that *P. spumarius* abundance may be lower in the understorey of woodland than other habitats for example as in heathland habitats composed principally of heather (*Erica* sp.). Any final conclusions should consider that woodland surveys did not take in to account spatial distribution of *P. spumarius* as canopies of mature trees are difficult to reliably sample.

Spain - Balearic Islands

Three species of Aphrophoridae were detected in the Balearics: *Philaenus spumarius*, *Neophilaenus campestris* and *Neophilaenus lineatus*. Results from Task 1 (see section 3.3.1. 6) indicated that in Majorca, nymphs of *P. spumarius* were more abundant in the cover vegetation of olive crops, followed by vineyard and almond. Nymphs of this species were present in the cover vegetation of all crops from early March to the end of May. Results on the temporal distribution of nymphs showed that nymphs N1-N2 and N3 were more frequent in March- early April, while N4 and N5 were more frequent in late April- May. In the case of *Neophilaenus* spp., the highest abundance of nymphs was detected in olive and almond crops. In general, nymphs of *Neophilaenus* spp. were present from the 1rst- 2nd week of March to the 4th week of April in vineyard and olive crops, while nymphs seem to be absent earlier (3rd week of April) in the almond crop. The youngest nymphs (N2) of *Neophilaenus* were found from early March to early April, while N4 and N5 were found mainly in late April. Position of nymphs in the plant did not followed any pattern. In general nymphs of *P. spumarius* were more abundant in the upper- middle part of the plant, while *Neophilaenus* spp. were more abundant in the bottom part. In regard to host- plant preference, nymphs of *P. spumarius* were found in a wide variety of species of plants, mainly from the family Asteraceae (i.e. *Chrysantemum* spp. and *Sonchus* spp.), while nymphs of *Neophilaenus* spp. were found exclusively in Poaceae species.

The general pattern of seasonality of adults *P. spumarius* recorded in Majorca showed differences between vegetation types at sampling locations (herbaceous cover, tree, shrubs species surrounding the crop, border vegetation). The highest abundance of adults was

recorded in May and October in the cover vegetation. Presence of adults increased in trees in June, while presence in the border vegetation of the crop increased in August and decreased around October. In the case of *Neophilaenus* spp., the highest abundance of adults was detected in the cover plants in May and November, however its presence in trees and border vegetation can be considered negligible.

In the case of the seasonality per crops, the seasonal pattern of adults of *P. spumarius* in the cover vegetation was similar in all crops with a peak of adults in May and a second one between October-November. In terms of abundance, cover vegetation in almond crop showed lower abundance of adults compared to that in olive and vineyard crops. However, abundance of adults was higher on olive and almond trees compared to negligible numbers of adults observed on vines The seasonality of adults in the border vegetation was unclear depending on the year. In general, they are present from July to November. The seasonal pattern for *Neophilaenus* spp. was similar to *P. spumarius*, but *Neophilaenus* species seem to be present in a lower density in all crops.

From the sampling conducted in summer and autumn in Ibiza, *P. spumarius* adults were more abundant in the border vegetation of all crops, while in November they were more abundant in the cover vegetation. Adults were sporadically detected in olive trees and vineyard plants. *Neophilaenus* spp. was more abundant in the cover vegetation of almond and vineyard but in low numbers. In Formentera, adults of *P. spumarius* were detected only in olive trees. In Minorca, adults of *P. spumarius* were collected from olive trees and vineyard in summer, meanwhile in autumn, adults were only collected from cover vegetation. Adults of *Neophilaenus* spp. were found in very low abundance in vineyard in summer and autumn in cover vegetation of almond crops respectively.

Results on the DNA- barcoding analysis of the vectors (258 specimens: 185 *P. spumarius* and 73 *Neophilaenus campestris*) showed that both species are clustered in well supported monophyletic clades and its identification is therefore confirmed both morphologically and molecularly.

The transmission test showed that from 125 vectors collected from the field, 11.32% of them were positive to *X. fastidiosa*. Inoculation to plants (*M. sativa*) with *X. fastidiosa* by field collected insects was confirmed since three of the plants were positive 30 days after inoculation and one plant 60 days after inoculation. In this case, it was confirmed that field collected adults of *P. spumarius* were able to effectively inoculate the bacteria to uninfected plants that became positive to *X. fastidiosa*.

2.4.2. Molecular identification of vectors

A basic search on BLAST and BOLD was carried out to determine what sequences if any are available online for identification of the more important vector and other potential vectors (Table 6).

Confirmed vectors or those identified as high risk	CO1	ITS2	CytB
Aphrophora alni	25	1	1
Aphrophora salicina	1	*	*
Cercopis vulnerata	*	*	*
Cicadella viridis	15	*	*
Neophilaenus campestris	2	1	1
Philaenus italosignus	3	*	1
Philaenus spumarius	>100	3	>100
Other species			
Aphrodes spp.	>100		
Lepyronia spp.	>100		

Mesoptyelus sp.	1		

Table 6. Number of available records found on Genbank for vectors which have been confirmed as vectors or are identified as having high potential of acting as vector (EFSA, 2015)[15]. Included are three species that can be confused with *Philaenus* spp.

Alignment of these sequences alongside sequences generated from specimens collected as part of the project demonstrate it is possible to use CO1 barcoding to identify the *Philaenus spumarius* from other members of the genus as well as other species often confused with *Philaenus* spp. Other vectors such as *Neophilaenus campestris* can also be identified using BOLD and Genbank online databases. (Figure 11).

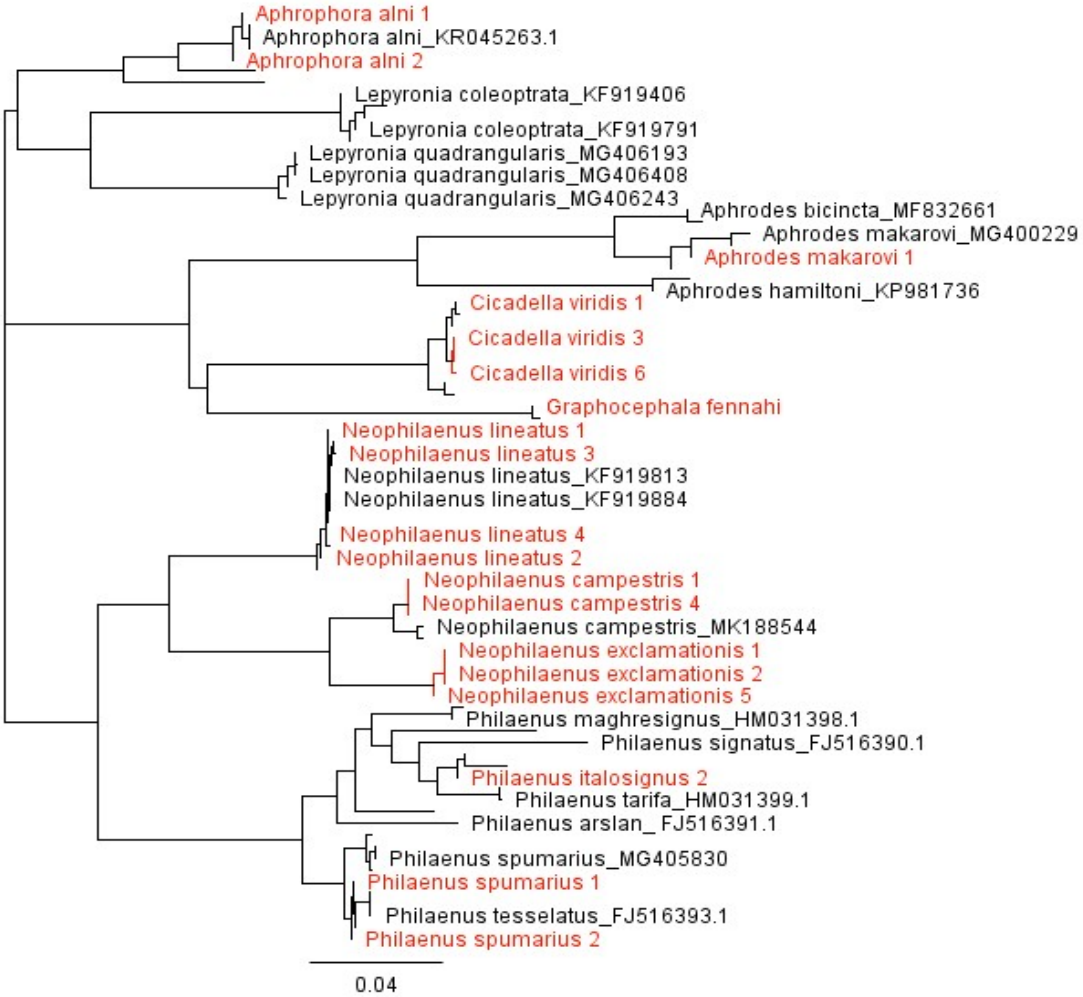

Figure 11. Neighbourhood-joining tree of CO1 of potential vectors (as available from Genbank, 2019). Samples from consortium members highlighted in red.

Sequences from other gene regions, such as ELF and CytB, can also be used for confirmation of molecular identification and are available on GenBank for *Philaenus* species. It should be noted that it is more difficult to differentiate between *P. spumarius* and *P. tessalutus*. Geographically *P. tessaltus* appears to be limited to the Iberian Peninsula, however

[15] EFSA PLH Panel (EFSA Panel on Plant Health), 2015. Scientific Opinion on the risks to plant health posed by Xylella fastidiosa in the EU territory, with the identification and evaluation of risk reduction options. doi:10.2903/j.efsa.2015.3989

mitochondrial DNA alone cannot be used to differentiate between the two and comparison of other gene regions is required to distinguish between the two species.
Therefore, to aid molecular identification of other *Philaenus* species within Europe and Mediterranean regions it is recommended that more specimens of other species within the *Philaenus* genus are sequenced for the Folmer CO1 region. Although several sequences are currently available in Genbank, the majority of these sequences only span 300-400 bp and may not lie within the 5' region of the mitochondrial Cytochrome c oxidase subunit I gene generally used for CO1 barcoding creating the potential to cause confusion. Future work should also target less represented individuals such as *Cercopis* spp, *Neophilaenus* spp., *Aphrophora* spp. and *Cicadella* spp. as well as including *Mesoptyelus* sp. which can be confused with P. spumarius. Work should include producing reference sequences for other gene regions such as ITS2 and ELF to aid with future identification of vectors.

Development of tests for the molecular identification of the main vectors
Development of specific tests for molecular identification of vectors focused on the two main vectors *Philaenus spumarius* and *Neophilaenus campestris*. Sequences generated for the ITS2 gene region for *P. spumarius* and other Aphrophoridae were aligned alongside other *Philaenus* spp. available on Genbank (Figure 12) and based on this alignment primers and probes were designed (PhiSpu_ITS2_F: 5' – TCATAACCCCACGTTTGTCC – 3', PhiSpu_ITS2_R: 5' – CAATTGTTCCGCATCGTACG – 3', PhiSpu_ITS2_P: FAM- 5' – GCCCACAACCGCCACGACCA -3' BHQ1).

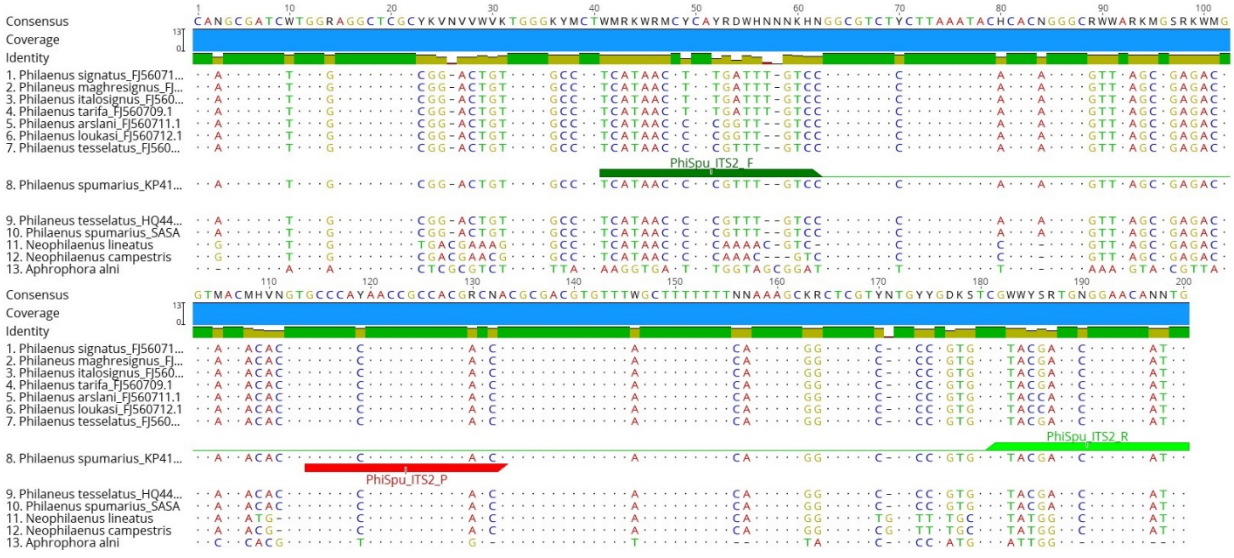

Figure 12. Alignment of ITS2 sequences from confirmed vectors and other closely related species. Primers and probe indicated in green and red respectively.

The *Philaenus spumarius* test was validated against species of interest as well as a selection of non-target insects (Table 7). The test was checked for repeatability and reproducibility.
Philaenus spumarius material (50 pg/µL and 5 pg/µL) and non-target species (15 ng/µL) were tested in triplicate over three separate runs. A consistent positive signal was achieved, and no amplification was observed for non-target species. The same test was also performed using Takyon™ Rox Probe MasterMix (Eurogentec) using different equipment (7900 Applied Biosystems) and results were consistent with previous tests.

Table 7. Average CT values obtained from real time PCR assay for *Philaenus spumarius* specific assay

Species	JumpStart (Taq ReadyMix)	Takyon blue
Neophilaenus lineatus	Undetermined	Undetermined
Neophilaneus campestris	Undetermined	Undetermined
Aphrophora alni	Undetermined	Undetermined
Evacanthus interruptus	Undetermined	Undetermined
Cicadella viridis	Undetermined	Undetermined
Philaenus spumarius (adult)	22.09	21.75
Philaenus spumarius (nymph)	28.13	27.93
Balclutha punctata	Undetermined	Undetermined
Neophilaenus lineatus (nymph)	39.17	Undetermined
Neophilaenus lineatus (adult)	Undetermined	Undetermined
Aphrophora alni	Undetermined	Undetermined
Elymana sulphurella	Undetermined	Undetermined
Philaenus spumarius (adult)	20.89	20.630
Philaenus spumarius (nymph)	29.18	28.828
Philaenus italosignus	Undetermined	nd
Philaenus italosignus	Undetermined	nd

For those *Philaenus* species for which DNA was not available *in silico*, checks were carried to eliminate any potential cross-reactions and it is likely that this test will not discriminate between *P. spumarius* and the closely related *P. tesselatus.* Therefore, to fully validate the test *in vitro*, testing of other *Philaenus* species is recommended and future work should focus on development of a test capable of differentiating between *P. spumarius* and P. *tesselatus*.
Development of a *Neophilaenus campestris* specific test has also proven to be problematic. To date it is possible to differentiate between *N. campestris* and *N. lineatus* using real-time PCR however the currently available test cross-reacts with *N. exclamtionis*. Not all species have nucleotide sequences available in Genbank, and as tests can only be validated empirically it cannot be confirmed whether other *Neophilaenus* species will also give positive amplification using these primers. Work is ongoing to validate the test on a larger number of non-target species in the *Neophilaenus* genus.

2.4.3. Detection of *X. fastidiosa* in vectors

Comparative efficiency analysis of PCR, Real-time PCR and LAMP assays in detecting *X. fastidiosa* in insect-vectors using three different extraction methods

PCR
Results of PCR tests conducted on total nucleic acids (TNAs) from insect vectors, extracted with three different methods (EM1-3), showed the presence of *X. fastidiosa* in both *P. spumarius* and *N. campestris*, with different levels of infections. PCR was able to detect *X. fastidiosa* in 20 *P. spumarius and 4 N. campestris* specimens only when applied on TNAs from EM3; whereas templates extracted from EM1 and EM2 didn't generate any positive reaction (Table 8).
Real-time PCR
Results of real-time PCR tests showed that none of the *P. spumarius* was positive for the presence of *X. fastidiosa* when TNAs from EM1 were used as template; however, 12 positive reactions have been detected in the templates of EM2 and 20 in those of the EM3. In the case

of *N. campestris*, only 3 insects were positive for *X. fastidiosa* using TNA from with EM2 and 4 in the EM3. Similarly, to PCR results, none of the TNAs from EM1 reacted positively in real-time PCR (Table 8).

LAMP

Results of LAMP tests showed differential positive reactions from TNAs extracted from insect-vectors by the three EM. LAMP was able to detect 8, 17 and 20 *X. fastidiosa* infected *P. spumarius* when applied on TNAs from EM1, 2 and 3, respectively (Table 8). In the case of *N. campestris*, LAMP has detected 1, 2 and 4 *X. fastidiosa* infected specimens from TNAs of EM1, 2 and 3, respectively (Table 8).

Table 8. Results of PCR, real-time PCR and LAMP tests applied on TNAs extracted by three different methods (EM) from *P. spumarius* and *N. campestris*. The specimens found positive to *X. fastidiosa* in the three techniques (a total of 24 specimens) were used as positive controls for extraction methods and detection techniques validation.

Insects species		PCR			Real-time PCR			LAMP		
		EM1	EM2	EM3	EM1	EM2	EM3	EM1	EM2	EM3
P. spumarius	Positive	-	-	20	-	12	20	8	17	20
	Negative	100	100	80	100	88	80	92	83	80
Total Infection %		**-**	**-**	**20**	**-**	**12**	**20**	**8**	**17**	**20**
N. campestris	Positive	-	-	4	-	3	4	1	2	4
	Negative	100	100	96	100	97	96	99	98	96
Total Infection %		**-**	**-**	**4**	**-**	**3**	**4**	**1**	**2**	**4**

Among the three methods tested for TNAs extraction from *P. spumarius* and *N. campestris*, the EM3 (using the CTAB protocol) proved to be the most suitable for extracting a sufficient amount of *X. fastidiosa* genomic DNA to be detectable by any of the molecular techniques used in this study. This result was supported by the overall identification of 24 *X. fastidiosa* infected insects that all resulted equally (100%) infected in the three detection techniques. Although the EM1 and EM2 were the simplest to perform and least expensive as few reagents were needed for their preparation; their outcomes remain precarious when used in different diagnostic techniques to detect *X. fastidiosa* in insect vectors. In contrast EM3 was the most efficient for bacterial DNA extraction but required greater effort than the other two extraction methods.

In general, the real-time PCR and LAMP showed to be more efficient to detect *X. fastidiosa* in insect-vectors, independently from the EM used, than the conventional PCR. However, when applied on the TNA from EM2, the real-time PCR showed to be more sensitive (12%), *i.e.* identification of 12 *X. fastidiosa* infected *P. spumarius* over none in PCR, and in the case of *N. campestris,* 3 specimens were detected over none in PCR. As expected, the LAMP has made the big difference at the sensitivity level, to detect *X. fastidiosa* from TNAs extracted with the EM1 whilst the other techniques have totally failed. The overall results showed that this technique was the most suitable to detect high numbers of *X. fastidiosa* infected insects; however, also in this case the best performance of this technique was obtained from EM3, and to a lesser extent with TNAs from EM2 and/or EM1.

Based on the results obtained in our study and conditions, the EM3 (CTAB protocol) is the most suitable extraction method for the obtention of high concentration of *X. fastidiosa* genomic DNA from insects that by its turn is accurately detected using LAMP test.

2.5. Conclusions and recommendations to policy makers

2.5.1. Vector surveys including evaluation of vector sampling and trapping techniques

Philaenus spumarius and *Neophilaenus campestris* are abundant and common in vineyards/olive groves however, other xylem feeding species should not be overlooked and may be of importance. For example, *Cicadella viridis* is more abundant in Netherlands, the United Kingdom, *etc*. compared to Mediterranean regions. The introduced Nearctic sharpshooter *Graphocephala fennahi* is also common in northern regions where rhododendron is grown and though nymphs are monophagous on this host, adults were found on a wide range of woody species.

In general, *P. spumarius* is more abundant in the canopy in southern European countries than other species such as *Neophilaenus* spp.

Based on the investigation into trapping methods, the consortium recommends the use of sweep netting as the best method for catching significant numbers of vectors with other trapping methods, such as sticky traps, being more suitable for monitoring for the presence of vectors within agricultural and horticultural environments and of less use for collecting vectors to screen for *Xylella*.

2.5.2. Molecular identification of vectors

DNA barcoding based on the 5' region of the mitochondrial Cytochrome c oxidase subunit I gene (CO1) using the Folmer primers is suitable for identifying potential vector species.

Improved database of CO1 sequences for less common potential vectors is still required as well as further specimens of *Philaenus* spp. and to aid with future molecular identification of closely related species such as *P. spumarius* and *P. tesselatus* reference sequences of other gene regions need to be generated.

A species-specific real-time PCR was developed during the project for *Philaenus spumarius*. Work is ongoing for the development and validation of a test for *Neophilaenus campestris*. International collaboration on this topic would allow sharing of reference material to ensure that the test has optimal inclusivity.

2.5.3. Detection of *X. fastidiosa* in vectors

Based on the results obtained, the CTAB DNA extraction protocol (EM3) is the most suitable for the obtention of high concentration of *X. fastidiosa* genomic DNA from vectors.

Overall, the real-time PCR and LAMP tests have proved to be more efficient than conventional PCR at detecting *X. fastidiosa* in insect vectors, independently from the extraction method used, and should then be recommended.

In general, *P. spumarius* showed higher ability to transmit *X. fastidiosa* compared to *N. campestris*.

2.6. Benefits from trans-national cooperation

3. The involvement of numerous partners within the project permitted sharing of sampling methodologies and improved knowledge of the biology of potential vectors in several European habitats. For the molecular aspect of the work, collaboration facilitated the exchange of vectors of both voucher specimens and DNA for the

development and validation of molecular tests. This contributed to the development of an EPPO Diagnostic Protocols on the known vectors of *X. fastidiosa* (PM 7/141 *Philaenus spumarius, Philaenus italosignus* and *Neophilaenus campestris*[16]). The work carried out through the project also contributed to policy discussions directly feeding into sampling strategies for vectors.Publications

3.1. Article(s) for publication in the EPPO Bulletin

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

Lester K, Murphy K, McCluskey A, Cairns R, Fraser K, Kenyon D. 2020. *Xylella fastidiosa*: an overview of research at SASA. Proceedings Crop Protection in Northern Britain 2020. pp45-50.

[16] https://onlinelibrary.wiley.com/doi/epdf/10.1111/epp.12610

4. Open Euphresco data

None.