Euphresco Final Report
Tracking vectors of bacteria and phytoplasmas threatening Europe's major crops (VECTRACROP)

Topic area
Phloem and xylem feeding insect vectors, fruit and field crops, bacteria and phytoplasmas of phytosanitary concern - **Topic Description 2015-D-168**

Topic title
Tracking vectors of bacteria and phytoplasmas threatening Europe's major crops (VECTRACROP)

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2. Short project description

Project summary, including aims and objectives
The main goal of the project was to generate more information on insect vectors of bacteria and phytoplasmas of phytosanitary concern, more specifically • on phloem and xylem feeding insects that should be tested as possible vectors for bacteria and phytoplasmas • on the fruit and field crop range that the vectored bacteria and phytoplasmas cause damage to • on the capability of the insects to vector specific bacteria and phytoplasmas of phytosanitary concern • on the specific association between vector and plant pathogen The main objectives of the project were to gain insight into the following questions: • Should yet-unknown phloem and xylem feeding insects (Auchenorryncha) be regarded as vectors of the pathogens in selected major crops in Europe? • What monitoring and trapping techniques are optimal to efficiently survey the phloem and xylem feeding insect vectors? • Are there reliable and validated diagnostic tools available to detect/monitor the prokaryotic plant pathogens inside the vectors? • Can we generate extra information on the transmission pathways by monitoring phloem and xylem feeding insect vectors in and around selected infested fields? • Can we generate extra information on the host range of the prokaryotic pathogens by performing vector-transmission trials between different test crops? • Can we reduce the number of treatments of pesticides due the better understanding of relation vector/pathogen/environment and thereby enable a better integrated management of vectors and diseases in the crops. The five research partners ILVO-BE and CRA-W-BE, ANSES-FR, INIAV-PT and INRA-MO dealt with several of these objectives using the following approach: The Belgian partners undertook a survey using various pathogen monitoring and trapping techniques in the field to collect phloem and xylem-feeding insects (*Auchenorrhyncha*) which could potentially act as vectors of '*Candidatus* Liberibacter spp.' and '*Canditatus* Phytoplasma' in the following susceptible crops: fruit trees (*Malus*, *Pyrus* and *Prunus*), *Apiaceae* (carrot, celery and potentially wild species near commercial fields). The study concentrated on Belgian infected orchards and organic crops (both known infestations and new outbreaks) and was then expanded to crops located in the (adjacent) vector-friendly environments. An interdisciplinary team of entomologists and molecular pathologists used morphological keys and molecular tools to identify the collected vectors and analyzed these as to whether they were transporting the selected pathogenic prokaryote. Through various transmission experiments in controlled greenhouse trials, the identified vectors were then used to characterize the host range of the pathogenic bacteria, in view of the evaluation of risk

represented by the pathogen and the vector for economically important crops grown in Belgium.

The Portuguese partner undertook a survey of potential Auchenorrhynca vectors of *Candidatus* Phytoplasma rubi (RS) on *Rubus* plants (both cultivated and wild) and on associated weeds inside the *Rubus* plots and in the surroundings, in an experimental agriculture station where the pathogen presence was already detected (in 2013). Insects' bio-ecological data were registered. Insects were taxonomically identified, both with morphological and molecular tools. Weeds as insect alternative hosts were identified. The presence of the pathogen inside the collected insects was assessed. Nested PCR, RFLP and sequencing and Real-time PCR were used to test all plants and insect vectors.

The French partner was mainly involved in monitoring xylem feeding insects, potential vectors of *Xylella fastidiosa*, in and outside the epidemic area, or on a wider scale, in targeted non-infested crops, and also surveyed psyllid species potential vectors of '*Ca.* Liberibacter solanacearum' on carrot and potato fields. ANSES built a survey network with the help of stakeholders from the French agricultural profession. The field survey was limited to mainland France and Corsica on specific crops and if relevant, also on adjacent wild plants. The main production areas were targeted. The extensive field surveys were conducted on a regular basis from spring to autumn in different regions of France. Several collection methods were used and compared: sweep net, stem tap, yellow sticky traps and/or yellow plates.
The identification of xylem-fluid feeders and psyllid species was done by morphological means complemented with molecular tests. This study allows a better knowledge of common potential vector species according to the region (north, center or south of France).
The identification of *Xylella* in insects was carried out by the bacteriology and virology unit of Angers on some samples depending of the time and resources available for that.

Since from the targeted pathogens, only the '*Ca.* Liberibacter solanacearum*'* was reported in Morocco, the main tasks of the Moroccan partner focused on the uninfected crops olive, grapevine, solanaceae and apiaceae (orchard agro-ecosystem and vegetable field agro-ecosystem). The survey of potential Auchenorrhynca and Psylloidea vectors was done on host crops and surrounding plants especially weeds. Several collecting traps were tested in order to have an overall idea about appropriate tools.
The laboratory of virology (INRA-Kénitra) conducted a large scale survey to investigate the presence of *Xylella fastidiosa* on olive and grapevine in the regions of Haouz, Doukkala, Meknes, Gharb and Berkane. The same tree crops, in addition to pear and apple, also investigated the presence of phytoplasma pathogens. Furthermore, other field surveys were carried out in the same regions on carrot in order to monitor the presence and distribution of '*Ca.* Liberibacter solanacearum', which was reported in the country in 2014. Molecular diagnostic tools (PCR, real-time PCR and sequencing) were used for the detection of these pathogens in plant tissues and in potential insect vectors.

Project duration:	01/03/16 – 31/05/18

Detailed project description

Overview of Work-packages

Work-packages (WP)	
No. of WP	**Title: Monitoring of potential vectors in and around targeted crops**
1	Project management and co-ordination
2	Monitoring of potential vectors in and around targeted crops
3	Identification of potential vectors and transported pathogens
4	Transmission capacity of important vectors
5	Phenology and host range of (important) vectors
6	Recommendations and dissemination

Project deliverables

- Deliverable 1: Timely reports, annual meetings with project partners and widely visible dissemination of the project results through diverse output (pamphlets, database, publications) (ongoing up to month 24)

- Deliverable 2.1: Report evaluating trapping systems for phloem and xylem feeding insects (Month 12)

- Deliverable 2.2: Collection of phloem and xylem feeding insects that could be potential vectors of AS, AP, PD, ESFY and '*Ca.* L. solanacearum' in Belgium, RS in Portugal and *Xylella fastidiosa* and '*Ca*. L. solanacearum' in mainland France and Corsica (Month 21)

- Deliverable 2.3: Collection of phloem and xylem feeding insects that could be potential vectors for phytoplasmas and/or Liberibacter in Belgium, *X. fastidiosa* and '*Ca*. L. solanacearum' in mainland France and Corsica and potential vectors of RS in Portugal (Month 21).

- Deliverable 3.1: List of potential insect vectors for phytoplasmas and/or Liberibacter in the partner countries (Month 24)
- Deliverable 3.2: DNA database containing the DNA sequences of the identified vectors (Month 24)
- Deliverable 3.3: List of identified pathogens inside collected Auchenorhyncha and Psylloidea vectors in the partner countries (Month 24)
- Deliverable 4.1: Methodology regarding the insects rearing of certain Auchenorhyncha or Psylloidea insect vectors (Month 18)
- Deliverable 4.2: Report evaluating transmission capacity of Belgian insect vectors for AP and PD and possibly '*Ca.* L. solanacearum' and other phytoplasma or Liberibacter (Month 24)
- Deliverable 5.1: Report on phenology of important Belgian insect vectors for AP and PD and possibly '*Ca.* L. solanacearum' (Month 24)
- Deliverable 5.2: Report on phenology and host range of important phloem and xylem feeding insects occurring throughout the partner countries (Month 24)
- Deliverable 6.1: Report analyzing the probability of spread of the studied phytoplasma species and '*Ca.* L. solanacearum' by Hemiptera (Month 24)
- Deliverable 6.2: Report containing general recommendations on the management of the identified potential vectors (Month 24)

Kick-off Meeting (21 June 2016; ILVO, Belgium) – TELECONFERENCE MEETING with the transnational project partners. National project guidance committee meeting, SPF, Brussels, Belgium.

- Define cooperative research aims, agree on time of reporting and specify the partners responsibilities;
- Outline the expected deliverables of each partner;
- Agree on the collaboration/exchange between partners working on the same WP during the project;

- Share the project program/tasks with the Belgian NPPO and stakeholders through a joint national project guidance committee meeting.

- Discuss Tech Transfer and dissemination.

Annual Meeting (May 2, 2017) - National project guidance committee meeting, SPF, Brussels, Belgium.

The steering committee (= project guidance committee) was informed on the intermediate results of the ongoing Belgian project tasks, followed by a discussion and suggestions to optimise the execution of the national project.

Final Meeting (July 2, 2018) - TELECONFERENCE MEETING with the transnational project partners. National project guidance committee meeting, SPF, Brussels, Belgium.

- Exchange results of year 1 and 2;

- Prepare final project report; discuss scientific and technical publications in preparation;

- Draft briefing EUPHRESCO reporting requirements;

- Discuss follow-up plans for continued cooperation and funding initiatives;

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2. List of abbreviations

AP	apple proliferation phytoplasma
PD	pear decline phytoplasma
ESFY	European stone fruit yellows phytoplasma
CaLso	'*Candidatus* Liberibacter solanacearum'
AY	Aster yellows phytoplasma
COI	Cytochrome c oxidase subunit 1 gene
ITS	Internal transcribed spacer
BOLD	Barcoding of life database

3. Executive summary

1. **Objectives –Project Research questions addressed in this study**

- Is it possible to identify new phloem and xylem feeding insect vectors (Auchenorryncha vectors) threatening selected major crops in Europe?
- What monitoring and trapping techniques are optimal to efficiently survey phloem and xylem feeding insect vectors?
- Is it possible to reliably monitor pathogens inside the vector based on validated diagnostic tools?
- Does monitoring potential phloem and xylem feeding insect vectors in and around selected infested fields deliver extra knowledge on the transmission pathways of phytoplasma, Liberibacter and related Auchenorrhyncha transmittable pathogens?
- Do transmission trials between (potentially) susceptible crops result in extra knowledge on vector transmission pathways?
- What is the status of *Xylella fastidiosa* in olive, grapevine and Citrus in Morocco ?

2. **Obtained results**

The phytoplasma and Liberibacter vector species this project is focusing on:

- Pear decline phytoplasma (‘*Candidatus* Phytoplasma pyri’, PD).

Most important known vectors: the pear psyllids *Cacopsylla pyricola & C. pyri* are assumed to be the key vector of PD. Also *Cacopsylla pyrisuga* may play a role in PD dispersal.

Cacopsylla pyricola *Cacopsylla pyri* *Cacopsylla pyrisuga*

- Apple proliferation phytoplasma (‘*Candidatus* Phytoplasma mali’, AP).



Most important known vectors: Two vectors have been reported to be capable of transmitting AP, namely *Cacopsylla melanoneura* and *Cacopsylla picta*.



Cacopsylla melanoneura *Cacopsylla picta*

*Is there a role for **Fieberiella florii** (Auchenorrhyncha) ?*
Transmission reports: Krczal et al. 1988; Tedeschi and Alma 2006.

- Aster yellows phytoplasma ‘(*Candidatus* Phytoplasma asteris’, AY).

This phytoplasma is transmitted by several leafhoppers generally belonging to the 4 genera *Macrosteles*, *Euscelis*, *Scaphytopius* and *Aphrodes*.


Macrosteles laevis


Euscelis incisus

Scaphotopius acutus


Aphrodes acutus

- Rubus stunt phytoplasma (‘*Candidatus* Phytoplasma rubi’, RS)
- *‘Candidatus* Liberibacter solanacearum’.

‘Ca. Liberibacter solanacearum’ species is transmitted to solanaceous species by the potato/tomato psyllid, *Bactericera cockerelli*. ‘*Ca*. L. solanacearum’ has also been detected in carrot (and celery) plants in several EU countries and is vectored by the carrot psyllid, *Trioza apicalis & B. trigonica (Can. Islands).*

Bactericera cockerelli

Trioza apicalis

Symptoms of CaLso on carrot

- *Xylella fastidiosa*

Although the psyllids belonging to the Psylloidea (suborder of the Sternorrhyncha) appear to be the important vectors for phytoplasma's and Liberibacter, very little is known about the role of the leafhoppers, planthoppers, cicadas, treehoppers and spittlebugs belonging to the Auchenorrhyncha suborder for several of these phytoplasma's and Liberibacter. Phloem and xylem feeding insects are the only organisms capable of taking up phytoplasma's from infested plants and most phloem-feeding insects belong to Auchenorrhyncha (Weintraub and Beanland, 2006).
In Belgium, the project focused on PD, AP and AY phytoplasma diseases and their vectors, in commercial production sites with known phytoplasma presence; this is in pear, apple and carrot, respectively. The online database "waarnemingen.be" in Belgium reports 409 different species belonging to the Auchenorrhyncha suborder that have been sighted up today. The main known vectors of the selected fruit tree associated phytoplasma pathogens (AP, PD) belong to the family of the psyllids. However, Tedeschi and Alma (2006) demonstrated that the leafhopper *Fieberiella florii*, belonging to the Auchenorrhyncha, is able to transmit the phytoplasma '*Candidatus* Phytoplasma mali'. In contrast, the main vectors of '*Candidatus* Phytoplasma asteris' belong to the Sternorrhyncha, Auchenorrhyncha and other Homoptera (Weintraub and Orenstein, 2004; Weintraub and Beanland, 2006; Duduk et al. 2008; Landa et al. 2013). These results suggest that, next to the known Sternorrhyncha, the Auchenorrhyncha vectors for certain phytoplasama, also other Auchenorryncha could possibly also play an important role in the transmission and spread of phytoplasmas (and possibly also '*Ca*. Liberibacter sp.') with predominantly psyllids as main (known) vector.
In addition, the French, and Morroccan partner focused on *Xylella fastidiosa* and its (potential) vectors. In Morocco, the initial task was a pathogen survey, whereas in France a large vector survey was aimed at. Facing some outbreaks with Rubus stunt phytoplasma, Potugal focused on a vector survey in commercial Rubus productions, adjacent hedges and weeds in the surroundings. Additionally, an Auchenorrhyncha survey was organized in avocado (Persea Americana in the region of Gharb and Loukkos between September and November 2018. The newly discovered presence of Penthimiola bella in avocado in Morrocco was described.

In Belgium, the comparison of the trapping systems was done on two locations where both apple and pear orchards were present, and carrot fields with known AY infestation. Sweep netting and beating techniques were efficient, yet mainly rely on the intensity of the monitoring (eg. the number of trees per orchard/field, and the number of beatings per tree (for apple and pear)). The most reliable method for a general Auchenorrhyncha monitoring (and insect monitoring in general) proved to be the use of yellow sticky traps. Also the use of glue on the plants themselves was tested in carrot. The method is useful if you want to know which insects landed on a specific plant, eg. an infected plant. However, glue is also likely to influence the feeding behavior and as such most likely not very useful to detect the pathogen inside the insect. Systematic sampling using yellow sticky plates was used in in pear (4), apple (2) and carrot fields (1) and yielded a lot of information on the presence of the Auchenorrhyncha, and thus (potential) vectors in these crops. On every selected field, 9 plates were placed in a grid pattern resulting in a checkerboard map for the insect presence. Every 2 to 3 weeks, the plates were replaced to yield information in a time series over the season. Similarly, in France, a common protocol for plot registration and method of sampling, including the tools used for sampling (sweeping net, yellow pan trap, etc.) were evaluated and implemented. In Portugal, yellow sticky plates were used in the commercial Rubus productions, whereas sweepnetting was used in the surrounding weeds.

In Belgium, standard molecular identification, based on the COI (and ITS2) and a blast against the BOLD database proved to be a fast and reliable tool to assist the morphological identification strategy. Based on the monitoring, graphs have been produced to visualize the results of the presence of the Auchenorrhyncha per field that was monitored. In France, morphological identification was used.
Additionally, in two of the carrot fields, also the presence of '*Candidatus* Liberibacter solanacearum' haplotype D was confirmed. The insect monitoring the shifted to the potential presence of psyllids, the known natural vectors of '*Ca*. L. solanacearum'. However, no *Trioza* sp. or *Bactericera* sp. were captured, and also in general, only very few psyllids were found in these '*Ca*. L. solanacearum' infected carrot plots. The remaining seed lots from those two plots was also tested and the cv Nerja seed tested positive for the presence of '*Ca*. L. solanacearum' at both locations. Yet, seed transmission is believed to play only a minor role in the spread of the pathogen.

Pathogen detection in the insects: in a selection of insects from infected orchards, AY phytoplasma was detected in *Macrosteles sexnotatus*, *Graphocephala fennahi*, and *Typhlocyba* sp. no insect tested positive for AP or PD (in apple or pear, respectively).

Insect populations of *Empoasca decipiens* (on *Malus* and *Faba*), *Zyginidia scuttelaris* (on *Corylus*) and *Cacopsylla pyri* (on *Pyrus*) were established, in preparation of the transmission experiments. Raising insect populations (*Empoasca* and *Cacopsylla*) initially succeeded, yet a population collapse resulted in the fact that no transmission trials could be set up.

Outdoor cages were placed on *Malus, Pyrus, Carpinus*, *Viburnum* (all in Melle) on trees (and shrubs on which eggs are observed. Identification and population dynamics are observed biweekly. The populations did not built up in the cages. As alternative method to study the population dynamics and the geospatial distribution of the insects in the monitored fields,

heatmaps were created per location and per insect, based on the results from the grid based monitoring of the insects in the selected fields.

Finally, the French partner analyzed the spatial information obtained from the survey using QGIS mapping. The QGIS software supports viewing, editing and analysis of geospatial data. The maps that were produced represented the geographical distributions of the main potential French vectors, using the INPN (National Inventory of Natural Heritage – Inventaire National du Patrimoine Naturel) data as reference data.

A first joint intermediate project "success story" was posted on the EUPHRESCO website. Additionally, a poster presentation was given at the 69th ISCP in Ghent. A disease note on the first detection of '*Ca*. L. solanacearum' and the aster yellows phytoplasma in carrot in Belgium is in preparation. A first publication in "Proeftuinnieuws" on the Auchenorrhyncha monitoring became available in 2017. In addition, oral presentations were given during the meetings or the transnational project consortium, and an oral presentation was also given at the 70th International Symposium on Crop Protection (ISCP). Finally, a scientific publication (which will be sent to a peer reviewed journal) on all project results, as well as more accessible publications for the sector (carrot and fruit) are also in preparation.

4. Description of the problem the research should solve

Several harmful phytoplasmas and bacteria such as *Xylella fastidiosa*, '*Candidatus* Liberibacter solanacearum' or fruit crop phytoplasmas are nowadays threatening important European crops such as potatoes, olives and fruit crops, with major economic impact. For these pathogens, some phloem and xylem feeding insects are already identified as vectors or candidate vectors whereas involvement of other Auchenorryncha in the transmission of the diseases has not yet been investigated.
Knowledge on vectors involved, their phytosanitary status in specific areas, their host range, alternative hosts, and influence of abiotic factors on the vector occurrence and disease transmission capacity is fragmented or lacking.
A comprehensive scientific insight is indispensable in order to improve risk evaluation and define effective regulation and phytosanitary management strategies adapted to local conditions for this type of vectored plant diseases.
Coordinated actions to join related plant bug researchers and involved parties would be beneficiary in the long run.

5. Research objectives

In order to address the above mentioned eminent problem, the research should preferably focus on the following tasks and deliverables:

- Mapping scientific knowledge and research projects on phloem and xylem feeding insect vectors (Auchenorryncha vectors) threatening selected major crops in Europe
- Identifying knowledge gaps and prioritise research activities on Auchenorryncha vectors and their interaction with pathogens, potential hosts and abiotic factors.
- Optimisation and knowledge building on phloem and xylem feeding insect vector monitoring and trapping techniques.
- Validated diagnostics tools for vector identification and identification of the pathogen inside the vector.
- Monitoring potential phloem and xylem feeding insect vectors in and around infested fields.
- Performing transmission trials towards susceptible crops.
- Research on life cycle and host range of these vectors depending on regional climate and seasonal variation.
- Vector plant host preference studies.
- Launching coordinated actions to join plant-insect researchers and parties involved in vector transmissible plant pathogen issues.
- Formulating recommendations for disease and insect management.

6. Materials & methods

WP1: Monitoring of potential vectors in and around targeted cops

1. *Belgium (ILVO & CRA-W)*

The following susceptible crops were inspected: fruit trees (*Malus*, *Pyrus* and *Prunus*), carrots, celeries, grapevines and potatoes. Beating and vacuum sampling was applied followed by a manual sorting to separate Hemiptera from insects of other taxonomic orders. Symptomatic plants were sampled and insects were collected in each visited plot.

Two types of sampling were performed:

Task 1:
In order to test trapping systems for phloem and xylem feeding insects, a Pro force suction machine will be tested for Auchenorrhyncha monitoring. In addition, a light trap system on batteries will be developed and tested for the same purpose. These methods will be complemented with methods where the lab is familiar with: pan traps using water and a surfactant (detergent) or another solvent (e.g. mono ethylene glycol), yellow sticky plates, beat-netting, sweep-netting and directly spraying a glue on the plant stems and leaves.

Task 2:
In order to gain information on Hemiptera phenology, two systematic samplings were carried out every two weeks. Two orchard infected with 16SrX phytoplasmas (apple and pear), and three carrot fields were sampled systematically. The apple, pear and carrot fields were orchards/fields with a previous history of known phytoplasma infections (apple proliferation, pear decline and aster yellows phytoplasma, respectively). Since no Liberibacter infection has been identified before, the same fields that were surveyed for phytoplasma were also surveyed for Liberibacter infections.

Task 3:
A random sample of crops (fruit trees, carrots, potatoes, celeries, grapevines) will be surveyed across Belgium. The diversity of surrounding ecosystems, small plots, organic crops and a good coverage of the Belgium territory will be favored to allow defining the status of the studied pathogens in the country. This part has not been started so far.

2. *Portugal (INIAV)*

On cultivated *Rubus* plants (inside plastic tunnels), on spontaneous ones (hedges of brambles) next to them, and also on neighboring weeds

- At the experimental Station of Fataca (Odemira, Portugal), in 2016 and 2017 At a commercial Rubus farm in the same region, in 2018
- From March to middle of July, every two weeks (during summer, temperatures are too high - few cicadellids are caught)

- Sampling devices:
  - -Coloured (blue and yellow) sticky traps used on Rubus plants (2016 - 2018)
  - -Sweep net on weeds (2016)

Figures: Pictures from the vector survey in Portugal using yellow sticky plates

Figure: picture of the weeds that were surveyed in the surrounding area

WP2: Identification and analysis of collected insects

1. *Belgium*

Task 1:
Collected insects were first identified based on morphological keys and with the help of Hemiptera external experts when necessary. However, seen the slow and difficult process, it was decided that for new insects, an immediate molecular identification test was easier than morphological identification. Once familiar with the insect species, identification was done based on morphological identification.

DNA extraction and PCR protocols were optimized in WP1 and then applied on individual DNA extracts.
Hemiptera collected from orchards and grape vines were be tested for *X. fastidiosa* (different project for ILVO) while those collected from carrots, and potatoes were tested for '*Ca*. L. solanacearum'. All collected Hemiptera will be tested for phytoplasmas using generic primers.

Task 2:
Phytoplasma infected orchards were first surveyed to set up all identification and detection tools.
Although detection methods already exist for all targeted pathogens (Firrao and Bazzi, 1994; Crosslin and Bester, 2009; Lorenz et al., 1995; Lee et al., 1998), their use on Hemiptera needs a prior validation which will be conducted on the first collections of insects available in the project. General nested PCR for phytoplasma detection (combination of P1/P7 with R16F2/R2 primer set) and the Li et al. 2009 detection method by real-time PCR were chosen as the most reliable methods to survey the pathogens.
Likewise, even if Hemiptera generic primers already exist (Ji et al., 2003), their application on the diversity of insects which was collected as well as their usefulness for the identification of insect species is unknown. A prior validation will thus also be carried out. The primers for the COI and ITS region were evaluated.
Moreover, in order to facilitate the comparison between molecular and morphological identifications, a non-destructive DNA extraction protocol was tested.
Although morphological criteria often provide a correct identification of insects, several drawbacks of this approach motivate the development of molecular identification tools: the difficulty to maintain the expertise in Entomology, the few internationally recognized standards available and the ambiguity of certain morphological criteria, especially for larval stadia, which lead to confusions.
Since DNA will be anyway extracted from collected insects, the sequencing of systematic maker gene(s) will help compare the results between experts, locations and projects.
To facilitate the comparison, nucleotide sequences generated in WP3 will be carefully validated through resequencing and sequence alignments prior to their submission in GenBank along with their respective data to constitute a collection of reference sequences of Belgian Hemiptera.
Both the NCBI GenBank and BOLD database for the actual identification based on the sequences was compared. The BOLD database proved to be more complete and more accurate and was further used in this study.

2. *France*

The vector survey was done by national partners of the French project partner (ANSES).

The following strategy was followed:

A common protocol for plot registration and method of sampling

In order to homogenize the registration of the sites followed and the methods of collection of Auchenorrhyncha potentially vectors of *Xf,* the PHL has developed a **common protocol** detailing the registration steps, the tools used for sampling (sweeping net, yellow pan trap...) and the insect vectors (atlas).
To confirm their registration and join our network, our possible partners had to answer a questionnaire in order to inform several information concerning their identity, the number and the type of site followed, the monitoring frequency, the sampling methods used and the environment of the plots... This allows us to reference all the sites monitored and to be in contact with our partners. The questionnaire is available by following this link:
https://docs.google.com/forms/d/1YirLXfrBjxvLBIL-IuUu_DQPc5ET6zCzpsRkYkxWJBg/edit

The observer was free to choose the site to be monitored, taking into account the large host range of the bacterium (all strains combined), the possibilities were therefore numerous. All the answers to the questionnaire were then recorded in an Excel file, where each site has a unique name and its information. The name of the site was then used as index in our database. Once the partner registration was completed, he can start sampling his registered sites. Before each sampling, he must first fill in a "**Sampling sheet**" where he indicates the date and the unique name of the site. We then recommended and provided several sampling methods and tools, in order to evaluate these different methods for catching potential vectors. There were four types of available sampling methods:

- Barber pitfall trap
- Sweeping net
- Yellow Pan trap
- Sticky trap

Moreover, the protocol provides valuable information on the use of sampling tools, the choice of a site and the recognition and identification of potentially vector species with the list and a photographic atlas of French vectors and a reconnaissance datasheet for *Philaenus spumarius* with an identification key. We therefore indicate to our partners which insects targeted by detailing their general silhouette and the average size of the vectors.
We regularly receive sampling tubes containing a variable number of insects, or sticky trap with trapped insects. Thanks to the protocol, collectors were able to properly target and capture Cicadomorpha. Then, our laboratory identifies them, using a recording system that compiles information on sampled vectors.

Receiving and recording samples in our database (using Access, Microsoft Office)

The collectors sent the captured insects in tubes, stored in alcohol 95. Each sample received a corresponding sampling sheet and we assign to it a "**sender reference**" which is constructed as follows:

1. **Plot's name**
2. **Date of sampling**
3. **Method of sampling use** (with the codes F: sweeping net, P: sticky trap, C: yellow pan trap, B: barber trap, A: other methods and I: unknown method)
4. **Environment where insects were collected** (C: In culture, H: in hedge, A: other)

Besides, the collector may indicate the presence or not of potential vector species, often according to his own knowledge of vectors. For this example, we would have two positive samples (with potential vectors) and four negative (without potential vectors). The first sample achieved with the sweeping net would have this reference:

- **CBGP** – **0308** – **F**001**A**
- Similarly, for the second sample, we would have CBGP – 0308 – **C**002**H**

The numbers "001" and "002" are simply identifying numbers, generate for each sample. Between the two samples, only the sampling technique (F, C) and the environment (A, H) change, the plot and the date remaining the same.

Once we have created the reference and assigned it to its sample, we record the sample in a traceability notebook where it is assigned a sample number.
In addition to its reference, we assign an unique number to the sample received for traceability, this number and other information are entered in a .mdb file on the **Access** software. We record in our database (mdb file) and for each sample, the date of capture, the sender reference, the type of crop specific to the parcel (given when the parcel was registered). In Access, we use a table "XYLELLA_TABLE PIVOT" which lists all the samples and their associated plots, which makes it possible to link the Excel file with all sites 'information' and the Access database with the results of the analysis, the identified insects.
This "query" can be exported from access to excel and allows us to link the insects analysed within a sample to the specific information of the sample in question, such as the date of capture, the type of crop and the environment of the site. And this with the final aim of extracting information on the phenology of insects and their environment.

How we identify insect?

Morphological identification involves the use of dichotomous keys available in the literature on these groups of insects: observation of external morphological characteristics associated with the study of genitalia. But there is no synthetic work allowing the identification of all the species present in France. For leafhoppers in general, volumes of French fauna are available (Ribaut, 1952, 1986; Della Giustina, 1983, 1989), and published in 1989 for the more recent ones, they do not take into account species introduced since that date. More recent German works are available, but they do not cover Mediterranean species (Biederman & Niedringhaus, 2009; Kunz et al., 2011; Holzinger et al.,2003…). A documentary collection of publications is essential to complete these gaps.

So, we use stereomicroscope to observe the external morphology of the insect.
In order to observe the genitalia of the insects under optical microscope, we must go through several stages:

1. Extraction of the last segments of the abdomen or genitalia of the insect using pliers
2. The extracted genitalia are placed in a watch glass, Potassium hydroxide (KOH) is added. We then place the watch glass on a hot plate. We leave the watch glass for 20 minutes, it dissolves the tissues and keeps only the exoskeleton.
3. Once the time has passed, we put the genitalia in distilled water for about twenty minutes to wash the KOH
4. Then we make a dehydration with an alcohol bath 70° (5min) and 96° (5min)
5. We finally transfer the genitalia in a solution of lavender oil to complete the preparation. Then, the genitalia are transferred on a slide with a small drop of Canada balm, a cover slip is applied on the slide. The slide is then placed in an oven heated to approximately 50°C. This final stage allows the good conservation of the insect's genitalia and the subsequent creation of reference collections (image 19).

Then the slide can be easily observed under an optical microscope

The analyzed and identified insects are conserved in alcohol tubes (70° for non-vectors of Xf and 95° for potential vectors in order to preserve their DNA for future molecular analyses that will allow detection of the bacterium in the insect) with the sample identification number to which they belong, written in Chinese ink or pencil on a tag placed in the tube.

Mapping

In order to realize maps and to analyze spatial information, we used QGIS (2.4.0 – Chugiak version), a free and open-source cross-platform desktop geographic information system (GIS) application that supports viewing, editing, and analysis of geospatial data.
We thus utilized different types of data and files format as:

- **Geographic coordinates**: all our geographical coordinates are from partners' data and enable us to create graphical maps. We use the **World Geodetic System** (WGS84) as reference coordinate system. We thus use our tabular data and save it as a text file which contains at least 2 columns with the latitude (X) and longitude (Y). We then import these coordinates in QGIS with this txt (or CSV) file as "delimited text layer".
- **Shapefile** (.shp), a popular geospatial **vector data** format that allows you to store geometric location and associated attribute information. Indeed, the shp format can spatially describe vector features: points, lines, and polygons, representing, for example, rivers, and lakes. For example, we used free shp to obtain the contours of the French regions (https://www.data.gouv.fr/fr/datasets/contours-des-regions-francaises-sur-openstreetmap/). This format lacks the capacity to store topological information.
- **Raster data**, different from vector data. Vector data has discrete features constructed out of vertices, and connected with lines and/or areas. Raster data, however, is like any image. Although it may portray various properties of objects in the real world, these objects don't exist as separate objects; rather, they are represented using pixels of various different color values

- **Heatmap** (image TIFF) is a visualization tool for dense point data. Heat maps are used to easily identify clusters where there is a high concentration of activity. The Heatmap plugin uses Kernel Density Estimation to create a density (heatmap) raster of an input point vector layer. The density is calculated based on the number of points in a location, with larger numbers of clustered points resulting in larger values. Heat maps allow easy identification of "hotspots" and clustering of points.

For the maps representing the geographical distributions of the main potential French vectors, we used the INPN data as reference data. The INPN is in charge of an inventory under the scientific responsibility of the National Museum of Natural History. The information collected by the INPN comes from many national programs and data provided by a range of partners. The data made available by the INPN reflect the state of knowledge or the availability of inventories. Under no circumstances can they be considered exhaustive.

3. *Portugal*

Portugal also used morphological insect identification. Due to limited experience and high difficulty, the help of Dr. Michael R. Wilson, from the National Museum of Wales, UK was obtained.

WP 3: Transmission capacity of important vectors

Belgium

Task 1: Insect rearing populations
Insects of interest will be caught and put on host plants in insect proof cages. The population will be monitored with the purpose of organising transmission trials in the second project year

Task 2: After rearing the insect populations of interest, they were put on infected trees/plants, (apple, pear and carrot). After feeding for a fixed period of time, they are transferred to phytoplasma negative trees. However, this task could not be completed – see results section.

WP4 Phenology and host range of important vectors

Belgium

Outdoor cage observations are organised by placing sleeve cages over branches of infected and non-infected host trees on which insects of interest have been observed. This will be complemented with a systematic and random sampling and linking these results to temperature and rainfall data.

WP5: Formulation of recommendations and dissemination of results

Belgium

Task1:
Based on the results generated under the project and depending on the diseases found, recommendations on the management will be formulated.

7. Obtained results

1. *Belgium*

WP1: monitoring of potential vectors in and around targeted crops

<u>Task 1.1 Trapping systems for phloem and xylem feeding insects (ILVO and CRAW)</u>

Evaluation of the use of the following trapping methods:

- a portable air suction machine, ILVO possesses a "Pro Force" machine (manufactured by Taneka)
- light interception traps
- sweep-netting technique
- beat-netting technique
- pan traps using water and a surfactant (detergent) or another solvent (e.g. mono ethylene glycol)
- adhesive techniques:
    - yellow sticky traps
    - directly spraying a glue on the plant stems and leaves

These techniques were evaluated based on:

- Success rate of capturing insects belonging to the *Auchenorhyncha* and *Psylloidea*
- Specificity for both targeted groups, i.e. compared to the portion of captured non-target insects
- Sampling period

Light interception trap set up

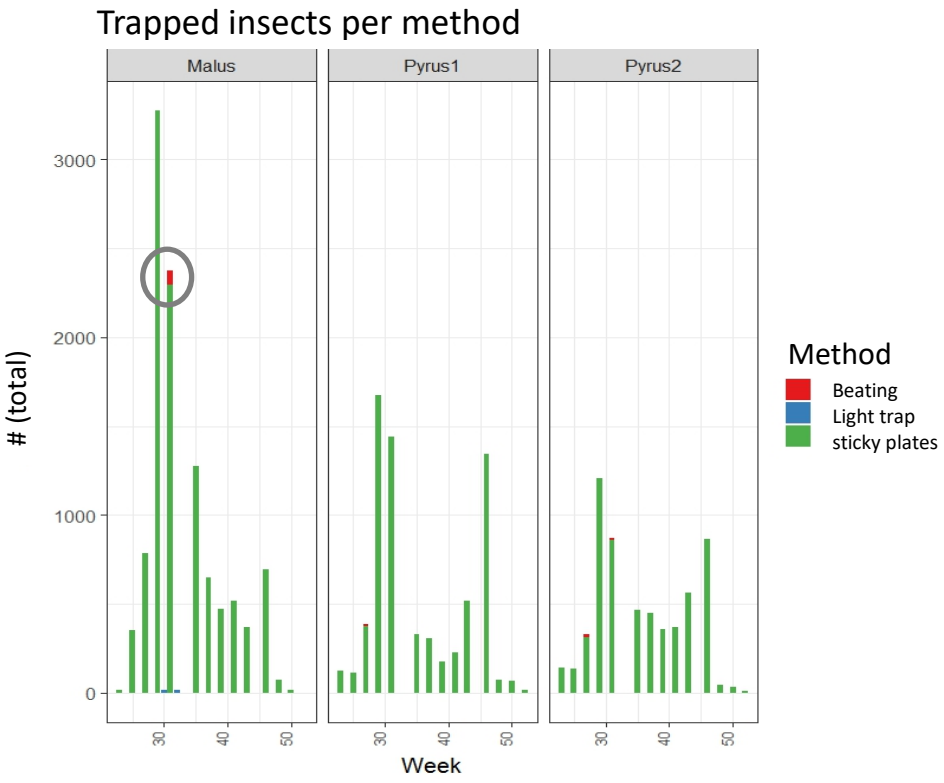
'Pro Force' portable air suction machine

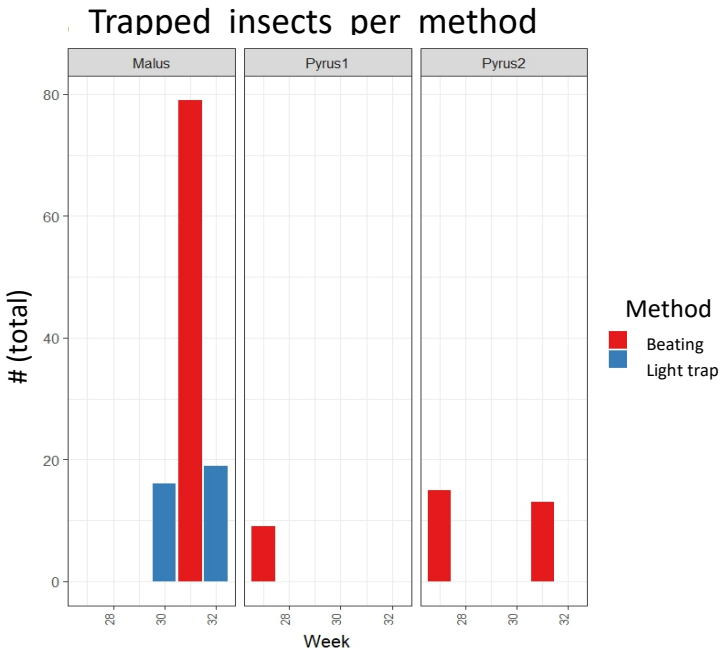
Beat-netting technique

Directly spraying a glue on the plant stems and leaves

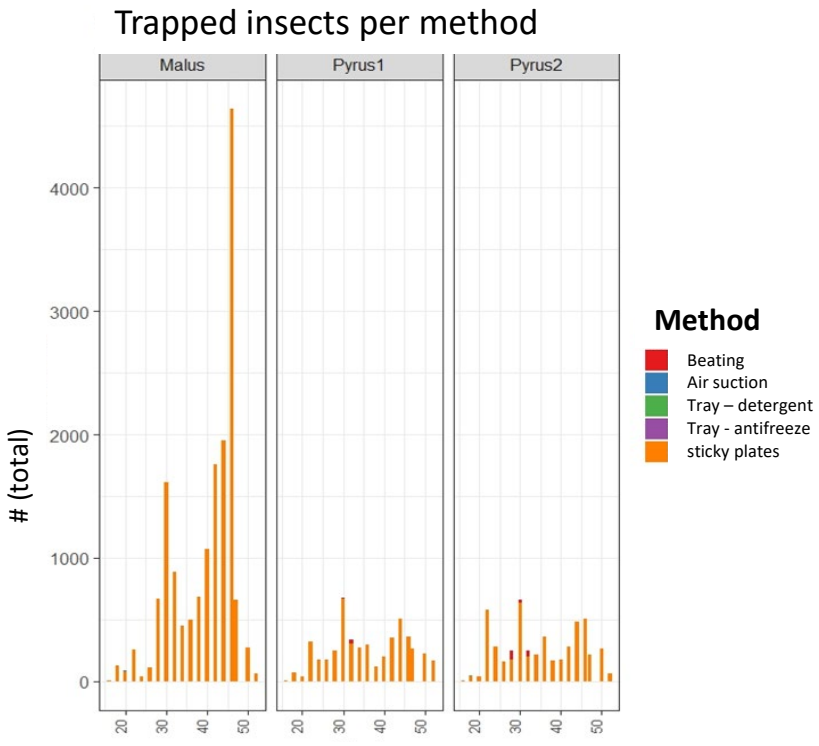
Comparison trapping methods in apple and pear orchards

Location Melle: <u>Beating – light traps – sticky plates (yellow):</u>

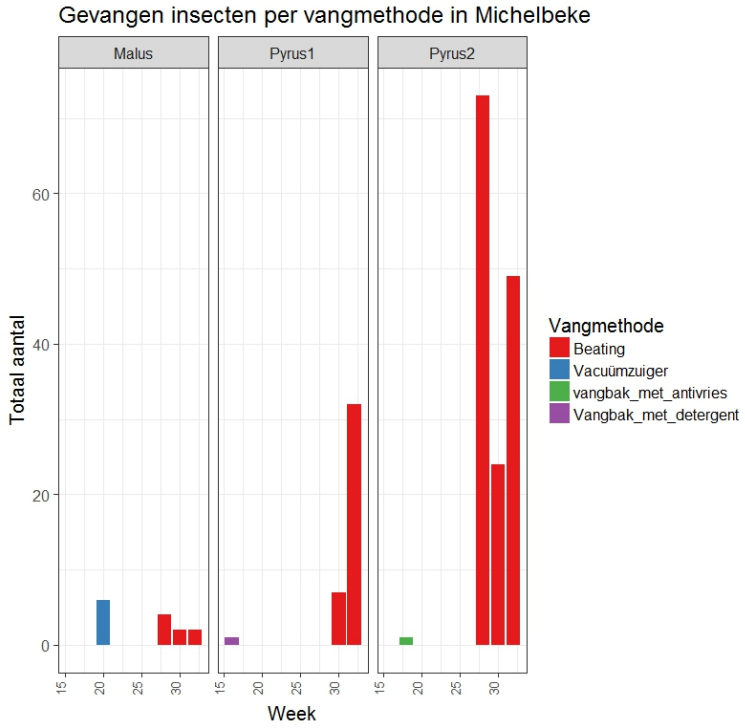




Location Michelbeke : Beating – vacuum air suction – trays (with detergent or antifreeze) – sticky plates



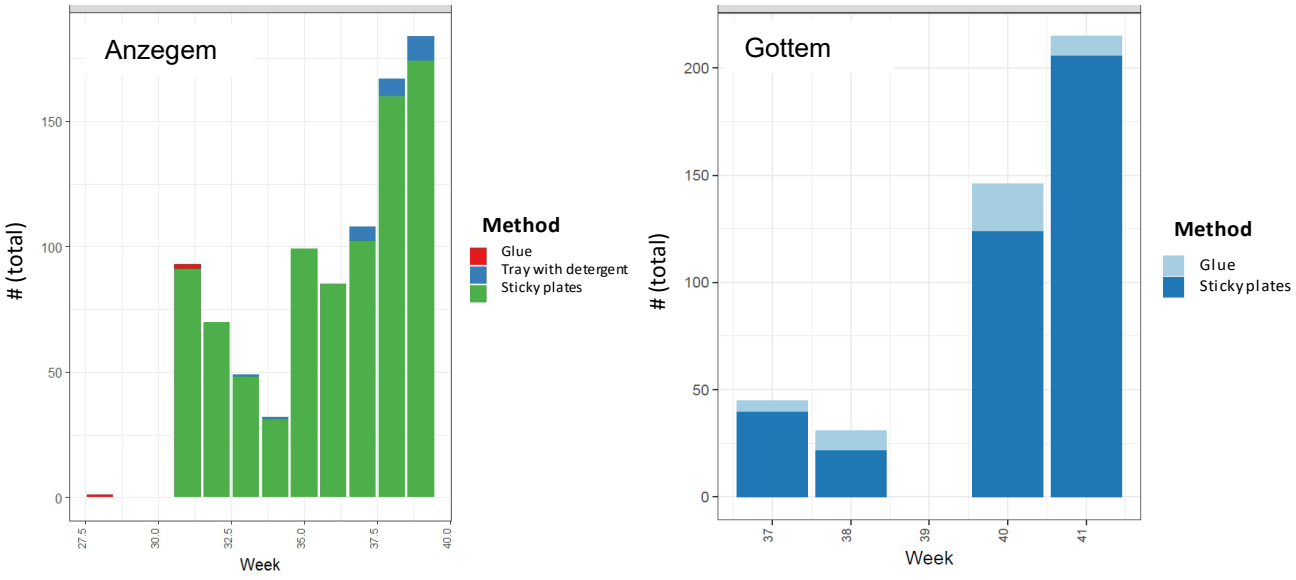
Trapped insects per method



Comparison trapping methods in carrot

Location Anzegem: Yellow sticky plates, trays with detergent, and glue (on the plants)

Location Gottem: Yellow sticky plates and glue (on the plants)



Location Jandrain-Jandrenouille: Sweep/funnel-netting and portable air suction machine

Statistical analysis of the number of Auchenorrhyncha individuals collected with both techniques showed that portable air suction machine gave a much better sensibility than sweep/funnel-netting ($P<0.001$).

Evaluation of the use of the following trapping methods:

- Adhesive techniques: yellow sticky traps are the most reliable insect monitoring technique. This method was further used by ILVO in the intensive monitoring of the Auchenorrhyncha insects in the targeted apple and pear orchards and carrot fields.
-
- Beat-netting is known to be one of the best sampling methods in orchard although a sufficient number of beats have to be performed which make it more labor-intensive than yellow sticky traps. This method was further used by CRA-W in orchards.

- Portable air suction machine (more useful for field crops such as carrots, than in tree orchards), beating and sweep-netting is useful when living insects need to be trapped (for morphological identification, for insect rearing purposes, for pathogen testing on the insects, etc.). These techniques could also be used for monitoring, yet standardization is needed, and to obtain a clear view of the insect presence, these techniques are quite labor intensive.

- Direct glue on the plants gives a view of what landed (/fed ?) on that particular plant, but is less useful for monitoring the population.

Task 1.2 Systematic sampling of phloem and xylem feeding insects in infested orchards/fields (ILVO and CRA-W)

Both pear-apple orchards and carrot fields are being studied both in the Flemish and Walloon region of Belgium:

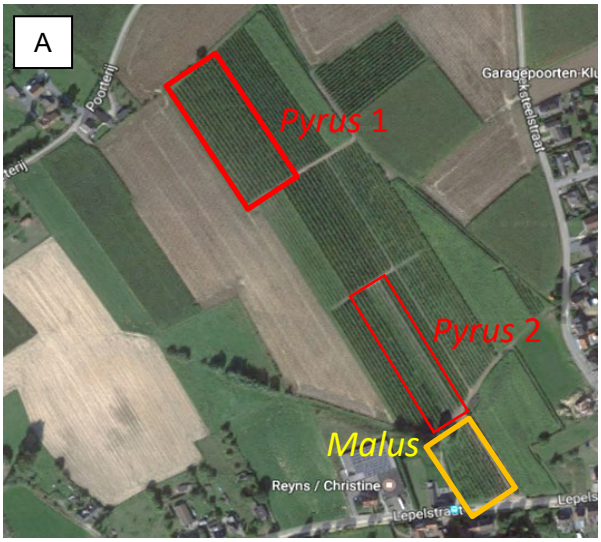
- AP and PD in infected apple and pear commercial and private orchards.

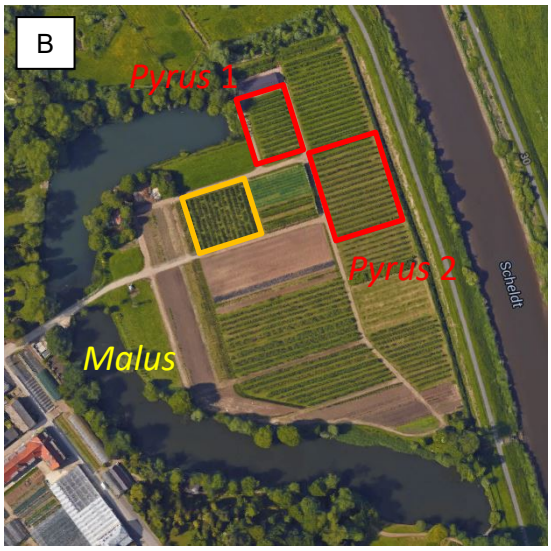
- *'Candidatus* Phytoplasma asteris' infected carrot fields (ad hoc decision during the project, based on a selection of fields of the pathogen monitoring project and an additional test on the presence of the AY phytoplasma).

- No specific monitoring for Liberibacter vectors since no outbreak has been found before the start of the project. However, '*Ca*. Liberibacter' monitoring is continued on the chosen locations that are monitored for AY and their (potential) vectors.

As mentioned above, the yellow sticky traps are widely used to monitor the insects.

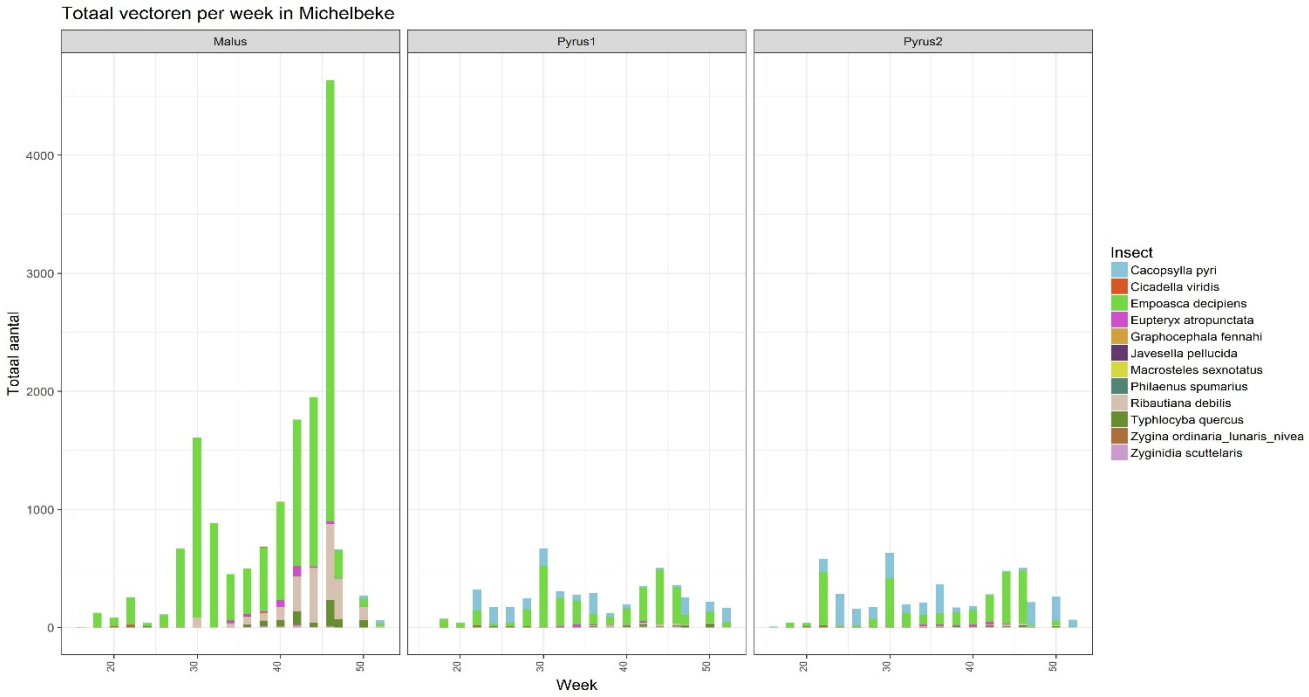
Selected Apple and pear fields are located in Michelbeke (A) and Melle (B).



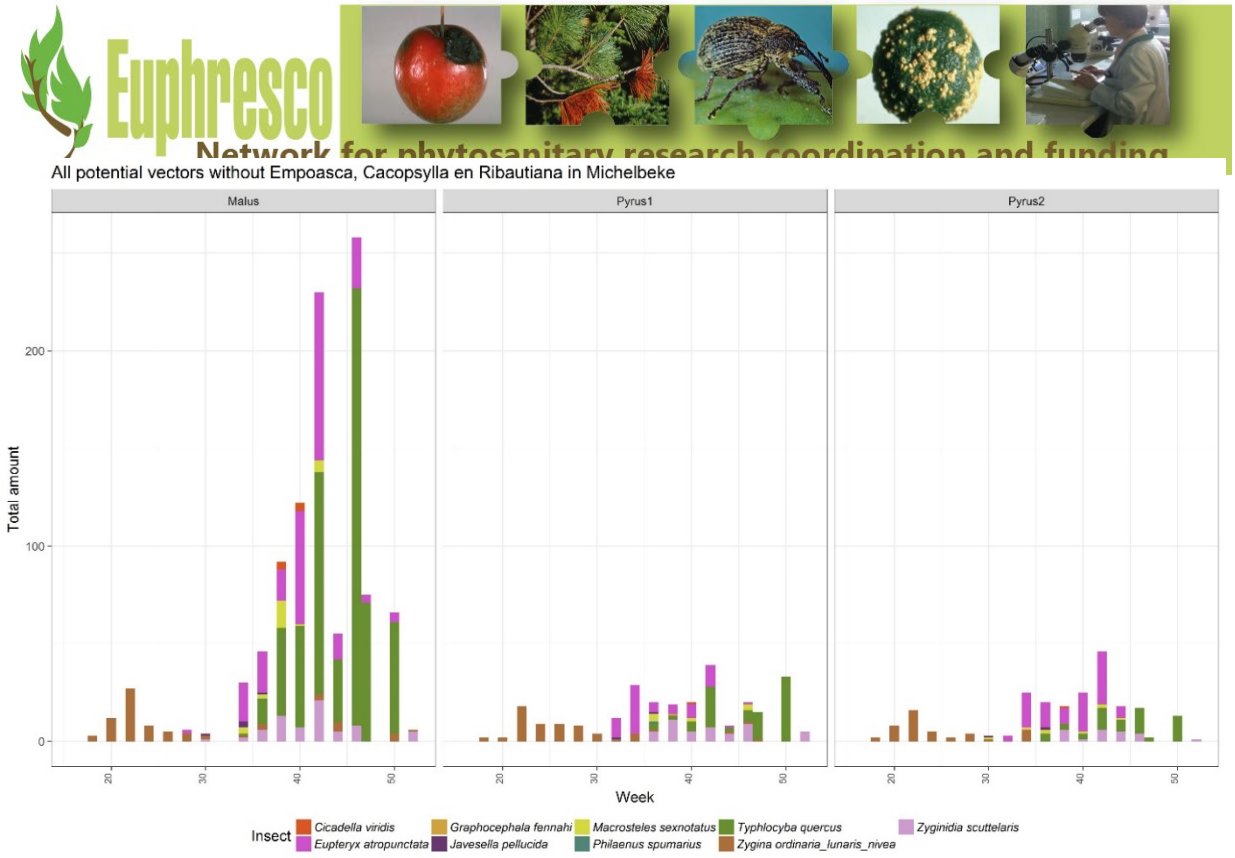


Survey in apple and pear – location Michelbeke

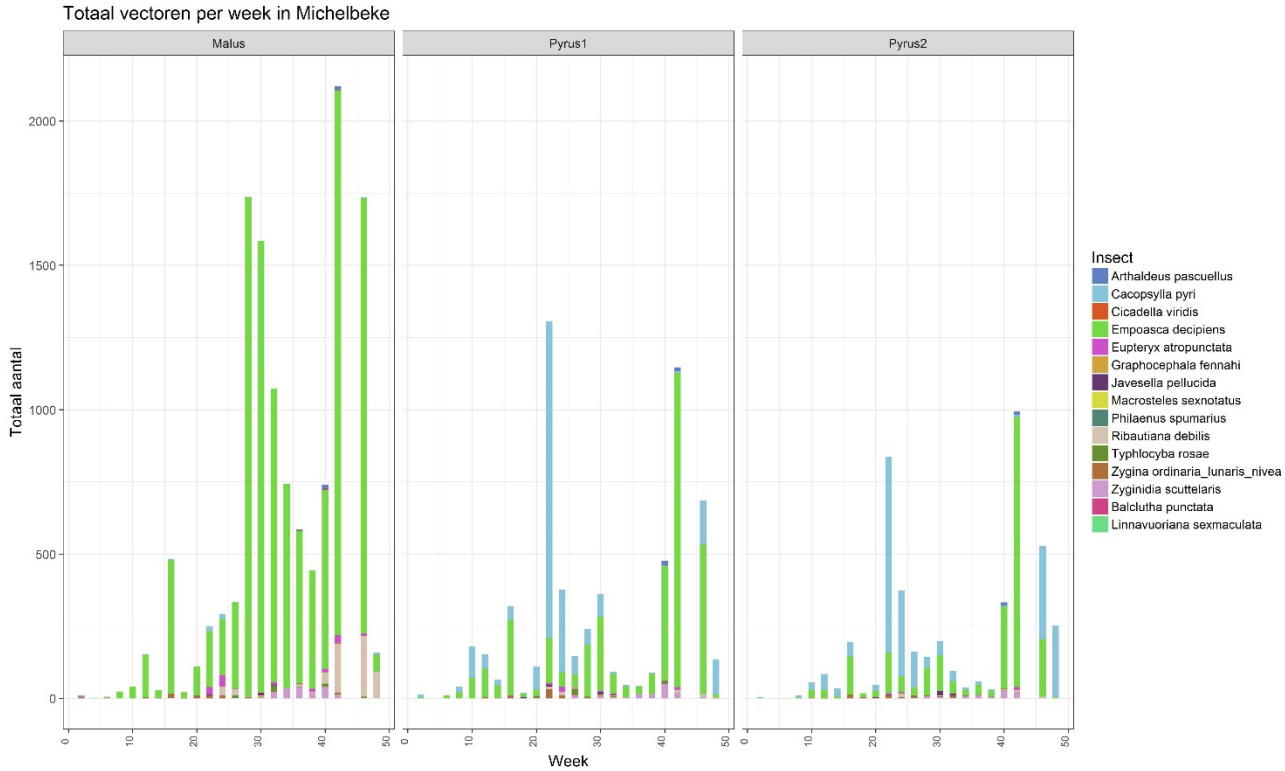
Most prevalent trapped insects in 2016



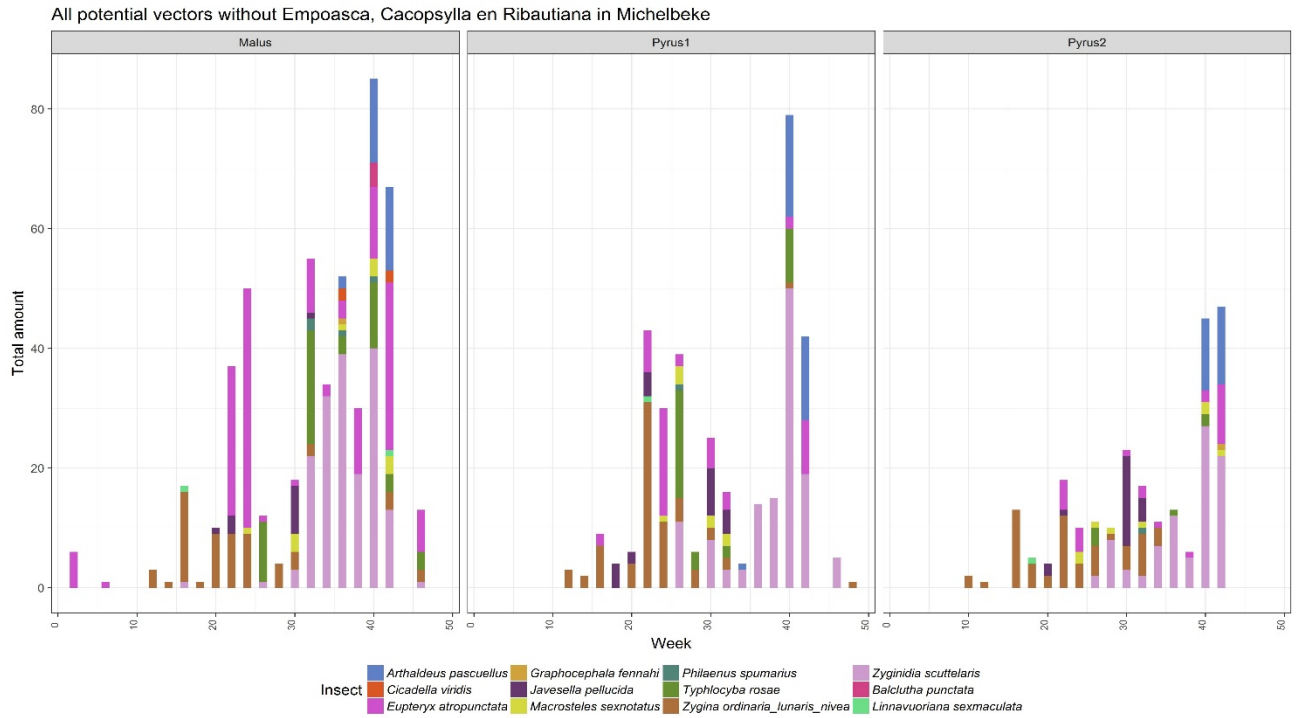
Trapped insects in 2016 <u>without</u> the most prevalent *Empoasca*, *Ribautiana* and *Cacopsylla* species



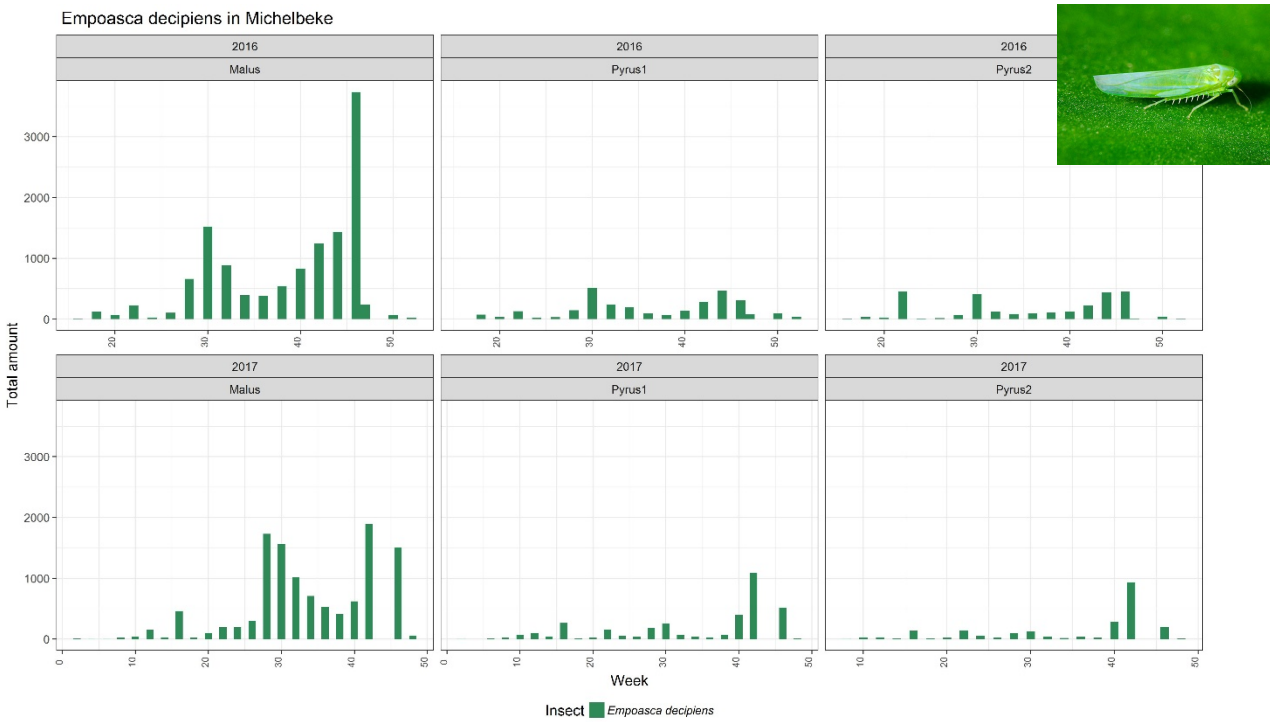
Most prevalent trapped insects in 2017



Trapped insects in 2017 <u>without</u> the most prevalent *Empoasca*, *Ribautiana* and *Cacopsylla* species



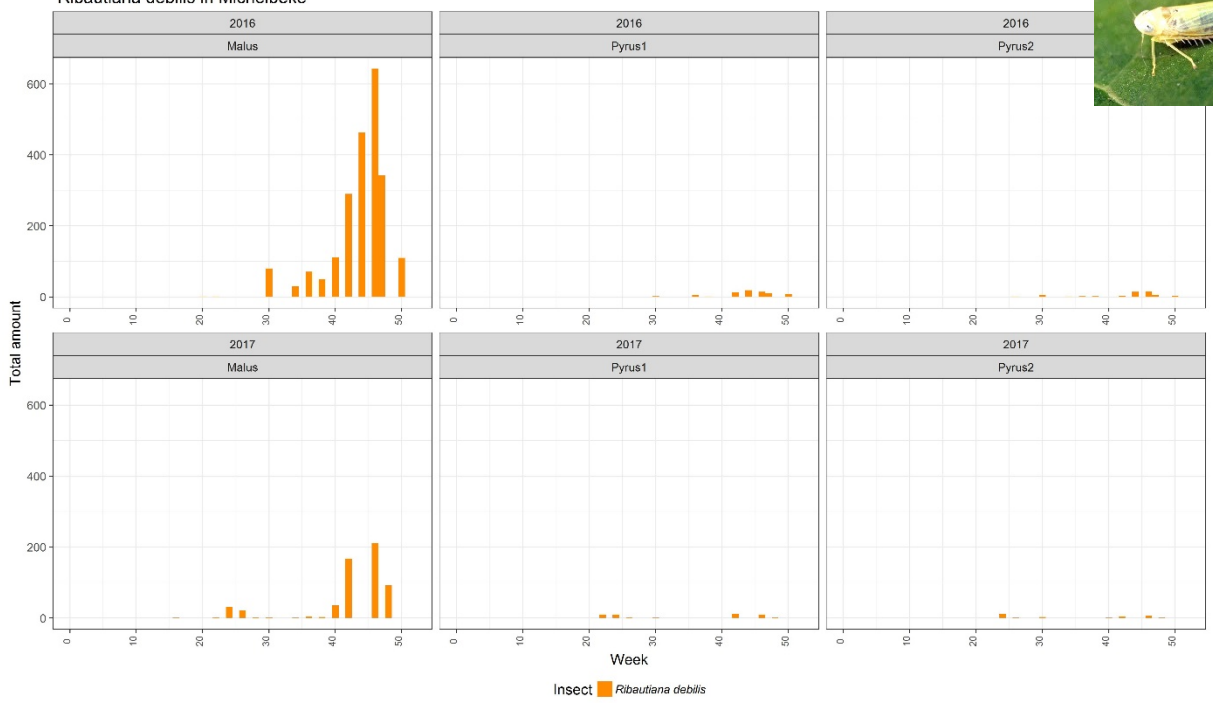
Survey results per individual insect



Euphresco

Network for phytosanitary research coordination and funding

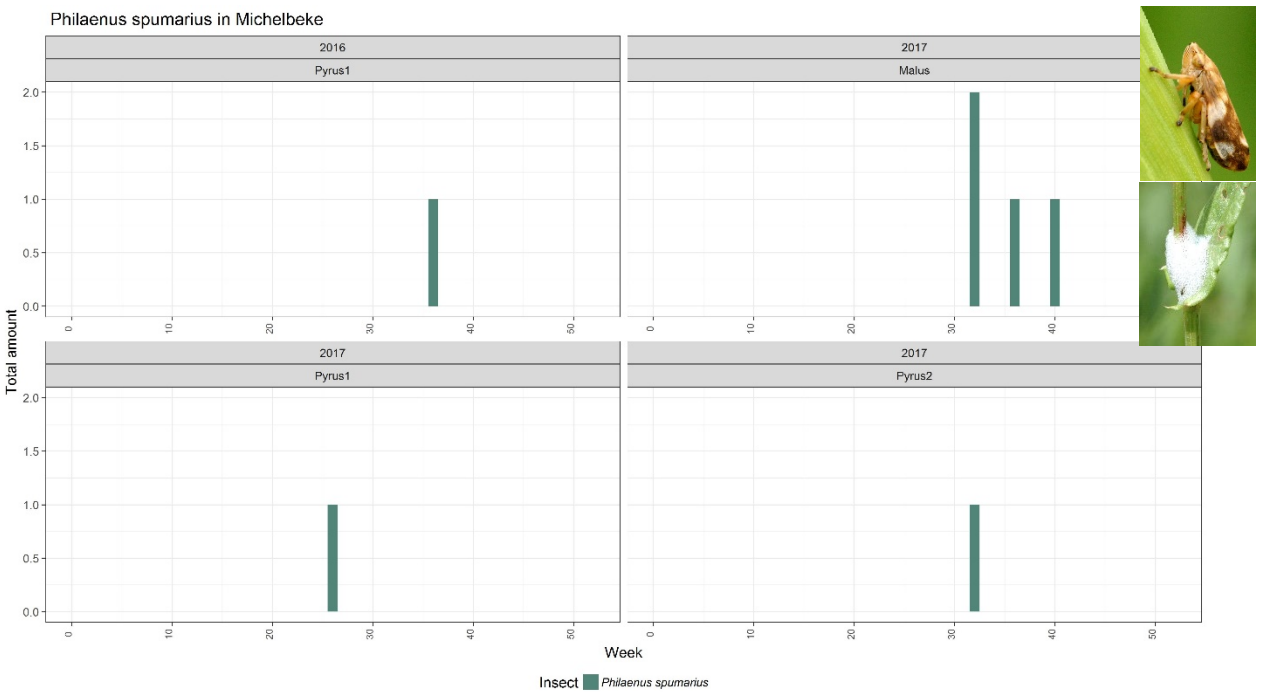
Ribautiana debilis in Michelbeke

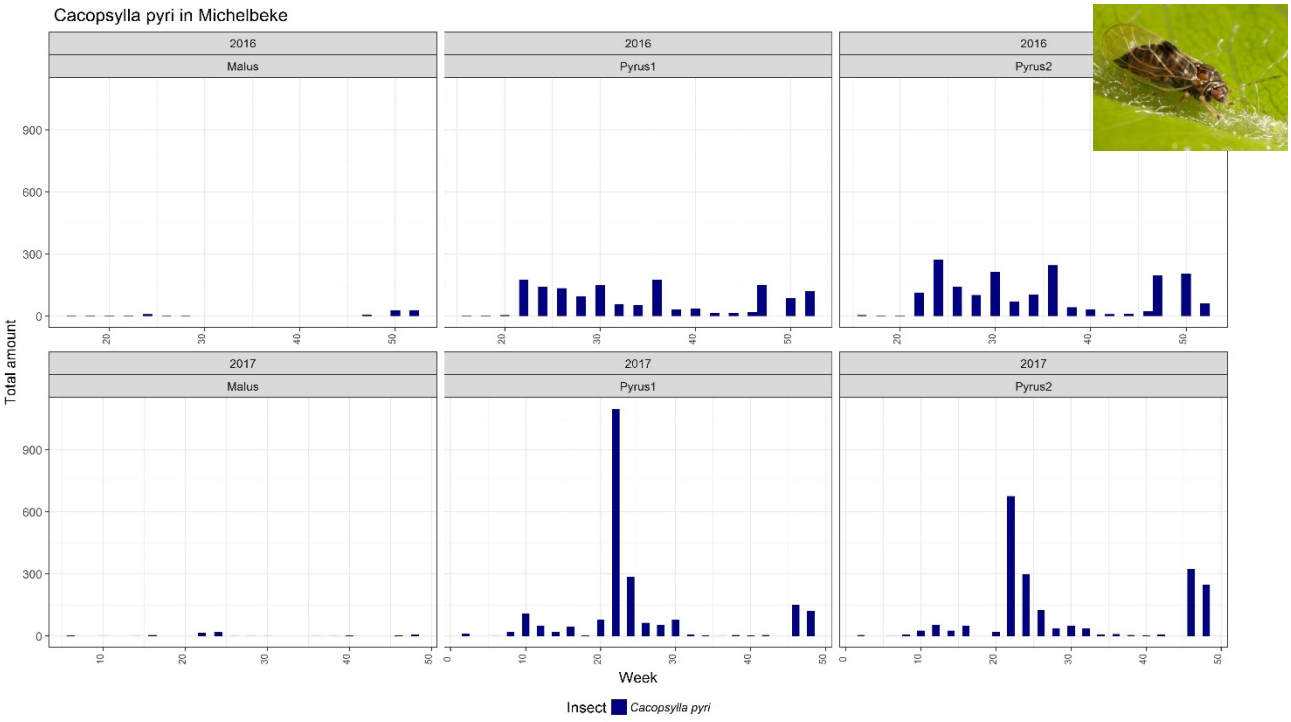


Macrosteles sexnotatus in Michelbeke

Euphresco

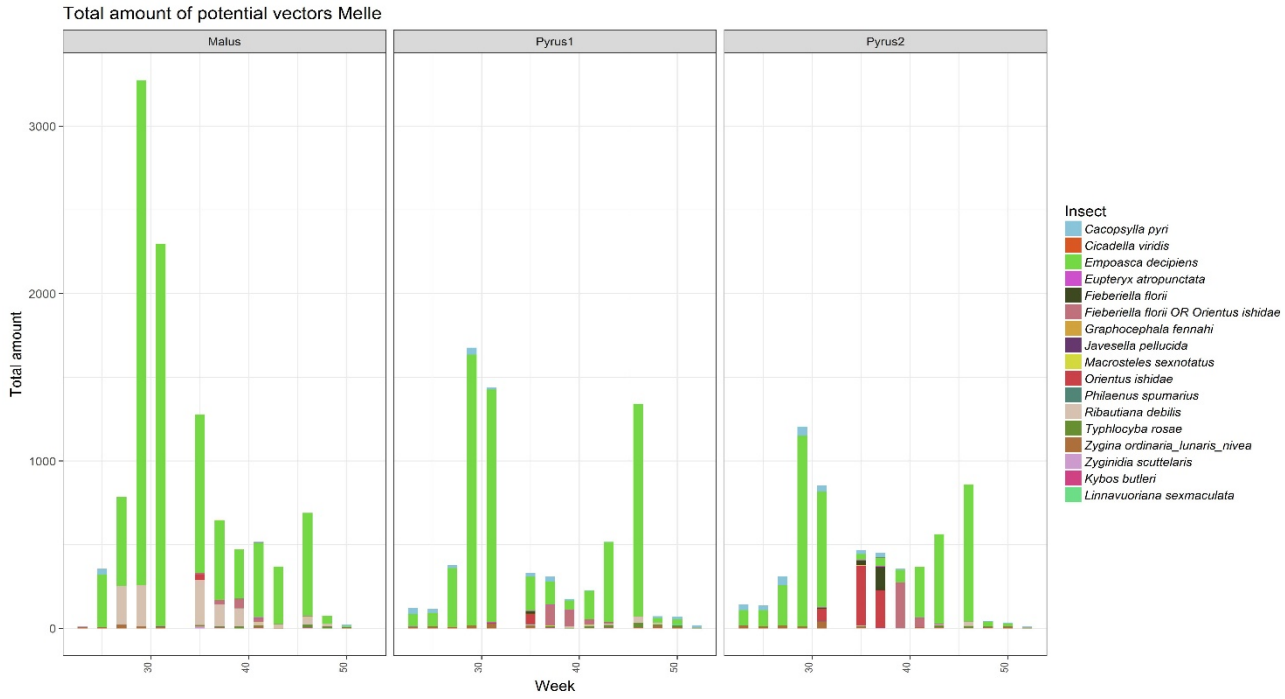
Network for phytosanitary research coordination and funding



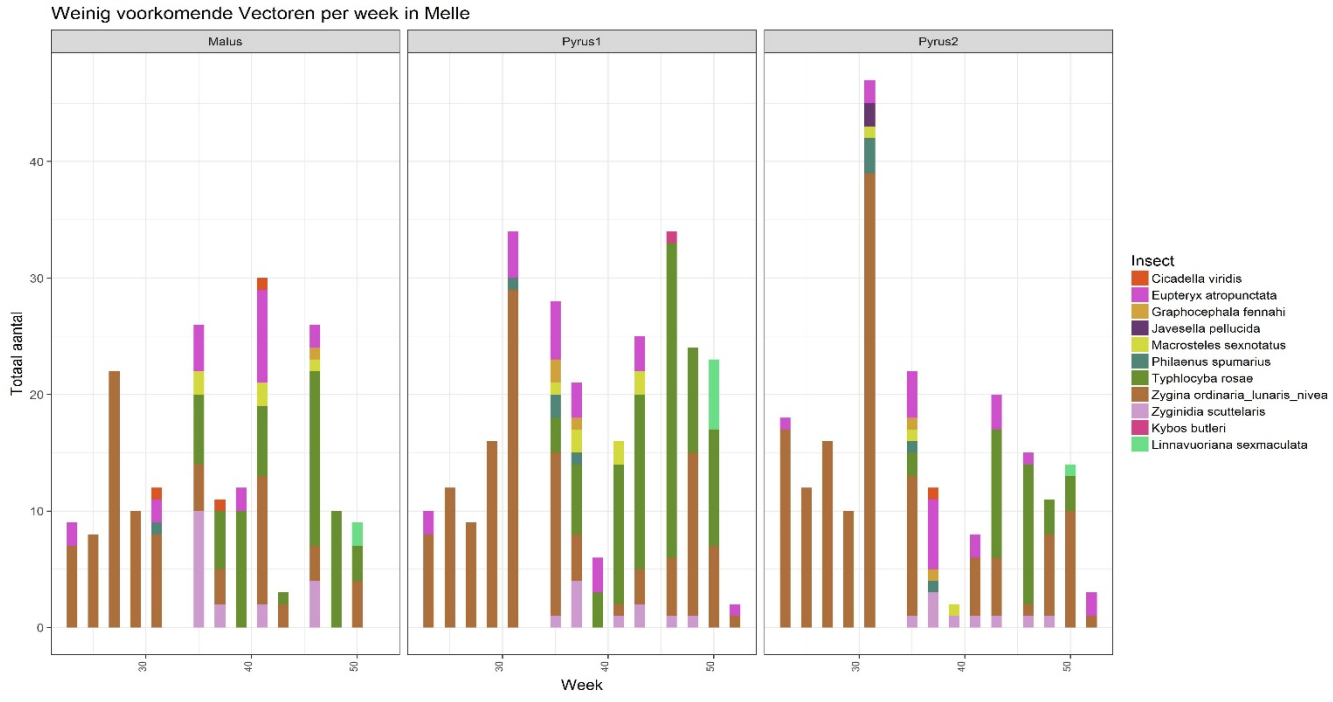


Survey in apple and pear – location Melle

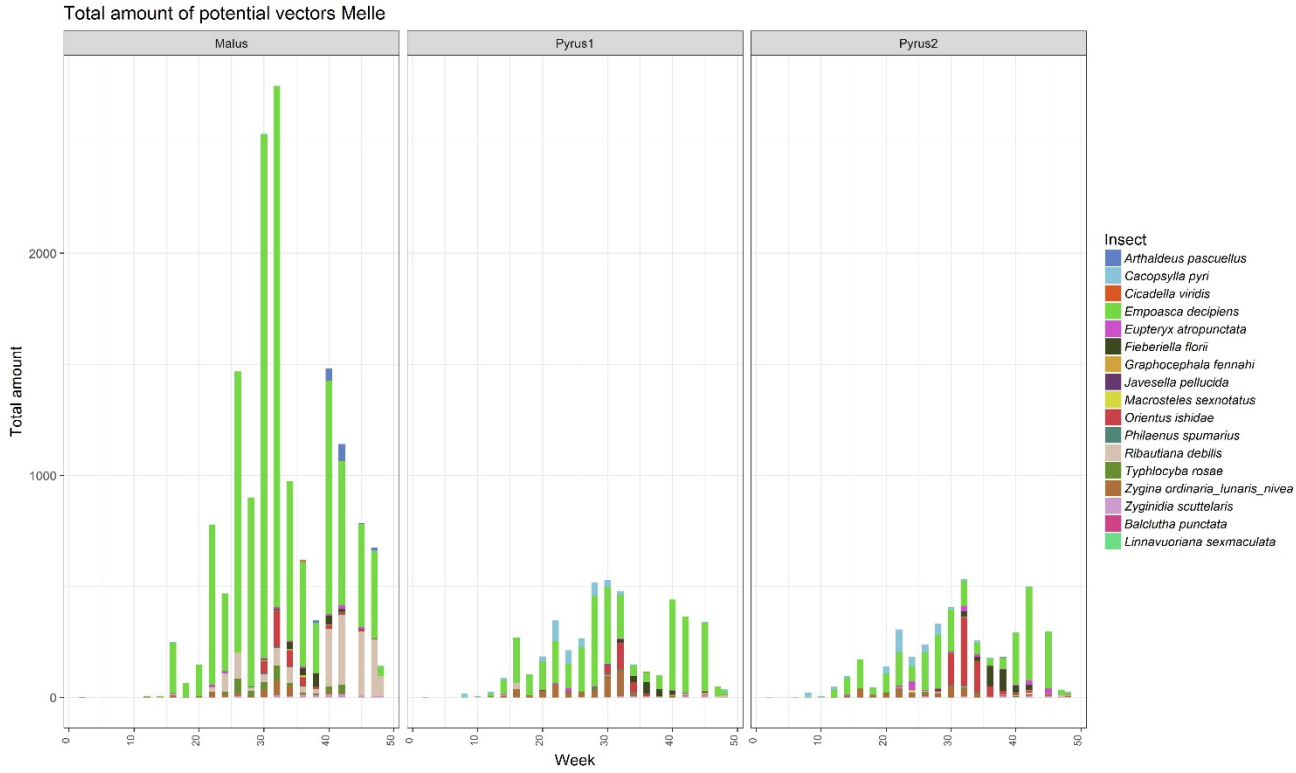
Most prevalent trapped insects in 2016



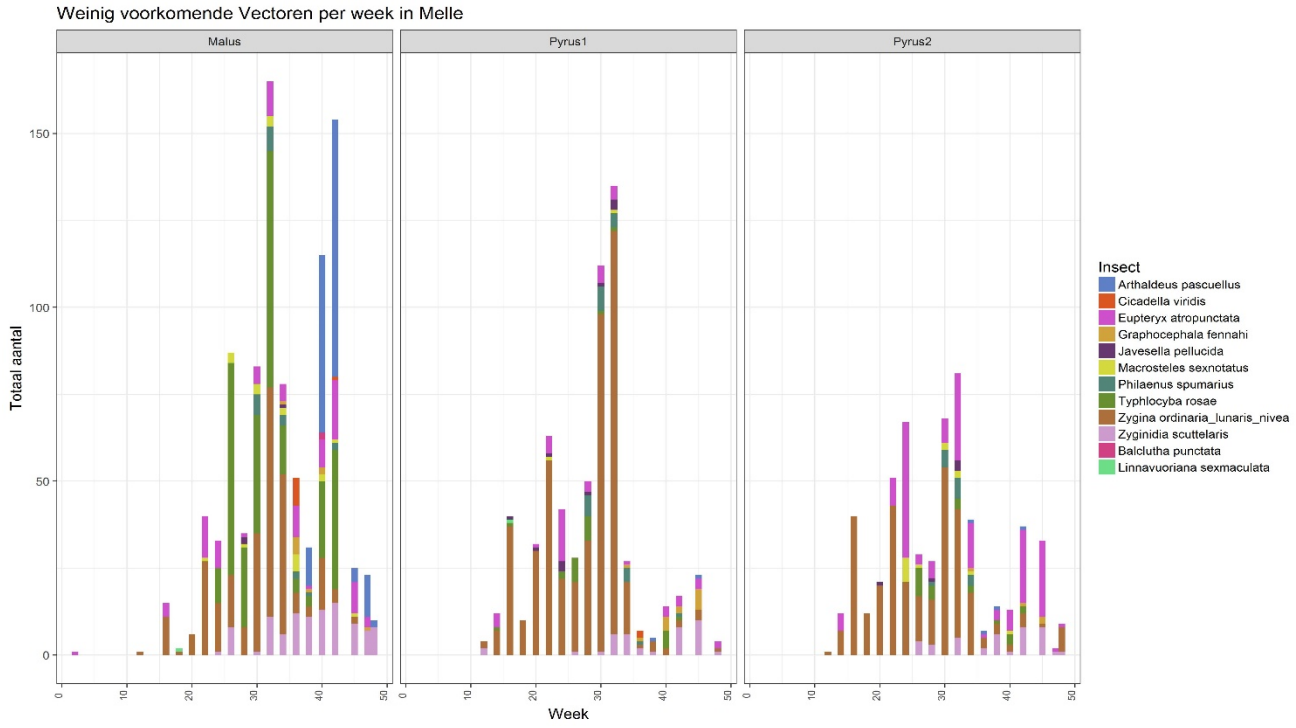
Trapped insects in 2016 <u>without</u> the most prevalent *Empoasca*, *Ribautiana, Orientus and Fieberiella* species



Most prevalent trapped insects in 2017

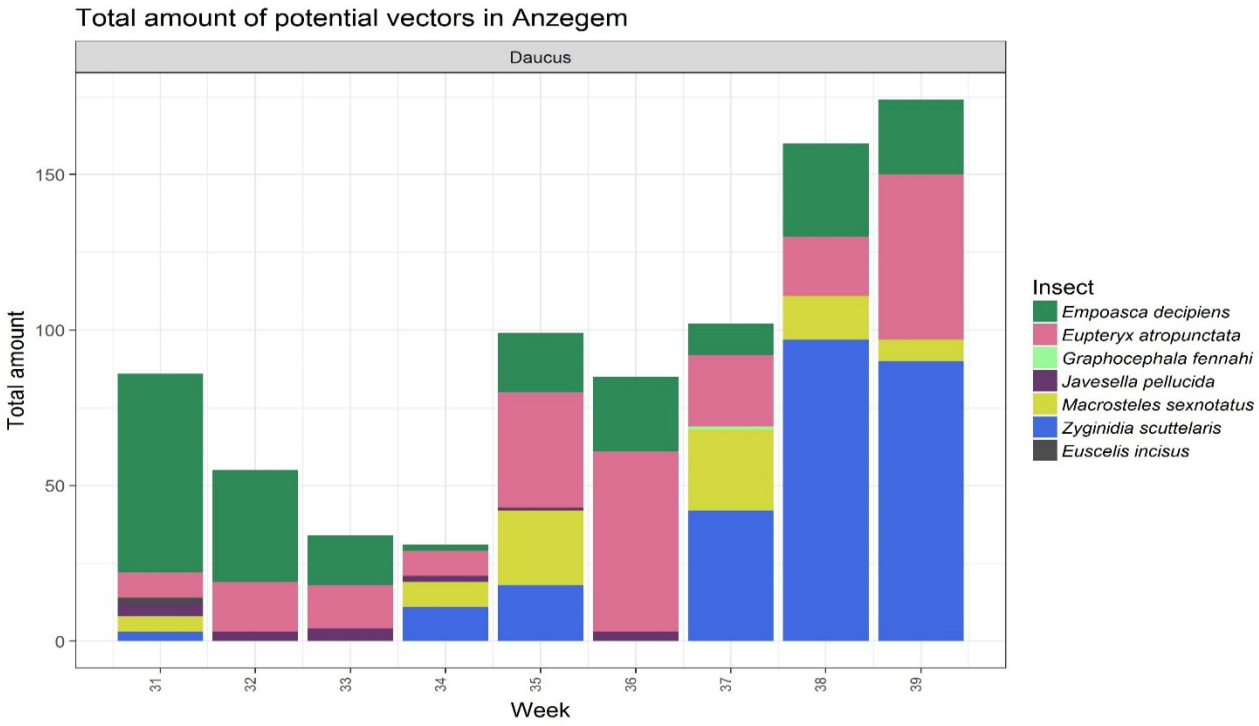


Trapped insects in 2017 <u>without</u> the most prevalent *Empoasca*, *Ribautiana, Orientus and Fieberiella* species

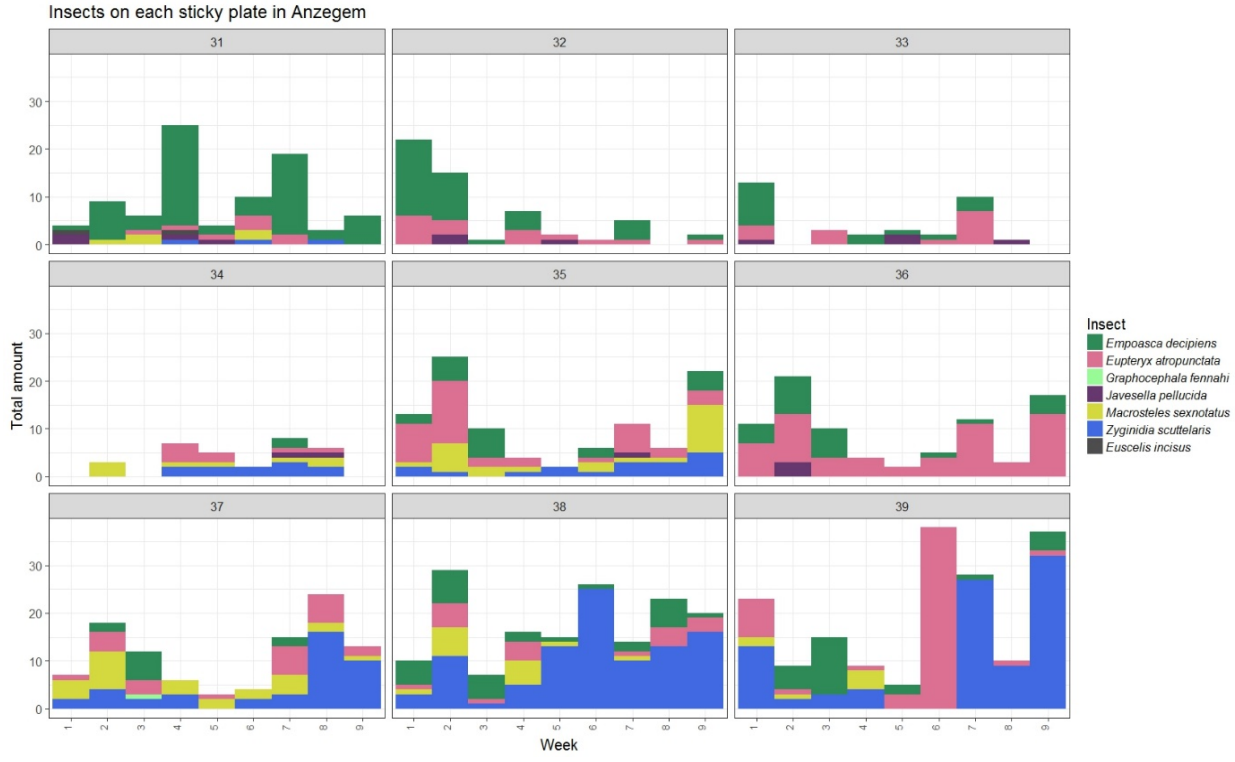


Monitoring in carrot - location: Anzegem

At this location, an infection of '*Candidatus* Phytoplasma asteris' was present during the survey.



Overview of the trapped insects per week.



The most prevailing insects in Anzegem:





Macrosteles sexnotatus *Empoasca decipiens* *Eupteryx atropunctata* *Javessella pelucida* *Zyginidia scuttelaris*

Survey in apple, pear and *Prunus*– location Gembloux and Saint-Denis Bovesse

Auchenorrhyncha were hand-collected in the three selected orchards of *Prunus*, *Malus* and *Pyrus* located in Gembloux. Systematic sampling was performed every week by collecting 60 leaves/tree/tree species on 4 infected trees per tree species. The results are shown in the figure below.

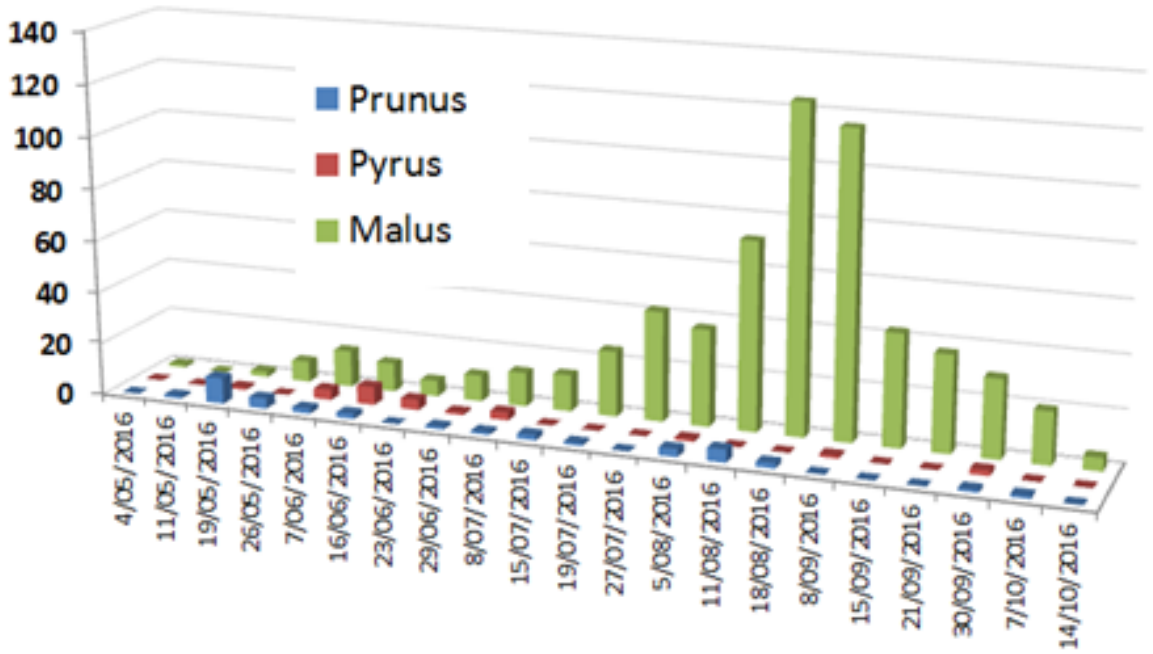
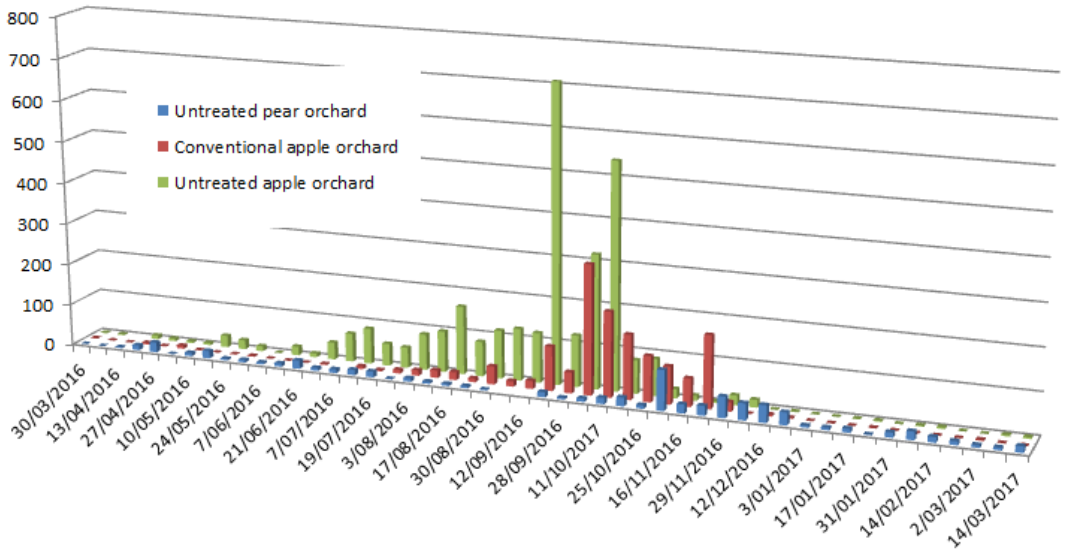


Fig. Number of Auchenorrhyncha individuals collected by hand during weekly samplings in *Prunus*, *Pyrus* and *Malus* orchards located in Gembloux.

The results showed that most of Auchenorrhyncha individuals were collected in the Malus orchard. Most of the individuals were Cicadellidae larvae which unfortunately prevented further identification. In total, 690 individuals were collected.

On the other hand, systematic samplings were also carried out by beat-netting. Therefore, weekly samplings were performed on 80 trees in 3 different orchards: one conventional apple orchard, one untreated apple orchard and one pear orchard. The results are shown in the figure



below.

Fig. Number of Auchenorrhyncha individuals collected by beat-netting in three different orchards: one untreated pear orchard, one conventional apple orchard and one untreated apple orchard.

Results showed that most Auchenorrhyncha were collected in the untreated apple orchard followed by the conventional apple orchard while in untreated pear orchard few individuals were collected. Although, this would require further repetitions, the phytosanitary treatments mainly applied during the growing season up to the harvest seems to have an impact on the population of the collected insects.
Collected individuals were then morphologically identified and the results are presented in the following table:

Family	Genus/species	Number
Aphophoridae	Aphrophora alni	9
Aphophoridae	Aphrophora sp.	4
Cercopidae	Cercopis sp.	1
Cicadellidae	unidentified adult	227
Cicadellidae	Alnetioidia alneti	80
Cicadellidae	Arboridia sp.	4
Cicadellidae	Edwardsiana avellanae	1
Cicadellidae	Edwardsiana sp.	604

Family	Genus/species	Number
Cicadellidae	Non identifiable	101
Cicadellidae	Philaenus spumarius	20
Cicadellidae	Ribautiana debilis	1711
Cicadellidae	Teneral NI	57
Cicadellidae	Typhlocyba quercus	1
Cicadellidae	Zygina flammigera	369
Cicadellidae	Zygina sp.	4
Cicadellidae	Zygina tiliae	1

Cicadellidae	Empoasca sp.	2008
Cicadellidae	Empoasca vitis	9
Cicadellidae	Eurhadina sp.	1
Cicadellidae	Fruticidia bisignata	13
Cicadellidae	Zygina rosea larve	1
Cicadellidae	Zygina sp. larve	39
Cicadellidae	unidentified larvae	635
Cicadellidae	Ledra aurita	8
Cicadellidae	Linnavuoriana sexmaculata	1
Cicadellidae	Linnavuoriana sp.	2
Cicadellidae	Zyginella pulchra	1
Cicadellidae	Zyginidia scutellaris	17
Deltocephalinae	Balclutha punctata	1
Deltocephalinae	unidentified	1
Cixiidae	Cixius nervosus	1
Cixiidae	unidentified	4
Cixiidae	Tachycixius pilosus	2
Cixiidae	unidentified	1
Delphacidae	unidentified	2
	Total	5941

To gain further insight into the phenology of these species, their abundance in each sampled orchard and for each sampling dates were plotted in the following figs.

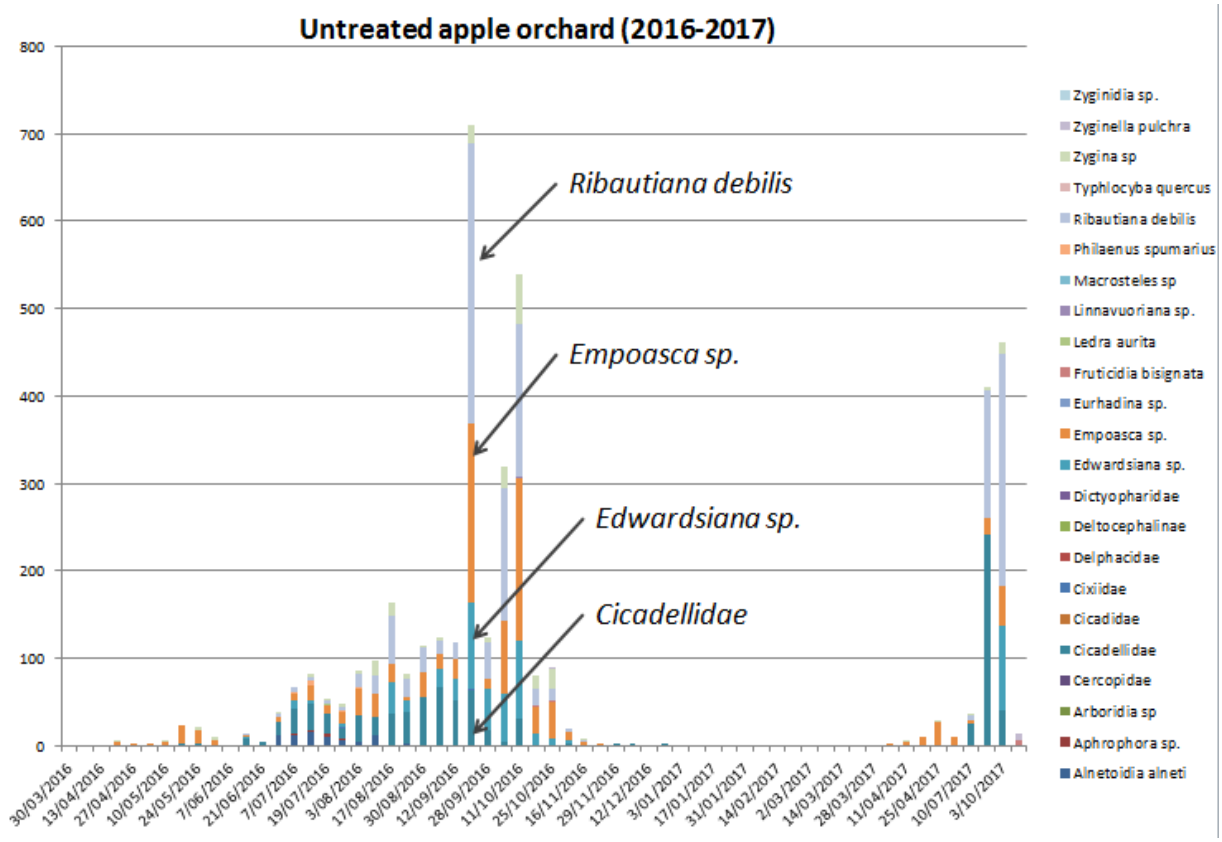


Fig. Auchenorrhyncha individuals collected by beat-netting in an untreated apple orchard located in Gembloux on a weekly basis in 2016 and at insects population peaks in 2017. Most abundant species and genera are highlighted with arrows.

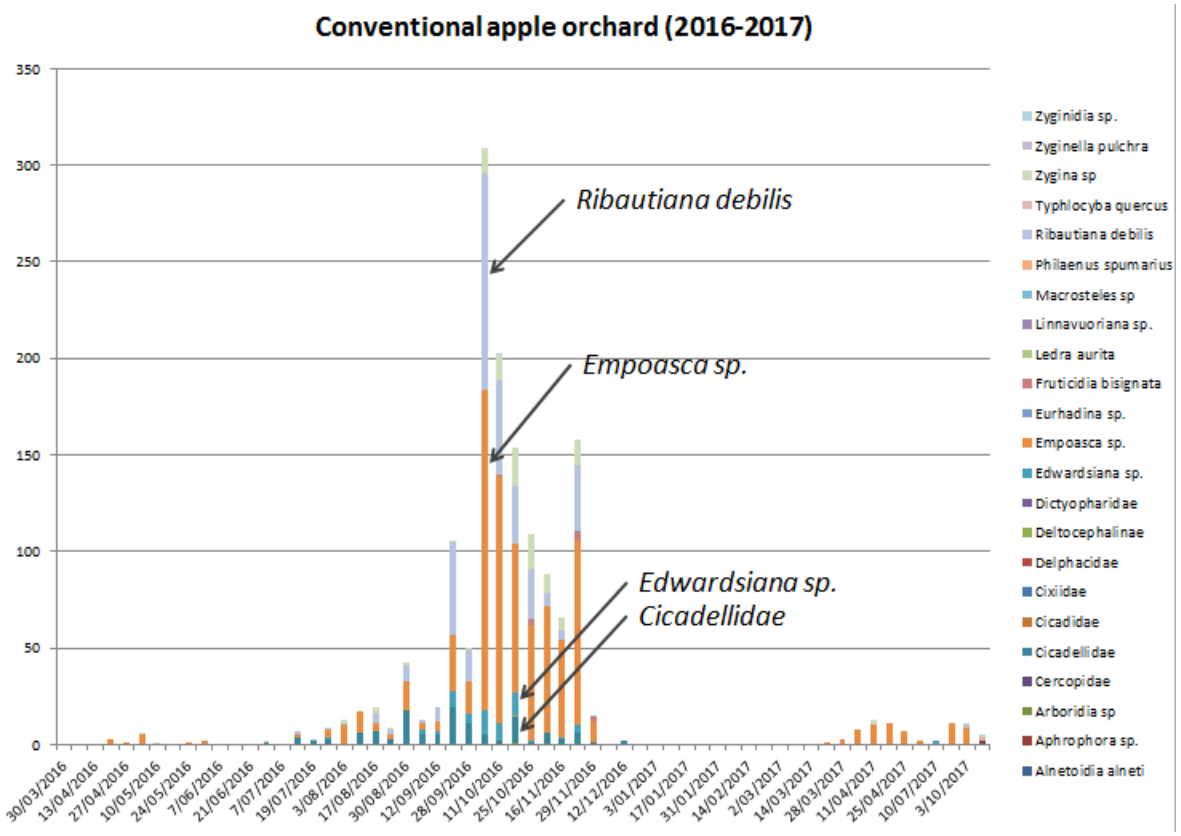


Fig. Auchenorrhyncha individuals collected by beat-netting in a conventional apple orchard located in Gembloux on a weekly basis in 2016 and at insects population peaks in 2017. Most abundant species and genera are highlighted with arrows.

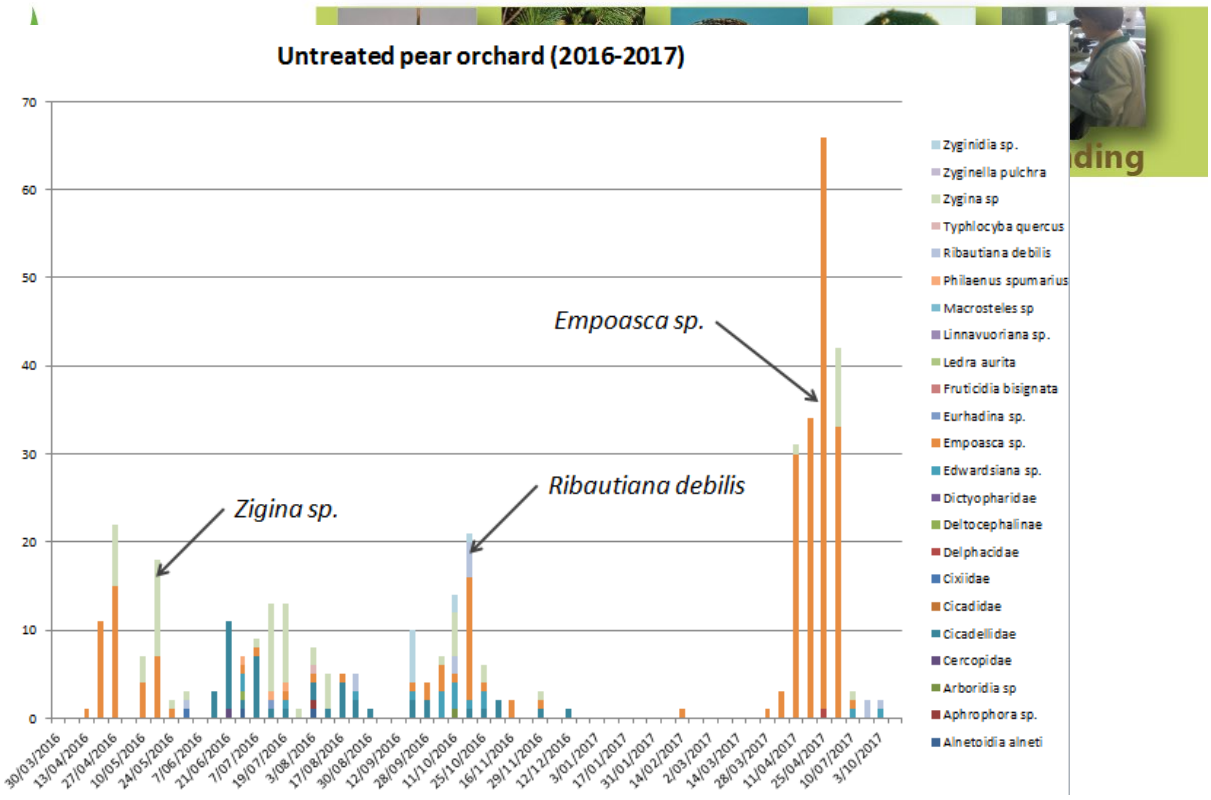
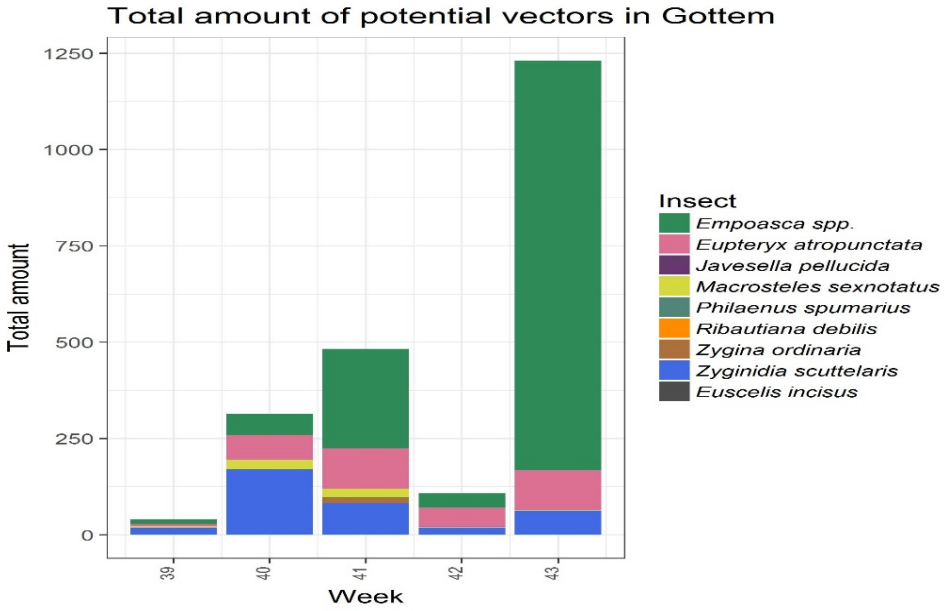


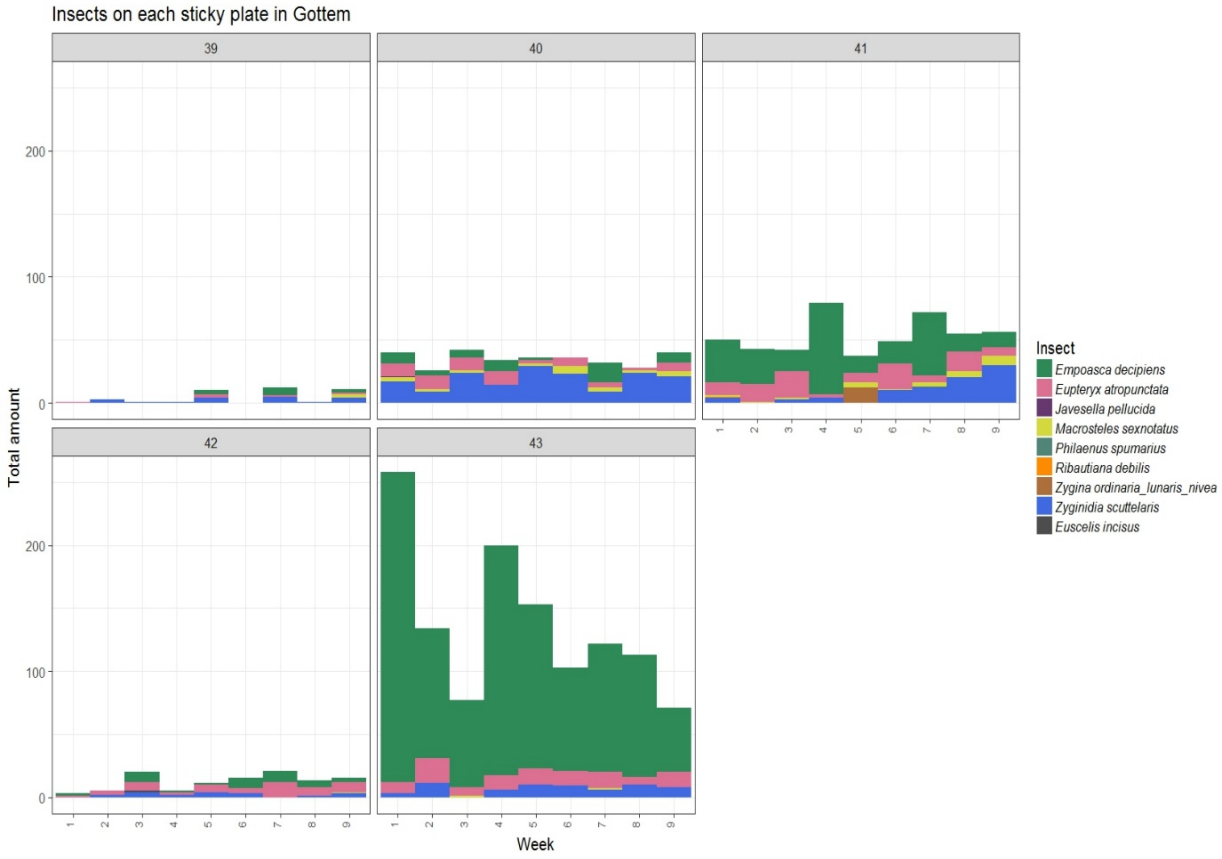
Fig. Auchenorrhyncha individuals collected by beat-netting in an untreated pear orchard located in Gembloux on a weekly basis in 2016 and at insects population peaks in 2017. Most abundant species and genera are highlighted with arrows.

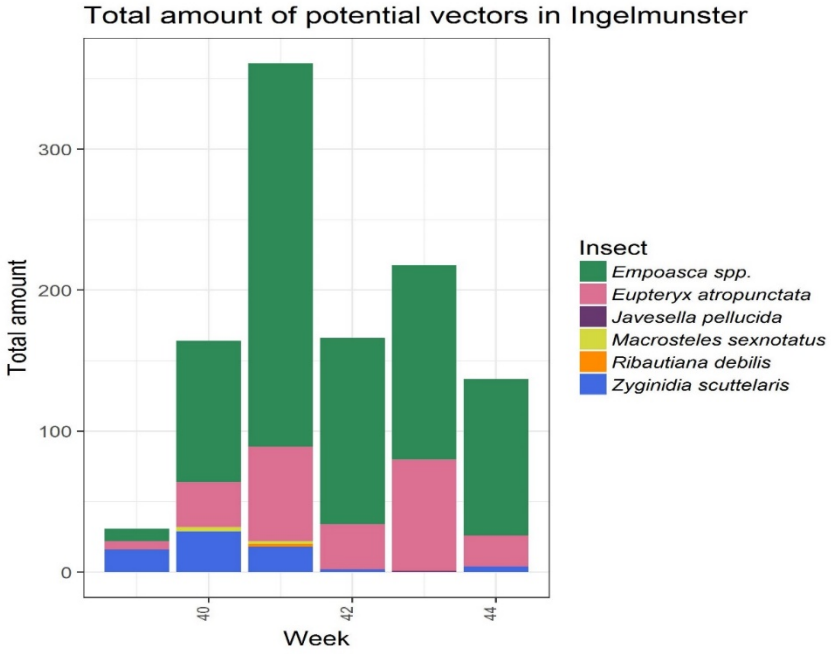
Monitoring in carrot - location: Gottem

At this location, an infection of '*Candidatus* Phytoplasma asteris' was present during the survey. Towards the end of 2017, also '*Candidatus* Liberibacter solanacearum' was found in several plants at this location.



Overview of the trapped insects per week

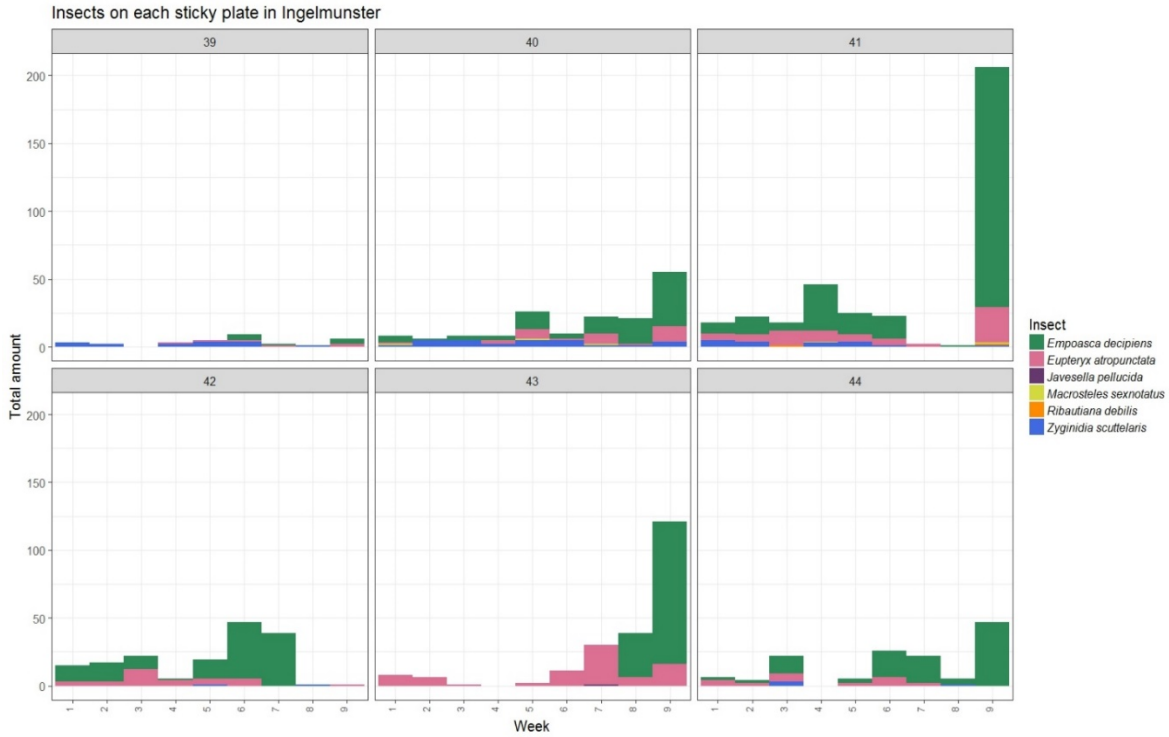

Insects on each sticky plate in Gottem
39
40
41
42
43
Total amount
Week
Insect
Empoasca decipiens
Eupteryx atropunctata
Javesella pellucida
Macrosteles sexnotatus
Philaenus spumarius
Ribautiana debilis
Zygina ordinaria_lunaris_nivea
Zyginidia scuttelaris
Euscelis incisus


Total amount of potential vectors in Ingelmunster
Total amount
Week
Insect
Empoasca spp.
Eupteryx atropunctata
Javesella pellucida
Macrosteles sexnotatus
Ribautiana debilis
Zyginidia scuttelaris

Monitoring in carrot - location: Ingelmunster

At this location, an infection of '*Candidatus* Phytoplasma asteris' was present during the survey. Towards the end of 2017, also '*Candidatus* Liberibacter solanacearum' was found in several plants at this location. For *Empoasca decipiens*, the opposite is the case. *Eupterix atropunctata* is almost always present in considerate amounts throughout the season.

Overview of the trapped insects per week



Both in Ingelmunster and Gottem, the two locations where also a '*Candidatus* Liberibacter solanacearum' infection was detected, the same prevailing insects as in Anzegem were trapped. However, where *Zyginidia scuttelaris* was found mainly late in the season in Anzegem, both in Gottem and Ingelmunster, this insect was mainly trapped earlier in the season.

Only a few psyllids (potential vectors of '*Ca*. L. solanacearum') were trapped. No *Trioza apicalis*, nor *Bactericera trigonica*, the known vectors of '*Ca*. L. solanacearum' in carrot were found in both infected carrot fields (Gottem or Anzegem).

Task 1.3. Sampling in the surrounding environment of infested orchards and fields (CRA-W)

When it comes to orchards, due to the removal of external AP infected rows in the orchard initially planned to be sampled in this task 1.3., and because several alternative sites were found to be negative for phytoplasmas, data previously generated in an extensive sampling campaign were further analyzed. Concerning carrot fields, surroundings and infested orchards were compared using two different sampling methods: sweep/funnel-netting and portable air suction machine.

Apple orchards and their surroundings:

Although Auchenorrhyncha were present in the samplings analyzed, too few individuals were present to compare the abundance of the corresponding species. On the other hand, since no Auchenorrhyncha were found to be positive for AP in the framework of this project, and since psyllids seem thus to be the most probable vectors of this disease in our country, the present work focused on these insect species. Moreover, very few data concerning AP vectors are available for our country so data have to be generated to allow drawing up recommendations in the framework of this project.
The data used came from the beat-netting of several replications (locations) of three types of apple orchards: cider, dessert and untreated as well as from their surrounding hedges consisting for the most part of hawthorn (*Crateagus* sp.).
The bar plots hereafter show the results of these statistical analyses for the nine most prevalent psyllid species found in the data sets.

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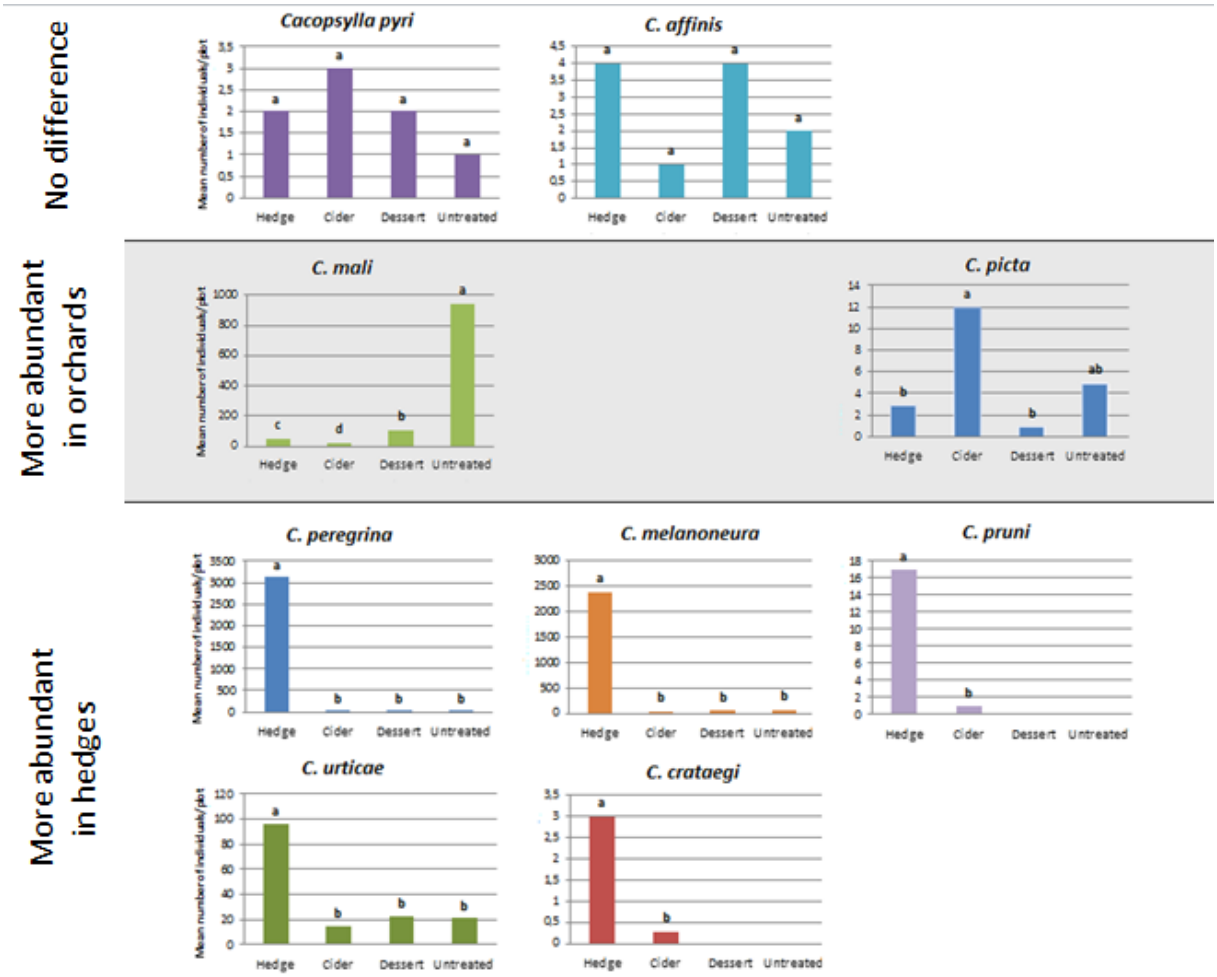


These results show that three groups can be distinguished according to the difference of abundance between orchards and their surrounding hedges. In the first group which concern *Cacopsylla pyri* and *C. affinis* (top of the figure), no difference was found between hedges and orchards. For the second group containing *C. mali* and the known vector of AP: *C. picta* (middle of the figure), the abundance of species was found to be significantly higher in at least one of the orchard type compared to hedges. In the third group (bottom of the figure), six *Cacopsylla* species were found to be significantly more abundant in hedges compared to orchards. In this latter group, *C. melanoneura* known as another AP vector was found to be highly prevalent in hawthorn hedges.

Carrot organic fields and their surroundings:

Samplings in carrots fields were carried out following different modalities presented in the table hereafter.

Years	2016	2017
Number of field rows per statistical object	3	4
Number of border rows per statistical object	1	2
Length of rows	20 m	
Trapping methods	Sweep/funnel-netting and portable air suction machine	portable air suction machine
Location	Walloon Brabant Belgium	

The results of these experiments showed that significantly more Auchenorrhyncha individuals were collected in the borders than in the fields (P<0.001) (see graphs hereafter).

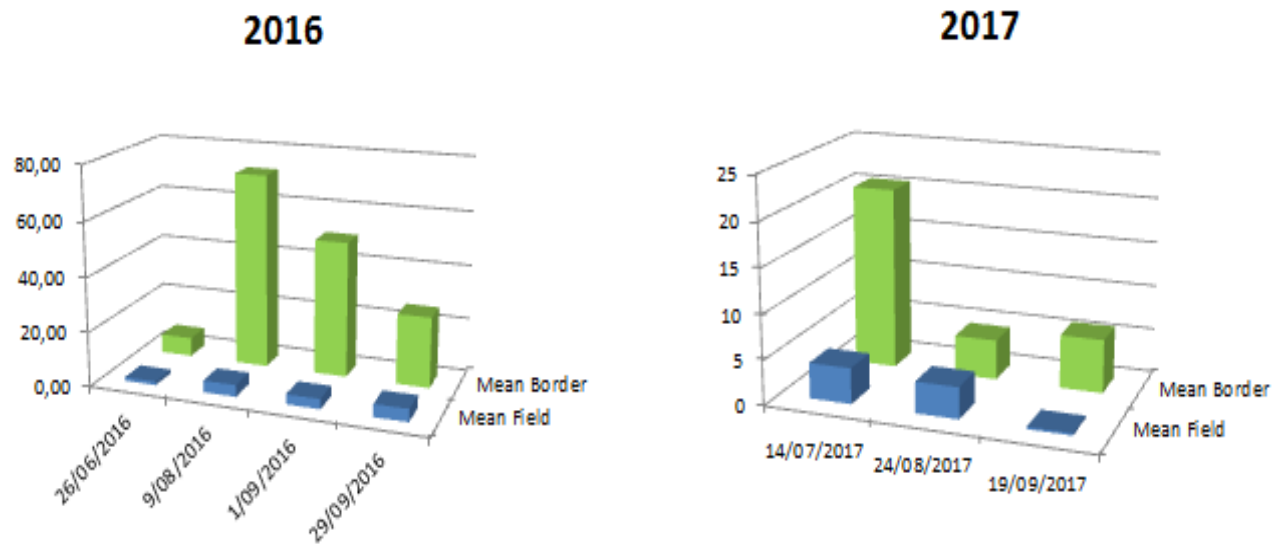


Figure 1: Number of potential vectors trapped with portable air suction machine per place and per date for the two years of the project.

In terms of population diversity, the population of Auchenorrhyncha was also found to be more diverse in the borders (see table hereafter).

Suborder	Super-family	Family	Species	Total	
				Field 2017	Border 2017
Auchenorrhyncha		Cicadellidae	Ribautiana debilis	0	8
Auchenorrhyncha		Cicadellidae	Macrosteles sp.	6	3
Auchenorrhyncha		Cicadellidae	Zyginidia sp.	18	12
Auchenorrhyncha		Cicadellidae	NI	1	3
Auchenorrhyncha		Delphacidae	NI	1	0
Auchenorrhyncha		Deltocephalinae	NI	0	22
Auchenorrhyncha		Aphrophoridae	NI	0	2
Auchenorrhyncha			NI	2	5
Sternorrhyncha	Psylloidea		Cacopsylla pyri	0	15
Sternorrhyncha	Psylloidea	Aphalarinae		3	0

WP2 Identification of potential vectors and transported pathogens

Task 2.1 Identification of collected vectors and setup of DNA database (ILVO and CRA-W)

The Auchenorhyncha and Psylloidea individuals found during WP1 were identified

- Morphologically: (online) morphological keys, reference material, Hemiptera experts. However, closely related species are very difficult to distinct morphologically and species identification generally rely on taxonomic traits related to the male genitalia, leading to mere identification at family or genus levels for captured nymphs and females
- By means of molecular methods: internal transcribed spacer 2 (ITS2) region of the ribosomal operon and the cytochrome oxidase I gene (COI), start off from molecular protocols developed during the European FP7 QBOL for Hemiptera and compare amplicons with online DNA databases (e.g. Genbank, BOLD, QBank)

The practical results can be found throughout the report. The identification of all insects that appear in the survey graphs were confirmed by sequencing.

Task 2.2 Detection of pathogens in collected potential vectors (ILVO and CRA-W)

- Start examining the captured insects for pathogens at the end of August.
- Samples of plants crushed and DNA extracted by means of the CTAB extraction method according to ANSES, or the DNeasy Plant extraction kit.
- Samples of individual identified insects collected from infected trees crushed and DNA extracted by means of the CTAB extraction method according to ANSES, or the Nucleospin Plant II extraction kit.
- Generic phytoplasma detection:
  - Generic detection method like the nested PCR (Deng & Hiruki, 1991, Schneider et al. 1995) followed by the primer set fU5/rU3 (Lorenz et al., 1995) or R16F2/R2 (Lee et al. 1995).
  - If quantification is required, the generic real-time PCR method of Christensen et al. (2004) will be used.
- *'Candidatus* Liberibacter solanacearum' detection:
  - Diagnostic tests already used in FOD FYLIBER and Euphresco PHYLIB project.
  - For the standard detection the primer set LsoF/OI2c (Liefting, L. W. et al. 2008, Li et al. 2009) and the real-time PCR method of Li et al. (2009) were used. The last method was determined to be the most performant and was validated for diagnostic use. The procedure was audited and an ISO17025 accreditation for the Li et al. (2009) diagnostic real-time PCR procedure was obtained by the lab.
- Further characterization of both phytoplasmas and Liberibacter: see primer table 1.

Table 1. Primer table for phytoplasma and '*Ca*. L. solanacearum' characterisation.

Primer name	Sequence 5'-3'	Gene(s)	Amplicon size (bp)	Reference
Phytoplasma				
P1 P7	AAGAGTTTGATCCTGGCTCAGGATT CGTCCTTCATCGGCTCTT	16S-23S rRNA IGS & 23S rRNA	1784	Deng & Hiruki 1991 Schneider et al. 1995
R16F2n R16R2	GAAACGACTGCTAAGACTGG TGACGGGCGGTGTGTACAAACCCCG	16rRNA	1248	Gundersen & Lee 1996 Lee et al. 1993
fU5 rU3	CGGCAATGGAGGAAACT TTCAGCTACTCTTTGTAACA	16rRNA	882	Lorenz et al. 1995
'*Ca*. L. solanacearum'				
OI2c OA2	GCCTCGCGACTTCGCAACCCAT GCGCTTATTTTTAATAGGAGCGGCA	16S rRNA	1168	Jagoueix et al., 1996 Liefting et al., 2009
CL514F CL514R	CTCTAAGATTTCGGTTGGTT TATATCTATCGTTGCACCAG	*rplJ/rplL*	669	Munyaneza et al. 2009
LsoF	GTCGAGCGCTTATTTTTAATAGGA	16SrRNA	79	

HLBr HLBp	CTACCTTTTTCTACGGGATAACGC FAM-AGACGGGTGAGTAACGCG-BHQ			Li et al. 2009 Li et al. 2006
Lso adk F Lso adk R	GCGCCACACTAACATCTCCTTCC CGCAGCAGTATGAGGGCC	*Adk* & flanking region	770	Ravindran et al. 2011
Lp Frag 4-(1611F) LP Frag 4-480R	GGTTGATGGGGTCATTTGAG CACGGTACTGGTTCACTATCGGTC	16S-23S rRNA IGS & 23S rRNA		Hansen et al. 2008

Table of the insects that tested positive for phytoplasmas

Code	Genus	Species	fU5-rU3 Pos	R16F2-R16R2 Pos	fU5-R16R2	Orchard	Field
TM083	*Philaenus*	*spumarius*	x	x		Melle	Pyrus 2
TM134	*Graphocephala*	*fennahi*		x		Melle	Malus
TM143	*Cicadella*	*viridis*		x		Melle	Pyrus 1
TM145	not identified			x		Melle	Pyrus 2
TM149	*Typhlocyba*	*quercus*	x	x		Melle	Malus
TM150	not identified		x	x		Melle	Malus
TM151	*Macrosteles*	*sexnotatus*	x	x		Melle	Pyrus 1
TM152	not identified			x		Melle	Pyrus 2
TM156	not identified		x			Melle	Malus
TAn02	*Javesella*	*pellucida*		x		Anzegem	Daucus
TAn04	*Macrosteles*	*sexnotatus*	x	x		Anzegem	Daucus
TAn05	*Eupteryx*	*atropuncata*		x		Anzegem	Daucus
TAn06	*Macrosteles*	*sexnotatus*	x	x		Anzegem	Daucus
TAn14	*Macrosteles*	*sexnotatus*		x		Anzegem	Daucus
5-4-2016	Mix of Hemiptera				x	Gembloux	untreated Malus
14-6-2016	Mix of Hemiptera				x	Gembloux	untreated Malus
28-3-2017	*Cacopsylla picta*				x	Gembloux	untreated Malus
11-4-2017	*Unidentified Psylloidea larvae*				x	Gembloux	untreated Malus
4-10-2016	*Unidentified Psylloidea adult*				x	Gembloux	untreated Malus

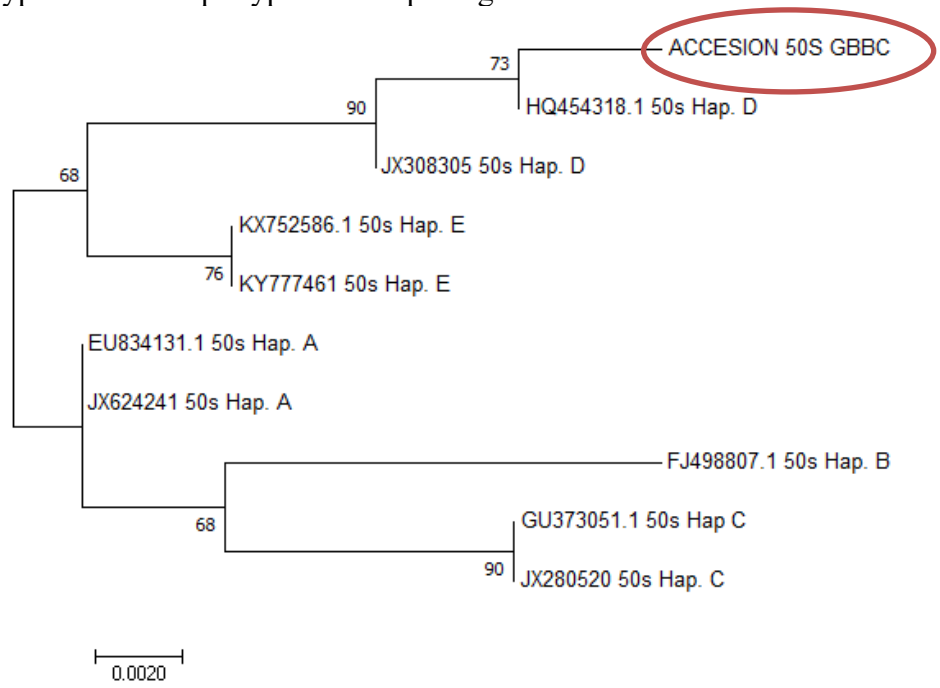
Only the insects that tested positive for either both PCR methods (fU5/rU3 primer set and R16F2/R2 primer set) or fU5/R16R2 were also confirmed positive by sequencing. So, finally, *Macrosteles sexnotatus* is the only insect that tested positive tested positive in carrot. The phytoplasma confirmed to be an aster yellows phytoplasma ('*Ca*. P. asteris'), identical to the isolate found in the carrot plants itself. *Macrosteles sexnotatus* also tested positive in the pear orchard. However, the phytoplasma in those insects were also identified as an AY phytoplasma. This phytoplasma not found in the pear or apple trees in the surveyed orchards. It is suspected that the phytoplasma is present in the herbs/weeds which are found in and around the apple/pear orchards.
This is also the case for the other insects that tested positive for phytoplasma, namely *Philaenus spumarius* and *Typhlocyba quercus*. Also from both those insects, also originating from the traps in the apple and pear orchards, only aster yellows phytoplasma was recovered. Only aster yellows phytoplasma was also found in mixes of Hemiptera collected by beat-netting in Malus orchards of Gembloux. It is not the first time that *Philaenus spumarius* tests positive for harbouring AY phytoplasma. Its role in phytoplasma transmission is under question, and can definitely not be ruled out (Ivanauskas et al. 2014). No information is available on the transmission capacity of phytoplasma diseases by any *Typhlocyba* species. Further investigation is needed.
The only insects found positive for 16SrX group phytoplasmas in the frame of this project were all Psylloidea.

Besides the pathogen tests on the trapped insects, also plant samples were taken on a regular base to confirm the continuous presence of the pathogen in the fields.

For Michelbeke and Melle, the presence of AP and PD phytoplasma were monitored in that way.
For Anzegem, Gottem and Ingelmunster, this was the case for the presence of '*Ca*. P. asteris'. However, from time to time, the carrot samples were also tested for the presence of 'Ca. L. solanacearum'. In the fall of 2017, this pathogen was indeed found at the locations of Gottem, Jandrain-Jandrenouille and Ingelmunster. It was not possible to allocate the symptoms to either 'Ca. P. asteris' or '*Ca*. L. solanacearum', since both pathogens appeared to be present in considerate amounts, even in mixed infections (Fig).
In Gottem and Ingelmunster, the remaining seed lots were also tested for the presence of both phytoplasma and '*Ca*. L. solanacearum'. In the location in Gottem, cvs. Nerja (Bejo), Triton (Clause) and Romance (Nunhems) were sown. No information could be obtained from the grower on which infected plants belonged to which cultivar, since the reduced vegetative growth of the carrots made it impossible to distinguish the cultivars in the field. In the location in Ingelmunster, only cv. Nerja was produced. From location Gottem, remaining seeds from the cvs. Triton and Nerja could be sampled and tested. This was also the case for the remaining part of the Nerja seed lot in Ingelmunster. Whereas seed lot Triton tested negative, the cv Nerja seed lots from both locations tested positive for the presence of 'Ca. L. solanacearum'. All '*Ca*. L. solanacearum' isolates (plants and seed lots from both locations) were identified as belonging to haplotype D (Fig.).

Fig. During harvest, a considerate percentage of poor quality carrots are manually sorted out (A); leaf yellowing and reddening in the field (B); excessive secondary ("hairy") roots formation (C).

Phylogenetic tree indicating the '*Ca*. L. solanacearum' haplotype. Haplotype D is one of the typical carrot haplotypes of this pathogen.



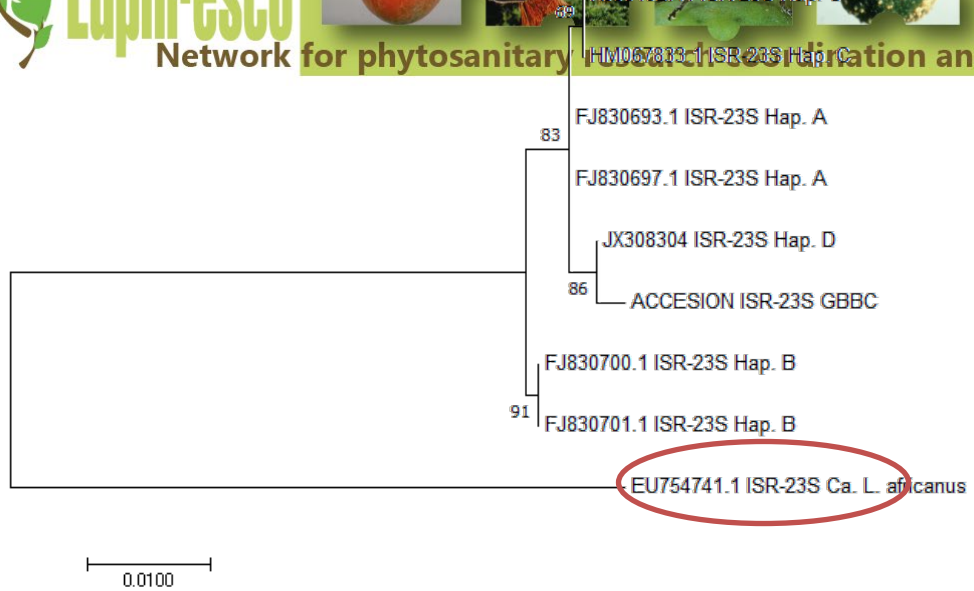


Figure. Molecular Phylogenetic analysis by Maximum Likelihood method
The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model [1]. The tree with the highest log likelihood (-493.34) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 nucleotide sequences. There were a total of 307 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [2].

Task 3.1 Insect rearing populations (ILVO)

Setup insect rearing populations of potentially important phloem and xylem feeding vectors: *Cacopsylla pyri* and *Empoasca decipiens* cultures were initiated.

Insect monitoring in the field:

Task 3.2 Insects infected in artificial conditions (ILVO)

Several populations of *Empoasca decipiens* and *Cacopsylla pyri* were reared on infected plants (AY infected carrot) and trees (PD infected pear). Only in one experiment, 20 Empoasca descipiens species were put on an AP infected tree (isolate accession 3/6 – JKI, Germany) and after 10 days, 10 insects were transferred to a healthy apple tree and 10 were tested for phytoplasma presence. All 10 insects that were tested, tested negative for phytoplasma presence. All 10 insects that were put on a healthy apple tree suddenly died. Unfortunately, all other transmission tests failed due to population collapse (populations of *E. descipiens*, *F. florii* and *Cacopsylla pyri*. This task could not be fully completed.

WP4 Phenology and host range of (important) vectors

Task 4.1. Outdoor cages were placed on *Malus*, *Pyrus*, *Carpinus*, *Viburnum* (all in Melle) on trees (and shrubs on which eggs are observed. Identification and population dynamics are observed biweekly. On selected phytoplasma negative *Malus* trees, the same was done with captured adult insects that are put inside the cages, tightened around a branch.
This method did not allow us to follow the population built up. No useful data were obtained on leaf- or planthopper populations in these cages. Both in the transmission experiments in the greenhouses, and the outdoor experiments in cages, the populations suddenly collapsed for unknown reasons.

Task 4.2. Host preference – olfactometer experiments.

Due to time contraints, this task was dropped and not completed. The choice not to do this task is partly due to the huge amount of surveyed insects that had to be processed (which took more time than expected), and partly also due to the unexpected identification of '*Candidatus* Liberibacter solanacearum' in two carrot fields. Because of the fact the '*Ca*. L. solanacearum' is a regulated pathogen, a lot of time went into additional monitoring of this pathogen, field visits, psyllid monitoring (re-assessing all sticky plates for the presence of psyllids), pathogen characterization, etc.

Task 4.3. Data generated by systematic and random sampling

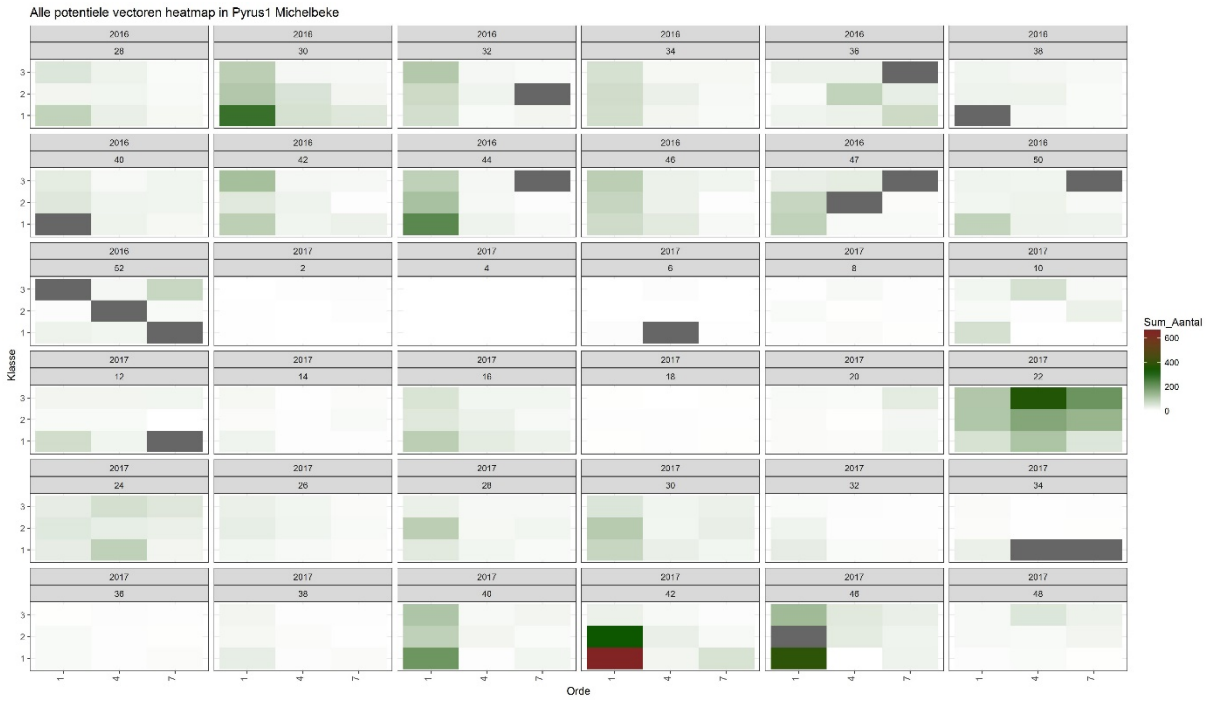
The phenology could still be monitored by the fact that a consistent monitoring of 9 geographically spread sections of each surveyed field resulted in data for each individual section, allowing to produce biweekly heatmaps for all captured insects of interest.
It is also possible to make the link between the survey data and the meteorological data. Graphs will be included in the final report.

Based on the placement of the yellow sticky traps, heat maps of the vector presence within the plots were produced. These heat maps give information on the spatial distribution in the field and to a lesser extend also on the way the insects enter the plot and spread within the plot.

1. ***Heatmaps for the location Michelbeke***

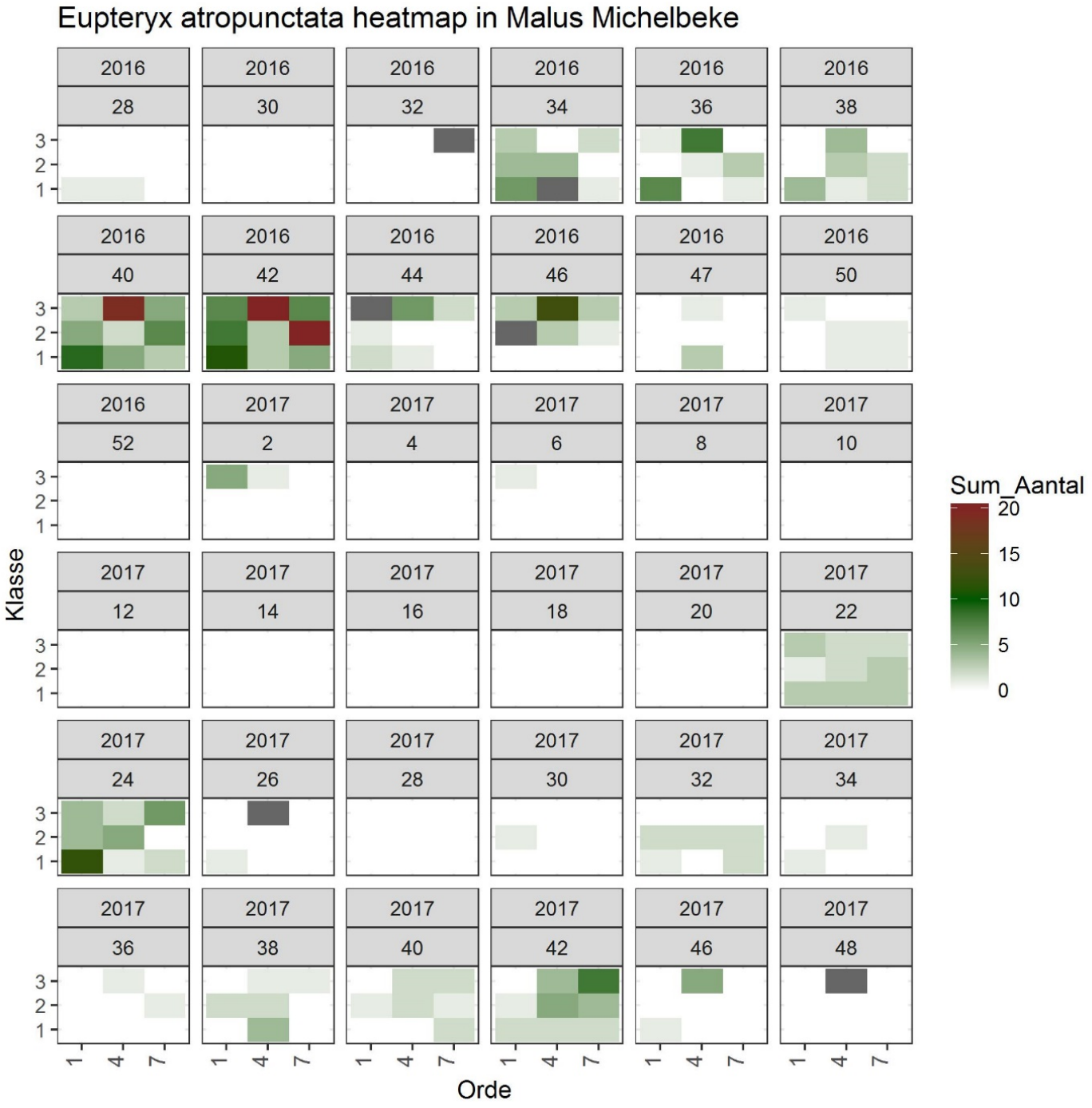
All potential vectors in *Malus*

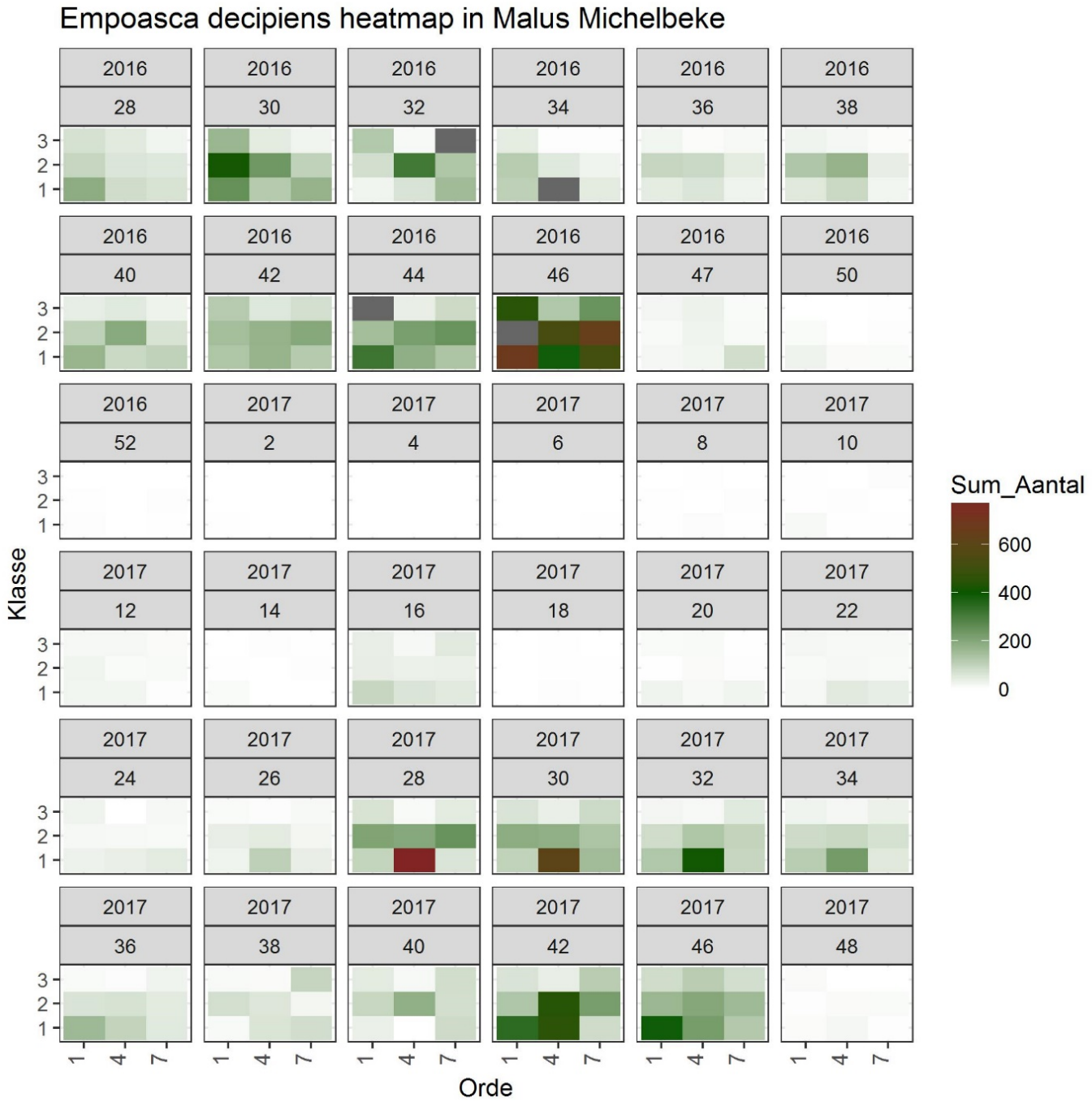
All potential vectors in *Pyrus* 1

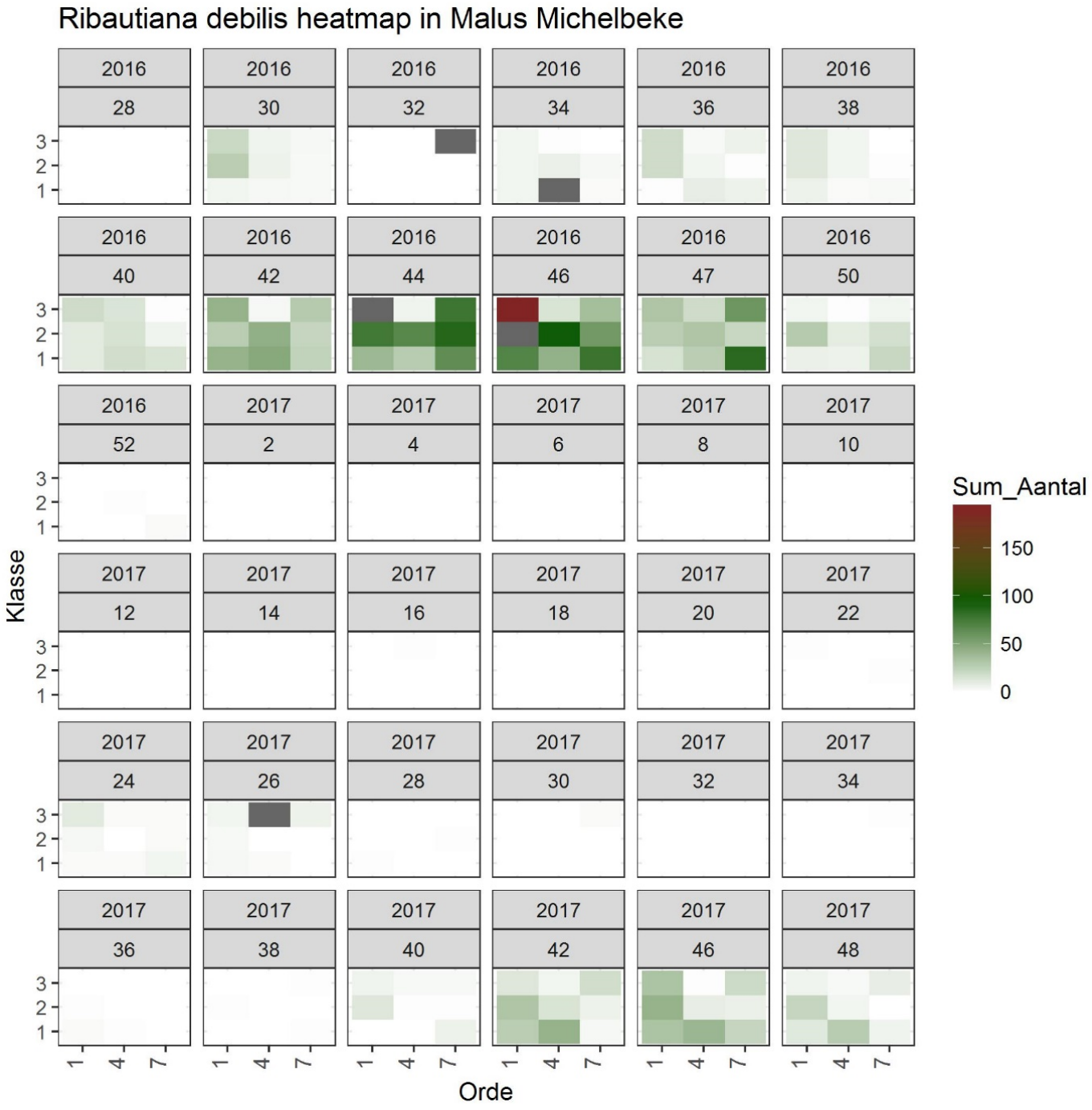


All potential vectors in *Pyrus* 2

Individual heatmaps for the most prevailing insects in ***Malus*** in Michelbeke
These heatmaps have also been produced for the Pyrus 1 and 2 fields in Michelbeke, as well as for the *Malus* and *Pyrus* fields in Melle. They will be added to this report as supplementary material (in digital format).




Empoasca decipiens heatmap in Malus Michelbeke
2016 28
2016 30
2016 32
2016 34
2016 36
2016 38
2016 40
2016 42
2016 44
2016 46
2016 47
2016 50
2016 52
2017 2
2017 4
2017 6
2017 8
2017 10
2017 12
2017 14
2017 16
2017 18
2017 20
2017 22
2017 24
2017 26
2017 28
2017 30
2017 32
2017 34
2017 36
2017 38
2017 40
2017 42
2017 46
2017 48
Klasse
3
2
1
Orde
1
4
7
Sum_Aantal
600
400
200
0


Ribautiana debilis heatmap in Malus Michelbeke
2016 28
2016 30
2016 32
2016 34
2016 36
2016 38
2016 40
2016 42
2016 44
2016 46
2016 47
2016 50
2016 52
2017 2
2017 4
2017 6
2017 8
2017 10
2017 12
2017 14
2017 16
2017 18
2017 20
2017 22
2017 24
2017 26
2017 28
2017 30
2017 32
2017 34
2017 36
2017 38
2017 40
2017 42
2017 46
2017 48
Klasse
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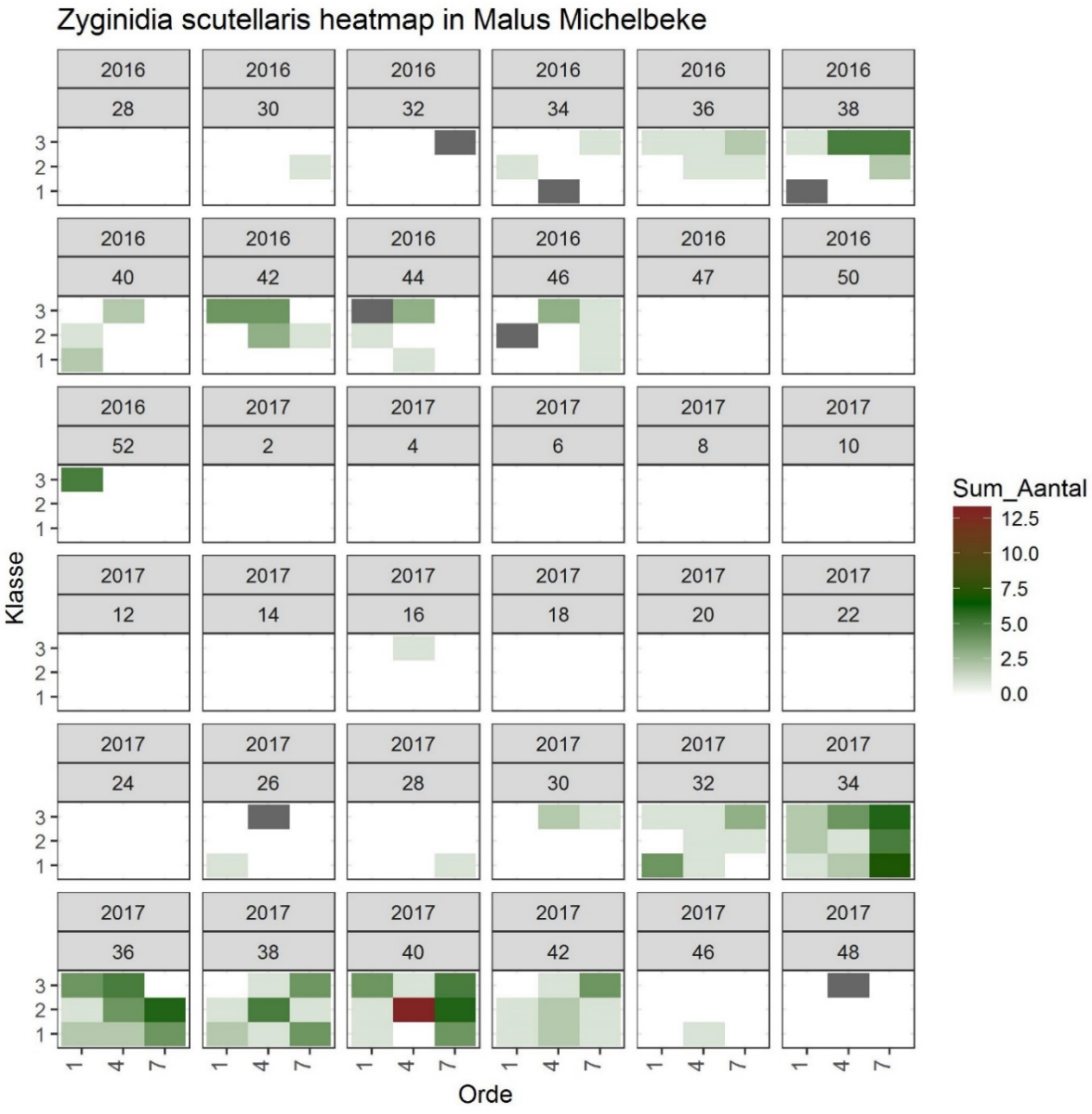
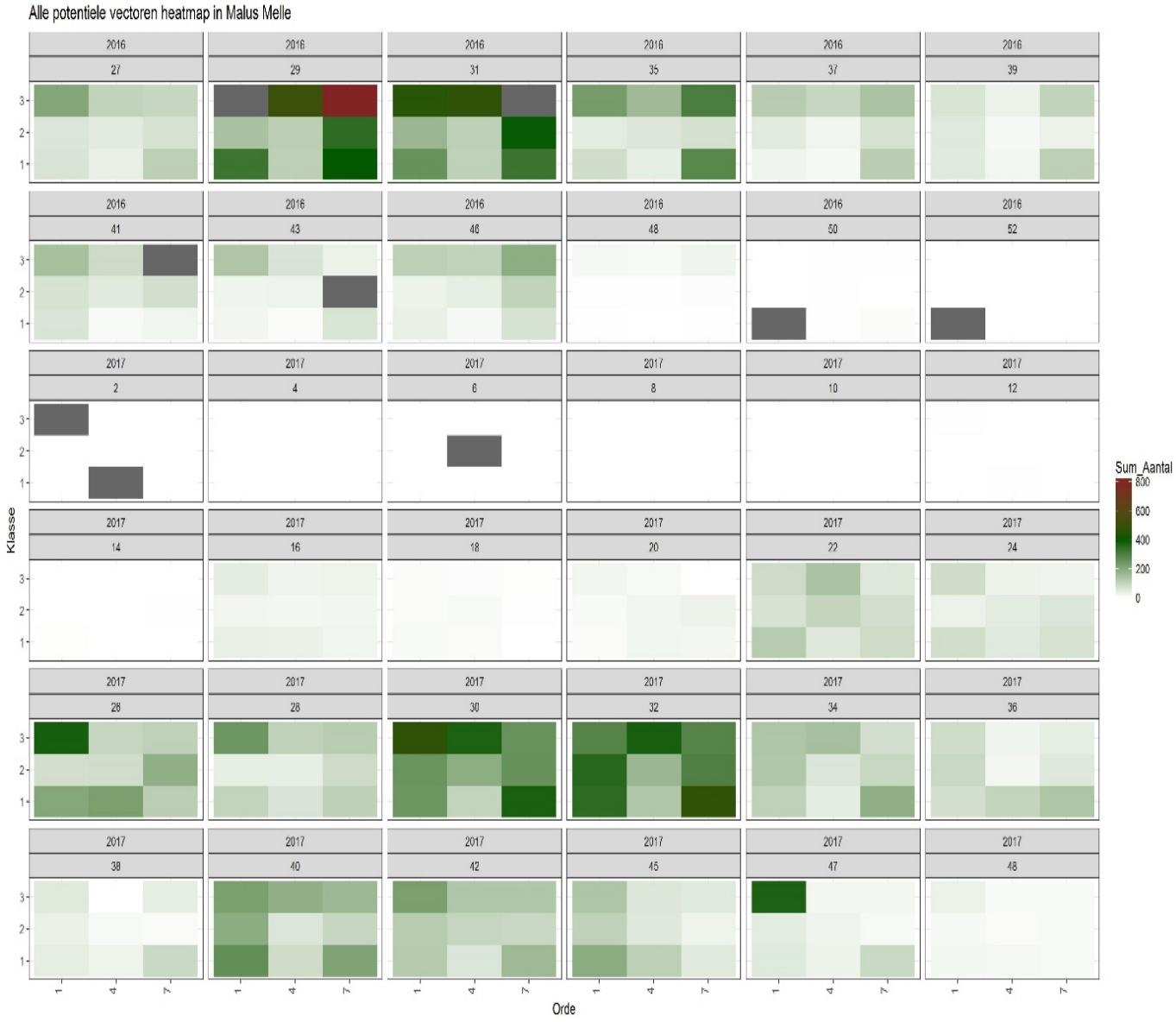
Orde
1
4
7
Sum_Aantal
150
100
50
0

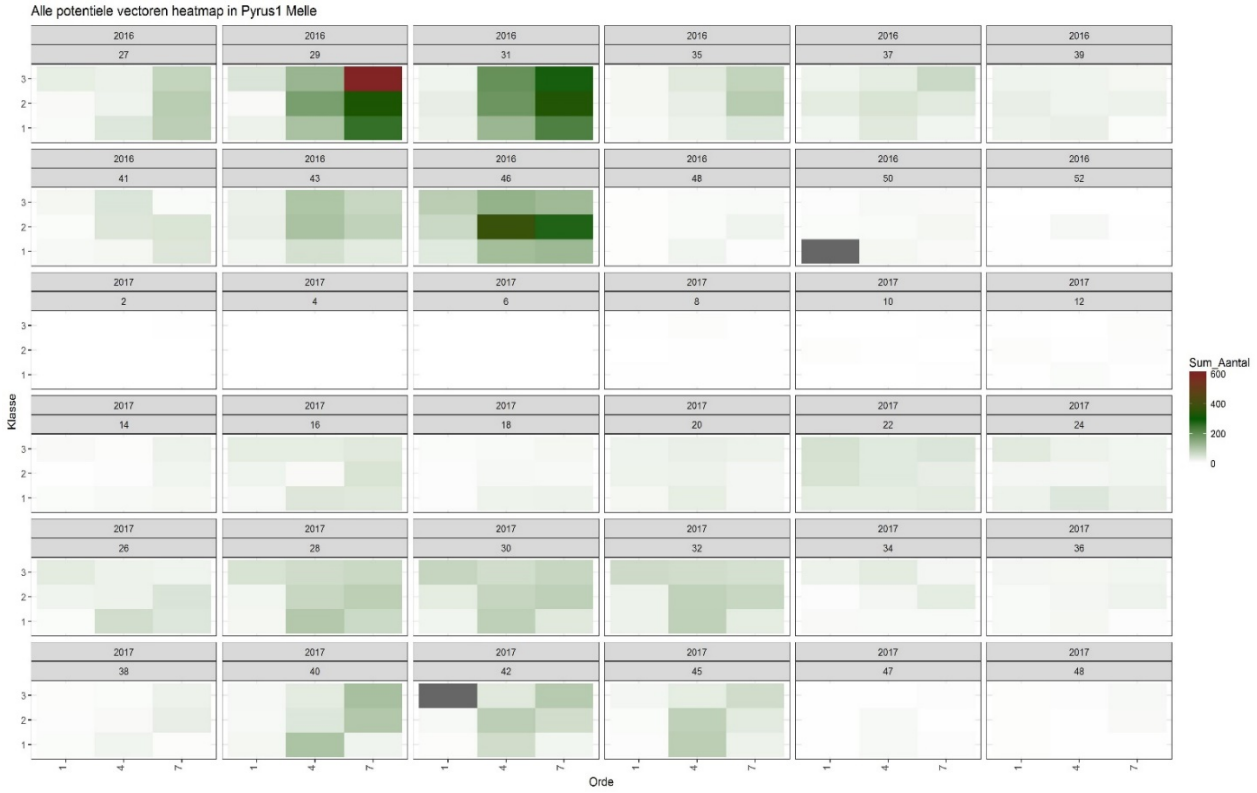

Zyginidia scutellaris heatmap in Malus Michelbeke
2016 28
2016 30
2016 32
2016 34
2016 36
2016 38
2016 40
2016 42
2016 44
2016 46
2016 47
2016 50
2016 52
2017 2
2017 4
2017 6
2017 8
2017 10
2017 12
2017 14
2017 16
2017 18
2017 20
2017 22
2017 24
2017 26
2017 28
2017 30
2017 32
2017 34
2017 36
2017 38
2017 40
2017 42
2017 46
2017 48
Klasse
3
2
1
1
4
7
Orde
Sum_Aantal
12.5
10.0
7.5
5.0
2.5
0.0

2. *Heatmaps for the location Melle*

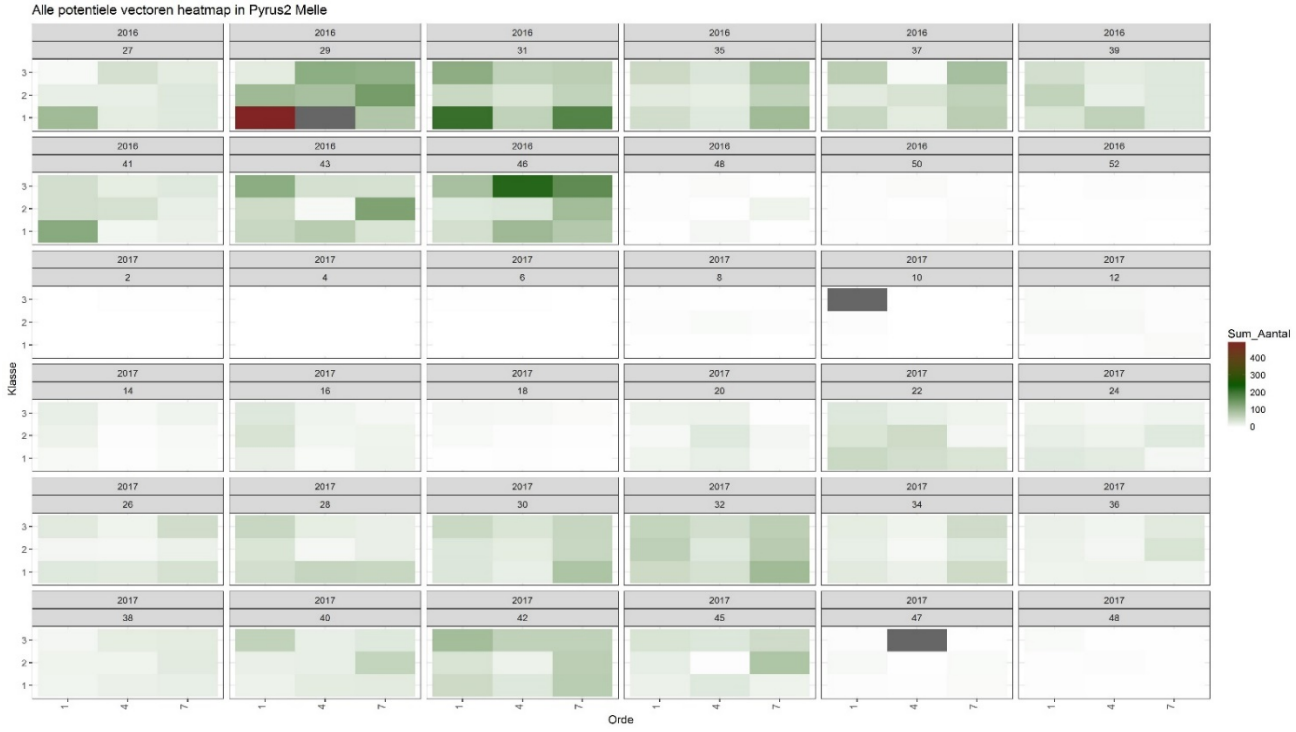
All potential vectors in *Malus*



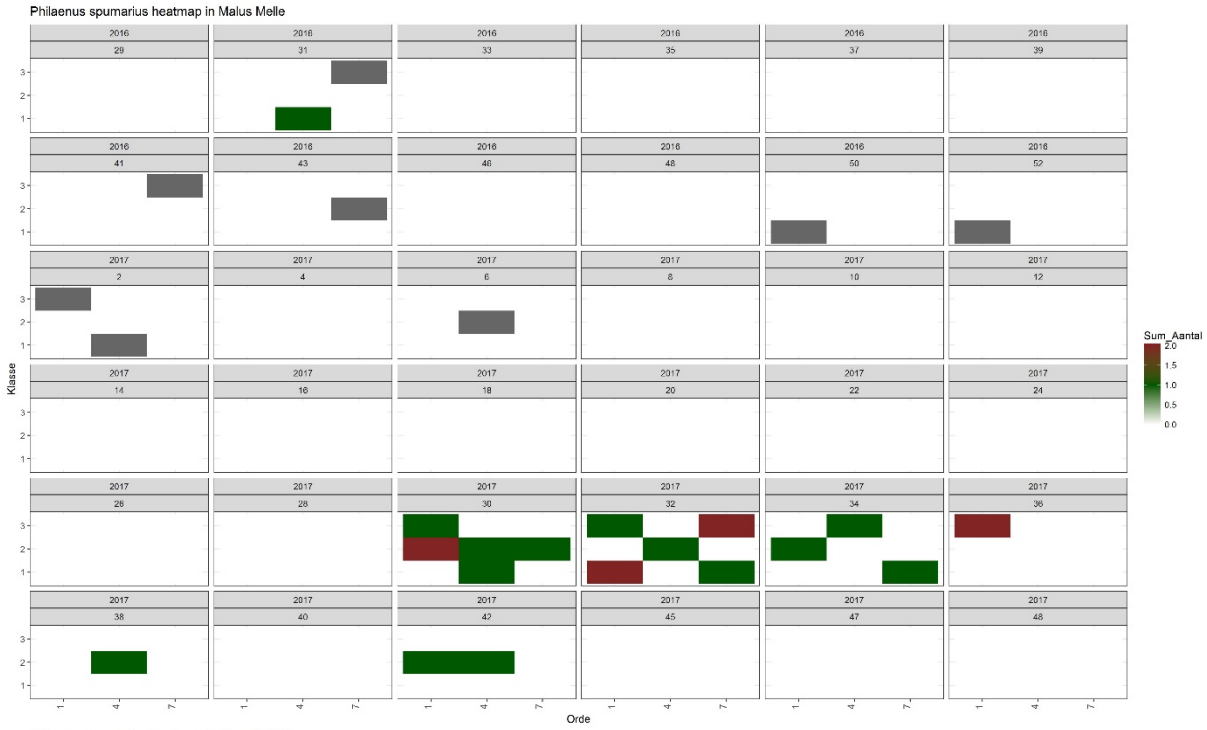
All potential vectors in *Pyrus* 1

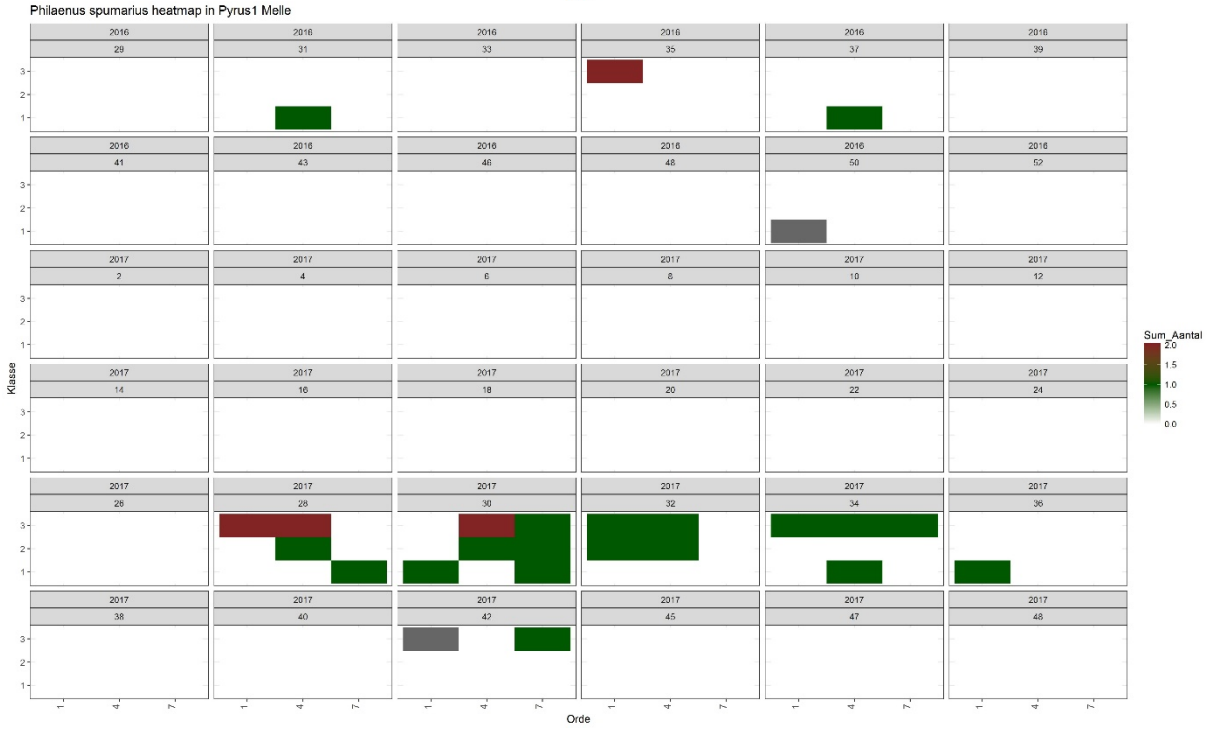


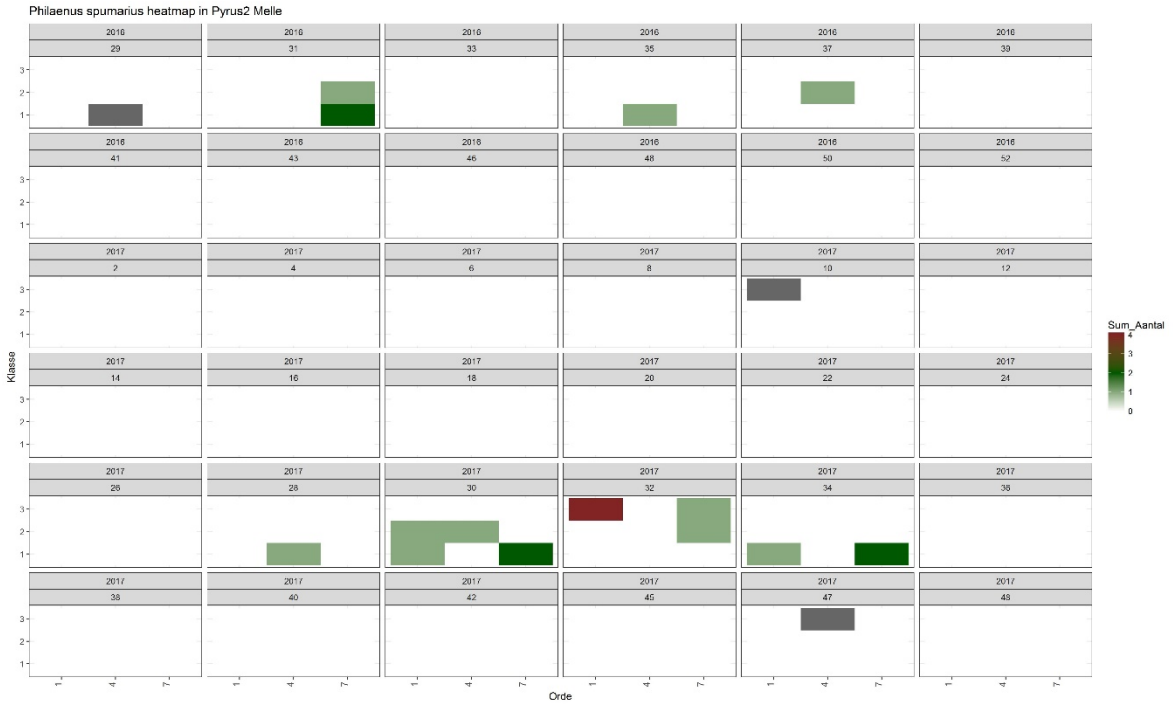
All potential vectors in *Pyrus* 2



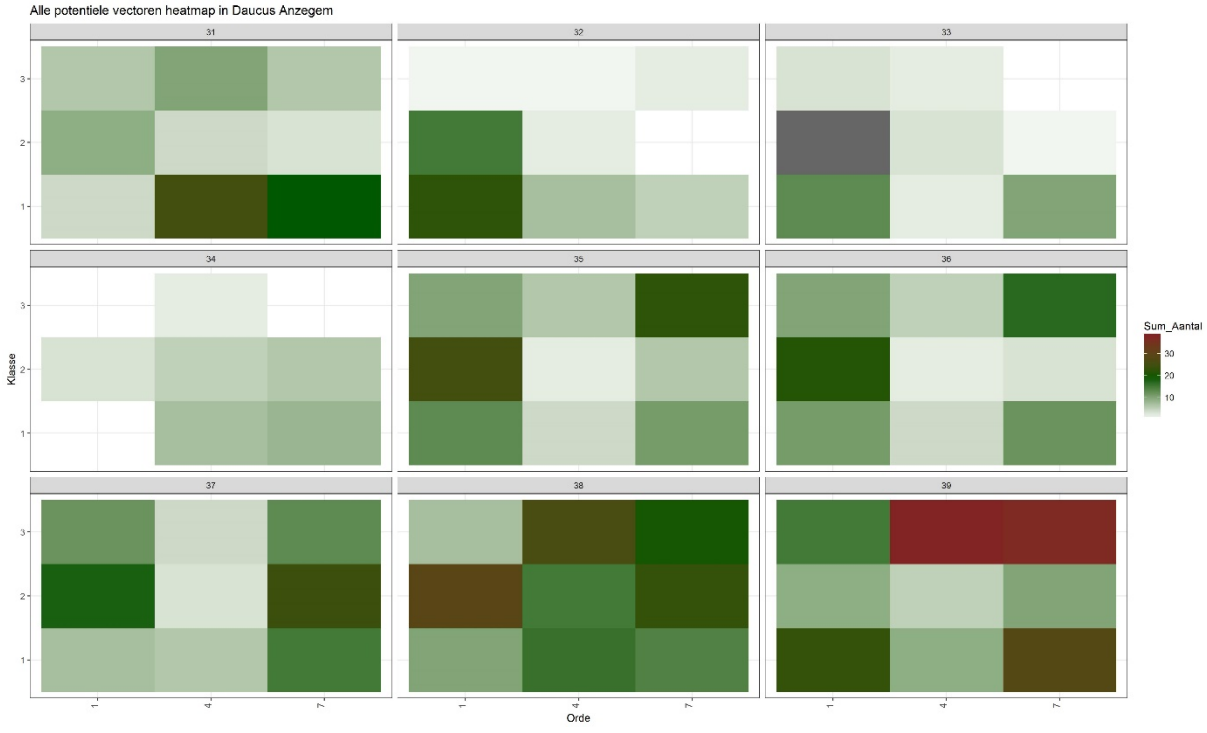
Individual heatmap for the insects that tested positive for phytoplasma, and all other heatmaps, produced for the insects trapped in the apple and two pear orchards, will be added to this report as supplementary material (in digital format). As an example, only those for *Philaenus spumarius*, an insect that tested positive for AY phytoplasma during the survey.



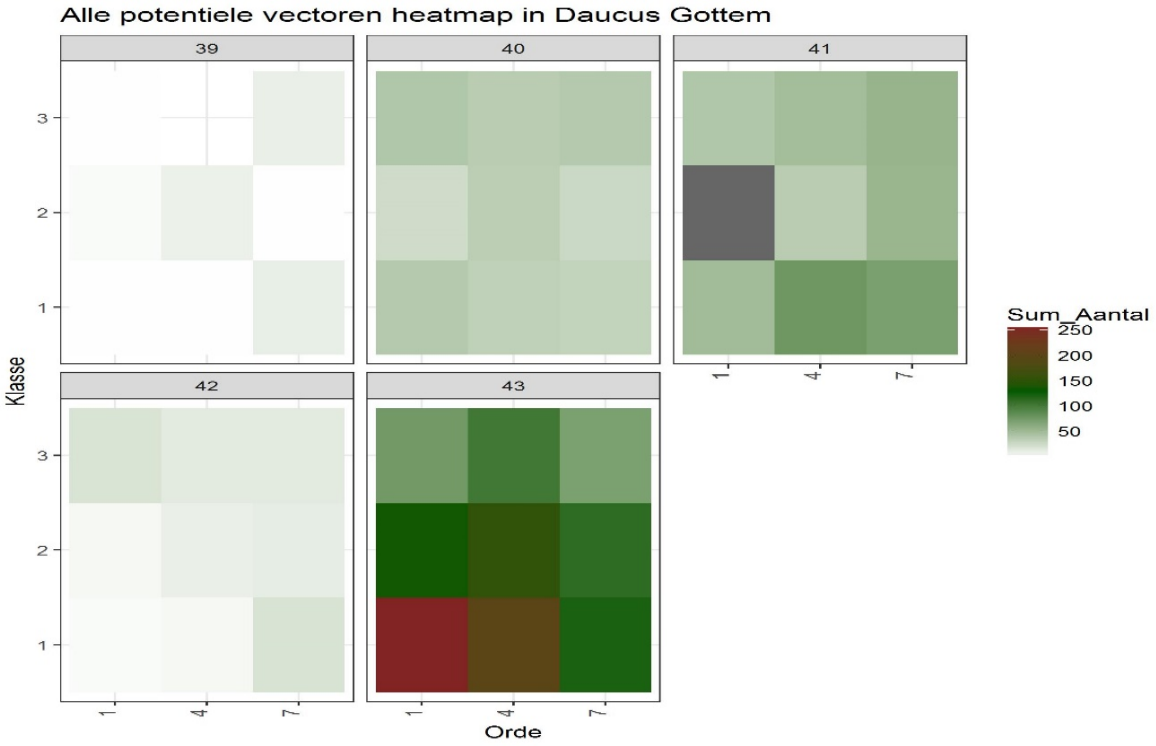


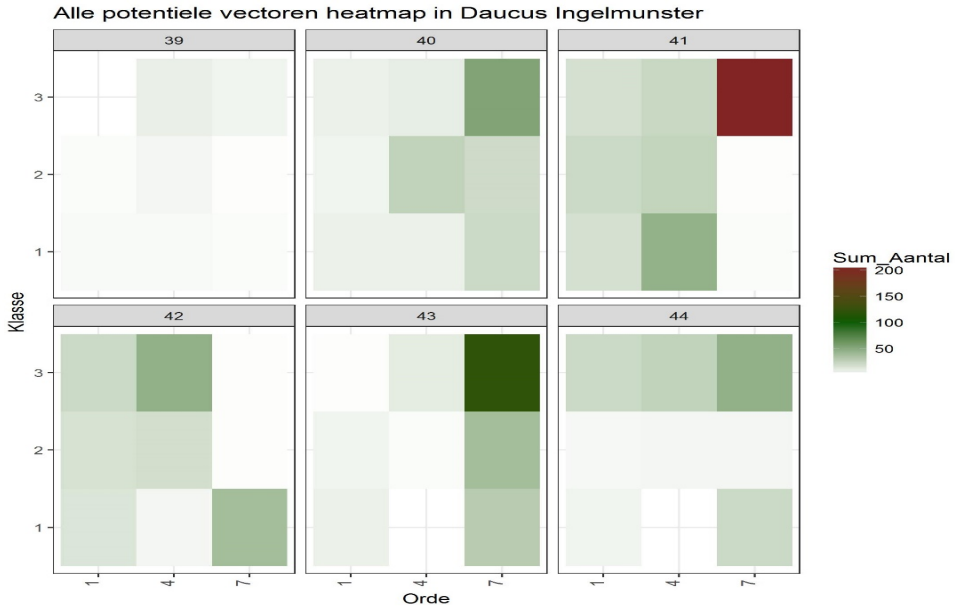


Heatmaps for the potential vectors in carrot (2016)



Heatmaps for the potential vectors in carrot (2017)

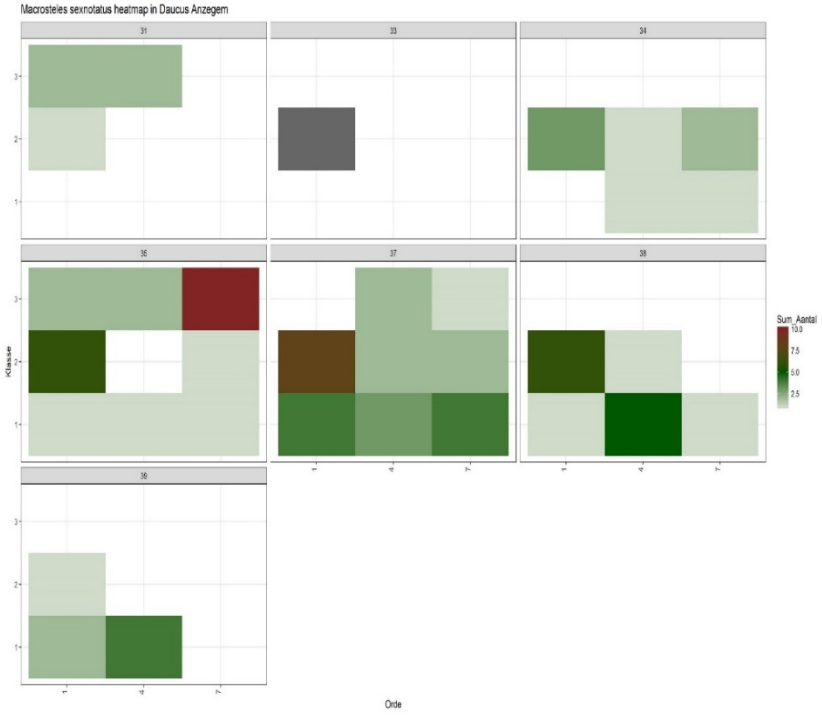




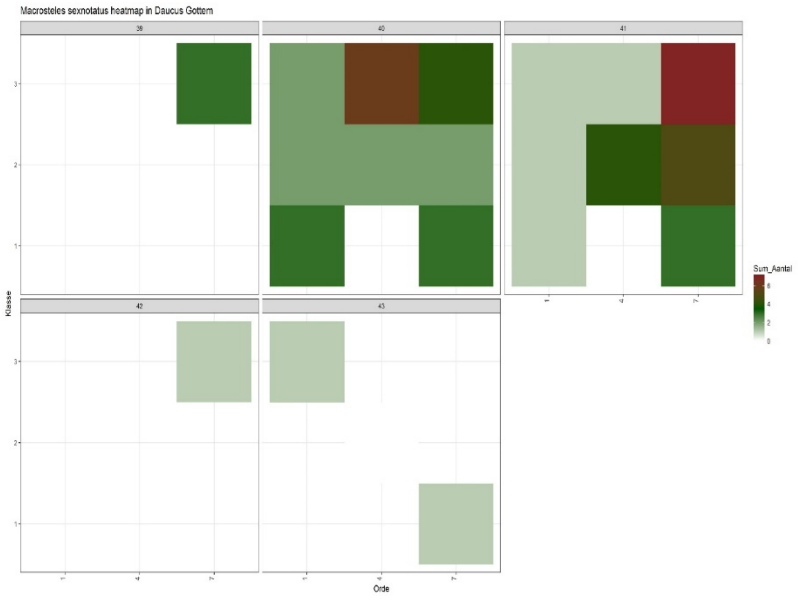
Heatmaps for individual insects in the carrot fields

Below an example of the heatmaps for individual insects in carrot fields. We chose *Macrosteles sexnotatus* as an example, since this insect can be considered as the main vector for the spread of the aster yellows phytoplasma in our carrot fields.

Macrosteles sexnotatus survey in 2016



Macrosteles sexnotatus survey in 2017

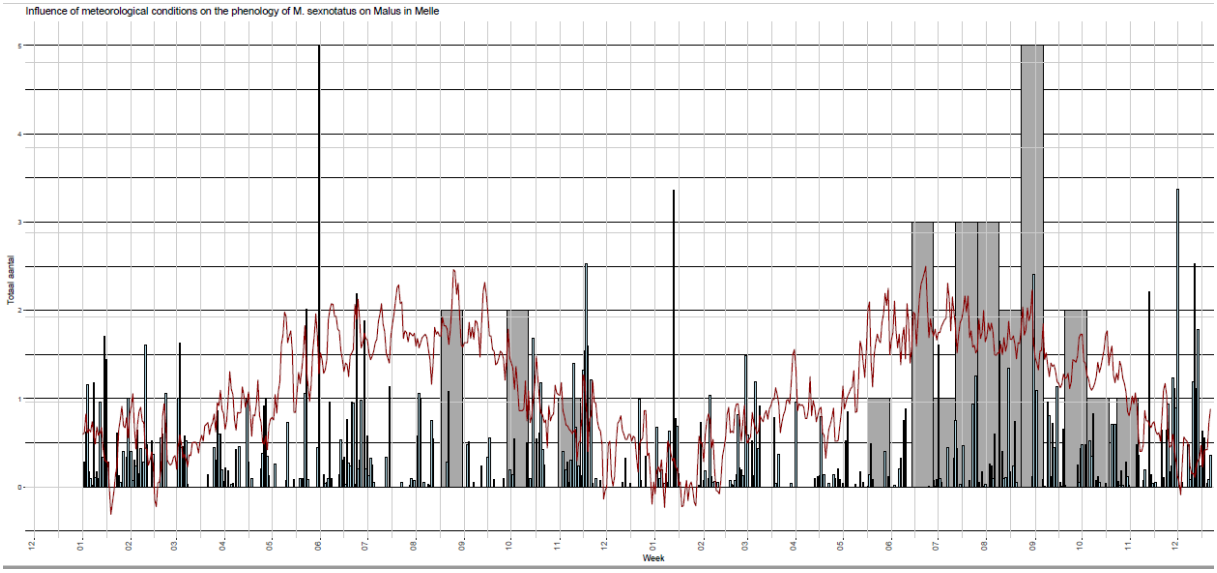


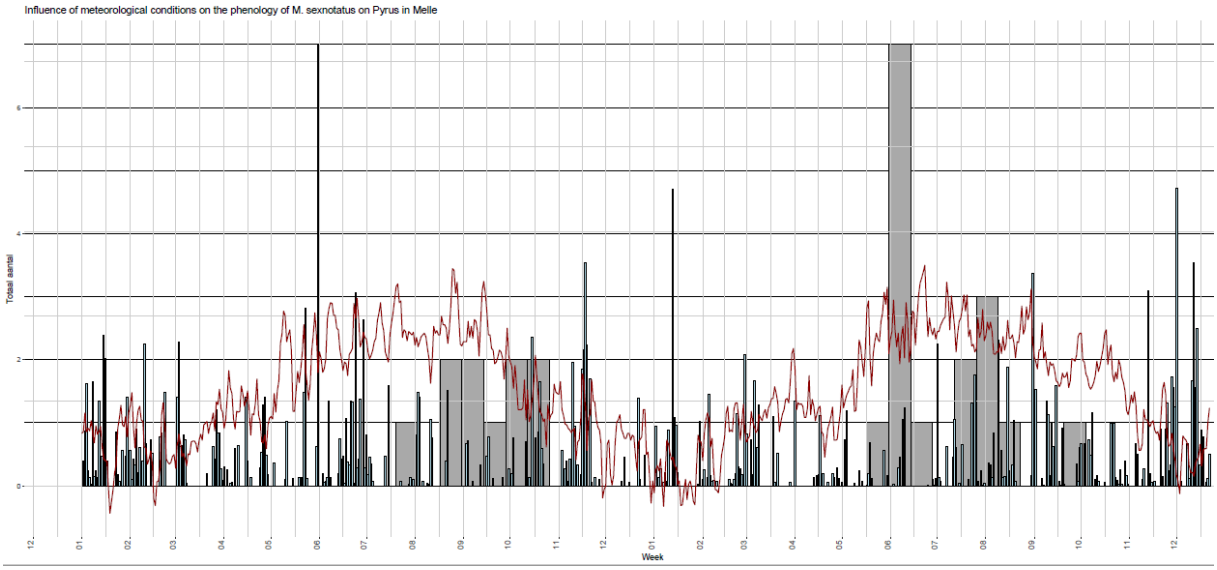
Insect population dynamics linked to meteorological data

Insects trapped in the apple and pear orchards at the location Melle

Legend for all graphs:
grey bars – # of trapped insects
green bars – rainfall
red line: temperature

Macrosteles sexnotatus

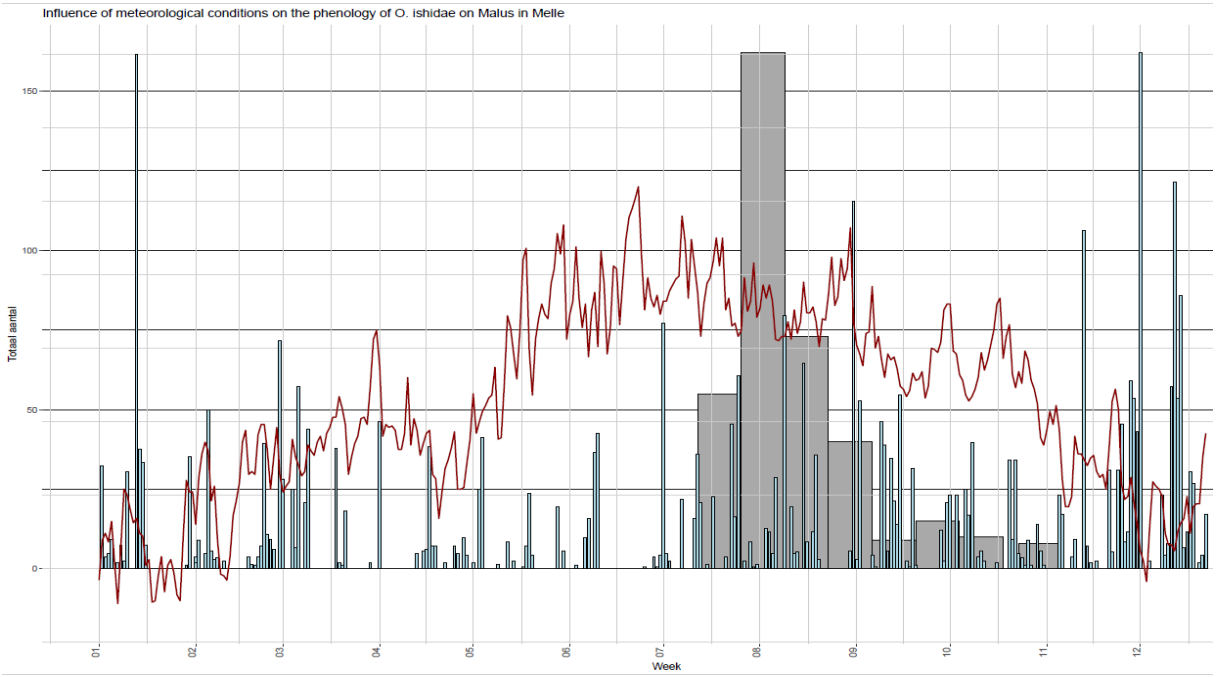


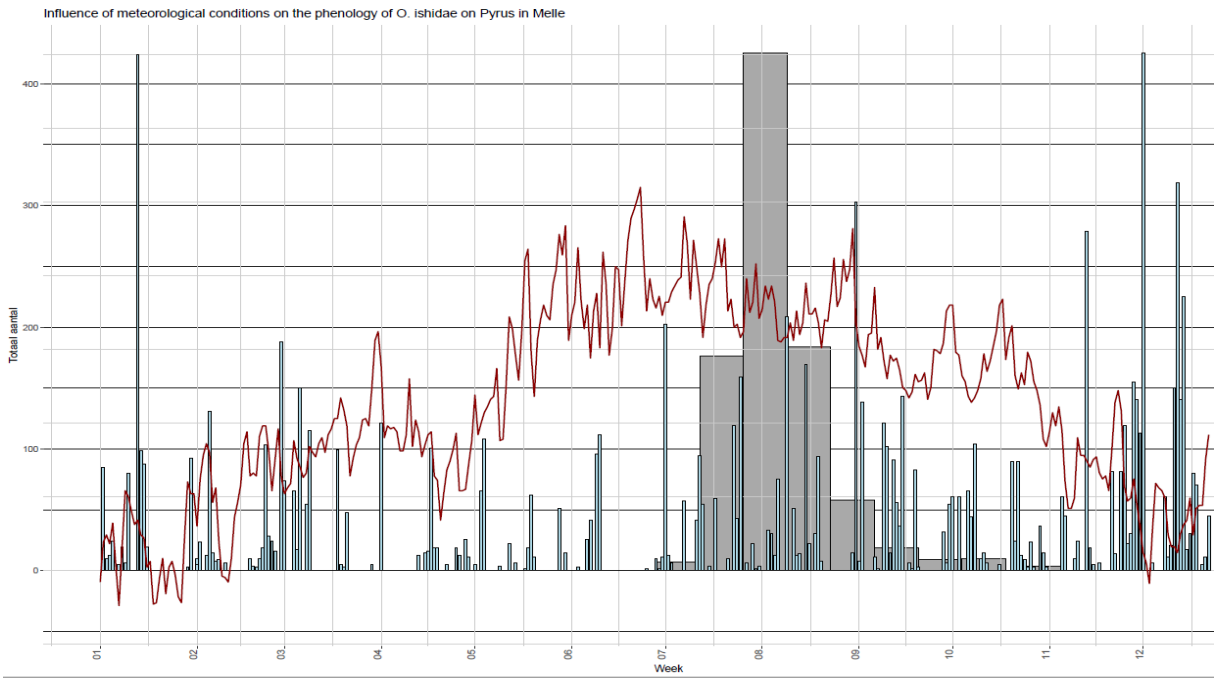


No clear conclusions can be made. During the second year, more *Macrosteles* insects were found on the sticky plates, and this already from earlier in the season. The temperature in 2017

was also higher, earlier in the season, explaining this difference. There does not seem to be a link with the rainfall. In addition, *Macrosteles* does not seem to prefer one or the other host plant. In 2016, the insects were more abundant in pear, whereas in 2017, it was the opposite and more insects were trapped in apple.

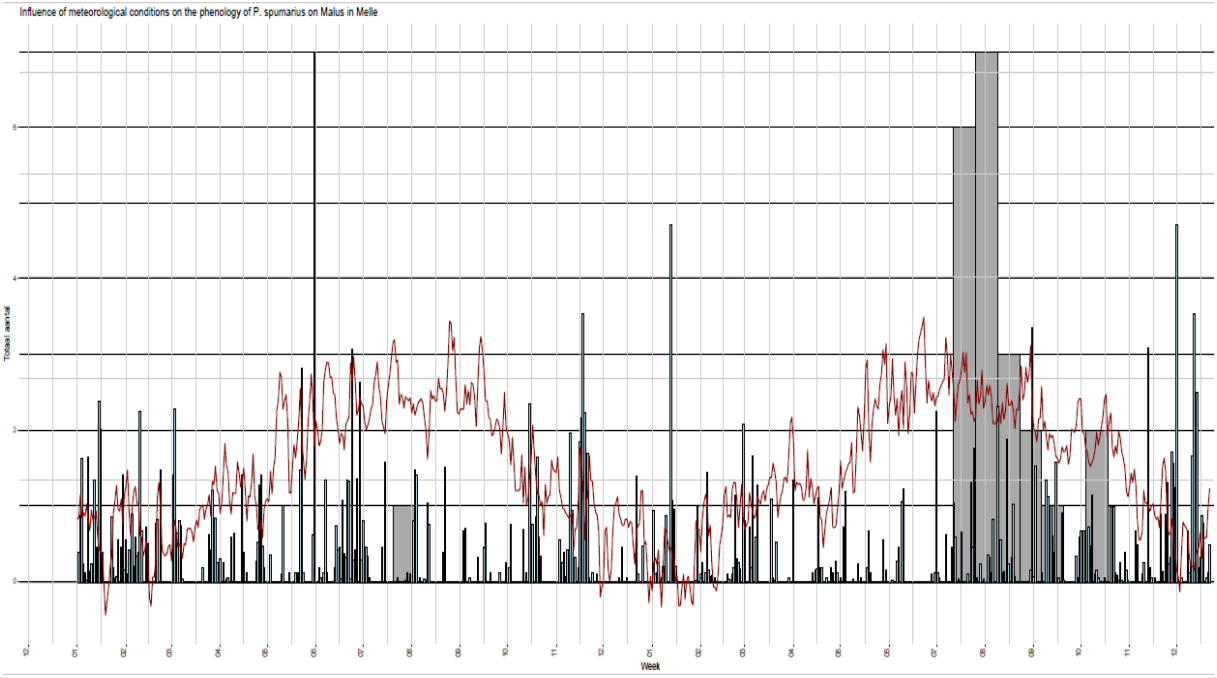
Orientus ishidae in 2017

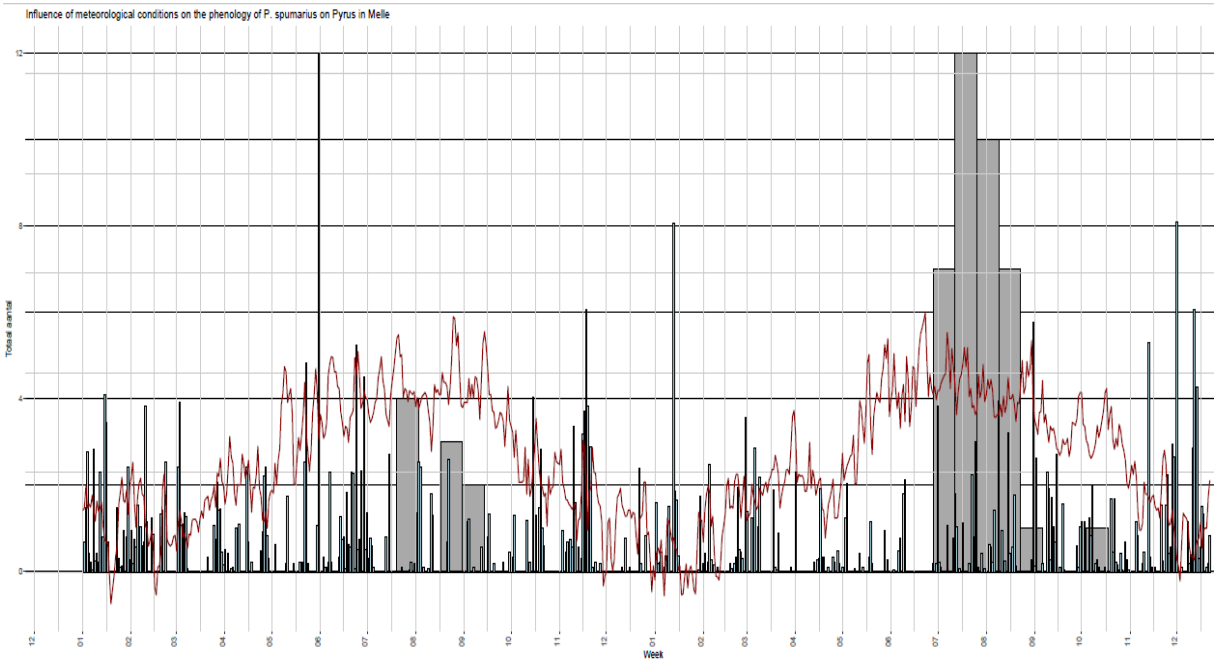




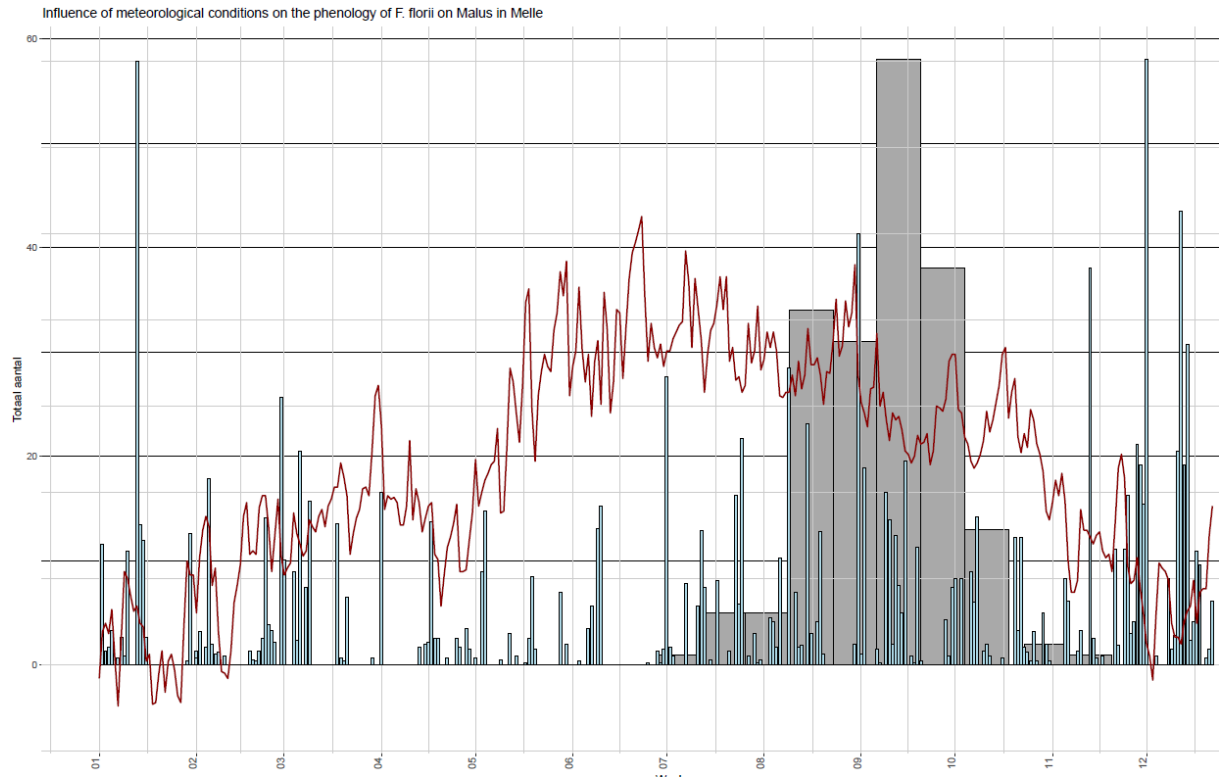
Also *Orientus* does not have a preference for apple or pear. The population dynamics were almost identical for both orchards (the numbers appear double for the pear orchards, yet this is because the amount of insects trapped on the 2 pear orchards were taken together (double the amount of sticky traps than in apple. (this is also the case for the other insects from the apple/pear locations)

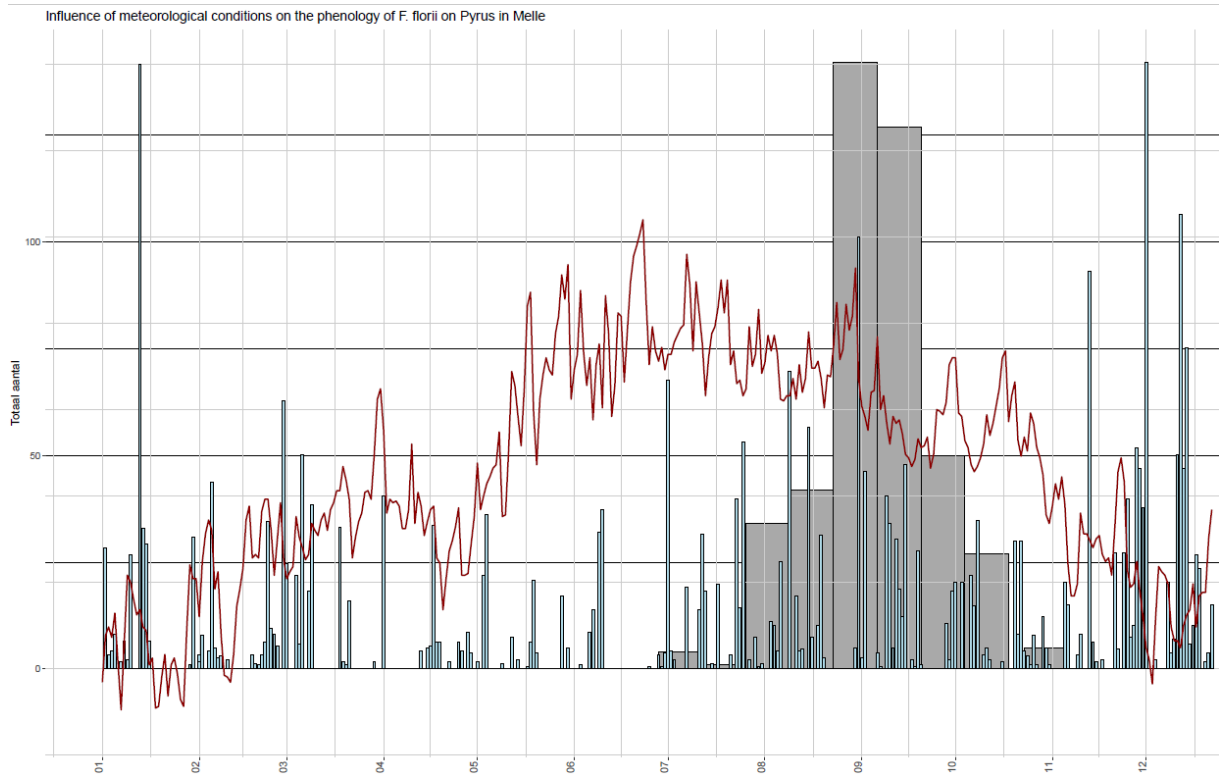
Philaenus spumarius



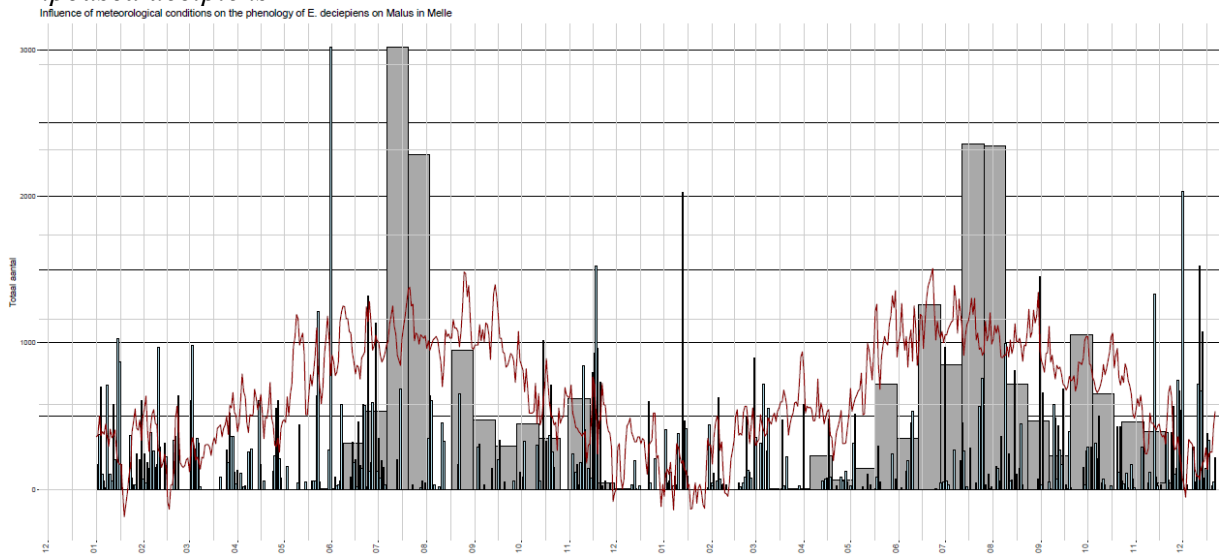


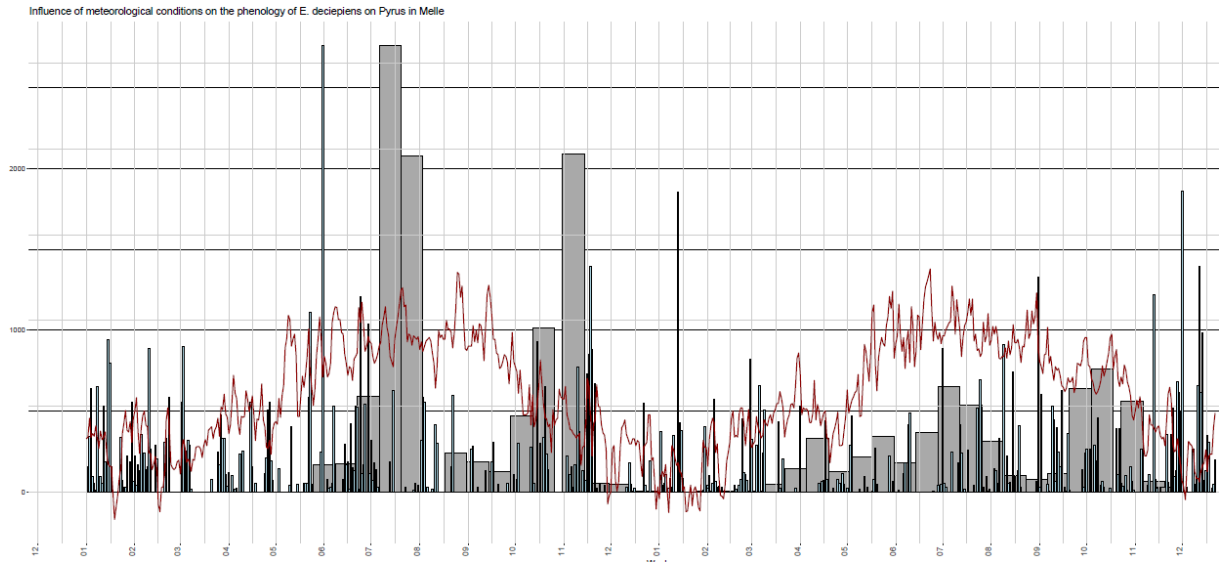
Fieberiella florii in 2017





Empoasca decipiens





WP5 – Recommendations and dissemination

Task 5.1 Assessment focusing on dissemination risks through Hemiptera transmission (ILVO and CRA-W)

Based on literature and on the results of WP2 (insect monitoring), WP3 (transmission capacity) and WP4 (phenology and host range), the probability of spread of studied phytoplasma species and '*Ca*. L. solanacearum' by Hemiptera is assessed.

In orchards, population and diversity of Auchenorrhyncha are especially high in untreated apple and peak during the September/October period. However, few Auchenorrhyncha

individuals were found to carry phytoplasmas. Moreover, none was found to carry 16SrX group phytoplasmas. Consequently, psyllids seem to be the most probable vectors of these orchard pathogens in our conditions. Psylloidae distribution within and in the surroundings of orchards showed that a combination of phytosanitary treatments/fruit destination/tree trainings/varieties and hedges significantly impact the abundance of two known vectors of AP but further studies need to be done to weigh the influence of each factor separately.

In carrots, population and diversity of Auchenorrhyncha were found to be higher in the field borders. Auchenorrhyncha individuals were found to carry 16Sr I (AY) photoplasmas in fields infected with phytoplasmas of the same 16Sr group strongly suggesting that these insects are the causative vectors.

Pesticide-based control measures for apples, pears and carrots, based on the population dynamics of the potential phytoplasma vectors identified during this study, are presented below:

Apple

Fytoweb has notified the registration of two active substances against the apple tree *Cacopsylla mali*: deltamethrin and lambda-cyhalothrin. Both pesticides belong to the group of pyrethroids and may be used up to twice a year (with a safety period of 7 days). Pyrethroids are also approved for use against aphids, but if the target is aphid control only products with other modes of action are preferred, such as spirotetramat, azadirachtine, pirimicarb, neonicotinoids (after flowering!) and paraffin oil. Keep deltamethrin or lambda-cyhalothrin as a backup for the specific control of the psyllids *C. mali*, *C. pyri* and *C. melanura*.
In view of the status of *Fieberiella florii* as a potential vector of *Candidatus* Phytoplasma mali (Tedeschi & Alma, 2006), it is recommended to include this Auchenorrhyncha species when drawing up control measures. However, no control agent against cicadas has been registered for use in apple cultivation, but lambda-cyhalothrin has a proven cicadas control effect. All treatments against *C. mali* applied between the beginning of July and the beginning of November, the period of *F. florii* activity in apple, help to control this cicada.

Pear

Regarding the control of the potential vectors of *Candidatus* Phytoplasma pyri in pear, for the time being only the pear psyllid *Cacopsylla pyri* and the pear sucker *C. pyricola* should be taken into account. Many products are registered in pear against these two psyllids: abamectin, thiamethoxam, spinosad/spinetoram, spirodiclofen, spirotetramat, deltamethrin, aluminium silicate, potassium hydrogen carbonate and thiacloprid. Adult pear psyllids and pear suckers are found all year round in pear trees, with the highest observed numbers at the end of May - beginning of June. Control is crucial during this peak period. Taking into account the application stage of the registered crop protection agents listed above, these can also be used at other periods of increased *Cacopsylla* spp. activity, e.g. from mid-November to the end of November.

Carrot

Macrosteles spp., *Typhlocyba quercus* and *Phylaenus spumarius* were identified as vectors of AY phytoplasma. In view of the possible transfer from AY to carrots, it is advisable to take these Auchenorrhyncha species into account concerning the pest control strategy in carrot. However, no pesticides against cicadas or psyllids have been approved for use in carrot cultivation (open air). For the control of other plant sucking insects in carrots, aphids in particular, a number of pesticides has been registered: lambda-cyhalothrin, spirotetramat, pyrethrines, thiacloprid, deltamethrin and pirimicarb. In view of the proven effect of lambda-cyhalothrin against cicadas, it could be considered to prefer the use of this aphid pesticide during the period of cicada activity, i.e. from mid-May to mid-November.

Additional information on authorized control agents against cicadas

According to Fytoweb, control agents are registered against cicadas for only two groups of crops: grapevines (table grapes and for wine production) and ornamental plants. In grapevines, only chemical control agents with lambda-cyhalothrin and indoxacarb are authorised for use against cicadas. In outdoor horticulture the following active substances are registered for cicadas control: lambda-cyhalothrin, thiacloprid and indoxacarb.

Task 5.2 Formulation of guidelines for the management of insect transmissions (ILVO and CRA-W)

By considering the occurrence and the phenology of infected insects, the different available options of vector integrated control will be theoretically assessed in the final report. An attempt will be made to generate general recommendations on the management of the identified potential vectors.

In orchards, the results of this project indicate that psyllids are the main vectors of 16SrX phytoplasmas and that Auchenorrhynchas, although highly present especially in apple orchard, play a minor role. The actual impact of phytoplasmas on the yield and crop value is really challenging to determine. However, it happens in extreme cases that producers have to remove entire infested rows of trees because the yield drops below the break-even point. To our knowledge the risk is higher in the outer rows of orchards seemingly because of insect transmission from the surrounding but this information need further experimentally validation. It is difficult, in the current stage of knowledge, to see the impact of hedges. Although, the PA vector *C. melanoneura* is highly present in hawthorn hedges, the most damaging and proved infection of AP we became aware of happened in outer rows of an apple orchard that was not protected by a hedge.

In carrot, it is difficult to measure the actual impact of AY on the yield and value of the crop but this impact seems not negligible since carrots positive for phytoplamas showed altered phenotypes. However, further studies would be needed to determine if measures can be apply to decrease potential yield losses.

Task 5.3 Reporting and dissemination (ILVO and CRA-W)

- Participation in Euphresco teleconference meetings (May 2016 & July 2018)
- Final project reports for FOD
- Presentations at Symposia and Conferences
- Scientific publications

2. *France*

Network synthesis

Typology of survey

For the typology of the sites, we will only consider sites registered by the PHL and discard sites provided by the CPIE (for which we lack information), for **166 sites** in total.
For the sites registered with the PHL in 2017 (figure 1) and 2018 (figure 2), we can see a slowdown in monitoring between the two years: 156 sites were monitored in 2017 while only 42 were monitored in 2018 (2018 includes 10 new sites).

The 166 sites were the subject of **1053** samplings. A sampling corresponds to a site, a date, a location (crop or hedge) and a collection method. This corresponds to an average of about six sampling per site (all year combined).

The sites were chosen under various conditions. The main crop categories sampled are "**viticulture**" (**19.3%**), "**orchards**" (10.2%) and "**great crop**" (9%) (figure 3). The "**diverse crop**" category (**49.4**%) includes other crop types less visited (including olive groves) and especially sites without declared cultivation. It can be scrubland, forest edges, lawns....

Sampling methods

The main sampling method was the **sweeping net** (used in **59%** of the total survey). **Yellow sticky trap** and **yellow pan trap** were also tested (15.1% and 8.2% respectively). The **Barber trap** was little chosen (only 4.6%). The observers used **other sampling methods or methods not mentioned** in 11.3% of cases (figure 4).

Sample location/environment

For each of the 166 sites, it was possible for partners to sample inside a crop (e.g. vines) or inside the crop plus a sampling in the immediate environment of this crop (for example hedges, edges, riparian forests...). So for each visit and for each sampling method, partners carry out a simple sample (crop or hedge) or a double sample (crop + hedge).

The 1053 samples were mainly carried out in **agricultural crops**, (37.2%) but a high number of samples were also taken in the **immediate environment/hedges**, (25.5%). Lastly, several partners carried out occasional samplings in **non-agricultural or other areas** (22.5%) (figure 5).

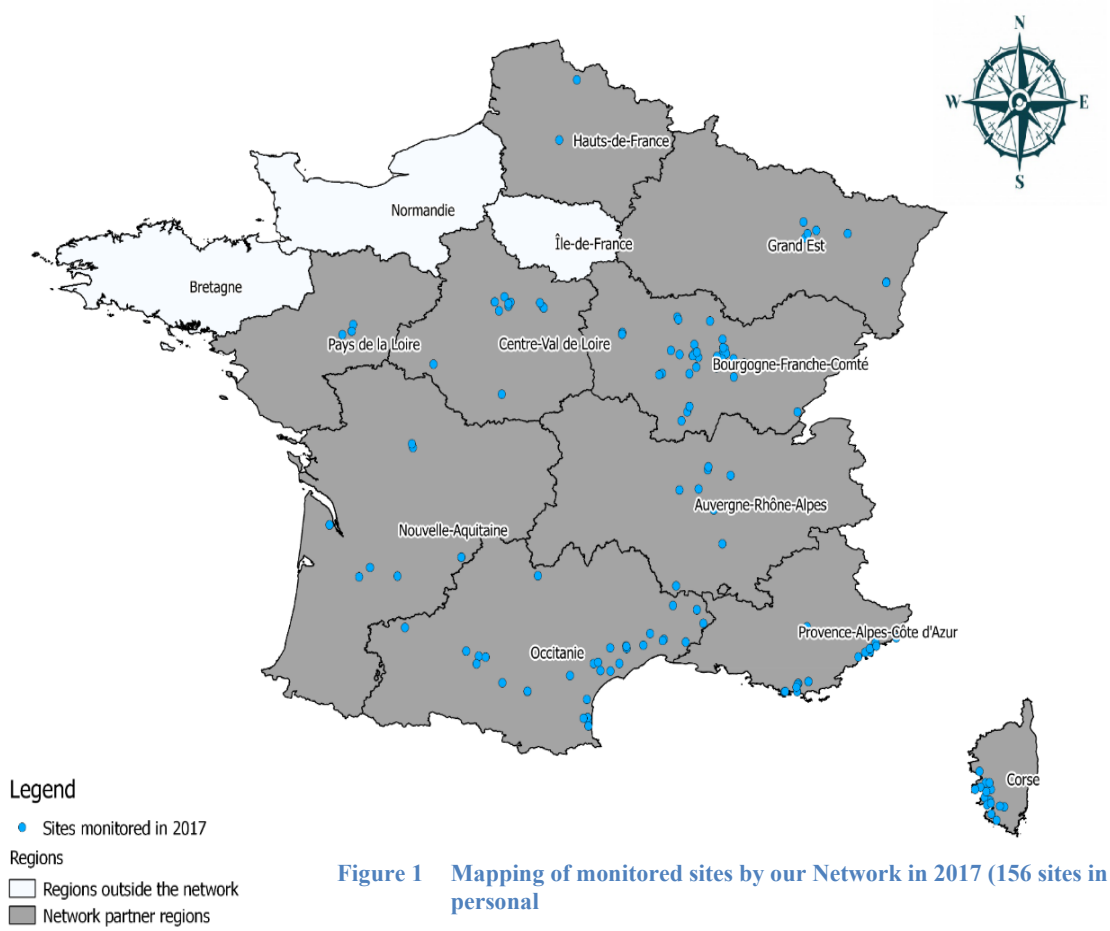


Figure 1 Mapping of monitored sites by our Network in 2017 (156 sites in total), source: personal

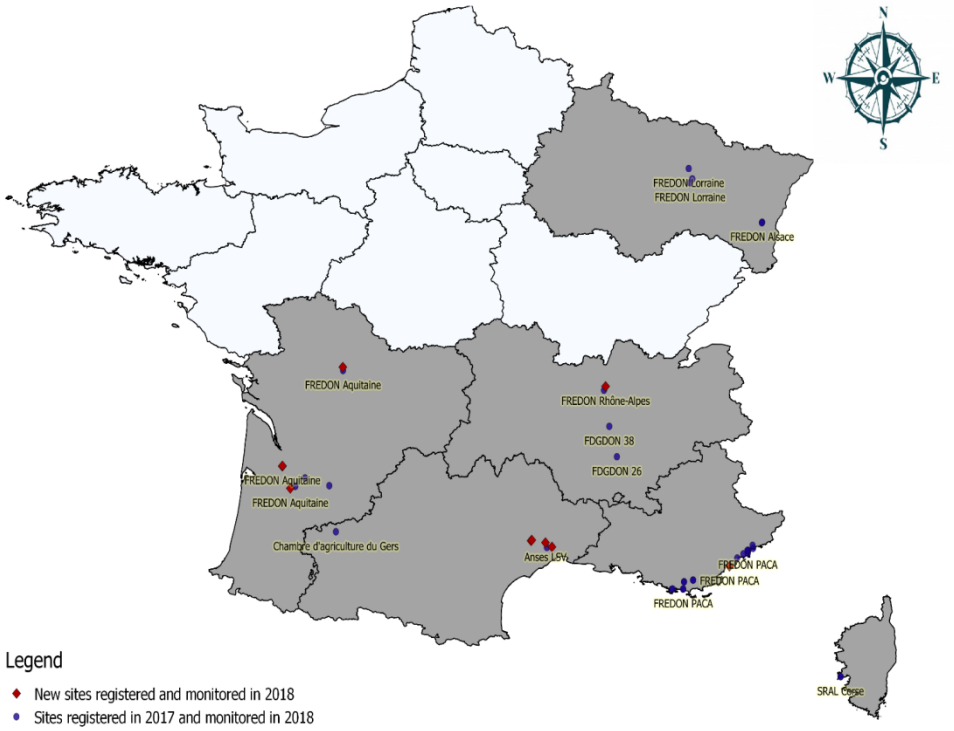


Figure 2 Mapping of monitored sites by our Network in 2018 (42 sites in total, renewal of the monitoring of already registered parcels and new parcels), source: personal

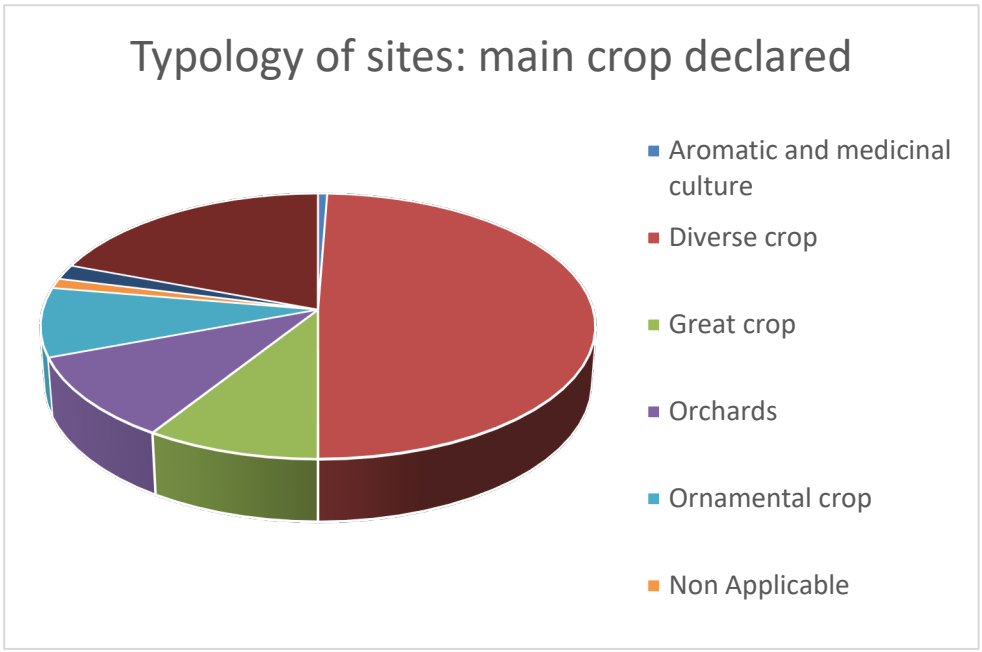


Figure 3 Main crops reported at sampling sites, source: personal

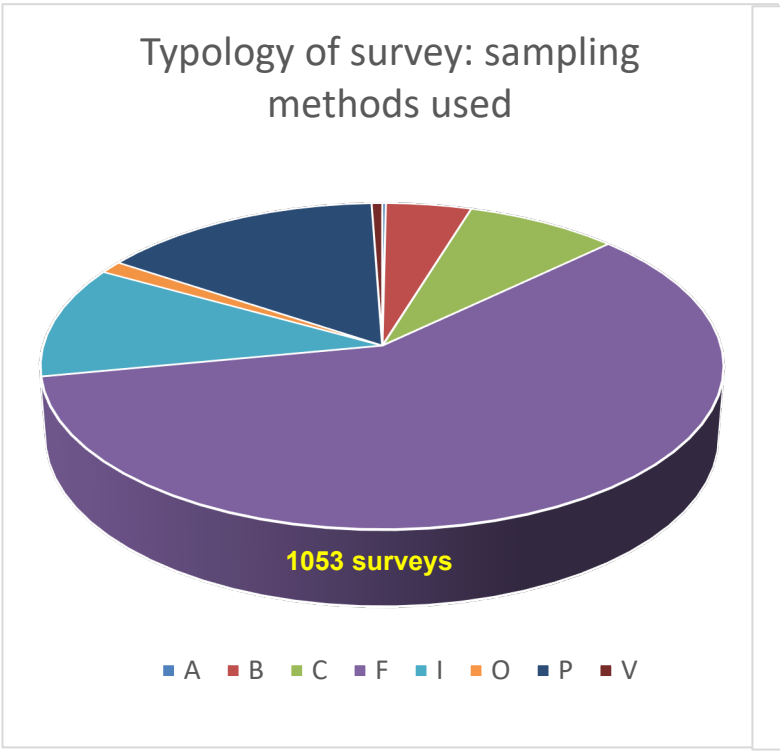


Figure 4 Sampling methods used with V: Glass trap, B: Barber trap, C: Yellow pan trap, F: Sweeping net, I: Unknown, O: On sight, P: Yellow sticky trap, A: Other, source: personal

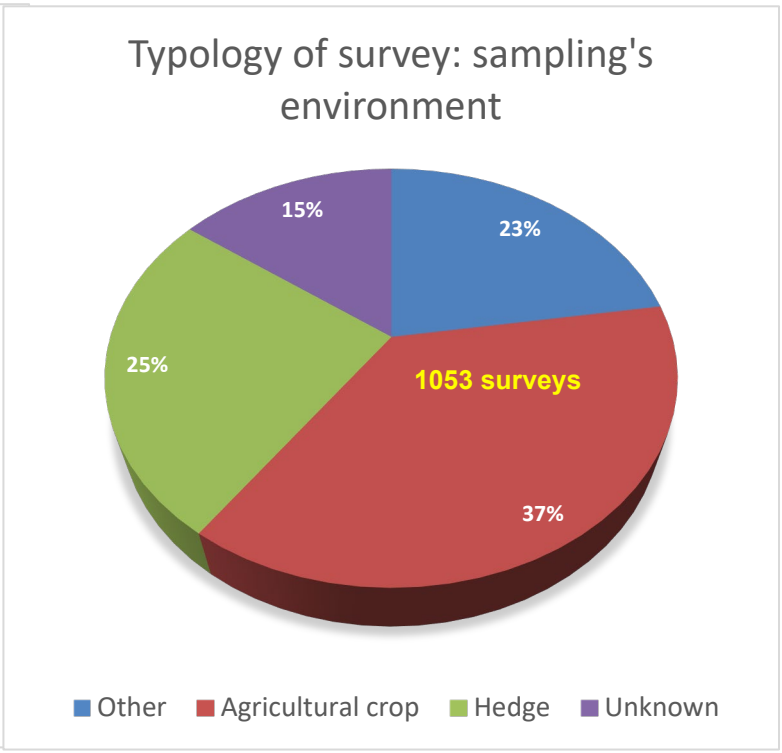


Figure 5 Sampling's environment, source: personal

Period of operation of the network and geographical origin of the samples

The 1053 samplings recorded since the beginning of the PHL's network, were made between January and December (figure 6), i.e. all year round (with a small number of records at the beginning and end of the year). The months with the most samplings are May (194 samplings) and June (206 samplings). The results of this report also include some historical samples (2011-2016) provided by some partners.

Of the 13 regions in metropolitan France, 10 have participated in the research network of vectors of *Xf*.

The top 3 participating regions are "**Occitanie**" (17.95% of total surveys), "**Nouvelle Aquitaine**" (16.14%) and "**PACA**" (16.14%), with 50.2% of the 1053 samplings (figure 7). This is correlated with the fact that *Xf* is present and under eradication in the PACA region and we know that south of France is an area at risk. Corsica which is also a region with outbreaks of the bacterium, accounts for 7.41% of all surveys. The Bourgogne - Franche Comté and Grand Est regions have also provided a significant number of samplings with respectively 13.58% and 11.02% of all the samplings carried out.

A heat map (see Materials and methods) of all the surveys over time allows to see the "sampling activity" since the start of the network (figure 8). The closer the sampling points are, the higher the concentration is. The "hot spots" that we see on the heat map well reflect the values of the histogram in figure 7.

Potential vector species within our network

The first objective of the network was **faunistic**: to identify and map **the most common** potential vector species, depending on the French regions. We know that 53 species are potentially vector species of *Xf* in metropolitan France. But of course, not all of them have the same epidemiological importance. A rare (i.e. apparently very sparsely distributed) species will have no epidemiological role, even if it is present throughout France. On the other hand, the phenology of a rare species across the country but locally abundant needs to be considered in the management of *Xf* in the area under consideration.

For the following wildlife results, we will include the data provided by the "*CPIE Loire Anjou*", located in the "Pays de la Loire" region (all their surveys are from this region, between 2001 and 2017).

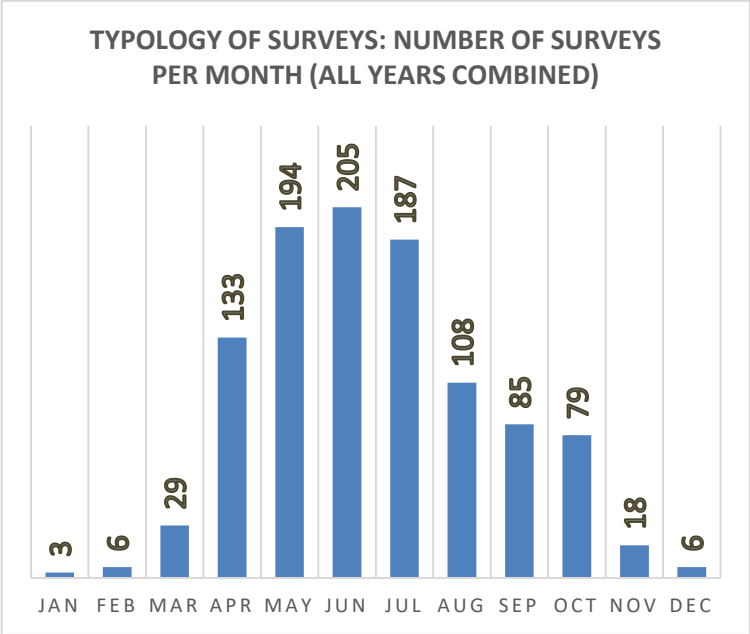


Figure 6 Number of Surveys per Month (all years combined)

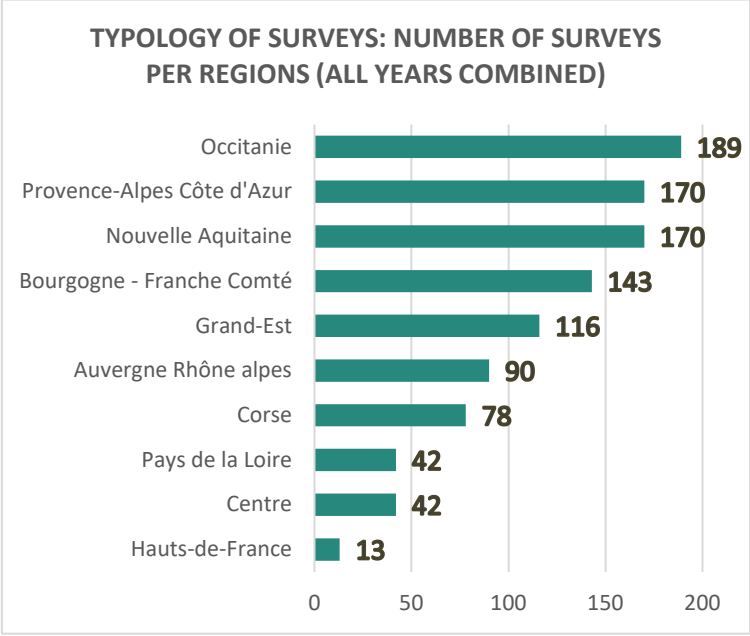


Figure 7 Number of Surveys per Regions (all years combined)

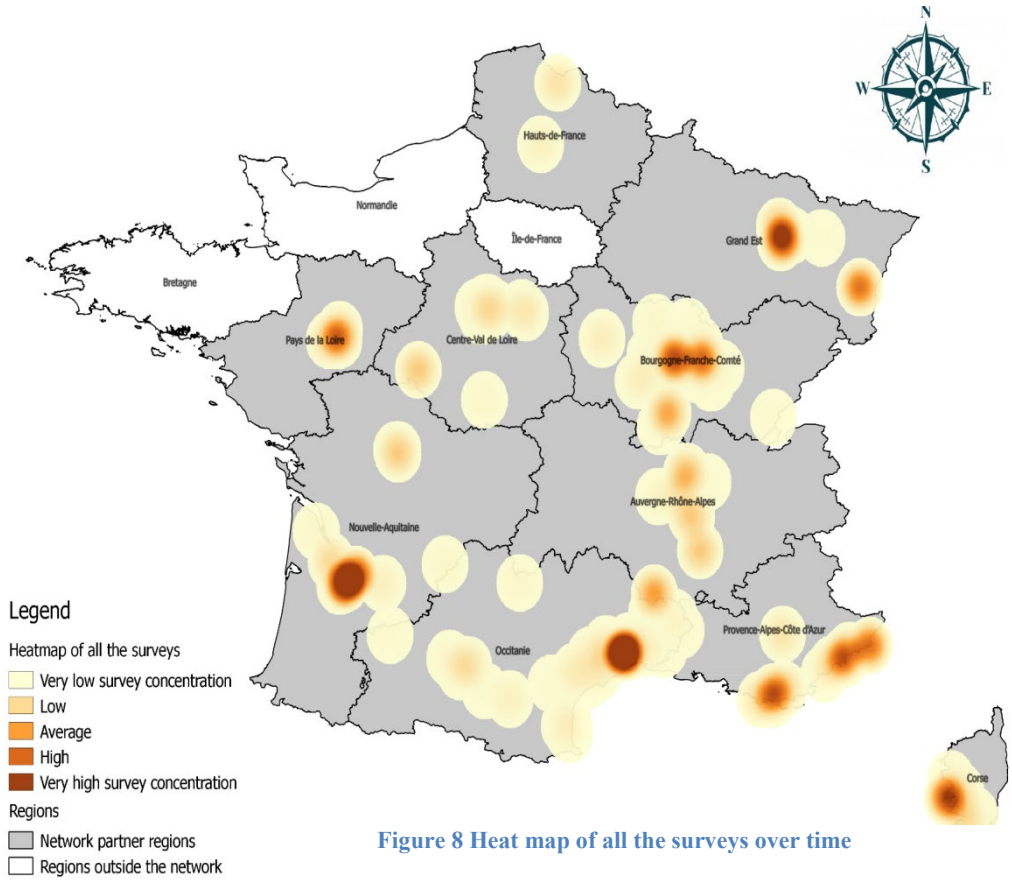


Figure 8 Heat map of all the surveys over time

Insect vectors 'families

We already know that the so-called "potential vector" insect families belong to the Cercopidae, Aphrophoridae, Cicadidae and the Cicadellidae (leafhoppers of the subfamily Cicadellinae and Ledrinae are involved in the transmission of *Xf*). The network partners collected a total of **24 010 insects** (without the CPIE data we would have 22 615 insects). In the field, it is quite easy to collect "Cicadomorpha" (= leafhoppers in the broad sense) by knowing their jumping behavior, size and general shape. But knowing that all Cicadomorpha are not vectors, then the distinction between potentially vector and non-vector species is far to be obvious. The silhouette and the size of specimens make it possible to undertake an initial screening. However, it is difficult to accurately estimate the size of an insect in the field. This is why the insects caught by the network partners belong to both categories (vectors and non-vectors, as shown in the figure 9). A majority of insects belonging to potentially vector families were collected (**82%**). However, 4319 specimens (18%) did not belong to a vector family. They were often Cicadomorpha belonging to morphologically close families (Issidae, Membracidae, Cixiidae, Delphacidae, Flatidae, Dictyopharidae...). These specimens have not been identified to the species level but are kept by our laboratory for potential future studies.

Among the **19 691 specimens belonging to a potentially vector family**, many larvae were collected. Larvae generally cannot be identified to the species level by a morphological method because until now, no methods are available. However, they can be identified at the family level. This is not a problem for Cercopidae, Aphrophoridae and Cicadidae as all species in these families are potential vectors. In contrast, for Cicadellidae, only few subfamilies are potentially vector considering families that contain **xylem feeders** (as Cicadellinae). Therefore, the status of larvae of Cicadellidae (which comprises 15 subfamilies in Europe) can often not be determined morphologically. Therefore, the status of Cicadellidae larvae is indicated as "unknown". However, for adults of Cicadellidae, it was possible to discriminate between potential vector species and non-vector species.

Potential vectors (as defined above i.e. **7405 individuals**) represent 52.2% of specimens belonging to a potentially vector family (occurrence) and 30.8% of all insects collected (figure 10). **Aphrophoridae** was the main trapped family, accounted for **72.1%** of the potential vectors collected, while Cicadellidae on the one hand and Cercopidae on the other represented only 10.5% and 16.7% of captures respectively.

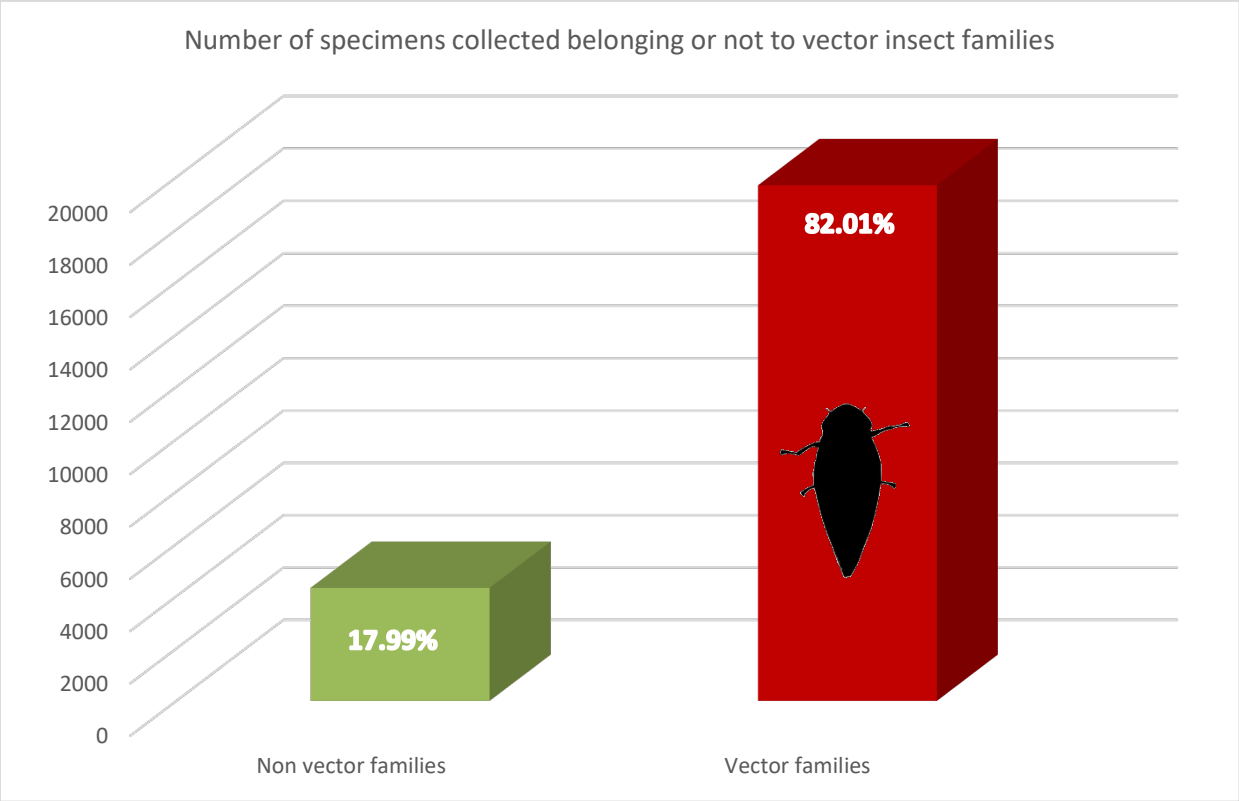


Figure 9 Number of insects belonging to vector families

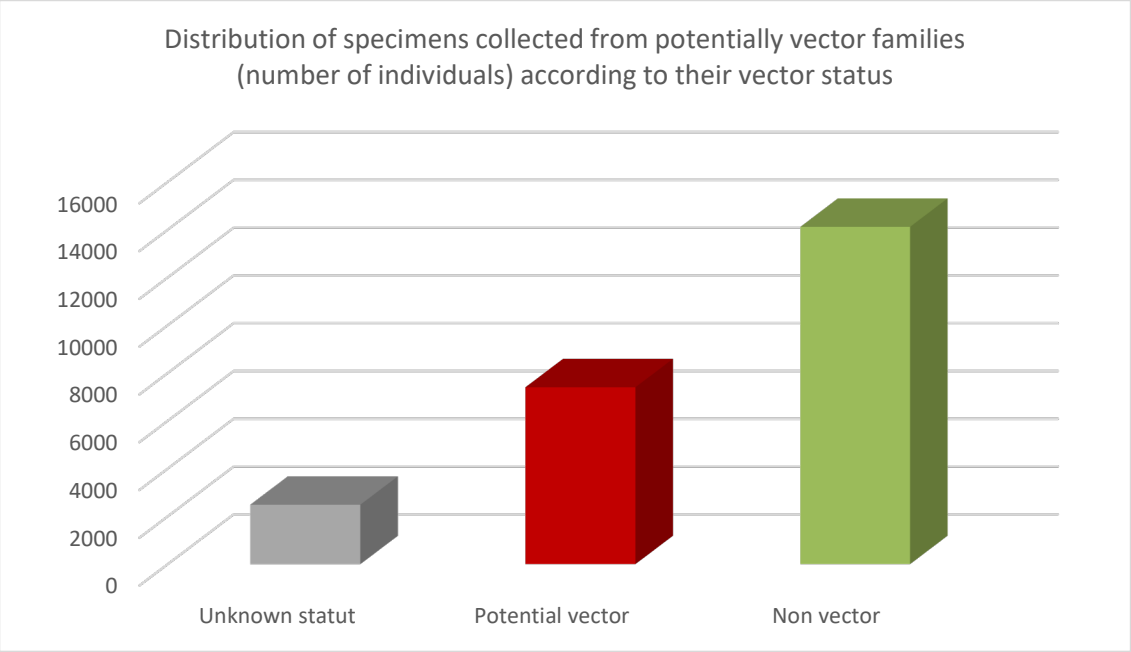


Figure 10 Vector status of the insect sampled

The different species were examined in more detail below. For each species with a significant number of specimens collected, a French distribution map is provided. It is based on network collections, which are indicated by colored points and by departmental zoning. The INPN's data are indicated in green while the network's data are indicated in red.

Cercopidae (false spittlebugs)

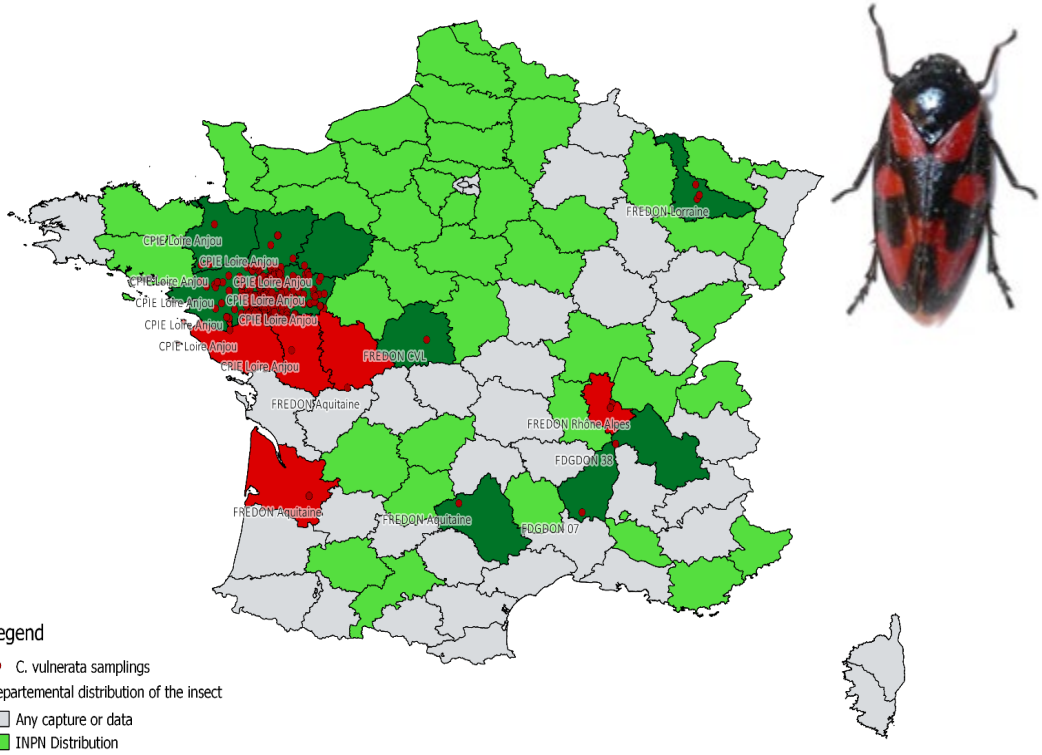
Cercopidae are characterized by brightly coloured patterns on the elytra (annex 9, image 23). They differ from true leafhoppers (Cicadellidae) in having the posterior tibia with one or two robust spines rather than rows of many thin spines (Biedermann and Niedringhaus, 2009) (annex 9 image 24). For adults, the shape of coloured red areas in relation to the dark background of the forewings generally makes possible to discriminate the different species. The forewing margin is black for *Cercopis* and *Triecphorella*, red for *Haematoloma* (genus). The appearance of the forehead, smooth or wrinkled, the presence of a transverse hull between the eye-spots, the color of the knees and the shape of the red spot on the forewing apex are the main characteristics that distinguish the species (Dusoulier, 2004). The diagnosis can be confirmed by observation of the male genitalia (annex 9, image 25 and 26).
This family includes 7 species in France (annex 2). As part of the sampling network, 2 species were collected (annex 7).

Cercopis vulnerata

This insect is commonly called "Red-and-black Froghopper". The nymphs are rarely seen, as they feed on underground roots.
It represents **12.5%** of all potential vector insect catches in the network (PHL + CPIE) and is present in **7.4%** of positive samples with at least one vector referenced (occurrence) for PHL specific samples (the data of the sampled insects provided by the CPIE are not integrated as "physical samples" by our laboratory and therefore have no specific reference). It is the **second** most sampled insect.
C. vulnerata occurs as a specie widely present in France even if the numbers collected are never very large (rarely exceed ten individuals). The maximum number of individuals caught at any one site is **29**. This specie is likely to play an epidemiological role for *Xylella* in areas where it is abundant. The geographical distribution of *Cercopis vulnerata* is represented in the figure 11

Cercopis intermedia

Very similar in appearance to *C. vulnerata*, this species has been collected less frequently. It differs in the presence of red knees and the red apical spot slightly arched (black knees and very arched spot in C. vulnerata). It accounts for **2.7%** of catches of all potential vectors (PHL) and is present in **5%** of positive samples with at least one vector (occurrence in PHL samples only). It was mainly found in the South of France, although it is also known from the North according to the INPN data.
It is never abundant in collections, often captured in a few units only. Its epidemiological role appears to be minor. The geographical distribution of *Cercopis vulnerata* is represented in the figure 12.



Cicad

As me :ophoridae by posterior tibias with rows of fine moving spurs. The venation of the forewing and hindwings, the distribution of various spots on the thorax and the vertex and especially the observation of male genitalia, are discriminating. For example, *Cicadella viridis* and *C. lasiocarpae* differ in the basic colour for males (night blue for the first one, yellow green for the second one) and the shape of the two spots on the vertex for females (polygonal in *C. viridis*, round in *C. lasiocarpae*) (Figure 13). However, for these two species, the male genitalia are nearly identical (Figure 14) (Biedermann and Niedringhaus, 2009).

The Cicadellidae family includes many species in France (several hundred in 15 subfamilies). Within the framework of the trapping network, 13 species of Cicadellidae belonging to 5 potentially vector subfamilies (Cicadellinae and Ledrinae) were caught, where the species *Cicadella viridis* was the most sampled. Other individuals from non-vector sub-families have not been identified (identification at the family level but not beyond).

Cicadella viridis

It is a very widespread species throughout France. The bicolored pronotum (yellow at the front and green at the rear) is distinctive. It can hardly be confused with *Cicadella lasiocarpae* (a very rare species) as previously reported. *C. viridis* is the **fourth** most collected species in the network with **8%** of all potential vectors and is present in **6.6%** of positive PHL samples with at least one vector. It is sometimes caught in large quantities (up to 100 individuals per sample, with a maximum of 139 individuals captured at one sampling). It is therefore likely to play a local epidemiological role in the vection of *Xf* provided that it is competent to transfer the bacterium. The geographical distribution of *C. viridis* is represented in the figure 15.

Other Cicadellidae

Other potential vectors were collected in the network (annex 7). *Errhomenus brachypterus* and *Ledra aurita* are the other two most sampled Cicadellidae and represent respectively 0.5% and 0.8% of the total insect vectors sampled. *E. brachypterus*, although captured in small numbers, is found in 2.8% of positive PHL 'samples with at least one vector, all located in the "Bourgogne" region. Likewise, *L. aurita*, a rather spectacular species, is rarely caught (2 individuals in "Bourgogne" and 49 in "Pays de la Loire" between 2004 and 2017). Because of their low abundance and frequency, these species will not have an epidemiological role in *Xf* vection.

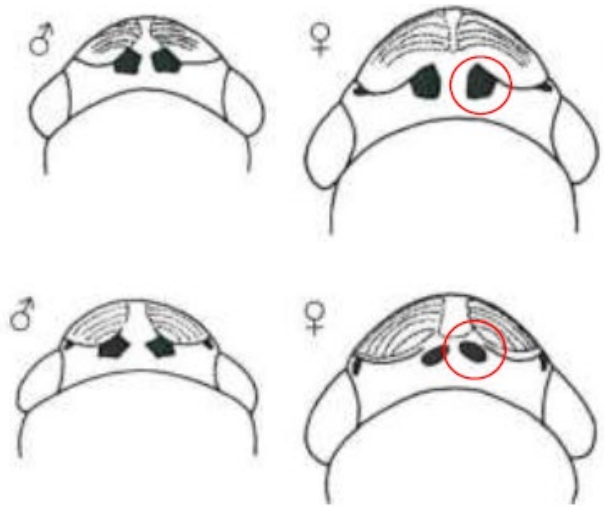


Figure 13 Shape of the two spots on the vertex for *Cicadella* females (polygonal in *C. viridis*, round in *C. lasiocarpae*), source: (Biedermann and Niedringhaus, 2009)

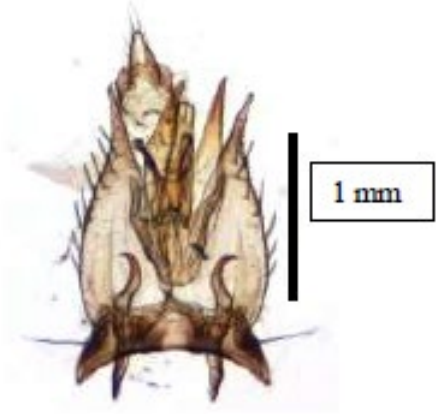


Image 14 Aedeagus of *C. viridis*, source: (Germain, 2016)

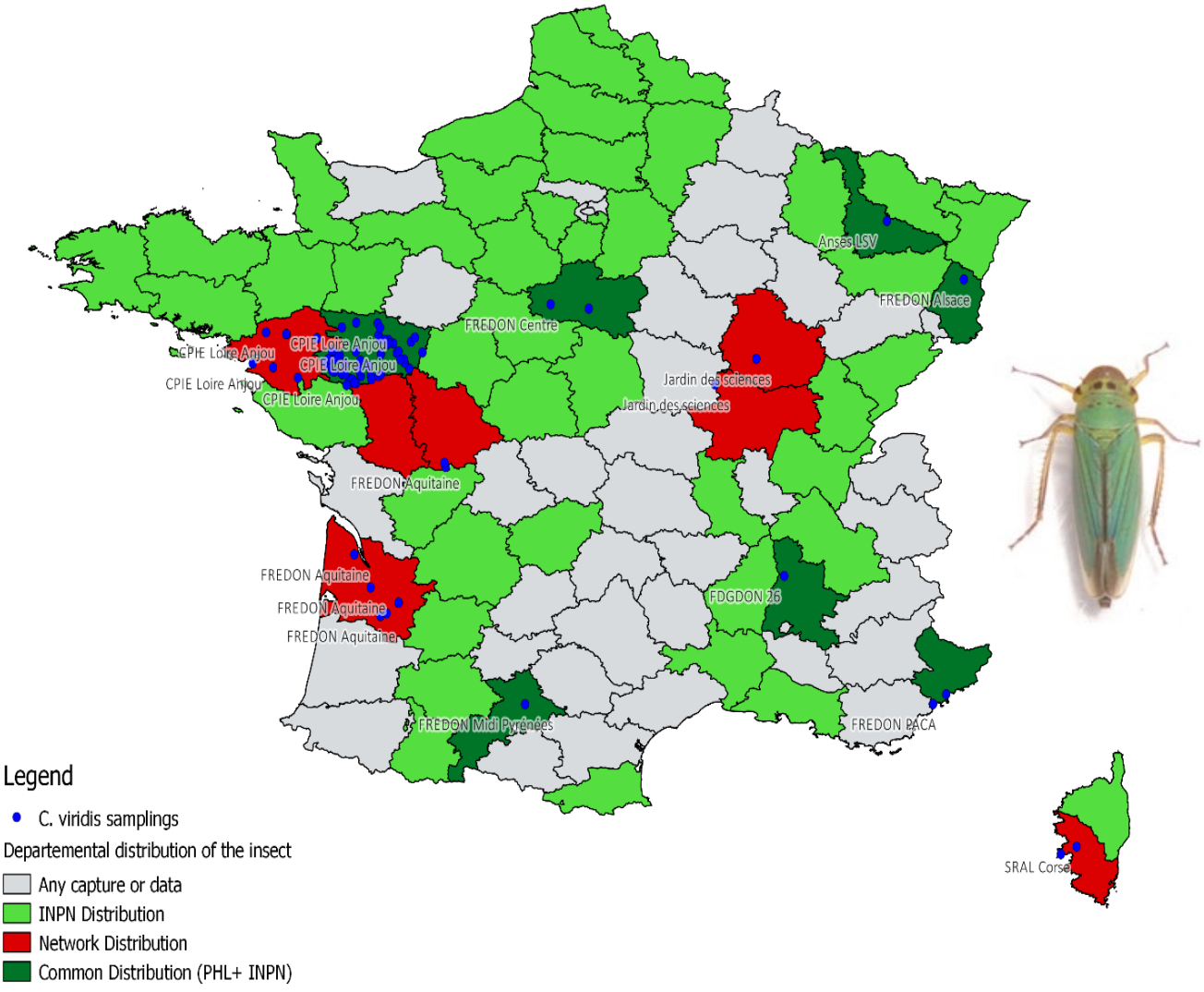


Figure 15 Geographical distribution of *Cicadella viridis*, source: Anses

Aphrophoridae (true spittlebugs)

They are generally oblong in shape. The biggest are *Aphrophora* (6/12mm), the smallest are *Neophilaenus* (5mm), the genus *Philaenus* being intermediate (4.4/6.8mm). *Lepyronia* (5.6/7mm) is globular in shape. Species identification necessarily involves the observation of genitalia. The PHL produced a reconnaissance sheet for *P. spumarius*, the species that could be most involved in *Xf* vection (see protocol in annex 12, "Fiche de Reconnaissance"), being currently confirmed as an effective vector of the bacterium... Aphrophoridae present in France are relatively easy to identify by their external morphology for genera and with genitalia for the species level. This would no longer be true if we were dealing with an invasive species. For example, in the genus *Philaenus*, only *P. spumarius* is present in metropolitan France but species present in Mediterranean countries could move northwards, like *P. italosignus*, another efficient vector of *Xf*. Morphologically, these species are differentiated by the observation of the ornamentations present at the apex of the aedeagus (annex 9, image 27). Fifteen species of Aphrophoridae are reported in France, 10 have been caught within the network (annex 2 and 7).

Philaenus spumarius

An Old World species introduced in North America and identified as a vector of *Xf* in California (Purcell, 1980). The common froghopper or *P. spumarius* is distinguished from other Aphrophoridae species by the entirely convex outer margin of the forewing, and the vertex plate, which does not have a median keel. One of the differences that separates *Neophilaenus* and *Philaenus* is the number of spines present on the apex of their posterior tibiae (see protocol in annex 12, "Fiche de Reconnaissance"). This is the species most collected in the network, both in number of individuals (**56.2%** of all potential vectors) and in % of positive PHL 'samples with at least one vector (**40%**). It is present everywhere in France, from North to South (figure 16). During the collections, it is possible to capture several hundreds of them without problem. Given its status as effective vector, distribution and abundance, it is confirmed that this species can play an epidemiological role in *Xf* transmission in France.

Neophilaenus campestris

This is the most collected Aphrophoridae after *P. spumarius*, and like the latter a confirmed vector of Xf (Cavalieri et al., 2018). It is morphologically close to the latter, but differs from it by the outside of the forewing with a concavity, a frontal plate with a median keel and especially by the presence of 12 spines on the apex of the posterior tibia in two rows. Coloring or size are useless to separate the 2 species in the field. This species represents **10.8%** of the potential vectors collected by the network and the *CPIE*. It was present in **14.2%** of PHL's samples where there was at least one potential vector. It was sometimes collected in large numbers (for example in Provence-Alpes Côte d'Azur) and it seems more frequent in the South of France. The INPN mapping seems very incomplete and the network data bring many new records (figure 17). Due to its abundance and distribution, it could have an epidemiological importance at least in the southern half of our country.

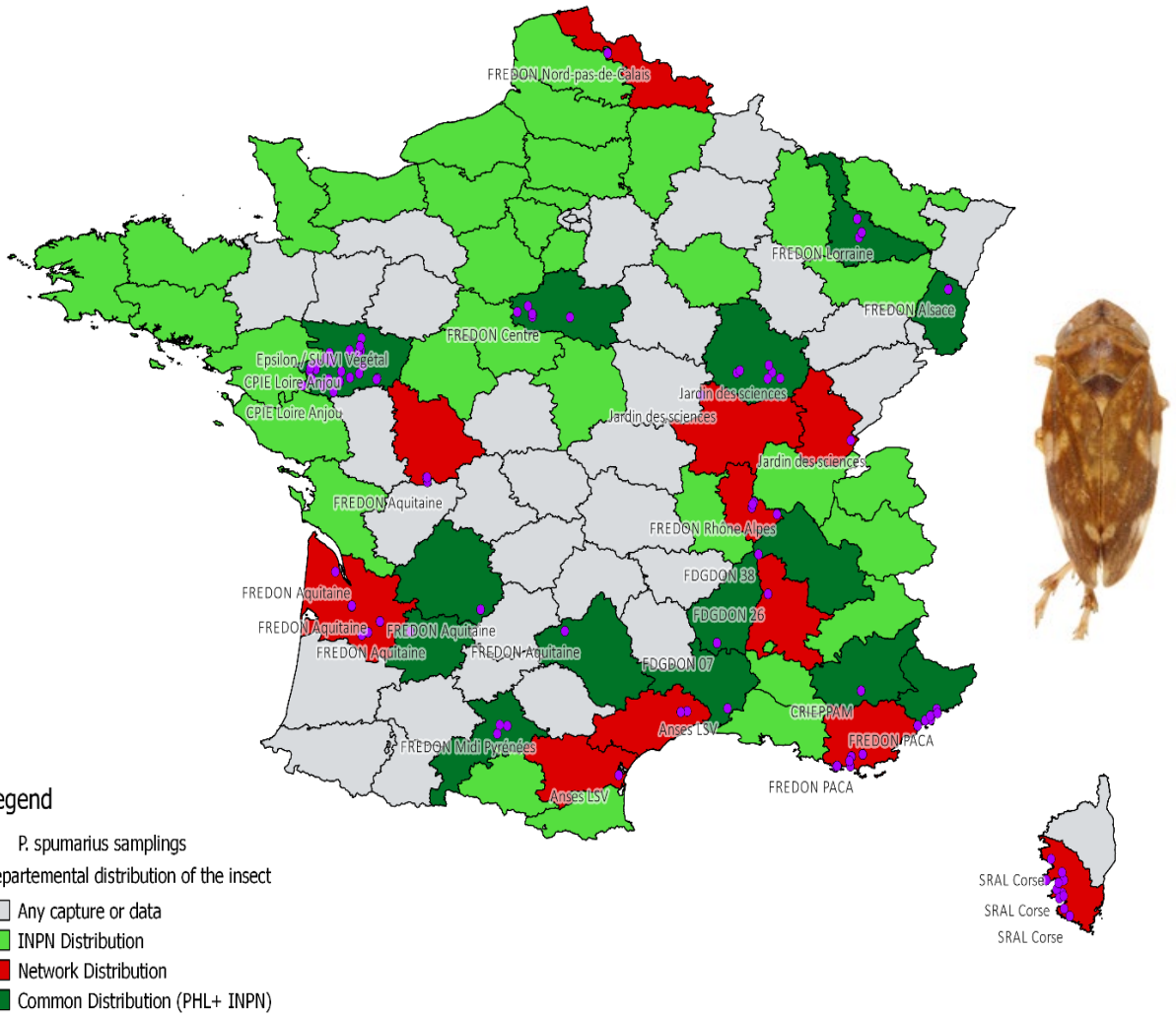


Figure 16 Geographical distribution of *Philaenus spumarius*, source: personal

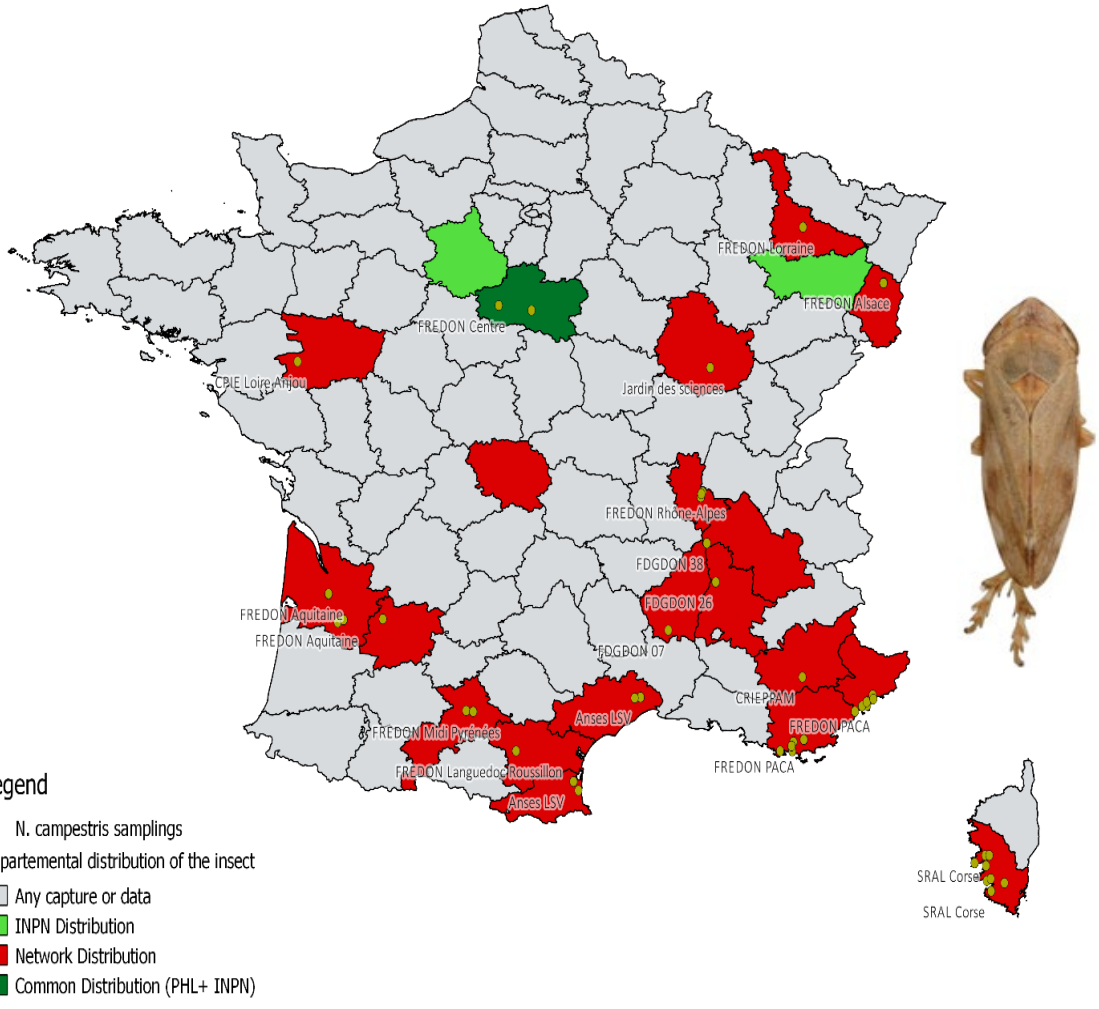


Figure 17 Geographical distribution of *Neophilaenus campestris*, source: Anses

Aphrophora alni

Due to its external appearance, it could easily be confused with *P. spumarius*. However, the presence of a median keel on the pronotum as on the front plate (visible at high magnification) makes it possible to differentiate it. It has a rather compact fusiform appearance, often larger than *P. spumarius* (6 to 9 mm against 5.3 to 7 mm). The basic colour is yellow-grey, the forewings with slight hairiness and two light spots, one of which is fairly wide in the middle and the other smaller in the hind.

In spite of its large size, it has been collected rather little in the network, in a very dispersed way and always in small quantities (a maximum of ten individuals). It represents only **1.8%** of potential vectors sampled and is present in only **5.7%** of PHL 'samples with potential vectors. It will not play any epidemiological role for *Xf*. The geographical distribution of *A. alni* is represented in the figure 18.

Neophilaenus and other Aphrophoridae

Several other species of Aphrophoridae have been caught in the network. *Neophilaenus lineatus* (**1.40%** of all potential vectors) and *N. minor* (0.26%), *Aphrophora salicina* (0.47%), *A. corticea* (0.05%), *A. pectoralis* (0.14%) and finally *Lepyronia coleoptrata* (0.19%). Given the small quantities, it is difficult to draw any conclusions on their geographical distribution, even with the INPN data which also seem very fragmentary, as the case for *N. lineatus* (figure 19). But whatever their distribution, their small abundance will not allow them to have an epidemiological role for *Xf*.

Cicadidae

Cicadas are among Xylella's potential vectors. Body size, wingspan, head shape, number of teeth on femurs, the color of the veins and various spots on the wings are distinctive features, but the sonograms and oscillograms of cymbalization are the most discriminating (Puissant, 2006).

Their abundance, size and host plants are risk factors. The sampling methods used in the network were not suitable to their capture. Only three sites in the Occitanie and PACA regions are sampled. Three very classical species have been identified (*Cicada orni*, *Cicadatra atra* and *Lyristes plebejus*). Cicadidae represent **0.78%** of all potential vectors sampled, no conclusion can be drawn from these few collections.

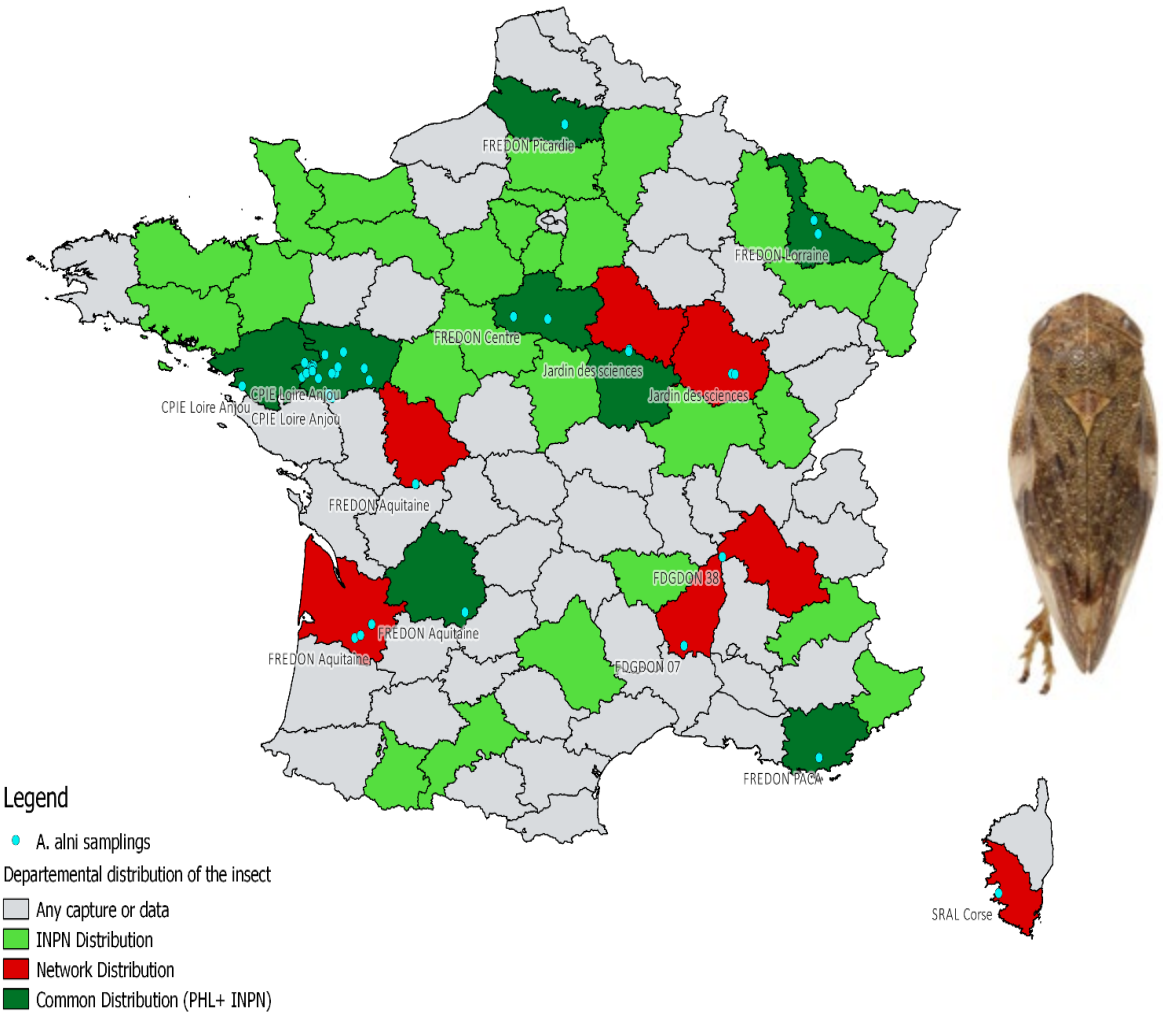


Figure 18 Geographical distribution of *Aphrophora alni*, source: Anses

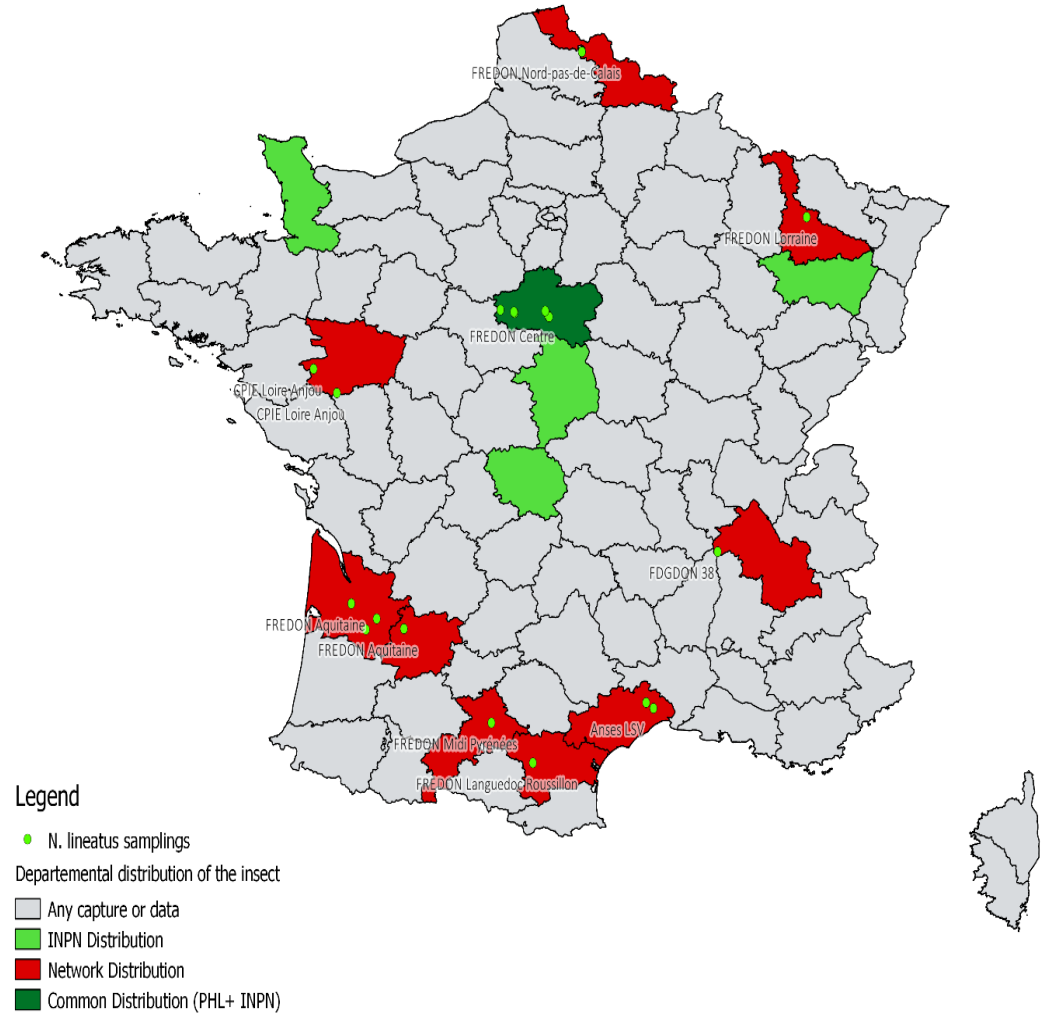


Figure 19 Geographical distribution of *Neophilaenus lineatus*, source: Anses

What are the best sampling methods?

The PHL network also aimed to assess the sampling techniques in order to issue recommendations to collector and observer. Four collection methods were made available to partners. The protocol put in place obviously does not allow a quantitative comparison of sampling because, for example, the duration of sweeping or the frequency of yellow pan trap surveys varied from one observer to another. Only qualitative conclusions can be drawn. For each sampling method, we compared the proportion of samples with and without potential vectors. The results above are **only based on the PHL's samples**, because the CPIE does not provide the information concerning the capture method.

It can be noted from table 1 below that the samples received (one site, one date, one collection method) often included a mixture of potential vectors and non-vectors. It shows that **no sampling method is entirely specific to potential vectors** of *Xf*. The apparently most suitable method is the **sweeping net** (**98.2%** of the samples received had at least one potential vector). But in 156.3% of cases, there were also non vector insects... The Barber trap with 39.6% of samples containing at least one potential vector also seems interesting. This is actually a special case because there were only 48 samples (as a reminder, 1053 in total) from this sampling method and the only potential vector species collected by this type of trap is *Errhomenus brachypterus*. It is a flightless Cicadellidae often present in Barber traps. Given its biology, it is highly unlikely to play an epidemiological role in the transmission of *Xf*. Otherwise, the Barber trap captures virtually no potential vectors. Other sampling methods are much less effective in assessing the presence of potential vectors. The yellow pan trap captures at least one vector in only 25.6% of the cases and the yellow sticky traps in 10.7% of the cases. These methods should not be used for this type of monitoring.

Life traits of the main species

To highlight some life traits, there must be sufficient collection for a given species. This is why only species caught in the PHL network with high numbers have been studied. Three species meet this criterion. These are *Philaenus spumarius* (4103 individuals, sampled in the PHL network), *Neophilaenus campestris* (801 individuals) and *Cicadella viridis* (444 individuals). We could have also studied *Cercopis vulnerata* which, with the CPIE samplings, has been collected in large numbers. However, the CPIE apart from a count does not provide us enough information like the type of crop or the sampling's immediate environmenton, and we have sampled only 191 specimens in the PHL network.

Are there differences between types of crops?

With regard to the environment of the potential vectors, it is interesting to check whether trends are visible according to the place of collection (crops, hedges, etc...). As regards the different type of crops, Philaenus spumarius is found in 61.4% of samples from ornamental crops and 49% on orchards but only in 30.7% of vine samples (figure 20). On the other hand, *Cicadella viridis* is more often present in vine samples (14.3%) than on other crops. It is on great crops that the 3 main species are the least frequent. We confirm here that *P. spumarius* is a species to be considered from an epidemiological point of view on ornamental plants. In addition, there is no clear trend for *Neophilaenus campestris*, which just seems to be a little more common in "orchards" and "aromatic and medicinal culture" in our network.

Table 1 Proportion of samples with each of the 3 insect categories (non-vector, vector or unknown status) as well as samples without arthropods. Sum of lines and percentage greater than 100 because a sample can contain several categories of insects

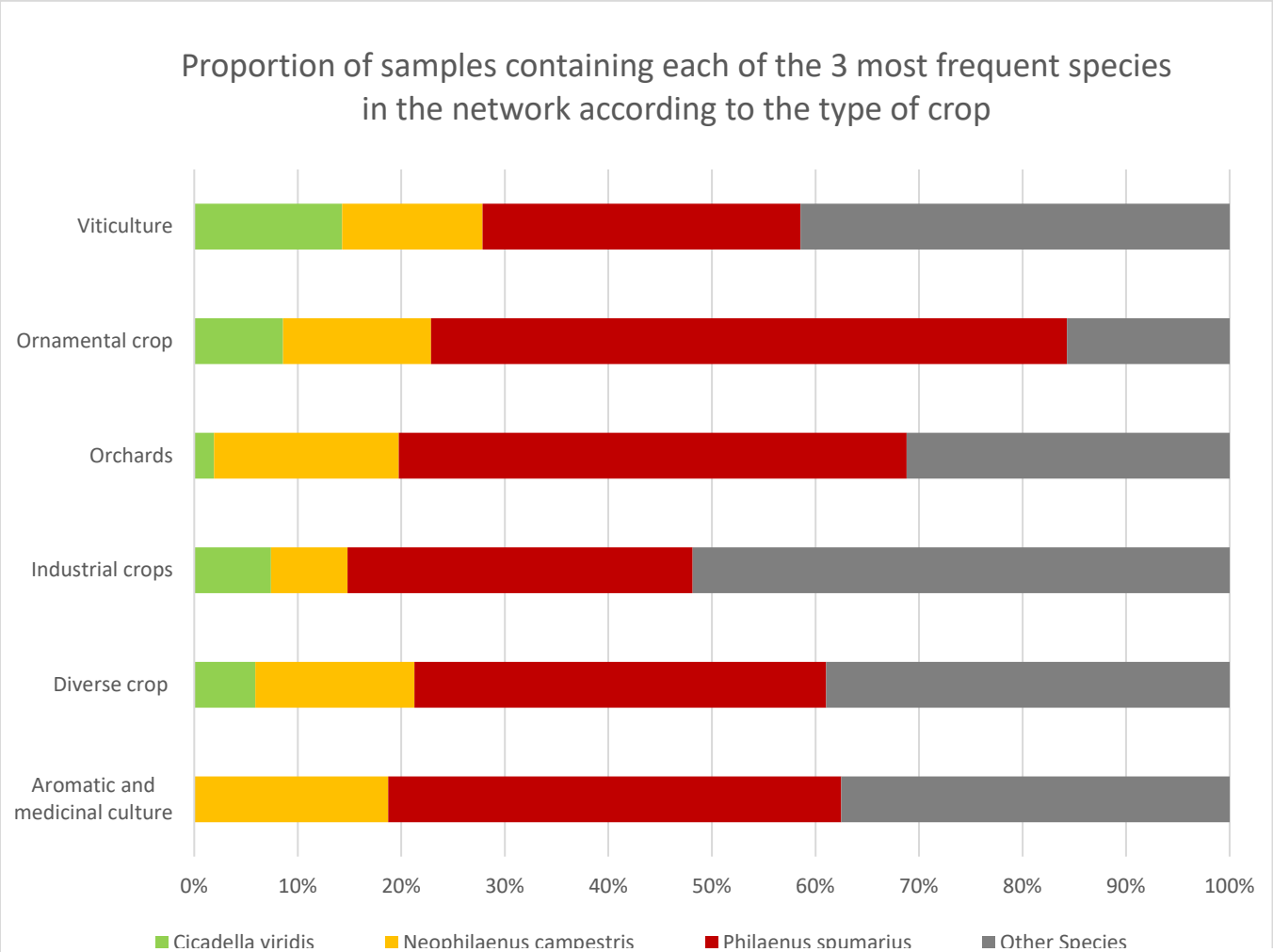
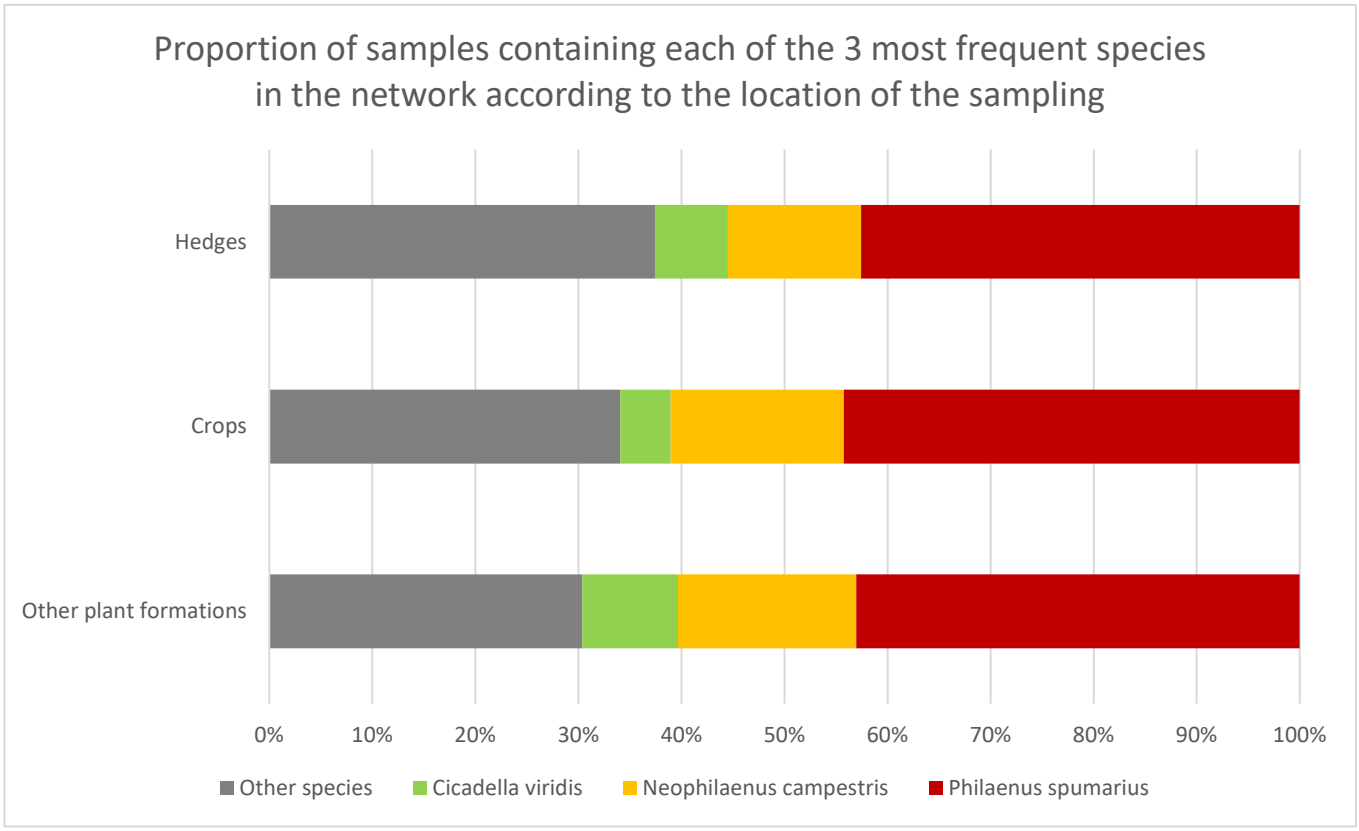


Figure 21 Proportion of samples (with the 3 main species) by immediate environment, source: Anses



Are there differences between crops and their immediate environment?

The collections were carried out in 3 main types of plant formations: in crop, in the crop environment (often hedges) and in other plant formations (for example lawns, forests, garrigues, etc...). For each of these plant formations, it is possible to assess the proportion of samples comprising each of the 3 main species, as well as the other potential vector species.
Figure 21 don't show any obvious difference between the proportions of each insect group according to the sampling environment. In all cases, the proportion of potential vectors belonging to the 3 main species remains the highest (in 66.5% of cases, when a potential vector is sampled in an environment (Crop, Hedges or Other formation), it belongs to one of the 3 main species.).

Periods of presence of potential vectors

For this variable, "period of presence", we used also the CPIE data but only they provide the sampling date.
The collection period (for samples collected within the CPIE and the PHL) range from January to December with a maximum of samplings between May and July (see figure 6). From these data, it is possible to visualize a period of adult activity for the 4 main highly effective species: *Philaenus spumarius, Neophilaenus campestris, Cercopis vulnerata* and *Cicadella viridis* as well as for all potential vector species.

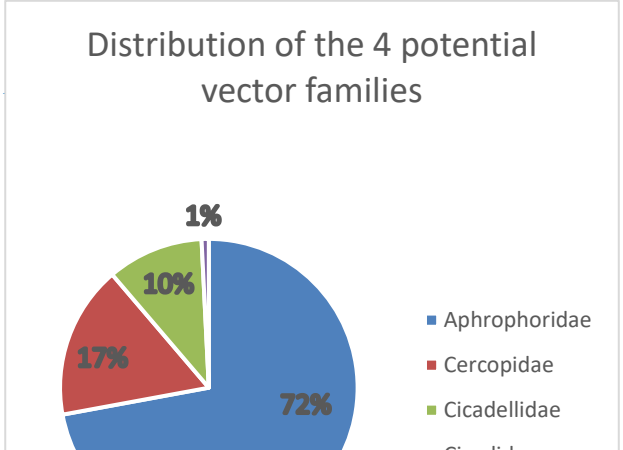


Table 2 Period of presence of potential vectors of 4 main species during the year, source: Anses

Network partners have reported the presence of "cuckoo sputum" (exudate secreted by spittlebug larvae) on their plots. But we received only few reports. The first spits were reported in early April and the last at the end of May. No regional trends can be detected from this small sample.

The tables above show the monthly number of positive samples for the presence of **adult** of all potential vector species (line 2) and the proportion of positive samples (contain at least one potential vector) in a given month in relation to the total number of samples in that month (line 3).
For example, in May, 285 of the 366 samples collected that month were positive for the presence of at least one potential vector species, which represents 78% of the May's collections. The first two positive samples were collected in **January** by the CPIE in the "Pays de la Loire" region. The maximum number of positive samples is therefore reached in May-June-July. Significant proportions of positive samples are still recorded in the fall even though the number of sampling is low at that time. The last capture took place in New Aquitaine in mid-December by the "FREDON Aquitaine".

All vector potential mixed

Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2	0	2	109	285	246	128	67	75	52	10	1
0%	0%	7%	52%	78%	71%	54%	50%	59%	55%	50%	17%

Philaenus spumarius

Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
1	0	1	8	71	86	48	24	34	35	7	1
0%	0%	3%	4%	19%	25%	20%	18%	27%	37%	35%	17%

Neophilaenus campestris

Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
0	0	0	2	42	30	2	2	7	16	1	0
0%	0%	0%	1%	11%	9%	1%	2%	5%	17%	5%	0%

Cicadella viridis

Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
0	0	0	0	4	27	16	16	36	16	2	0
0%	0%	0%	0%	1%	8%	7%	12%	28%	17%	10%	0%

Cercopis vulnerata

Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
0	0	0	84	186	117	7	1	0	0	0	0
0%	0%	0%	40%	51%	34%	3%	1%	0%	0%	0%	0%

Philaenus spumarius

The first capture is recorded in **January** in Pays de la Loire (CPIE) and significant proportions of positive samples are still recorded in November and December. This shows the importance of extending the sampling effort late in the year to better assess the risk of adults of *P. spumarius* in late autumn and early winter.

Neophilaenus campestris

As for the other main species, we notice a peak in captures in May and a disappearance in November. July and August seem to be unfavorable months for this species, which nevertheless appreciates dry grasslands (Biedermann and Niedringhaus, 2009).

Cicadella viridis

Note that *Cicadella viridis* is, unlike the other main species mentioned here, a bivoltine species and can produce two generations per year in wetlands (Biedermann and Niedringhaus, 2009). Of the 117 *C. viridis* positive samples, most were caught between June and October with a peak in September. The last capture took place in November. ***C. viridis* appears to emerge later** than the other species studied, perhaps due to its development in wet environments.

Cercopis vulnerata

There is a total of 395 positive sample for *C. vulnerata* with a total number of 923 specimens captured, the most were caught between April and June with no capture after August. Therefore *C. vulnerata* seems to be **the most early species**.

Discussion

The PHL Network had three priority research objectives, with:

- Faunistic objectives: mapping of the main species, lists of potential vectors according to regions and crops.
- Methodological objectives: recommendations for sampling potential *Xylella* insect vectors
- Biological objectives: biological elements for the most common species

All these objectives have been achieved and are detailed in the conclusion. However, there are parameters that are not taken into account in our research network, such as the host plants from which the insects were collected, so we could establish a host spectrum for the insects sampled. In the same way the climatic zones or the altitude of the sampling sites are not considered, it would certainly be possible to study these parameters using the QGIS software and the appropriate layers, always with the aim of determining the influence of the environment on the population abundance of the vectors.

The network makes it possible to obtain information on the French vectors different from those present in Europe or America, hence the importance of such a research network.

Another study was also conducted, which consisted in a study of two close species: *Neophilaenus lineatus* and *N. minor*, which are currently difficult to separate by morphological means. A preliminary study is presented in annex 10 where we undertake a morphological and molecular study with the individuals collected with our research network.

3. Portugal

Pictures of the devastating effect of symptomatic Rubus plant with associated 'Ca. Phytoplasma rubi' presence (wild and cultivated), in the South of Portugal (Odemira region). The problem is most devastating on blackberry production.

Taking into consideration that also wild Rubus plants in the region were infected, it was assumed that there was an insect vector in the region for this phytoplasma.
Literature is scarce about this issue, but there is the indication that the cicadellids (leafhoppers) Macropsis fuscula/ Macropsis scotti are vectors. Both exist in Portugal (*) .
However, other leafhopper species are mentioned in literature as phytoplasma vectors and so attention was given to all Auchenorrhyncha (Fulgoromorpha and Cicadomorpha).

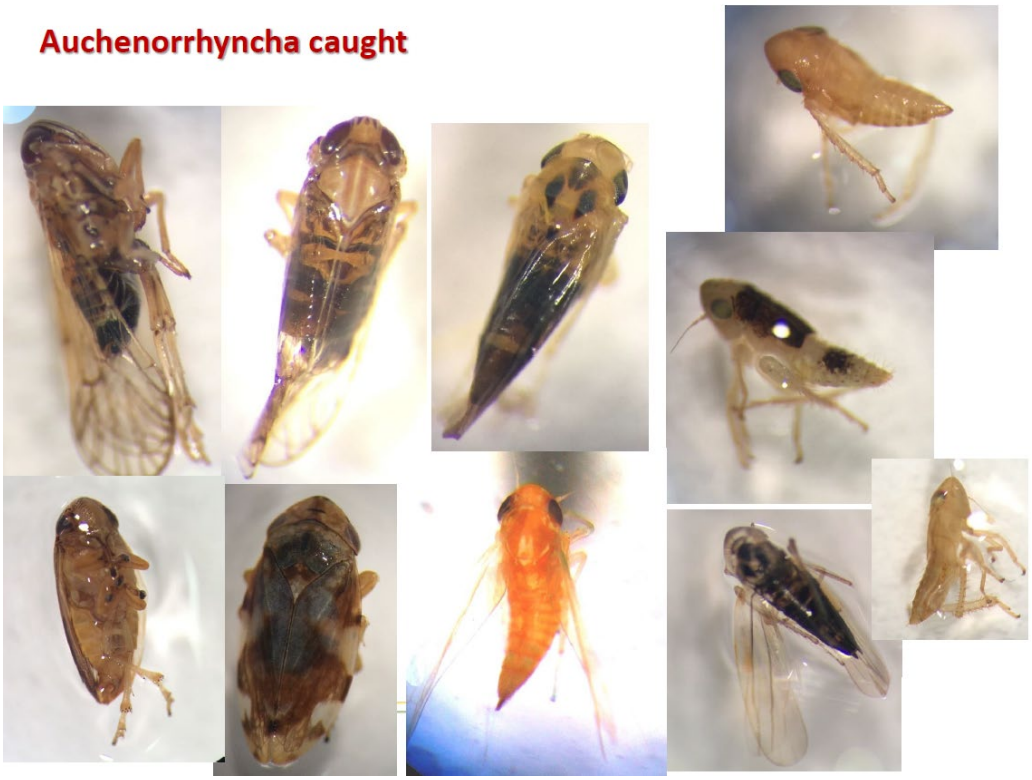
(*) References:
- Cadman, C.H. 1961. Raspberry viruses and virus diseases in Britain. Horticultural Research 1:47–61.
- de Fluiter, H.J. & van der Meer, F.A. 1953. Rubus stunt, a leafhopper-borne virus disease. Tijdschrift over Plantenziekten 59:195–197.
- Jach, M. 2013. Fauna Europaea: Macropsis Lewis 1834. Fauna Europaea version 2.6.2. http://www.faunaeur.org.
-Tonks, N.V. 1960. Life history and behaviour of the leafhopper Macropsis fuscula (Zett.) (Homoptera: Cicadellidae) in British Columbia. The Canadian Entomologist 92(9):707–713.
- Weintraub, P.G. & Beanland, L. 2006. Insect vectors of phytoplasmas. Annual Review of Entomology 51:91–111.

Macropsis sp.

During the Auchenorrhyncha survey in Rubus, a high plant- and leafhopper diversity was detected. In each year of the survey (2016-2017), more than 20 morphotypes were caught. In terms of abundance, Typhlocybinae were by far the most abundant taxonomic group. For the insect identification, the help of Dr. Michael R. Wilson from the National Museum of Wales was much appreciated.
By the end of the project, the presence of 'Ca. Phytoplasma rubi inside the insects (by nested PCR) was still in progress.

Pictures of the caught Auchenorrhyncha:

Auchenorrhyncha caught



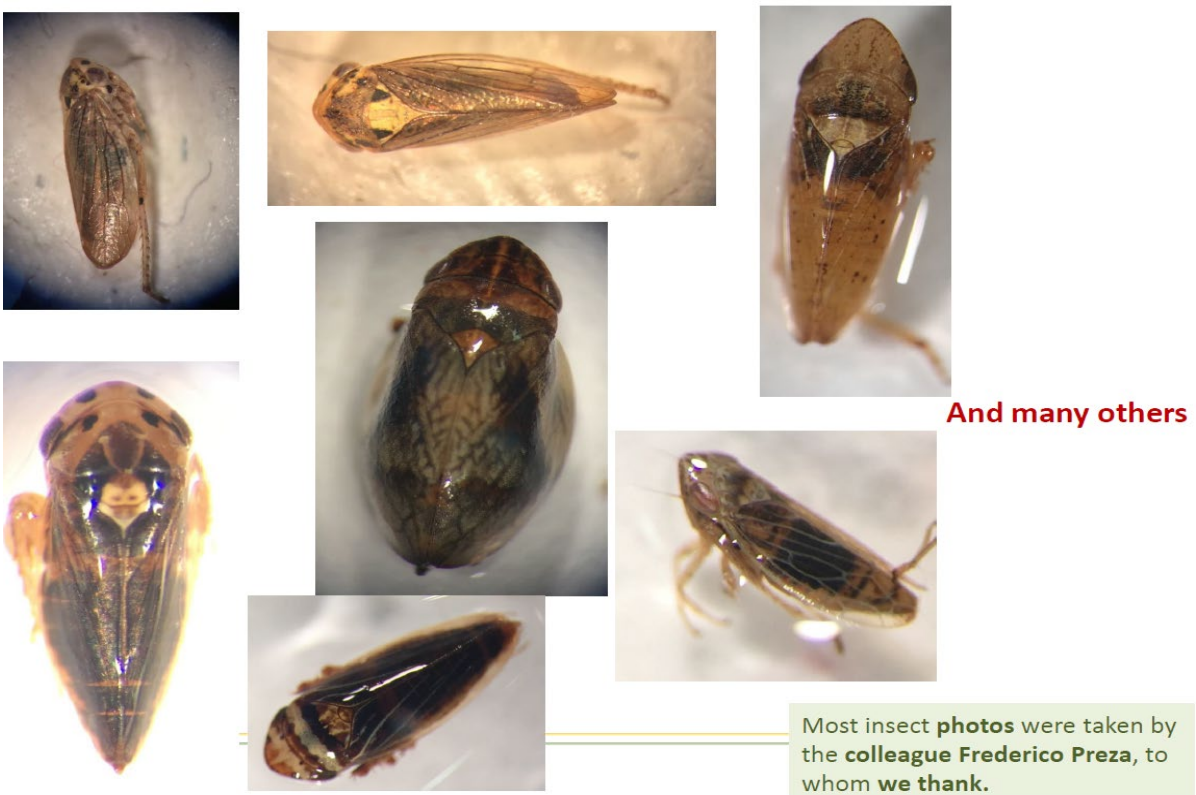


Table of the present Auchenorryncha in Rubus in Portugal:

Aphrophoridae	*Neophilaenus* sp.
	Philaenus sp.
	Philaenus spumarius
Cicadellidae	*Agallia* sp.
	Aphrodes sp.
	Exitianus sp.
	Hauptidea sp.
	Macropsis sp.
	Megopthalmus sp.
	Neoaliturus fenestratus
	Psammotettix sp.
	Ribautiana sp.
	Zyginidia sp.
Delphacidae	*Asiraca clavicornis*
	Toya sp.
	Toya propinqua
Tettigometridae	*Tettigometra* sp.

4. Morrocco

No specific results report was obtained by the Morrocan partner. However, a publication on the *X. fastidiosa* survey was sent, as well as a folder on the limited survey on Auchenorrhyncha in avocado in Morroco. Both publications are referenced under chapter 9 (Publicatoins).

8. Conclusions

1. Belgium (ILVO & CRA-W)

Diagnostic tools:

Methods of choice are

- Phytoplasma: nested PCR + sequencing for identification
- *'Ca*. Liberibacter': real-time PCR complemented by several endpoint PCR techniques.
- Insects: COI and ITS2 molecular test followed by identification through blast in the BOLD database

Monitoring technique of the insects

- General survey - method of choice: yellow sticky traps
- Specific capture of living insects: sweep-netting or beating.
- Monitoring of insects, landing on a specific target plant: glue

Confirmed vectors:
Macrosteles sexnotatus, Philaenus spumarius – both for Aster yellows phytoplasma
Potential new vectors*: Typhlocybe quercus, Empoasca decipiens, Fieberiella florii, Orientus ishidae, Eupterix atropunctata*

Auchenorryncha monitoring:
Both in the fruit orchards (apple and pear) and in the carrot fields, an extensive survey of the prevailing plant and leafhoppers could be done over a period of two years. The results give a nice overview of which Auchenorrhyncha species are visiting apple and pear orchards, and which species are frequenting carrot fields. Since most of the identification was done by means of molecular techniques, followed by sequencing, also a lot of new sequence data on these insects have become available. Since not a lot of sequence data are currently publically available through the NCBI or BOLD databases, the addition of these sequences will be a excellent contribution to these databases. During the identification process, the BOLD database seemed the most reliable database so far. The sequences will therefore be added to the BOLD database.
From the survey, we can say that *Macrosteles sexnotatus* was confirmed as vector for AY in the carrot fields. Also in the pear and apple orchards, *M. sexnotatus* was found positive

for AY, yet not for the infections in apple and pear (AP, resp. PD). Also *P. spumarius* was confirmed as an AY phytoplasma vector. However, the numbers of *P. spumarius* insects in the monitored fields were much lower. *Fieberiella florii* was also present (yet in low numbers) and can be considered as a potential vector. The phytoplasma was never confirmed in this insect, and the amount of *F. florii* that was trapped was also low. Even if phytoplasma presence was also not confirmed in *T. quercus, O. ishidae* and *E. decipiens*, these three insects are also of interest and form a potential risk. Finally, another insect of interest is the commonly found *Eupterix atropunctata*. It would be recommendable to include also this insect in future vector studies

This project was also a transnational project with partners in Portugal, Morocco and France. The Moroccan partner only worked on (vectors of) Xylella, and the Portugese partner on *Rubus* stunt vectors. The French partner, ANSES, also worked on *Xylella*, yet generated quite some survey data and sequences from the Auchenorrhyncha group, resulting from input from 132 sites (from 9 regions in France). Their main objective was identifying new/potential vectors for *Xylella fastidiosa*. The presentations of the transnational partners, given at the final project meeting, will be added as an addendum to this project report.

2. *France (ANSES)*

The networkcomposed of twenty five partners throughout metropolitan France, had three research objectives for a better knowledge of *Xylella*'s potential vectors. After two years of studies, we achieved the following results:

- **Faunistic objectives**: an initial list of potential vectors presents in crops or in the environment of crops is now available. Fifteen different species of potential vectors were collected by the partners. The mapping of the most common species is carried out. The 4 main species are *Philaenus spumarius*, *Cicadella viridis*, *Cercopis vulnerata* and *Neophilaenus campestris*. We confirm here that *P. spumarius* is the main potential vector species in France.
- **Methodological objectives**: as part of future recommendations on the methods for monitoring vectors in plots, we tested 4 methods for collecting insects. It should first be stressed that no method is specific to *Xf* vectors. The method which combines the best abundant harvest and specificity is the sweeping net. The Barber trap is only suitable for a soil leafhopper. Yellow pan trap and sticky traps are not sufficiently specific.
- **Biological/Phenological objectives**: the number of collection sites allows for the 4 main species to obtain some basic biological information:
    - *Philaenus spumarius* is found in our network more frequently in ornamental crops and orchards. It is sometimes present in large quantities. It is not caught more frequently in hedgerows than in crops. This is the species present as adults over the longest period (March to December in our network). This is undoubtedly the most epidemiologically significant potential vector.
    - *Neophilaenus campestris*: there is no clear trend for this species, which just seems to be a little more frequent in orchards and aromatic culture, as well in the crop as in its environment. Summer appears to be an unfavorable period for

its activity (as *Cercopis vulnerata*) because the catch deficit is greater for *N. campestris* than for *P. spumarius* and *C. viridis*. Given its abundance, it is a species to be monitored from an epidemiological point of view. Other species of the genus *Neophilaenus* are sometimes present, but in smaller numbers in the network.

- *Cicadella viridis*: is more often present in vine samples than on other crops. It also seems to be a little more frequent on "miscellaneous" plant (plants except crops or hedges) than other species. It appears later but is still collected in November
- *Cercopis vulnerata* seems to be a precocious species.

3. Portugal (INIAV)

A high diversity of Auchenorrhyncha were caught. By the end of the project, it was still unclear which of the plant and leafhoppers could be associated with the spread of the Rubus stunt phytoplasma.

4. Morocco

The bacterium *Xylella fastidiosa* is gram negative, xylem-inhabiting, devastating pathogen which causes various diseases on more than 300 plant hosts. Given the recent confirmed findings of X. fastidiosa in the European Union, this bacterium is becoming a serious threat to the Moroccan agricultural sector. A survey was conducted during May-September 2015 on the presence of X. fastidiosa in several commercial groves, covering olive, citrus and grapevine growing areas. In a few trees, severe symptoms which could be associated to the bacterium were observed. A total of 900 samples of different crops from different regions were randomly collected: 220 olive trees (cv. Picholine Marocaine) from two regions, 410 citrus trees belonging to 7 different cultivars collected in 4 regions and 270 grapevine plants belonging to 6 different cultivars from 3 regions; all these samples were tested for the presence of *X. fastidiosa* by using an ELISA commercial kit. The obtained results did not show any positive sample. These preliminary results are taken as an encouraging indication, considering that X. fastidiosa was not found in Morocco, at least in the surveyed crops. However, frequent extensive surveys in different regions are needed to prevent its entrance into the country.

For more information: see publication, mentioned under 9.

9. Publications

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Annexes

Poster ISCP 69, Ghent.

VECTRACROP poster EUPHRESCO – ZANADO

Presentations /publications of the EUPHRESCO project VECTRACROP partners.