Validated models for bees exposed to stressors III

Deliverable D9.7

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PoshBee

Pan-european assessment, monitoring, and mitigation of stressors on the health of bees

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Table of contents

Preface 4
Objective 4
Summary 4
1. Preparation of the material for solitary bee haemolymph collection and delivery 5
2. Reception of the samples, sorting, and preparation 6
2.1. Sample delivery 6
2.2. Reception of the samples, sorting, and traceability 6
3. MALDI BeeTyping: Data acquisition 7
4. MALDI BeeTyping: Data preparation and post-processing for statistical analysis 10
5. Conclusions 10
6. References 11

Preface

MALDI BeeTyping was developed by BioPark, starting in 2013, and is based on using pollinator haemolymph for monitoring bee health (Arafah et al., 2019). This "blood test" was inspired by MALDI-BioTyping®, a method daily used in clinical microbiology for the identification of bacteria. Transferring this approach to bee haemolymph arose from previous work conducted by Dr Philippe Bulet (CNRS partner, PoshBee WP9 leader) on the MALDI molecular mass profiling on haemolymph of the fruit fly *Drosophila melanogaster* (Uttenweiler-Joseph et al., 1998). Haemolymph provides information on the immune response of insects to pathogens. This innovative mass spectrometry approach was established in the former laboratory of Dr Bulet (IBMC, Strasbourg, France) in the '90s and transferred to the BioPark Team (Drs Karim Arafah and Sébastien Voisin) through the project HematoBeeTest® (HBT®, FEAGA 2013-2016). In brief, MALDI-BeeTyping is based on the analysis of the molecular mass fingerprints (MFPs) of peptides and proteins (<18 kDa) circulating in the bee haemolymph (Arafah et al., 2019).

Objective

This deliverable (D9.7) aims to provide a model for the use of **MALDI BeeTyping** as applied to the **solitary bee model, *Osmia bicornis***. D9.7 covers the three categories of experiments defined in the PoshBee project:

- **Field experiments**: solitary bees are living in their natural environment and are collected in different countries and field areas;
- **Semi-field experiments**: solitary bees are maintained in enclosures and are receiving field representative doses of pesticides;
- **Laboratory conditions**: solitary bees are submitted experimentally to pesticides and/or other stressors under controlled conditions.

This "blood test" on *O. bicornis* haemolymph will be applied to the different samples collected from field to laboratory experiments with pesticides or pathogens, and pesticides associated with other stressors.

This document reports the general model and workflow based on the first two PoshBee validated model deliverables (D9.5 and D9.6), devoted to *Apis mellifera* and to *Bombus terrestris*, respectively:

- Design of a specific solitary bee haemolymph collecting kit and validation of the SOP for *Osmia bicornis* haemolymph collection;
- Set-up of the procedure for traceability (barcode stickers, delivery form, sample database);
- Preparation of the packaging (coated and non-coated barcoded tubes, ice packs, collecting kits, delivery forms) and updated safety shipment requirements;
- Reception of the samples, sorting, quality and identity checks;
- Preparation of the solitary bee haemolymph samples for MFPs by MALDI BeeTyping according to the SOP;
- Acquisition of the MFPs using a manual mode, and integration in an *O. bicornis* database that merges the MFPs and the sample barcode, including a sub-classification according to the sample origin (field, semi-field and laboratory);
- Data curation (e.g., sample classification, spectra and peak settings, preparation of the MFP spectra, peak list generation) for statistical analysis (e.g., PCA analysis, R software, automatic classification and machine learning);
- Design of the analytical report.

Summary

Briefly, the haemolymph tissue is the circulating body fluid in invertebrates, analogous to human blood. The aim of MALDI BeeTyping (see the workflow imaged in Figure 1) is to analyse the *O. bicornis* haemolymph, in an approach we developed on *A. mellifera* samples and applied to *Bombus terrestris* haemolymph to follow the impact of stressors on bee health.

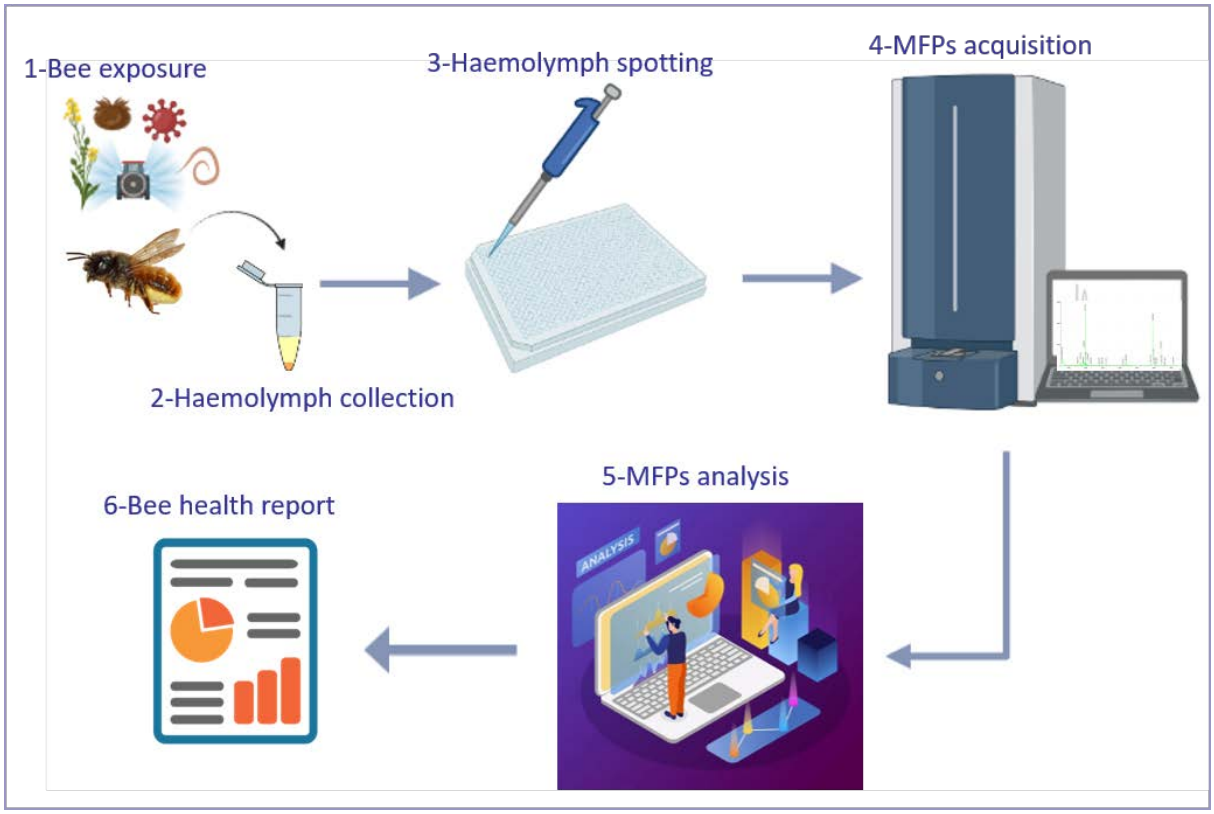

Figure 1. General workflow of MALDI BeeTyping analysis on *O. bicornis* haemolymph. The workflow describes six steps: (1) Bee exposure to various stressors (chemicals, pathogens, nutrition), (2) haemolymph collection from an individual solitary bee, (3) haemolymph spotting (diluted sample), (4) MFPs acquisition and (5) analysis, and (6) the bee health report generation.

O. bicornis haemolymph was collected according to the SOP delivered within PoshBee deliverable D1.1 Protocol for field sampling, further adapted for semi-field and laboratory sampling through the specific solitary bee kit delivered to the experimenters. The kit is derived from the one developed for honey bee haemolymph samples and has been optimised for *O. bicornis*. Each individual haemolymph sample collected from the field, semi-field or laboratory condition has been submitted to MALDI BeeTyping analysis to provide MFPs. This haemolymph test based on MALDI profiling will serve to follow solitary bee health in the in the same way as has been developed for the honey bee and implementation for bumble bees. The present deliverable details the validated model for the application of the MALDI BioTyping method on *O. bicornis* haemolymph, aiming to evaluate the impact of agrochemicals on the health status of solitary bees. This model includes the different steps listed above to acquire individual MFPs from (i) haemolymph collection (steps 1,2 see the general workflow, Fig.1), (ii) sampling delivery and reception, (iii) sample preparation for analysis (step 3), and (iv) data acquisition (step 4), processing (step 5) and reporting (step 6). This model was developed, applied and validated on laboratory, semi-field and field samples.

This validated model for bees exposed to stressors concerns O. bicornis*. Two models were built in 2019 and 2020 for* A. mellifera *and* B. terrestris, respectively*. The present model may be applied to other solitary bee species if approximately the same size as* O. bicornis, *potentially including some of the novel species investigated within Poshbee.*

1. Preparation of the material for *O. bicornis* haemolymph collection and delivery

Traceability is a prerequisite for sample analysis, data management, and merging these in an integrative database is one of the project objectives. To ensure the identity of each individual sample

collected by project partners, we use a barcoding label for each individual bee sample (the same barcode for haemolymph and body, but with a different colour, red for haemolymph and black for the body). For each partner involved in the field, semi-field and laboratory sampling, we provide on request the solitary bee kits for haemolymph collection. In addition, an adjusted number of pre-coated collecting tubes (pre-coating being a prerequisite to prevent proteolysis and melanisation) are added to the parcel. The capillary preparation was also adapted to the strength of the solitary bee cuticle. As a safety issue, we provide an additional set of coated and classic tubes (approx. 5%). Each parcel includes a proforma form and a detailed Standard Operating Procedure (SOP) to collect solitary bee haemolymph for field, semi-field and laboratory experiments. The coated tubes (for haemolymph) and classic tubes (for body) are delivered in cold conditions (ice packs) and the partners are informed by e-mail before any shipment.

2. Reception of the samples, sorting, and preparation

The *O. bicornis* haemolymph samples were obtained from the different partners, in accordance with the D1.1 protocol provided to them by PoshBee partner BioPark (BIOP) for the field sampling. Samples were accompanied by an official document for sample importation by BIOP (Agreement n°DDPP/SPAE/2020-03833 renewed in December 2020, valid for two years). Training and demonstration, regarding haemolymph sampling according to the SOP, were delivered to the different partners engaged in bee sampling in the Bologna Workshop. The Work Package (WP) 9 video was produced by BIOP and CNRS partners in September 2021 and edited by Pensoft and is available online on the PoshBee Project YouTube channel under the title “Poshbee research: Bee haemolymph analysis using MALDI BeeTyping”. The following conditions for effective storage and delivery of the bee haemolymph samples are recommended: freezer at -20°C and dry ice for sample storage and delivery, respectively.

2.1. Sample delivery

Sample delivery will strongly determine the quality of MFP data. The different partners sent the parcels in dry ice, ensuring preservation of the integrity of the haemolymph samples. Good communication is a prerequisite to secure the samples (exact dates of sending, safety form, sampling list and observations) and their traceability.

2.2. Reception of the samples, sorting, and traceability

On arrival, the parcels are checked to assess the integrity of the delivery in terms of information forms/sample lists and the precise number of samples. The partners at the origin of the sampling are informed by e-mail when the parcel(s) arrived at BioPark. When necessary, we request clarification and additional information linked to the established sample list provided by the experimenters to integrate them (if any) in our general PoshBee sample database.

On receipt of this supplementary file, the traceability barcode stickers are checked and the two sets of tubes (haemolymph and body) classified. At this stage, the samples are checked individually in order to detect any abnormality (e.g., colour, viscosity, presence of physical contaminants).

When necessary, feedback, including any observation that may require clarification, is delivered to the partner who provided the samples to get any additional information prior to analysis.

Sample information is tracked by BIOP using the individual sample barcodes. All samples are classified in a dedicated “Sample Excel datasheet” (Table 1).

Table 1. Example of data recorded for *O. bicornis* semi-field 2020 experiment. This table contains the required information on the collected samples such as country, time of collection, plant, tube number, collector, comments about the samples, date of reception at BioPark, date of MALDI analysis, number of pools for LC-MS/MS and date of LC-MS/MS analysis. CHE: Switzerland, T1: collection before pesticide application, BUC: Buckwheat (*Fagopyrum esculentum*), SEN: *Sinapis arvensis*, PHA: *Phacelia tanacetifolia* and DAS: Dalel ASKRI.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Country	Time (T1/T2/T3/T4)	Species	Gender	Plant	Tube N°	Barcode	Collection date	Collector	Comments	Received	MALDI	LC_Pool n°	LC-MS/MS
2	CHE	T1	Osmia	Female	BUC	1	08503	11/06/2020	DAS		17/09/2020	15/04/2021		
3	CHE	T1	Osmia	Female	BUC	2	08504	11/06/2020	DAS		17/09/2020	15/04/2021		
4	CHE	T1	Osmia	Female	BUC	3	08510	11/06/2020	DAS		17/09/2020	15/04/2021		
5	CHE	T1	Osmia	Female	BUC	4	08511	11/06/2020	DAS		17/09/2020	15/04/2021		
6	CHE	T1	Osmia	Female	BUC	5	08514	11/06/2020	DAS		17/09/2020	15/04/2021		
7	CHE	T1	Osmia	Female	BUC	6	08522	11/06/2020	DAS		17/09/2020	15/04/2021		
8	CHE	T1	Osmia	Female	BUC	7	08525	11/06/2020	DAS		17/09/2020	15/04/2021		
9	CHE	T1	Osmia	Female	BUC	8	08527	11/06/2020	DAS		17/09/2020	15/04/2021		
10	CHE	T1	Osmia	Female	BUC	9	08533	11/06/2020	DAS		17/09/2020	15/04/2021		
11	CHE	T1	Osmia	Female	BUC	10	08538	11/06/2020	DAS		17/09/2020	15/04/2021		
12	CHE	T1	Osmia	Female	BUC	11	08501	11/06/2020	DAS		17/09/2020	15/04/2021		
13	CHE	T1	Osmia	Female	BUC	12	08506	11/06/2020	DAS		17/09/2020	15/04/2021		
14	CHE	T1	Osmia	Female	BUC	13	08507	11/06/2020	DAS		17/09/2020	15/04/2021		
15	CHE	T1	Osmia	Female	BUC	14	08509	11/06/2020	DAS		17/09/2020	15/04/2021		
16	CHE	T1	Osmia	Female	BUC	15	08512	11/06/2020	DAS		17/09/2020	15/04/2021		
17	CHE	T1	Osmia	Female	SEN	16	08484	11/06/2020	DAS		17/09/2020	15/04/2021		
18	CHE	T1	Osmia	Female	SEN	17	08485	11/06/2020	DAS	Not much	17/09/2020	15/04/2021		
19	CHE	T1	Osmia	Female	SEN	18	08486	11/06/2020	DAS		17/09/2020	15/04/2021		
20	CHE	T1	Osmia	Female	SEN	19	08496	11/06/2020	DAS		17/09/2020	15/04/2021		
21	CHE	T1	Osmia	Female	SEN	20	08500	11/06/2020	DAS		17/09/2020	15/04/2021		
22	CHE	T1	Osmia	Female	SEN	21	08487	11/06/2020	DAS		17/09/2020	15/04/2021		
23	CHE	T1	Osmia	Female	SEN	22	08492	11/06/2020	DAS		17/09/2020	15/04/2021		
24	CHE	T1	Osmia	Female	SEN	23	08494	11/06/2020	DAS		17/09/2020	15/04/2021		
25	CHE	T1	Osmia	Female	SEN	24	08497	11/06/2020	DAS	Not much	17/09/2020	15/04/2021		
26	CHE	T1	Osmia	Female	SEN	25	08498	11/06/2020	DAS		17/09/2020	15/04/2021		
27	CHE	T1	Osmia	Female	SEN	26	08502	11/06/2020	DAS		17/09/2020	15/04/2021		
28	CHE	T1	Osmia	Female	SEN	27	08505	11/06/2020	DAS		17/09/2020	15/04/2021		
29	CHE	T1	Osmia	Female	SEN	28	08508	11/06/2020	DAS		17/09/2020	15/04/2021		
30	CHE	T1	Osmia	Female	SEN	29	08513	11/06/2020	DAS	Yellow	17/09/2020	15/04/2021		
31	CHE	T1	Osmia	Female	SEN	30	08516	11/06/2020	DAS	Not much	17/09/2020	15/04/2021		
32	CHE	T1	Osmia	Female	PHA	31	08490	11/06/2020	DAS		17/09/2020	16/04/2021		
33	CHE	T1	Osmia	Female	PHA	32	08491	11/06/2020	DAS		17/09/2020	16/04/2021		
34	CHE	T1	Osmia	Female	PHA	33	08493	11/06/2020	DAS		17/09/2020	16/04/2021		
35	CHE	T1	Osmia	Female	PHA	34	08481	11/06/2020	DAS		17/09/2020	16/04/2021		
36	CHE	T1	Osmia	Female	PHA	35	08489	11/06/2020	DAS		17/09/2020	16/04/2021		
37	CHE	T1	Osmia	Female	PHA	36	08482	11/06/2020	DAS		17/09/2020	16/04/2021		

WP7 Osmia 2020 CHE

3. MALDI BeeTyping: Data acquisition

Following receipt, we proceed to the establishment of the "*Sample Excel datasheet*" for traceability, and preservation of the samples at -20°C. The samples are thawed prior to the MALDI BeeTyping analysis. Sample preparation, as described by the specific SOP, consists of a ten-fold dilution of the solitary bee haemolymph. All analyses are done on an AutoFlex III Smartbeam® MALDI-TOF-MS (Bruker GmbH, Germany) with the FlexControl 3.4 and FlexAnalysis 3.4 software, for mass spectrum acquisition and data analysis, respectively. Analyses are performed in a linear/positive mode. The positive linear mode enables the capture of positive ions in the mass range selected (*m/z* 600 to 18,000).

Each diluted haemolymph sample is spotted three times on a reusable MALDI plate (MTP 384 target plate polished steel BC) and data are acquired once for each spot (N = three technical replicates) in manual acquisition mode. The calibration is performed using a mixture of Apiscal (a homemade calibration kit) and Protmix I provided by Bruker to evaluate the performance and optimum operative condition of the mass spectrometer (spectral resolution and reproducibility, analytical sensitivity, mass accuracy). The calibration procedure follows the dedicated SOP. The sample size allows us to use a single MALDI plate to analyse several conditions at once. In the figure below, we present differential spectra between two different experimental conditions (Figure 2).

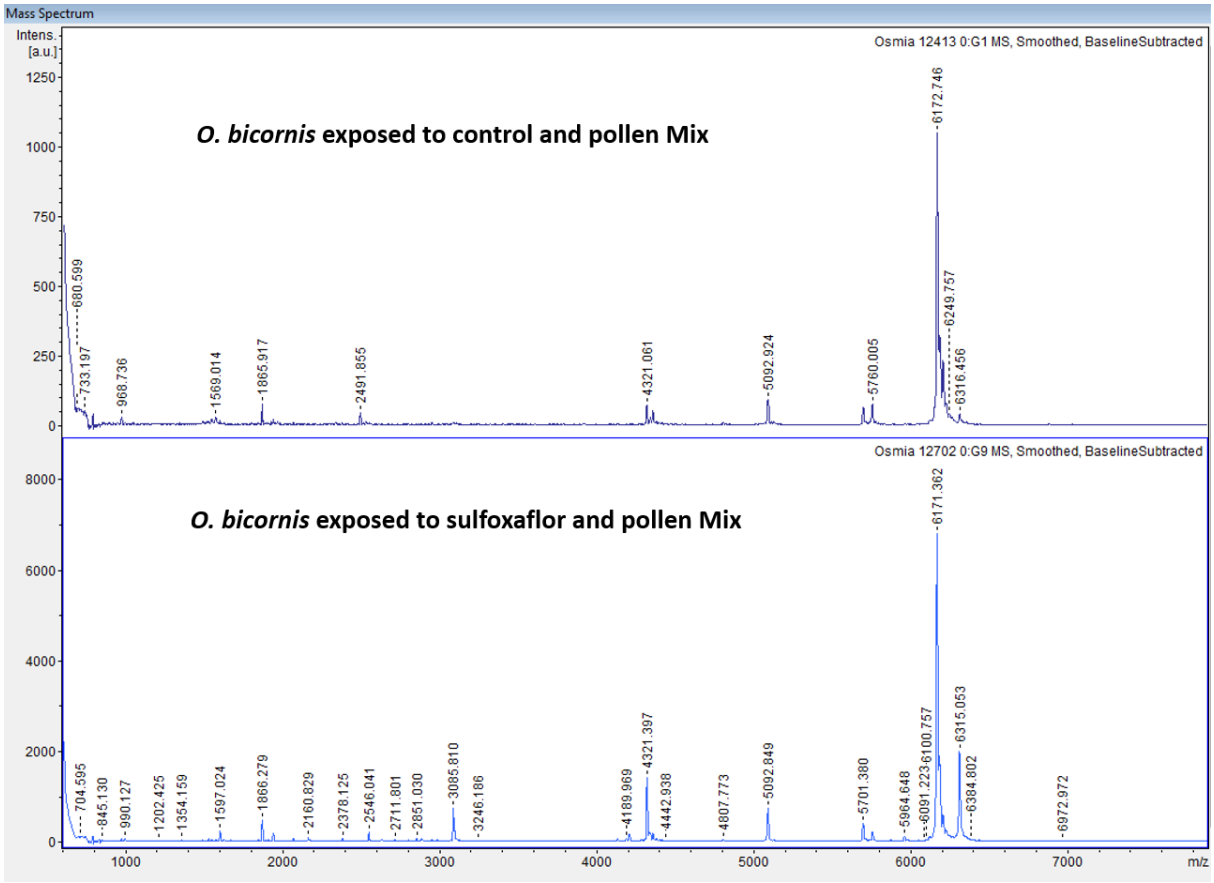

Figure 2. An example: differential MFPs of haemolymph composition of *O. bicornis* between two different experimental conditions, control and pollen mix and sulfoxaflor and pollen mix. Experiment performed by PoshBee partner WBF-Agroscope within the framework of WP5 (study of the effects of agrochemical-nutrition interactions on bee health in the laboratory). The MFPs were acquired using FlexControl 3.4 software, smoothed and baseline subtracted and analysed with FlexAnalysis 3.4.

The MALDI BeeTyping was validated on the main models of the project but also extended to the novel wild species investigated by PoshBee partners (University of Mons, Belgium and Martin Luther University, Germany) for risk assessment. The following figures, presented in the next part, show examples of different MFPs acquired on haemolymph collected from the main three species of the project *Apis*, *Bombus* and *Osmia* (Figure 3) and the novel species (*Colletes hederae, Anthophora plumipes, Halictus scabiosae*) investigated within PoshBee (Figure 4).

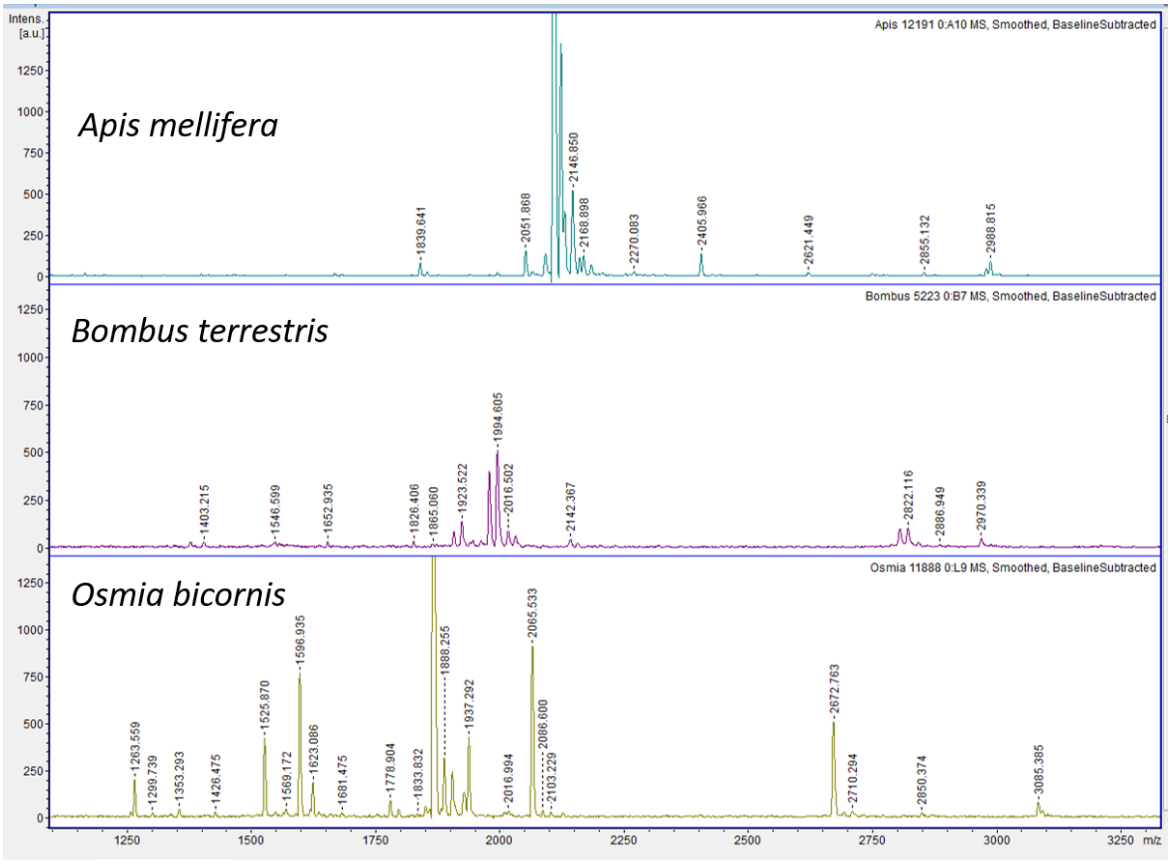

Figure 3. Differential MFPs of haemolymph collected from the main three species investigated within PoshBee, *A. mellifera*, *B. terrestris* and *O. bicornis*. The bee samples were prepared by different PoshBee partners (Atlantic Pollination Ltd, UK; Red Beehive Company, UK; Royal Holloway University London, UK; Martin Luther University, Germany) within the framework of WPs 6 and 7 which are related to the effects of agrochemical-pathogen interactions on bee health in the laboratory and the effects of chemicals and their interactions with other stressors on bees tested in semi-field and field experiments, respectively. The MFPs were acquired using FlexControl 3.4 software, smoothed and baseline subtracted and analysed with FlexAnalysis 3.4.

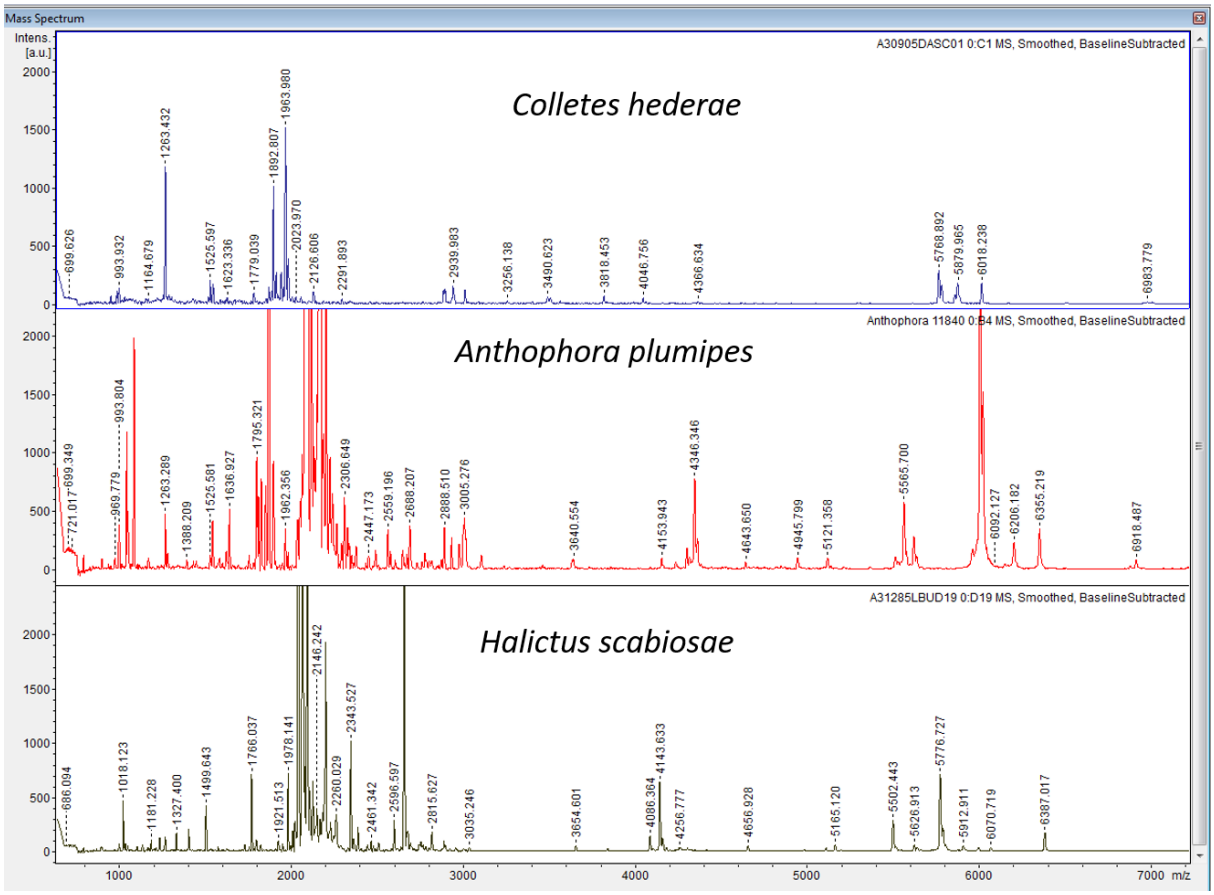

Figure 4. Differential MFPs of haemolymph composition between three of the novel wild species for risk assessment investigated within the framework of PoshBee; *Colletes hederae, Anthophora plumipes,* and *Halictus scabiosae*. Experiments performed by different PoshBee partners (Atlantic Pollination Ltd, UK; Red Beehive Company, UK; Royal Holloway University London, UK; Martin Luther University, Germany) within the framework of WPs 6 and 7. The MFPs were acquired using FlexControl 3.4 software, smoothed and baseline subtracted and presented with FlexAnalysis 3.4.

4. MALDI BeeTyping: Data preparation and post-processing for statistical analysis

The raw data obtained by MALDI BeeTyping are classified according to the different experimental conditions and processed using appropriate settings (e.g., spectra and peak settings) using the ClinProtools Software 2.2 from Bruker (Germany). A post-processing step involving spectral normalization of all calculated peak areas is also performed prior to statistical analysis. From the three replicates per sample, an average spectrum is generated and used for statistical analysis. Following this treatment, the average spectra are used to build Principal Component Analysis (PCA). The PCA can generate 3D score plots, variance per PCA component, and a peak list sorted according to the normality of the distribution and the appropriate statistical test of significance to discriminate experimental sample populations (namely experimental classes) using supervised/unsupervised PCA. In addition, machine learning-based algorithms are used to build a computational model of spectral recognition and classify the samples according to different parameters (e.g., stressor type, intensity of bee exposure to agrochemicals). Mass peak lists were also generated using FlexAnalysis software to integrate data in RStudio for statistical analyses.

5. Conclusions

In this deliverable D9.7, an optimized workflow for generating MFPs by MALDI profiling, from the solitary bee *Osmia bicornis*, has been established. This approach is derived from the well-known MALDI

BioTyping (FDA and EMA approved) used in clinical microbiology for microbiological identification. This approach is referred to as MALDI BeeTyping and is usable on *Osmia bicornis* haemolymph to monitor the impact of stressors through a simple “blood” analysis. The validation of this scenario was performed on a representative experimental case study. This validated model of MALDI BeeTyping is now applicable to laboratory, semi-field and field samples in different stress conditions (biotic and/or abiotic).

6. References

Arafah K, Voisin SN, Masson V, Alaux C, Le Conte Y, Bocquet M, Bulet P. MALDI-MS profiling to address honey bee health status under bacterial challenge through computational modeling. Proteomics. 2019 Dec;19(23):e1900268. doi: 10.1002/pmic.201900268. Epub 2019 Nov 11. PMID: 31657522.

Uttenweiler-Joseph S, Moniatte M, Lagueux M, Van Dorsselaer A, Hoffmann JA, Bulet P. Differential display of peptides induced during the immune response of *Drosophila*: a matrix-assisted laser desorption ionization time-of-flight mass spectrometry study. Proc Natl Acad Sci U S A. 1998 Sep 15;95(19):11342-7. doi: 10.1073/pnas.95.19.11342. PMID: 9736738; PMCID: PMC21644.