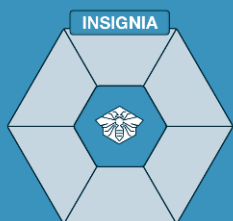


Guideline for apicultural citizen science to apply the honey bee colony for bio-monitoring of the environment



INSIGNIA Consortium 2018 – 2021





Guideline for Apicultural citizen science to apply the honey bee colony for bio-monitoring of the environment

Authors

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This guideline is the outcome of the INSIGNIA study "Environmental monitoring of pesticide use through honeybees", concerning the scientific development of a substantiated Apicultural Citizen Scientist protocol, including data analysis, non-invasive sampling for the honey bee colony, in-hive pesticide passive sampler development, molecular detection of pollen origin, pesticide risk exposure modelling for honey bee colonies, and modelling of pollen availability and diversity for bees.



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Content

1. Rationale
2. Study set-up
3. Protocols
4. Methods

Accounting

This guideline is a general guideline for the application of honey bee colonies for bio-monitoring and how to organise an Apicultural citizen science study. The study objective determines the toolbox for the beekeepers. This guideline has been designed and tested for its practical and scientific merits in the INSIGNIA study 2018-2021. This resulted in a best practice guide for Apicultural citizen science studies for bio-monitoring with honey bee colonies.

The supporting INSIGNIA studies, reports, and publications are available on the INSIGNIA website (<https://www.insignia-bee.eu>) - publications. This website will be maintained for seven (7) years after the end of the INSIGNIA study 2018-2021.

Reading guide

This guideline consists of:

1. *Rationale* with the background of the application of the honey bee colony as a monitoring tool, and the key outcomes of the INSIGNIA study.
2. *Study set-up*. This chapter describes the outline of the study, linking relationships, research integrity, data integrity, and communication processes.
3. *Protocols* for the beekeeper in the role of Apiculturalist Citizen Scientist. In this edition 1, April 2021, of the guideline, the protocols for bio-monitoring for pesticides and bio-monitoring for pollen diversity, with their specific toolboxes, are presented. The format of the protocols for different subjects is identical, to facilitate combining protocols for specific monitoring subjects in a study plan.
4. *Methods* applied in the laboratories for pesticide residue analysis, pollen ITS2 metabarcoding, the statistical methods, and the description of the exposure risk and pollen availability models.

Acknowledgement

The INSIGNIA Consortium thanks Steven Laan of AI-catcher, The Netherlands, for his critical evaluation of improvements of the flowcharts, icon drawings in the protocol and general layout of the Guideline.

1 - Rationale





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1 Honey bees for bio-monitoring

The link between the honey bee colony and bio-monitoring is the comprehensive interaction between the colony and the environment. Bees depend entirely for their food and in-hive resistance on the nectar, pollen, water, and propolis available in an approximately three to six kilometre radius (28 to 113 km²) around the colony. A smaller radius of about 1 to 1.5 km (3 to 7 km²) is preferred and longer foraging distances have also been recorded (Garbuzov et al., 2015; Beekman & Ratnieks, 2000). Along with this collection, and due to the bees' behaviour on the flowers, contaminants, both solid in small and ultra-small particles and as a liquid, are inadvertently collected and brought into the hive. In the foraging season of the colony, about a quarter of the colony forages on average ten times a day and visits multiple flowers (Steen, et al, 2012; Winston, 1991). This is the power of the big numbers that the honey bee colony offers with its large forager cohort and the accumulation of what is collected in a central spot, the hive.

As a result, the colony reflects both the nutrient availability as well as the environmental pollution status of the foraging area (Chauzat et al., 2006; Conti & Botrè, 2001; Bromenshenk et al., 1985; Steen, et al., 2016). The pollutants collected are not restricted to pollutants in nectar, pollen, water and propolis taken from the soil by the plants themselves, but also include pollutants deposited on the flowers, leaves, buds, and surface water by airborne processes, soil dusting, dust deposition and by human activities such as the application of plant protection products. To give an impression of what is deposited daily, an example is the dust on cars and their windscreens. This is the visible part of the deposits, which is only a fraction of the total deposition.

2 In-hive processes

The many thousands of foraging bees from one honey bee colony transport mainly nectar and pollen to a single location, the hive. Annually, a colony collects on average of 100 to 125 kg of nectar and 25 to 30 kg of pollen. Both nectar and pollen are collected in portions of 20 to 30 mg. This requires four to five times more nectar foragers than pollen foragers in the hive. Inside the hive, nectar, pollen, xenobiotics and plant pathogens are exchanged among the in-hive bees via trophallaxis, auto-, and allogrooming, and physical contact, and they are accumulated over time in the different bee hive compartments (DeGrandi-Hoffman et al., 1986; Paalhaar et al., 2007; Eyer et al., 2016; Steen et al., 2018). In the hive, the incoming food follows the nectar route or the pollen route for storage, consumption and application, and the contaminants attached to or dissolved in the food follow the same routes. The water route is different, as water is not stored. The wax route is not exactly a route, as beeswax can bind pesticides and immobilise the contamination as does propolis. Finally, the loose particles route follows a maximal 2-weeks route on the bees in the hive. As each route has its typical circulation inside the hive and final storage, this must be taken into consideration when planning sampling strategies for assessment of contaminants in the hive.

2.1 Nectar route

Incoming nectar is handed over by trophallaxis (food sharing) to in-hive nectar-transporting bees. Part of this fresh nectar is fed directly to the foraging bees to fuel new flights, part is distributed throughout the in-hive bees and larvae and the remainder is stored in the cells (Brandstetter et al., 1988; DeGrandi-Hoffmann & Hagler, 2000; Brodschneider et al., 2007). Part of the stored nectar is not or only slightly concentrated and is the daily fuel of the colony. Due to the trophallaxis of freshly collected nectar, within hours this nectar circulates throughout the colony. Another part is concentrated into honey which has about 80% sugar content for winter stores (Eyer et al., 2016). The



honey is processed by exposing nectar on the tongue to air currents produced by wing fanning. This evaporation process is repeated many times before the nectar reaches its final sugar concentration. Each time the partially processed nectar is returned into a cell until the final sugar concentration is reached. Consequently, nectar collected on different days becomes mixed, and the honey in the capped cells, with about 80% sugar content, is the result of this mixing process.

2.2 Pollen route

Pollen is stored straight away by the pollen foragers, as beebread. The pollen is mainly consumed by the nurse bees. Bees prefer freshly collected pollen and therefore the time for which beebread is stored is short. Within one week about three-quarters of these stores are consumed, and in 2 weeks the major part is consumed during the reproduction periods of the colony (Anderson et al., 2014; Carroll et al., 2017; Roessink & van der Steen, 2021). In the process of collecting pollen, executed during foraging and the home flight of the forager honey bee, besides pollen, other particles are brushed into the corbicula (pollen basket) as well.

2.3 Water route

Honey bee colonies collect about 25 kg water per year to dilute larval food and cool the brood nest (Nicolson, 2009; Kühnholz & Seeley, 1997). This water is collected as surface water from ditches, puddles, ponds, mud, rivers and lakes. The link between water, pesticides and honey bees is the contamination of this surface water, direct by contamination deposition, run off from sprayed fields, drift deposition and indirect via livestock farming and companion animals. Biocides are applied to these animals to control endo- and ectoparasites. This goes via feed and direct application on the skin/coat of the animals to control fleas and ticks. Parent pesticides and their metabolites of pesticides applied via the feed are excreted with the urine and faeces. Slurry, containing these excretions containing biocides or metabolites, is distributed in large amounts on the soil for fertilisation. Part of these substances will run off to surface water. Another part will be immobilized. Some biocides show very low mobility in the soil (high Koc) and will remain in the soil, bound to the clay particles, while others like Amitraz are very mobile (low Koc) and rinse off to the surface water (UNAF, 2018). Perkins et al. (2020) studied Fipronil and its metabolites and Imidacloprid from pet treatments in English rivers. These substances were found in up to 99% and 66% of the samples tested, respectively. The chronic risk quotient, the ratio of the amounts exposed to and the toxicity, indicates a high risk to the aquatic ecosystem. This also indicates a significant risk that bees pick up these substances by water collection. Although not stored and partially consumed, the biocides will be among the xenobiotic molecules circulating in the colony. The importance of this route of exposure for pesticides and biocides is also a topic in the expert groups working on the bee guidance (EFSA) and pollinator guidance (ECHA) and is potentially part of the guidances. The definitions of pesticide and biocide overlap. A 'pesticide' is something that prevents, destroys, or controls a harmful organism ('pest') or disease, or protects plants or plant products during production, storage and transport. The term includes, amongst others: herbicides, fungicides, insecticides, acaricides, nematocides, molluscicides, rodenticides, growth regulators, repellents, rodenticides and biocides (<https://ec.europa.eu/food/plant/pesticides>). Biocides, which are intended for non-plant issues to control pests and disease carriers such as insects, rats and mice do not fall within the remit of EFSA. This is an ECHA topic.



2.4 Wax route

Beeswax is a lipophilic matrix that binds non-polar substances. The vast majority of pesticides and other organic pollutants have a lipophilic nature, and therefore they are susceptible to migrating to the wax inside the bee hive and hence accumulate. A large number of pesticide residues can be accumulated and retained in wax for long periods of time due to a lack of elimination, reaching concentration values up to mg kg^{-1} (Lozano et al., 2019). However, the physicochemical properties and chemical stability of each substance influence the extent to which it accumulates, with significant variations among different contaminants.

2.5 Propolis route

Propolis is a lipophilic, resin-like material which bees collect from plant exudate and is used mainly for sealing the bee hive. Although it contains some wax, it behaves differently and is used differently in the bee hive. The value of propolis to medicine and human health has long been recognized in that it is an antimicrobial, antioxidant, anti-inflammatory and antitumour product (Burdock, 1998; Bankova et al., 2000). Due to its lipophilic capacity, stickiness and its exposure to the atmosphere while on the plants as well as inside the honey bee colony, propolis accumulates higher amounts of acaricides, pesticides and heavy metals when compared to honey or pollen or even wax (Pareja et al., 2011; Maragou et al., 2016, 2017; Gérez et al., 2017), a fact that makes propolis a very good matrix for environmental monitoring studies. Honey bees collect propolis almost all year round, but higher quantities are collected when the colony is in need and there are particular plants or flowering buds available. Special plastic sheets are available in the market for collecting propolis, where the bees collect high amounts of propolis which are clean from wax and other hive debris. The amounts of propolis collected are strongly colony and region dependent.

2.6 Loose particles' route

Another group of contaminants and micro-organisms, although not bound to the food, find their way throughout the colony. These contaminants, like particulate matter (fine dust), plant pathogens and molecules that remain on the bee's body after direct contact with flowers during food collection, or along with water collection, will circulate in the colony through trophallaxis and auto- or allogrooming. Whereas pollen collectors brush the pollen and particles actively into the corbiculae during foraging, nectar foragers do not do that (Westerkamp, 1991). Based on pollen distribution studies, within a few hours all of the in-hive bees carry the collected particles to a greater or lesser extent (Paalhaar et al., 2007; Free & Williams, 1972; DeGrandi Hoffman et al., 1986). The residence time in the colony is not known for all these materials. It is known that the residence time vital of *Erwinia* bacteria on honey bees is some days (de Wael, 1988; Alexandrova et al., 2002). The residence time of pollen on the bees in the hive is at least 10 days (Michielsen, 2017). This 10-days period is an indication of the in-hive residence time of loose particles. Taking this residence time into account, the sampling frequency in a bee-monitoring program must therefore be at least bi-weekly.

3 Bee-monitoring matrices

A matrix is a substance that carries e.g. xenobiotics or plant pathogens and is not altered by these xenobiotics or micro-organisms. For example, nectar is a matrix of systemic pesticides and pesticide molecules deposited directly in the flower during spraying or as a result of drift. Pollen is a matrix of particles and molecules that are physically or chemically bound to its surface as a result of direct spraying or airborne deposition. Beeswax is a matrix of lipophilic molecules that enter the colony via nectar, pollen, or



directly via contaminated bees. The honey bee itself is a matrix of pollen grains, plant pathogens, and non-biological particulate matter stuck to the branched hairs and food collecting parts of the bee. The pollen forager is focused on combing as much pollen as possible into the corbiculae, and exhibits frequent auto-grooming during foraging, whereas the nectar forager exhibits non-frequent auto-grooming.

3.1 Bee matrices' efficacy for monitoring of pesticides

In a review by Johnson et al. (2010) it is clearly shown that pollen is by far the most important vehicle for pesticides to enter the colony. Pooling together all data in this paper, the median amount of pesticides in wax, pollen, bees and honey was 36, 61, 2 and 0.1 ppb respectively. In the studies where wax and pollen were analysed simultaneously (from the same colony), the median amount of pesticides in wax was 31 and in pollen 108 ppb. This difference in efficacy can be explained by the routes described. Honey is obviously a poor matrix because of the quick dilution of nectar in the sprayed flowers, the in-hive dilution with non-contaminated nectars and the temporal in-hive mixing of the nectars during honey processing (Schatz & Wallner, 2009). Also, bees themselves are not the most efficient matrices for bio-monitoring because of autogrooming during the collection flight, in-hive auto- and allogrooming, detoxification in the alimentary tract of the bees and the in-hive trophallaxis.

The practice and nature of sampling by the different matrices differs significantly. Every in-hive bee sample is by definition a mixture of all age cohorts of bees, which all have different levels of exposure (Steen et al., 2012). Forager bees are directly and the in-hive bees indirectly exposed by trophallaxis and physical contact. Also, hive entrance samples comprise bees that have visited different locations (Visscher & Seeley, 1982; Waddington et al., 1994). Wax is a rather efficient matrix. However, it has the disadvantage that it is selective in its binding capacity and it is, because of strong binding and the long in-hive residence time of months to years, an archive from which it is impossible to distinguish between recent and older pesticide influxes.

Pollen is the most efficient bee matrix. Working with trapped pollen, there is a tension between the duration of trapping and the impact this has on the colony; trapping for too long may have negative impact on the colony, whereas short activation of the pollen trap will result in snapshot data as the flowering period of most flowers varies from only days to some weeks. Because of non-invasive sampling considerations and of aiming to affect the bio-monitoring tool "honey bee colony" as little as possible, activation of the pollen trap should be limited to a maximum of four days, if weather conditions are such that no pollen is collected in a single day of collection. On the other hand, beebread is the accumulation of collected pollen. As most beebread is consumed in a 2-week period, it mostly holds recent information. However, beebread consumption depends strongly on the colony's condition, particularly the brood status (Crailsheim et al., 1992). As beebread storage is randomly distributed in the colony over the edges of the brood nest, it is very time consuming to collect this material, and it also has a disturbing impact on the colony. Furthermore it is much more invasive and time-consuming to collect compared to pollen trapping.



4 Non-biological passive samplers

Besides these biological matrices, non-biological samplers have been developed. The Beehold tube, a tube lined with sticky polyethylene glycol (PEG) on the inside through which bees enter the hive, is a matrix of particles found on the exterior of the entering bees (Steen et al., 2018). The APIStrip is an in-hive sampler. It is a polystyrene strip covered with Tenax (a porous absorbent polymer), which is placed between two combs in the centre of the colony. The Tenax is very firmly bound to the plastic strip, and binds pesticide molecules that circulate inside the colony on the bees' exterior, as loose particles and in gas form. In environmental research, these types of non-biological matrices are referred to as "passive samplers". The fabrication, and preparation for analysis of the APIStrip is described in [Murcia-Morales et al. \(2020\)](#). The mode of application is presented in this guideline in [chapter 3 Protocols, subchapter 3.2](#) Protocol for the Apiculturalist Citizen Scientist for honey bee colony monitoring for pesticides. The fabrication, mode of application and preparation for analysis for pollen and bacteria of the Beehold tube is described in Steen et al. (2018). The preparation for analysis of pesticides from the Beehold tube and from the APIStrip is presented in [Report year 1 \(2019\)](#) of the INSIGNIA pilot study on environmental monitoring of pesticide use through honeybees, p 38-39.

In Table 1.1, the in-hive storage times/ archive periods, in-hive routes, matrices and their suitability as a bee-monitoring matrix are summarised.

Table 1.1. Storage periods and consequences for sampling

	In-hive archive period	In-hive route	Matrix for bio-monitoring of contaminants or pollen availability
Nectar	0 to X days	Trophallaxis, physical exchange, temporal cell storage	Research for direct exposure to pesticides, not for bio-monitoring ¹
Honey	∞ days	No in-hive route, cell storage	
Pollen/ Beebread	5 days	Mouthparts, physical exchange, cell storage	Pesticides Pollen diversity
Water	0	Trophallaxis, physical exchange	Not a matrix because of no storage
Wax	∞ days		Some of the pesticides, depending on chemical characteristics ²
Propolis	∞ days		Some of the pesticides, depending on chemical characteristics and amounts collected ³
Loose Particles	up to 10 days	Physical exchange	Particulate matter, heavy metals, bee parasites, bee pathogens, micro plastics

¹ Due to pesticide degradation and dilution, pesticide levels decrease rapidly in temporally stored nectar storage (Schatz & Wallner, 2009). Honey stomach sampling shows direct exposure to this source.

² Wax is considered as a non-reliable pesticide matrix for bee-monitoring because of selective binding capacity to pesticides and dilution by new wax.

³ Propolis is considered as a non-reliable pesticide matrix for bee-monitoring because of its colony- season and regional differences in availability.



5 Invasive and non-invasive honey bee colony sampling

Sampling the honey bee colony can be done by various means, either invasively (at the expense of the colony) or non-invasively (without affecting the colony). As pollutants/xenobiotics bio-sampled by the honey bee colony form an intrinsic part of the bee food and the in-hive routes by trophallaxis and allo- or autogrooming, the xenobiotics are present in the food matrix and bee matrix. Invasive sampling involves taking bees or food to an extent that the colony itself is negatively affected. Non-invasive sampling does not negatively impact on the colony. Impact on the colony should be balanced with efficacy of the sampling. There are no established standards for taking bees from a colony without negatively affecting this superorganism, however recommended sampling is a maximum of 3% of a certain cohort in order to consider sampling as non-invasive if using a 3-weekly sampling frequency. Trapping pollen is to a certain extent invasive, but the colony can compensate for this loss of pollen. Furthermore, there is a huge difference in trapping efficacy of different designs of pollen traps, ranging from 10% (Free, 1967) to 54% (Vassiere et al., 1996).

5.1 Apiary sampling, colony sampling, sample pooling

Colonies in an apiary divide themselves over the surroundings with only partly overlapping foraging areas (Waddington et al., 1994). Consequently, it takes more than one colony to cover an area and the pooled apiary samples are thereby, by definition, a spatial sample.

Focusing on the environmental study of pesticides or other pollutants, the worldwide network of honey bee colonies provides the ultimate means to apply these colonies for bio-sampling, both for large-scale overview monitoring and also for detailed small-scale monitoring. Bio-monitoring means structured repeated bio-sampling. Focusing on large-scale studies, the land use and colony density determine the "region" and not the national borders. For example, it makes more sense to combine the Northern European plains of Denmark, the Netherlands, Flanders, and Northern Germany as one region; likewise for Alpine regions, and Mediterranean countries could be another region. Sampling points should be spread evenly over the region, depending on the study objective.

6 Apicultural citizen science

Citizen science is research, conducted partly or completely by citizens and non-professional scientists, often in collaboration with and under the direction of professional scientists and scientific institutions (WUR, Wikipedia). Apicultural citizen science is citizen science focused on beekeepers, who sample their colonies or make regular recordings of hive parameters such as the number of bees, colony weight, foraging periods etc. The main conditions for successful citizen science in general must be clear, unambiguously interpretable instructions about sampling, storage, and shipping etc. For Apicultural citizen science, colony conditions and beekeeping practices are additions to the general main conditions. Commitment of the beekeeper and support for the beekeeper are also essential.



7 The INSIGNIA study

7.1 Objective of the INSIGNIA study

The INSIGNIA study was a proposal submitted to an European Commission call on “*Environmental monitoring of pesticides use through honey bees*”. The aim of the study was to develop a best practice protocol for Apicultural citizen science and to conduct “*A pilot study on the best practices for a European wide monitoring program with honey bee colonies in an Apicultural citizen science (CS) setting to study pesticide use and exposure of honey bees and investigation of pollen sources*”.

The INSIGNIA consortium formulated the following goals:

- To develop and test the Apicultural citizen science protocol for applying honey bee colonies for bio-monitoring of pesticides and pollen in a citizen science study;
- To set-up and test the organisation of an Apicultural citizen science study;
- To develop non-invasive sampling and therefore to develop and test (in-hive) passive sampler(s);
- To test and optimise ITS2 metabarcoding for pollen identification;
- To model pesticide exposure and undertake risk mapping / risk modelling;
- To evaluate the CORINE database for its applicability to bees;
- To list pesticides detected in this study;
- To list bee-collected pollen origin;
- To make a list of applied non-legislated pesticides detected in this study.

These goals have been achieved and are summarised in Table 1.2. Table 1.2 lists the deliverables with rationalisation of the choices made, the results and scientific and layman publications. The table contains links to the documents accessible through the INSIGNIA website. This website will be open for seven (7) years after the closure of the INSIGNIA 2018-2021 study.

The study objectives, tests, outcomes, references and final recommendations of the 2020 study are presented in Table 1.2.

Table 1.2. Summary of deliverables and output

Study objectives	Tests	Outcomes	Recommendations	References/ deliverables
Pesticide degradation	In pollen room temperature versus freezer	freezer storage, cold transport	Freshly collected pollen for pesticide detection to be stored in freezer and cold transport	Deliverable 3.2. See link below Deliverable 1.2 See link below Report 2019 See link below
	In APIStrip, room temperature versus freezer	Dry and dark, room temperature	Wrapped in Al foil and dry and dark, room temperature storage and transport.	
ITS2 pollen	In pollen	Freezer storage, cold transport, 96% ethanol storage and transport	Freezer at CS location, cold transport to AC and lab. Possible transport from AC to lab in 96% ethanol	
	In pollen	Room temperature + silica-gel storage	Dark dry storage in plastic bag + silica-gel.	
Pesticide matrix	APIStrip	Best matrix	Recommended	
	Beehold tube	Low functioning matrix	Not recommended	
	Beebread	Good matrix, comparable to	Not recommended for practical sampling reasons	



1 - Rationale



Study objectives	Tests	Outcomes	Recommendations	References/deliverables
		APIStrip but less consistent		
	Trapped pollen	low functioning matrix	Not recommended	
Number of colonies for pooled apiary sample	3 colonies per apiary	Minimally 2 colonies	Minimally 2 colonies	Report 2019
Monitoring duration	6 months	Minimally 6 months based on pesticide findings	6 months	Report 2019
Sample frequency	Bi-weekly (fortnightly)	Minimally bi-weekly	Minimally bi-weekly	Report 2019
Sample transport	Post, Courier	Courier	Courier	Practical and reliable, country dependent
Sample labelling			Sampling date Sample code Matrix Beekeeper ID Colony ID	Outcome evaluation 2019 and 2020 study
APIStrip storage	Storage test	No difference in pesticide degradation as function of frozen or room temperature storage.	Room temperature storage and ambient transport	See link
Pollen storage	Preservation test			See link
APIStrip 2020 evaluation	APIStrip 2019-2020			See link

7.2 Important links

7.2.1 Deliverable 1.2

[Report on the best monitoring practice based on y1 to be ring-tested](#)

7.2.2 Deliverable 3.2

[Citizen science investigation for pesticides in apicultural products](#)

7.2.3 Report year 1

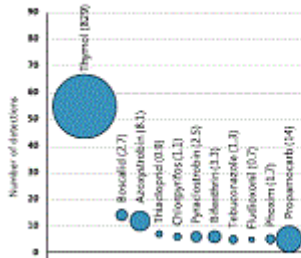
[Final Report Year 1](#)



REPORT YEAR 1 (2019) OF THE INSIGNIA PILOT STUDY ON ENVIRONMENTAL MONITORING OF PESTICIDE USE THROUGH HONEYBEES

7.2.4 Scientific publications

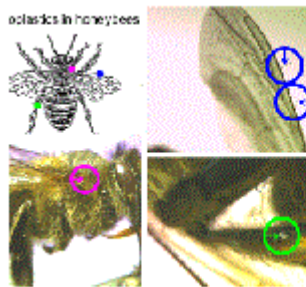
[APIStrip, a new tool for environmental contaminant sampling through honey bee colonies](#)



Publication. APIStrip, a new tool for environmental contaminant sampling through honeybee colonies.

2020. APIStrip, a new tool for environmental contaminant sampling through honeybee colonies. María Murcia-Morales, Jozef J.M. Van der Steen, Flemming Vejsnæs, Francisco José Díaz-Galiano, José Manuel Flores, Amadeo R. Fernández-Alba. *Science of the Total Environment* 729 (2020) 138948

[Honey bees as active samplers for microplastics](#)



Publication. Honeybees as active samplers for microplastics

2020. Honeybees as active samplers for microplastics. Carlos Edo, Amadeo R. Fernández-Alba, Flemming Vejsnæs, Jozef J.M. van der Steen, Francisca Fernández-Piñas, Roberto Rosal. *Science of The Total environment*. Volume 767, 1 May 2021, 144481

[An innovative home-made beebread collector as a tool for sampling and harvesting](#)

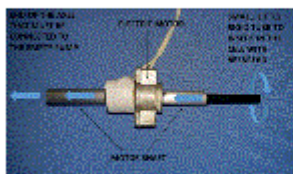


Figure 3. Beebread collector equipped with a small electric motor.

Publication. An innovative home-made beebread collector as a tool for sampling and harvesting.

Loglio, G., Formato, G., Pietropaoli, M., Jannoni-Sebastianini, R., Carreck, N., & van der Steen, J. (2019). An innovative home-made beebread collector as a tool for sampling and harvesting. *Bee World*, 96(1), 16-18

[How and why beekeepers participate in the INSIGNIA citizen science honey bee environmental monitoring project](#)

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RESEARCH ARTICLE

How and why beekeepers participate in the INSIGNIA citizen science honey bee environmental monitoring project

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Beekeepers as citizen scientist investigate the environment of their honey bees



11. INSIGNIA: Beekeepers as citizen scientists investigate the environment of their honey bees

Beekeepers can be valuable volunteers in large scale research studies. They own honey bee (*Apis mellifera*) colonies, have basic equipment, and are knowledgeable about apiculture. In the INSIGNIA project, citizen science beekeepers collected samples from their colonies over two seasons. Several protocols and different sampling devices for beekeeper participation in monitoring the environment for pesticides and bee forage sources were developed and tested, including all

steps until sample analysis in the laboratory. The study was accompanied by studies investigating the motivations and skills of beekeepers volunteering as citizen scientists. The samples and information collected with the help of citizen scientists will be used to model the biodiversity of pollen sources, risk mapping for pollinators and environmental pollution in different environments.

7.3 The set-up and testing

The set-up and testing of the organisation of an Apiculturalist Citizen Scientist study

In the INSIGNIA study, we developed a reliable well-functioning interaction process between the apiculturalist and the scientists. It is a format in which the Study Director (SD) is the pivot between the sponsor, the Apiculturalist Coordinator (AC), the laboratories, the statisticians and modellers and the stakeholders. The AC is the pivot between the SD and Apiculturalist Citizen Scientist (CS). The data integrity and data communications are process related tasks assigned to particular participants to be the Data Curator (DC) and Communication Curator (CC).

The practical elaborations of the goals set, are presented in

- Study set-up
- Protocols
- Methods

7.4 Non-invasive sampling

Non- invasive sampling and the development and testing of in-hive passive sampler(s) (2019 study)

In 2019 we tested two bee-matrices and two in-hive samplers in four countries with four to five apiaries with three colonies per apiary in ten bi-weekly sampling rounds, from May to September. For non-invasive sampling and pesticide matrix testing, we applied two bee-matrices, namely trapped pollen and beebread. The two non-biological passive samplers were applied as pesticide matrices, namely the [Beehold tube and the APIStrip](#). The latter has been developed within the INSIGNIA study (Murcia-Morales et al., 2020).

In 2020, the 2019 sampling phase was followed by a large-scale ring-test study in nine countries (Latvia, Denmark, UK, Ireland, Belgium, France, Austria, Italy and Greece), with nine apiaries per country. In the 2020 study, the best and most practical pesticide matrix identified from the results of the sampling in 2019, the APIStrip, was used for pesticide monitoring, and trapped pollen was used for ITS2 metabarcoding. Prior to the 2019 sampling, the best practices of sample storage and shipping were tested. The best practice storage studies continued in 2020 and 2021 based on progressive insights.

In 2019, the trapped pollen, beebread, Beehold tubes and APIStrip were each analysed for pesticides per colony to collect data for statistical analysis to identify the best matrix



for pesticide detection and for identification of botanical origin of pollen via ITS2 metabarcoding.

According to the proposal in the Grant Agreement, in 2020 we applied pooling of the samples from different colonies within the apiary. The rationale behind this apiary-pooling was that the focus of this bio-monitoring study is on the environment, and as honey bee colonies in an apiary each cover different foraging areas, the apiary provides more comprehensive and accurate information about the environment than a single colony.

The number of colonies, sampling frequency and best matrices were chosen based on the individual colony data of the statistical analyses for 2019. Based on the individual colony data, we decided to sample 2 colonies per apiary in a bi-weekly sampling scheme for pesticides with the APIStrip in-hive exposure, and for pollen with a one-day pollen trapping every two weeks. In the 2020 sampling, the pollen samples of both colonies were pooled per sampling date. The details of pooling and shipping to the labs are described in chapter 2-Study set-up and 3-Protocols.

7.5 Pesticide residue analysis and pollen ITS2 metabarcoding

7.5.1 The pesticide residue analyses

In both laboratories of the Benaki Phytopathological Institute (Greece) and the University of Almeria (Spain), the residue extractions and analyses were performed identically on all samples. The division of samples between laboratories was agreed prior to the beginning of the sampling rounds. The extraction of the APIStrip was performed in acetonitrile according to the established procedure (Murcia-Morales et al., 2020). For the vast majority of compounds, the limits of quantification were set as 0.5 nanograms per gram of Tenax (parts per billion, ppb). When a pesticide residue was detected, but the calculated concentration was below 0.5 ppb, it was reported as "< LOQ", meaning that it was present in the sample at trace levels. The analysis list comprises 272 pesticides, included in the multiresidue methods of both laboratories. The details/ analysis protocols are presented in chapter 4-Methods.

7.5.2 Botanical identification of mixed-pollen samples by ITS2 metabarcoding

Molecular identification of the botanical origin of pollen, in mixed bee-collected pollen pellets, beebread and in the non-biological matrix Beehold tube, was performed by DNA metabarcoding using high-throughput sequencing (HTS) with the nuclear barcoding marker ITS2 (internal transcribed spacer 2 regions of nuclear ribosomal DNA). To that end, first, DNA was extracted from pollen using the NucleoSpin Food kit of Macherey-Nagel with an in-house-made combination of zirconia beads of varying sizes to assure exine rupture of pollen grains of varying sizes. The DNA extracts were then analysed using the ITS2 metabarcoding protocol. Briefly, DNA extracts were PCR-amplified with the universal primers ITS-S2F (Chen et al., 2010) and ITS-S4R (White et al., 1990), as part of an oligo scaffold that incorporates the MiSeq-specific adapters and the unique indexes for multiplexing. PCRs were run in triplicate to account for PCR bias. Library preparation for HTS was performed using a dual-indexing approach. The mixed pollen samples were sequenced on the Illumina MiSeq platform using 2×250 cycles v2 chemistry for pools by approximately 380 samples. Analysis of sequence reads and taxa assignments were performed using an improved and updated ITS2 reference database and a bioinformatics pipeline modified from Sickel et al. (2015). The reference database includes ITS2 sequences representing bee plants collected by the INSIGNIA consortium. Prior to large-scale implementation of this metabarcoding protocol to samples collected



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by citizen scientists in 2019 and 2020, sample storage / transportation, DNA extraction, PCR conditions and primers were assessed and the protocols were optimised accordingly. The protocols are presented in chapter 4-Methods.

The ITS2 reference library

The ITS2 sequence reads generated in the Illumina MiSeq platform for each mixed-pollen sample were aligned with the sequences of the reference library, and taxonomic classification was performed using the UTEX and RDP classifiers embedded in the bioinformatics pipeline (Sickel et al., 2015). The success and accuracy of taxonomic identification depends, to a large extent, on the quality and breadth of the reference library; ideally, accurately curated sequences of every single plant species visited by the colonies under study would be represented in the reference library. Additionally, the more geographically suited that the reference library is, the more accurate can be the classification at the species level; ideally, the library would only include ITS2 sequences for the plant taxa occurring in the area visited by the colonies under study. To address these issues, first the ITS2 reference library assembled by Sickel et al. (2015) was updated with ITS2 sequences recently deposited in GenBank. Due to higher plant diversity, and possibly to lower sequencing efforts, Mediterranean species are probably underrepresented in GenBank. Although a greater breadth of the ITS2 database is expected for northern Europe, not all species are represented in GenBank. Therefore, in an attempt to improve taxonomic classification of the mixed-pollen samples, bee plants (list prepared by INSIGNIA) identified to the species level were gathered from across Europe by ACs and shipped dried to IPB for Sanger-sequencing of ITS2. The newly developed sequences were added to the ITS2 reference library. Secondly, the GBIF (<https://www.gbif.org/>) and the EurMed Plant (<https://www.emplantbase.org/home.html>) databases were used for generating the lists of plant taxa occurring in the INSIGNIA countries.



7.5.3 Pesticide exposure risk mapping

Risk modelling / evaluation of the CORINE database for its applicability to bees

In order to process the acquired monitoring data into risk maps, two modelling approaches were tested. Firstly, an approach applying mechanistic modelling of landscape-level pollen foraging of honey bees was used. This was based on the [CORINE](#) database, and an assessment was made of how well landscape-level exposure risk modelling can be performed using CORINE data. An adapted version of the [nectar foraging model](#) of Baveco et al. (2016) was used to analyse the landscape around the hive location for the distance-weighted amount of CORINE landcover classes that were potential sources of pollen or pesticides returned to a hive, as indicated by the results of the monitoring campaign. Secondly, a statistical / machine-learning based approach was used where the relationships between CORINE landscape characteristics and exposure risk and pollen diversity were directly obtained from the data collected in INSIGNIA. With the model, predictions can be made for a new location at the country-scale. Both modelling approaches provide the means to produce risk maps of honey bee pesticide exposure, as well as maps of pollen diversity. In addition, within the application of these two modelling approaches the potential of the CORINE data set for use in such detailed spatial modelling was evaluated.

7.5.4 The Apiculturalist Citizen Scientist (CS), sociological study and profile

The mission and definition of citizen science is vividly debated, but normally, mainly scientists and policy stakeholders have voice in this debate. In contrast, in INSIGNIA we drew attention to how the citizen scientists themselves actively construct their own roles within the project, in relation to both their histories and the project's scientists. Drawing on a set of in-depth interviews with participating Austrian beekeepers in the 2019 study season, we showed that even within a small, relatively homogeneous sample of participants there is considerable diversity in how the citizen scientists conceptualise their roles.

In the 2020 study season several traits and attitudes of 69 volunteering beekeepers from ten countries were surveyed. Beekeepers volunteering to participate in the INSIGNIA study were mostly male and older than 55 years. Participants can be rated as experienced in beekeeping, as shown by the number of colonies they manage (on average 70) and a mean of circa 20 years of beekeeping experience. Knowing the target group and their motivation will help in designing successful citizen science studies and recruiting volunteers. We found that the motivation of beekeepers was similar to that of other studies on environmental volunteer motivations, with helping the environment and contributing to scientific knowledge being strong motivators. Our results suggest that receiving laboratory analysis results of the samples from colonies is the most meaningful way of appreciation for beekeepers, but not their primary reason for participation. Citizen scientists ranked environment, beekeeping and science as the areas where they expected the study to be most impactful. A citizen scientist beekeeper in this study on average spent circa 12 working hours on sampling throughout the season. After the study, most volunteers would participate in similar studies or recommend participation to other beekeepers. We suggest that beekeepers have a great potential to help research in many ways. The next steps for citizen science in honey bee research would be to create more collaborative or co-created research projects, where beekeepers are also involved in higher-level tasks like identifying research questions or study design.

7.6 Communications

7.6.1 In-consortium

The in-consortium communication has been organised by frequent virtual meetings about general and specific subjects, which has proved extremely useful for keeping the study on track, strengthening the network, and for planning and coordination. The minutes of these meetings have been approved by the participants and then uploaded in the INSIGNIA Cloud. A notification of this upload was sent to all participants. The cloud was used for hosting a blog, and for relevant documentation, publications and administrative documents.

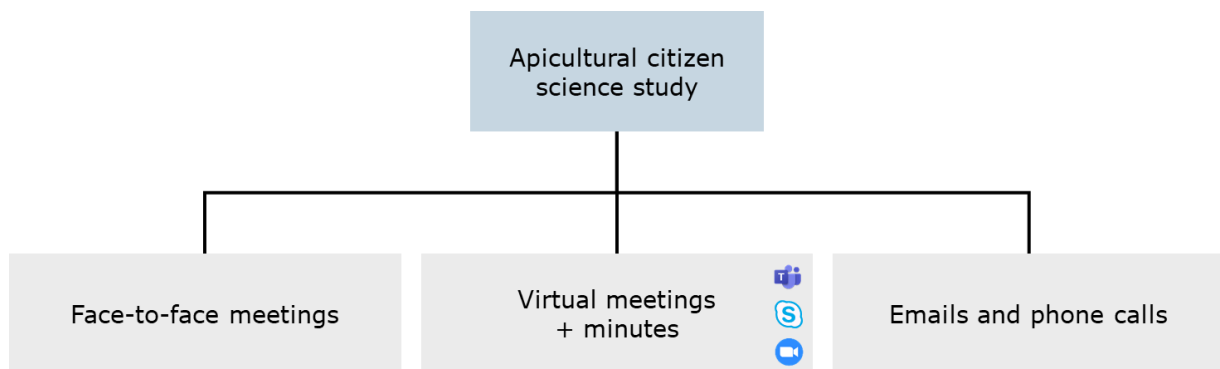


Figure 1.1. In-consortium communication

7.6.2 Beekeeper Instruction - tutorials and other means of instruction

From the Apiculturalist Coordinator (AC) to the Citizen Scientist beekeepers (CS)

It is the responsibility of the Apiculturalist Coordinator (AC) to ensure that the Citizen Scientist beekeepers (CS) are fully trained and understand exactly what they have to do. In the INSIGNIA study, instruction was achieved through an integrated approach via the written CS Picture Manual, instructional tutorial videos, face to face meetings, virtual training sessions, emails and telephone calls. The CS were recruited for their high level of general beekeeping experience and competence, but nonetheless INSIGNIA required them to learn additional skills, especially in relation to the care taken in collecting, labelling and storing samples.

In both 2019 and 2020 a number of instructional videos were produced, showing in detail the exact procedures required for sample collection, and these were placed on the INSIGNIA YouTube Channel. These were originally in English, but several alternative versions were produced in other local languages such as Danish, German, Italian and Latvian. The approach taken by the individual ACs depended on circumstances and varied from country to country. If the CS all lived fairly close to the AC, training through meetings to discuss what was required was the preferred approach; in some cases this was on an individual basis or alternatively in groups, depending on the geographical location. If the CS were more widely distributed this was less feasible, and telephone instruction took place.

In 2020 the global Covid-19 pandemic in most cases prevented local meetings of the CS in each country, but virtual training and regular feedback sessions did take place using Zoom, Skype and Microsoft Teams. Throughout the study, the ACs made themselves available to answer any queries from the CSs by telephone, email or through comments made in the regular LimeSurvey questionnaires.

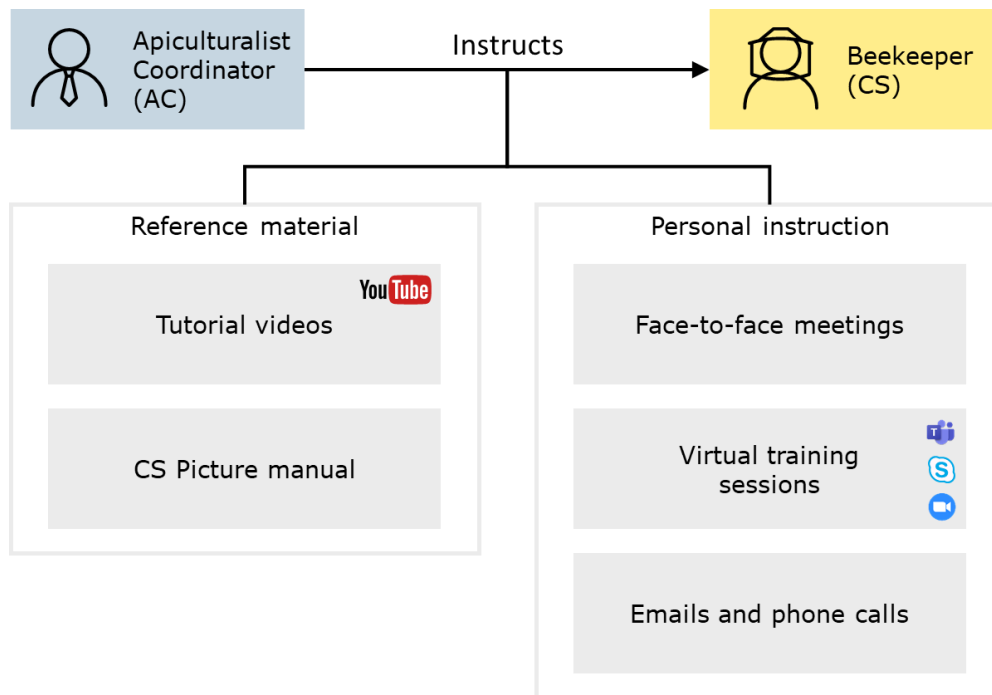


Figure 1.2. Communication from AC to CS

7.6.3 AMA sessions (Ask Me Anything)

These AMA sessions, intended to keep the participating beekeepers updated, were complementary events to the regular contact of the beekeepers with the AC. The AMA meetings were virtual meetings held with all the beekeepers participating as Apiculturalist Citizen Scientists in INSIGNIA in each country. The subject matter of these meetings included objectives of the study, updates on results and / or whatever was relevant to the citizen science. These proved to be useful for helping to ensure the ongoing motivation and commitment of the beekeeper citizen scientists.

7.6.4 Communication with stakeholders by means of social media and journals

In a beekeeping citizen science study such as this, the stakeholders include other beekeepers, the general public and decision makers. In INSIGNIA, a strategy for dissemination to these stakeholders was established from the outset. The website <https://www.insignia-bee.eu> was set up as one of the first study deliverables, to become the core of the strategy. As well as information about the study and the members of the INSIGNIA consortium, the website had an active blog and links to social media including Instagram, Facebook and YouTube. The regular blog entries and social media posts were written by many members of the INSIGNIA team, and were intended to inform both the participating CSs, and other stakeholders such as other beekeepers and the general public.



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Lectures, presentations and talks about the study have been given by various team members at a range of meetings, from international platforms such as the Apimondia International Apicultural Congress in Canada, the Apimondia Bee Health Symposium in Italy and the COLOSS Asia Conferences in Thailand and Japan, through national and regional beekeepers meetings to local meetings of individual beekeepers' associations. Several articles arising from the study have been written and then translated into many local languages for publication in many local beekeeping journals.

A regular series of "Notes for stakeholders" have also been produced on a range of topics, and were distributed via a mailing list comprising beekeeping associations, environmental journalists, editors of beekeeping magazines, relevant government ministries, and relevant non-governmental organisations.

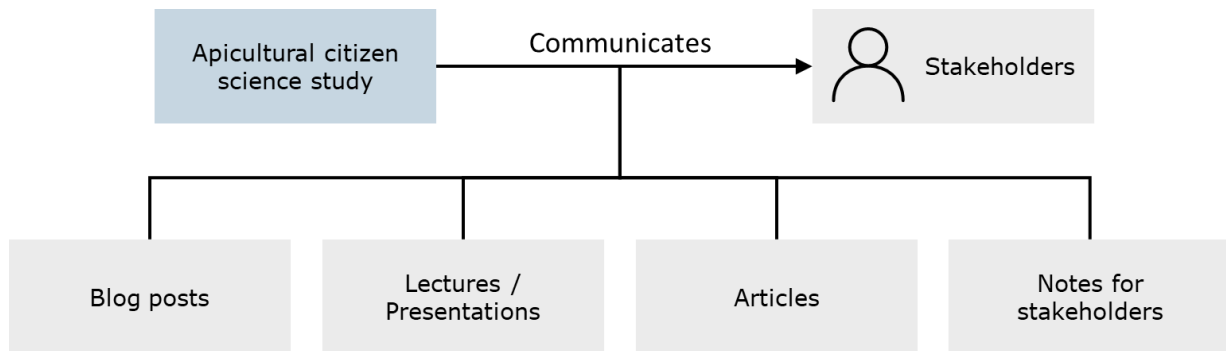


Figure 1.3. Consortium outward communication



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2 - Study set-up

Tasks - responsibilities - workflows of the Study Director, Apiculturalist Coordinator, Data Curator and Communication Curator





2 - Study set-up



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Introduction

The Study Director (SD) has the responsibility for the study in all its aspects. The SD coordinates and participates in writing the study plan with the Apiculturalist Coordinator (AC), the laboratory specialists, statisticians and modellers involved, according to scientific standards. He / she is the pivot between the sponsor, the AC, the analytical laboratories, the statisticians and modellers and the stakeholders. This function comprises responsibilities for study coordination, study progress, study quality, coordination of CS instruction, data integrity, data storage, reporting to the sponsor about study content and finances, organising the communication routes, both internal and external, and the final study report.

The Apiculturalist Coordinator (AC) is responsible for the practical implementation and the course of the citizen science study in a particular country or region. He / she is the pivot between the SD and the Apiculturalist Citizen Scientist (CS). The function responsibilities comprise the recruitment of the apiculturalist CS, the content and assembly of the toolbox, the sample quality in terms of labelling, storage, transfer from CS to AC and subsequently from the AC to the laboratories, the sample flow recording and layman reporting to the participating CS.

Data integrity and communication, both internal and external, are key processes in the Apicultural citizen science study. Although the SD is responsible for these processes and the AC for the practical implementation, these processes have such a comprehensive range that specific tasks should be delegated to control the process. The Data Curator and Communication Curator tasks and processes are described in a separate paragraph "Data integrity and communication".

The study plan process is worked out in the paragraph "Study Plan". The role of the SD and AC is depicted in a flow chart and process descriptions are shown in the paragraphs "Study Director" and "Apiculturalist Coordinator". Data integrity and communication processes are presented in the paragraph "Data integrity and communication".

List of abbreviations

SD: Study Director
AC: Apiculturalist Coordinator
DC: Data Curator
CC: Communication Curator
CS: Apiculturalist Citizen Scientist



1 Study Plan

Every research study starts with a question or hypothesis, formulated in a study proposal. A key process to turn a study proposal into a study plan is the question articulation, in other words, what exactly is the question or hypothesis? Next, this defined research question must be translated into a study plan.

In practice, a sponsor formulates a research question and an institute or consortium will, after verification of the definite study question, make the study plan.

In Apicultural citizen science, the study plan process is an interplay between a SD, an AC, and the scientific specialists (e.g. chemists, geneticists, molecular biologists, statisticians, modellers, environmentalists) involved in the study. The AC is the beekeeping specialist. In this interplay the practical possibility, scientific standards, financial and manpower possibilities are weighed, in order to come to a balanced study plan with timelines, responsibilities and processes. This study plan is submitted to the sponsor who posed the research question.

In an Apicultural citizen science study plan, the key process is the collaboration and interactions between the SD and the AC, a strict sampling or recording timeline, a well-defined protocol for the beekeepers, the actual citizen scientists, and an adequate toolbox for the beekeepers to do the job. The study plan comprises the layout of the study, the study course, the responsibilities on all levels - from beekeeper, via the AC through to the SD - and the processes like the instructions, communication, recordings and reporting, research integrity and overall data integrity.

2 Research integrity*

Research integrity means conducting research in a way which allows others to have trust and confidence in the methods used and the findings that result from this. Researchers are expected to abide by the standards of research integrity:

- Honesty in all aspects of research including
 - Presentation of research goals, intentions and findings;
 - Reporting on research methods and procedures;
 - Gathering data;
 - Using and acknowledging the work of other researchers;
 - Conveying valid interpretations and making justifiable claims based on research findings.
- Scrupulous care, thoroughness and excellence in research practice
 - In performing research and using appropriate methods;
 - In adhering to an agreed protocol where appropriate;
 - In drawing interpretations and conclusions from the research;
 - In communicating the results.
- Transparency and open communications
 - In declaring conflicts of interest;
 - In reporting of research data collection methods, including the re-use of data collected for other purposes;
 - In analysis and interpretation of data;
 - In making research findings widely available, including sharing negative results as appropriate;
 - In presenting the work to other researchers and to the general public.

- Care and respect for
 - All participants in and subjects of research, including humans, animals, the environment and cultural objects;
 - The stewardship of research and scholarship for future generations.

* *The definition of research integrity presented is quoted from the University of Bath, UK ([Definition of research integrity \(bath.ac.uk\)](http://bath.ac.uk))*

3 Study process

The study process is pictured in Figure 2.1, which shows the interactions between the key players in the process. These will be described in detail in the following paragraphs, the emphasis of this chapter being on the AC, with detailed instructions for best practice.

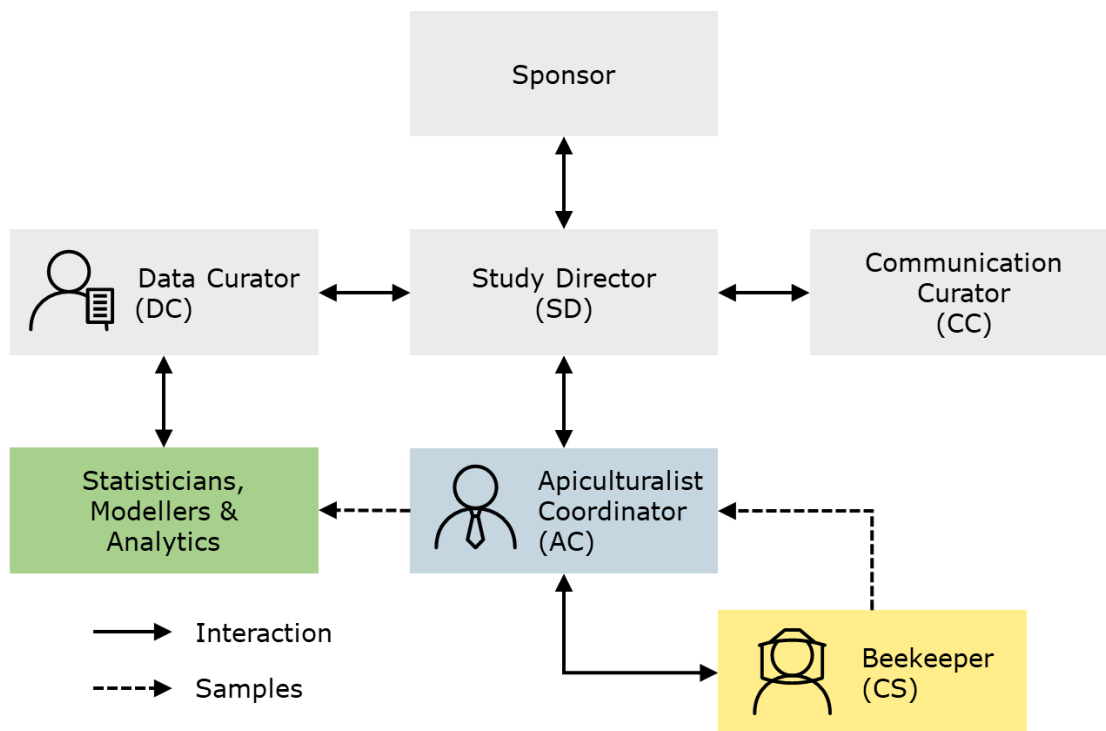


Figure 2.1. The study process and interactions of the key players

4 Study Director - tasks and responsibilities

The pivotal function of the SD is shown in Figure 2.2. The arrows are interactions that will be elaborated in bullet points.

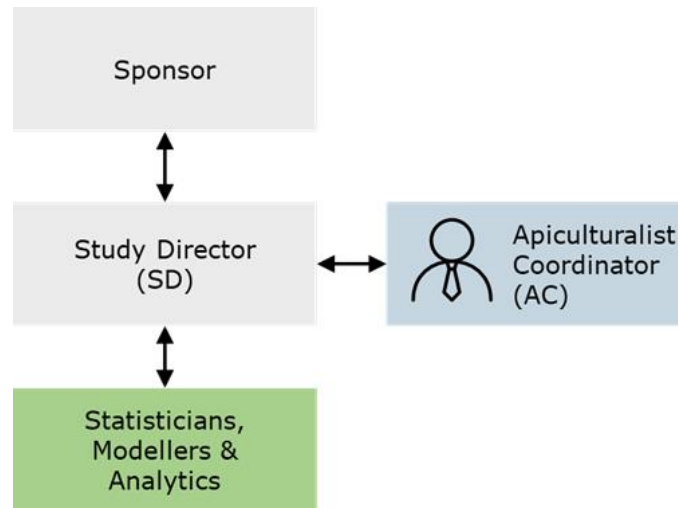


Figure 2.2. Links between the Study Director and other key players

The overall key task of the SD is to monitor the progress and the quality of the study in all aspects, to ensure data integrity and to take action when needed. He/she is also responsible for the coordination of the practical and scientific output and paper-writing process.

The SD function towards the sponsor comprises:

- Reporting according to a reporting plan / deliverable list, of
 - the study progress reports
 - the final report
 - the financial reports
 - other agreed deliverables
- Reporting in writing any deviations and amendments to the study plan, including the rationale of any amendment and the implications for the study plan and outcome;
- Monitoring the study progress and quality.

The SD function towards the AC includes:

- Frequent contact about all relevant study matters and study updates;
- Organising regular (virtual) meetings and making available the minutes on the study cloud or other relevant accessible storage tools;
- The internal communication in consultation with the Communication Curator;
- The coordination of the instruction of the beekeepers with all relevant tools;
- The direct contact with the beekeepers via the AC;
- All data integrity matters and updates concerning recordings and reporting in consultation with the Data Curator;
- The organisation of plenary meetings.



2 - Study set-up



The SD function towards the analytical laboratories, statisticians and modellers comprises:

- Frequent contact about the analytical process;
- Clarifying and resolving through discussion any issues that arise;
- The organisation and checks of the uniformity of the sample identifications;
- Providing the statisticians and modellers with the relevant data files, being entries of the data warehouse, in collaboration with the Data Curator;
- The organisation of the plenary meetings.

The SD function towards the stakeholders comprises:

- Organising the communication with the stakeholders, in consultation with the Communication Curator, via e.g. notes and bee journal articles;
- Being the addressee for the stakeholders.

5 Apiculturalist Coordinator - tasks and responsibilities

The AC is responsible for the practical implementation of the study plan and is the pivot in the Apicultural citizen science study, as shown in the flow chart in the section "Study process". In practice, the AC organises the local part of the study, that is the actual sampling in a particular area / region / country and all requirements for adequate sampling. The AC is a co-operator with the Data Curator and co-operator with the Communication Curator (paragraph "Data integrity and communication").

The AC interaction with the SD is shown in the SD paragraph.

The AC function towards the beekeeper comprises:

- Recruitment of the beekeepers;
- Assigning a CS number to each beekeeper;
- Providing the beekeepers with all information of the "why and how" of the study and its specific goals;
- Instruction about the study protocol with all appropriate tools;
- Providing the beekeepers with the toolbox to conduct the sampling and recordings;
- Being the contact person for the beekeepers;
- Informing the beekeepers about the study progress and results.

The specific AC tasks are elaborated in the subparagraphs below.

The AC function towards the laboratories comprises the following:

- During the study, the AC receives the samples taken by the beekeepers in pre-appointed time slots and under pre-appointed conditions (protocols). The AC checks the samples and labels and makes sure that the samples that will be sent to the laboratories have the correct label information;
- Sending of the samples, in conjunction with the analytical laboratories. The AC makes sure that the transport is carried out with a reliable postal service and /or courier, with tracking / tracing to follow the shipment;
- The AC keeps records of the sample materials sent, sample returns and transport to the laboratories, in the AC journal (record-keeping file);
- According to Good Laboratory Practice (GLP) the original label follows the sample to the laboratories, and these labels are stored by the laboratories along with the analytical results for a minimum duration of seven (7) years or other time period specified in the study plan;



2 - Study set-up



- The AC takes care of correct sample labelling and sample pooling if necessary;
- The AC is the intermediary between the laboratories and beekeepers in case of specific issues arising;
- The AC keeps correct recordings in the AC journal of the sample identification and sample flow.

The AC function towards the statisticians and modellers is:

- The analytical results are sent to the SD. Before sending the results to the statisticians and modellers, the SD will be in contact with the AC for a label/coding check;
- During the statistical analyses and modelling processes, the AC will provide the statisticians and modellers with any specific information needed, and will be available to answer any queries relevant to the data and /or analyses.

5.1 Apiculturalist Coordinator and Apiculturalist Citizen Scientist (AC and CS)

5.1.1 Recruitment

The beekeepers who will participate in the study as citizen scientists (CS) are selected and invited by the AC.

The basic conditions for a CS to participate are:

- Internet access and an email address;
- Having a minimum of 2 non-migrating honey bee colonies (well overwintered and of average strength as generally accepted in the particular region);
- Having time within the pre-determined sampling rounds to do the appointed pre-, post-, and actual sampling activities;
- Having an awareness of the environmental surroundings;
- Being reliable in sticking to the agreements and appointments;
- Being a skilled beekeeper;
- Having an affinity to science, as a pre-condition for involvement as a CS.

5.1.2 Citizen scientist number for sample- and data warehouse recordings

In consultation with the Data Curator, the CS data and CS number are stored in the data warehouse.

To the selected beekeepers a unique citizen scientist number (e.g. AT001, DK002, GB003, LV001,...) is assigned, as agreed in the study plan.

5.1.3 Information and contact

The quality and reliability of the data / results concerning pesticides, pollen or anything else sampled depends on the quality of the sampling. This constitutes the basis of any monitoring study. Therefore, the CS must be well informed about the "why and how" of the study set-up, its specific goals, the study progress and study results. It is up to the AC to inform the CS about these and to place the role of the CS in perspective as a key participant in the citizen scientist study. The basic condition for the relationship between the AC and CS to be successful is open and clear communication. All communication channels available in the study are at the disposal of the AC, including AMA ("ask me anything") meetings, WhatsApp groups, Signal groups, Facebook groups, or whatever is applicable to keep in touch with the CS. These will assist if questions arise from the CS, and will help to ensure the smooth running of the study and the continued and essential engagement of the CS.



5.1.4 Instruction of the CS

The importance of correct sampling, sample storage and sample transport is explained in the previous subparagraph. This can only be achieved by crystal clear instructions.

Therefore instruction tools must be made, which are:

- An instruction picture manual;
- Links to tutorial and instruction movies;
- Demonstrations or whatever is required for good instruction under the local conditions, ideally on-site education near to the beekeeper;

These instructions comprise:

- Strict (not to be misinterpretable) instruction for sample labelling / completion of pre-printed labels;
- Strict procedures for the actual sampling;
- Strict instructions for sample storage at the beekeeper's premises;
- Clear instructions plus adequate mailing boxes / envelopes for sample shipping to the AC.

The instructions and procedures are subject-specific and will be elaborated in the protocols in the Chapter "Protocols". Examples are available on the INSIGNIA website <https://www.insignia-bee.eu>.

5.1.5 Toolbox

Including sample tools, pre-printed labels and return-transport boxes/ envelopes.

The content of the "toolbox" is subject-specific and is specified in the "Protocols". The AC assembles the toolbox for the CS. This toolbox contains all materials needed for adequate sampling, sample storage, sample labelling and sample shipping.

A toolbox for bee-monitoring by the honey bee colony, designed for the CS contains:

- Tubes, bags and pencils. The pencil is mandatory because all ink-based pen writing can be erased by alcohol, water or other solvents;
- Non-biological passive samplers / matrices;
- Supporting material for storage;
- Pre-printed sample labels. This key aspect is elaborated in a sub-paragraph "Instruction for sample labelling";
- Envelopes / boxes for sending back to the AC by the CS. This key aspect is elaborated in a sub-paragraph "Samples from the CS to the AC".

5.1.6 Instructions for sample labelling

All efforts in the recruitment, sampling, analytics, statistics, modelling and reports are useless if there is not a solid, clear and unambiguous sample label that cannot be misinterpreted.

It is mandatory for all AC involved in the study to use the same coding system for the samples.

Any label should have the following information:

- A country or region abbreviation;
- Sampling subject / matrix;
- CS ID;
- Sampling date;
- Sampling round.

All this information is additionally summarised in the **sample ID** on the label.

The example below, applied in the INSIGNIA pesticide and pollen bee-monitoring study in 2020, meets these conditions. Also, for uniformity, the recommended practice is to provide agreed templates on the study platform.

The **label** consists of five different elements (figure below):

- The country code (e.g. Austria AT)
- The CS ID name and number (example: Name of beekeeper with CS ID 001)
- The sampling round (SR)
- The colony number
- The sample matrix

The sample ID completes the sample in the combination of the details: Country code - CS ID Number - SR - Colony Number - sample matrix abbreviation.

Figure 2.3. Pollen label

Figure 2.4. APIStrip label

In the INSIGNIA study

- The templates for the labels were available on the study platform (study cloud);
- An online tool was available through the study platform;
 - The labels must be in the correct language, therefore they need to be translated (to make sure the citizen scientists understand the label) and adapted for the particular country (country code, citizen scientist code, sampling round).
 - The labels need to be printed.
 - The printed labels need to be placed inside the sample bags rather than stuck on the outside. See the instruction / template in the box below.
- The CS beekeepers were asked to make a (cell phone) photo of the labelled sample before storing the samples. These photos were sent to their AC. The AC checks the labels and contacts the CS in case of missing or incorrect parts of the label.

The instructions must make it crystal clear what the CS must add to the labels, because the samples will be processed by the AC (e.g. pollen will be split and sent to the laboratory in ethanol; the other part will be stored in the AC's freezer). The CS instruction is elaborated in chapter 3-Protocols.



5.1.7 Samples from the CS to the AC

The AC will pre-organise the transport from the CS to the AC by providing adequate free-post package material and contact with a courier or postal service. This transport time must be short, preferably within 3 days, to avoid degradation of samples. A simple data logger can be added to record the transport conditions (Temperature (T) and Relative Humidity (RH)).

5.2 Sample handling by the AC after arrival from the CS

5.2.1 Label check

The samples need to be checked for damage and for missing labels:

- Has the CS filled in the sample ID? If yes, compare it to the other information on the label and check whether it was assigned correctly. If not, the sample ID needs to be allocated.

5.2.2 AC journal

The AC journal (e.g. offline Excel sheet, notebook) is kept by each AC, and combines the original information from the beekeepers and the information for the labs. It gives the opportunity to track the samples and every working step and has to be archived for the required time period (7 years) by the AC or otherwise if required in the study plan.

The requirements are:

- Correct recording of the pre-labels to be sent to the beekeepers and recording of the sending dates;
- Correct recording of arrival of samples from the beekeepers after sampling;
- Correct recording of the samples and sample handling after return from the beekeepers, as, for example:

➤ **NL001-SR01-P** = Netherlands, beekeeper 1; sampling round 1; pollen sample

If samples (from a given apiary) are pooled, this can be recorded in several ways. For example, for recording the pooling process of a pollen sample:

- **NL001-SR01-P** = NL001-SR1-1-P + NL 001-SR1-2-P
or
- **NL001-SR01-P** = NL001-SR1-1-P (not pooled because not enough pollen for pooling)
or
- **NL001-SR01-P** = NL 001-SR1-2-P (not pooled because not enough pollen for pooling)
or
- **NO NL001-SR01-P** sample because of holidays, private circumstances, etc.

- Correct recording of samples and sample IDs and dates sent to the laboratories;
- Recording of appointments and comments of the participants related to sampling;
- Recording of shipment to the laboratories.

The Data Curator (DC) will export the data of an Online Survey of the AC's country into that country-specific spreadsheet (=journal).

The AC is responsible for his/her own study journal. It is used to check the samples, the labs use it to add the day of sample arrival, to add notes and to add the lab code (if it differs from the original sample ID).

5.3 Pooling

Sample pooling might be a part of the study plan. Based on all pitfalls encountered in the INSIGNIA study, here is shown the detailed description which has proved to be essential in order to avoid the need for interpretation by the AC. The instruction drawings have proved to be essential for the proper pooling practice.

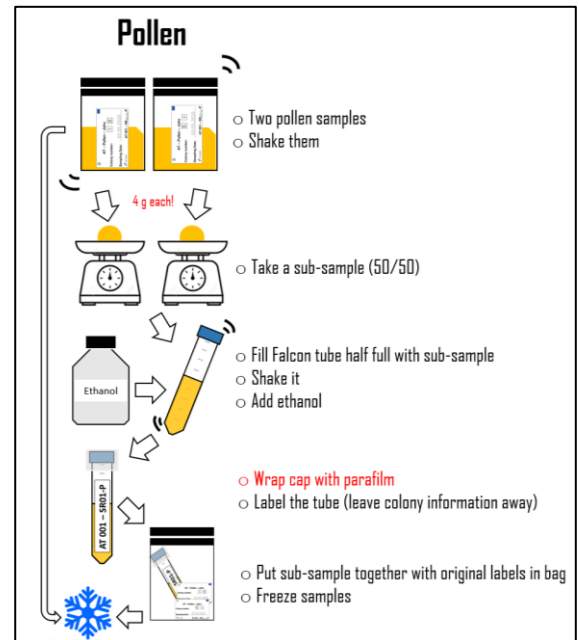
- APIStrips

No processing needed. Immediately check them, record the sample IDs, freeze the samples after receiving them and store them until shipment to the designated lab.

- Pollen

Pollen was collected from two colonies per sampling date per apiary. The two samples from the same apiary **must be pooled by the AC**.

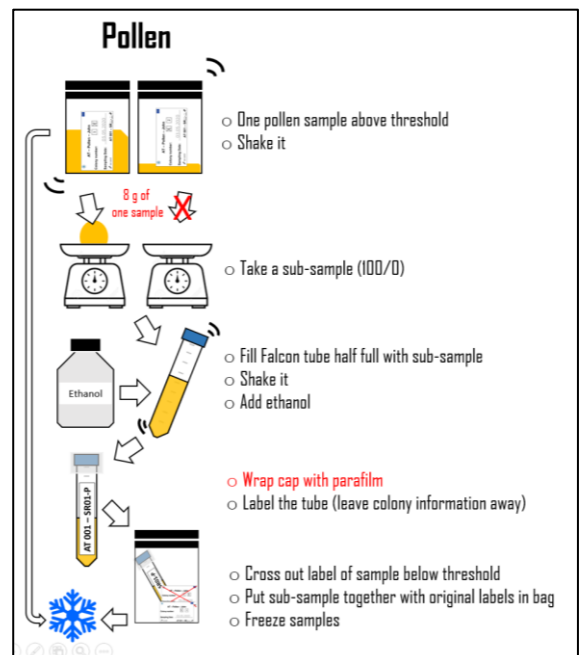
- Mix each colony sample thoroughly, by shaking the bags.
- **Take 4 g** (use a fine scale!) of each sample and add the pollen of both samples to a **15 ml Falcon tube** (= 50/50 pollen sample).
- Mix the 50/50 pollen mixture thoroughly.
- Add 96% ethanol until the tube is full and close the tube.
- Wrap the cap with parafilm to avoid leakage and therefore cross-contamination.
- Make a copy of the two original sample labels to keep for the AC records.
- Label the Falcon tube with the "new sample ID" (leave colony information off).
- Pack the Falcon tube together with the original labels in a sample bag – each Falcon tube in its own separate bag, seal the bag, freeze the original samples as backup and also freeze the sub-sample until shipment.



How to pool two unbalanced samples:

A minimum amount of 4 g per sample is needed.

- One of the two samples has less than 4 g so 8 g of the other sample are taken. The sample is not pooled (=colony level sample)! The corresponding original label of the insufficient sample will be crossed out and sent to the designated lab.
- Both of the two samples have less than 4 g. No sample can be analysed. Cross out both labels and send them to the designated lab.
- In both cases, make a record in the AC lab journal and in the data warehouse! Lab journal: see chapter VII below. Data warehouse: there is a column called "pooled or colony sample". Fill in Y (yes) for pooled and N (no) for colony sample. This is important for the labs.





5.4 Sample storage till shipment to the laboratories

Store all samples (samples and sub-samples) according to the instruction in the study plan until shipment to the laboratories.

5.5 Shipping samples from the AC to the laboratories

- Shipping is done in consultation with the laboratories and preferably by courier on a Monday (to avoid retention on the premises of the transport company over the weekend). Make sure that the pots, bags, containers and other sample containers are properly sealed;
- Print a list of the samples that will be shipped and add this accompanied information to the parcel;
- Mail the same list to the lab partners prior to shipment of the samples;
- Add a data-logger to record the transport conditions.

5.6 Laboratory sample identification

It is strongly recommended that the labs should also use a standardised system for spreadsheet recording of the analytical results for each sample and the sample ID reference number, for comparability between labs and to avoid confusion at the stage of data analysis.

6 Data integrity and communication

Both data integrity and external communication are responsibilities of the SD and the practical execution can be assigned or delegated to a participant; the Data Curator (DC) and the Communication Curator (CC).

The process of "data integrity" comprises that the:

- SD guards data safety and integrity of the data warehouse;
- SD assigns data management task to the DC;
- DC tasks comprise
 - to send alert emails prior to sampling to the beekeepers;
 - to send questionnaires via online questionnaire programs. The questionnaire and data collection tools will be addressed separately in a sub-chapter "The online survey questionnaire";
 - to upload the online questionnaire data in the data warehouse (in the study platform).
- AC checks labels and samples after return from the beekeeper;
- AC records samples and codes in his/her AC journal;
- AC sends the samples with the original label to the labs;
- Labs send results to SD;
- SD sends results to the AC for label checks;
- AC sends checked results back to SD;
- SD sends results to the DC;
- DC uploads the results of the lab analyses;
- DC compiles data sets for statistical analysis and modelling.



2 - Study set-up



The process of external communication comprises that:

- CC makes notes for external contacts;
- CC organises the writing of articles for bee journals based on the input by the SD and, via the SD, the laboratories, and input by AC, and input of the beekeepers via the AC;
- CC takes care of the website and website upload;
- CC takes care of / feeds the social media;
- CC makes, or has made, the format of the picture manuals, to be translated / adapted by the AC;
- CC makes, or has made, instruction video and other visual instruction material;
- CC organises that participants contribute to the study blog, notes and other output.

6.1 Online questionnaire survey program and data warehouse

During the whole year, and especially during the sample collection periods, the AC stays in close contact with the DC doing the online survey with the citizen scientists. The DC will start the process of reminding the participating citizen scientists prior to each sampling date, but the AC still has the end responsibility for the data entry process and the rest of the study in his/her country.

The data warehouse is filled in by the AC, and the management of the data warehouse is done by the DC. The DC can alert the AC to their duty for correct data input in the data warehouse.

The data warehouse is accessible on the study platform via <https://www.insignia-bee.eu> (replace insignia-bee.eu by the consortium website).

It is shared between the SD, AC, and the designated labs. The latter include information on sample arrival and the lab-sample-IDs (if they differ from the originals).

If applicable, the DC adds the sample ID for each sample to the data warehouse (framed in the picture below, as an example applied in the INSIGNIA study).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
	Country	Last name	Citizen Scientist ID	Lat	Long	Sampling date	Sampling round (SR01-SR10)	Date of arrival at AC	Date shipment AC to me/abarracking lab (lab 1)	Date lab arrival (lab 1)	Lab sample ID (lab 1)	Pollen Sample ID-col 1	Sample arrived at AC (Y/N)	Pollen Sample ID-col 2	Sample arrived at AC (Y/N)	Date shipment AC to pesticides lab (lab 2)	Date lab arrival (lab 2)	Lab-sample ID (lab 2)	APIStrip Sample ID-col 1	Sample arrived at AC (Y/N)	APIStrip Sample ID-col 2	Sample arrived at AC (Y/N)
1	Austria	Last name 1	AT001	47.0000	15.0000	30.04.-03.05.2020	SR01	19.06.2020	25.06.2020	29.06.2020	A402	AT001-SR01-1-P	Y	AT001-SR01-2-P	Y	25.06.2020	15.07.2020	AT 2-1-1	AT001-SR01-1-A	Y	AT001-SR01-2-A	Y
2	Austria	Last name 2	AT002	46.0000	13.0000	30.04.-03.05.2020	SR01	21.09.2020	09.11.2020	10.11.2020	A403	AT002-SR01-1-P	Y	AT002-SR01-2-P	Y	09.11.2020	15.07.2020	AT 6-1-1	AT002-SR01-1-A	Y	AT002-SR01-2-A	Y
4	Austria	Last name 3	AT003	48.0000	16.0000	30.04.-03.05.2020	SR01	19.06.2020	25.06.2020	29.06.2020	A418	AT003-SR01-1-P	Y	AT003-SR01-2-P	Y	25.06.2020	15.07.2020	AT 8-1-1	AT003-SR01-1-A	Y	AT003-SR01-2-A	Y
5	Austria	Last name 4	AT004	48.0000	16.0000	30.04.-03.05.2020	SR01	30.04.2020	25.06.2020	29.06.2020	A406	AT004-SR01-1-P	Y	AT004-SR01-2-P	Y	25.06.2020	15.07.2020	AT 3-1-1	AT004-SR01-1-A	Y	AT004-SR01-2-A	Y
6	Austria	Last name 5	AT005	46.0000	16.0000	30.04.-03.05.2020	SR01	08.07.2020	13.07.2020	15.07.2020	A407	AT005-SR01-1-P	Y	AT005-SR01-2-P	Y	13.07.2020	22.07.2020	AT 3-1-2	AT005-SR01-1-A	Y	AT005-SR01-2-A	Y
7	Austria	Last name 6	AT006	46.0000	14.0000	30.04.-03.05.2020	SR01	03.07.2020	13.07.2020	15.07.2020	A408	AT006-SR01-1-P	Y	AT006-SR01-2-P	Y	13.07.2020	22.07.2020	AT 3-1-3	AT006-SR01-1-A	Y	AT006-SR01-2-A	Y

Figure 2.5. Example of data records in the data warehouse



2 - Study set-up



6.1.1 Automated communication

The texts for automated communication with CSs will be translated into the required languages, depending on the participants in the study, by the AC. The online survey setup allows addition of other languages as needed. The AC makes sure that every participant in their country receives an online survey link for each sampling round. The AC is responsible for the process of data provision by the participants in consultation with the DC. Within the study platform there is a document containing all online survey questions /content that has to be translated into the languages by the AC.

6.1.2 The online survey questionnaire

The online survey questionnaire serves two purposes. Firstly it confirms which dates the CSs have collected samples, and secondly, it is a way to collect additional data about the conditions in the apiaries over the sampling season.

There are various systems available for making questionnaires. In the INSIGNIA project, the online LimeSurvey survey software was used, but there are other systems such as Google Formula and Survey Monkey. It is the responsibility of the DC to set up the questionnaire and send out emails to all CSs for each sampling round. Only if individual CSs fail to answer the questionnaire will the AC be involved, and they will then make direct contact to the relevant CS to ensure that the questionnaire will be answered.

The online survey tool LimeSurvey was a good choice for the INSIGNIA project since it can issue individual invitations with a "token". This means that during the whole process it is possible to keep track of all the responses from the beekeepers, issue reminders, and to notify the AC to contact non-respondents. LimeSurvey also has a very good mapping facility, for determining the GPS position of each participating apiary.

Information to be collected includes:

- Which samples have been collected in the sampling rounds, and the exact dates for the start and end of the subsequent sampling rounds.
- Information about the size of the colonies based on frame size and number of occupied bee lanes. This is important, as larger colonies will be expected to collect more food, nectar, and pollen, and consequently also pollutants.
- Finally, the questionnaire gives the CS the opportunity to report (chemical) varroa treatments and other relevant information.

The DC collects the answers and adds information to the data warehouse. The DC also ensures that sampling dates from the questionnaire and the physical samples are consistent.

6.1.3 Summary sample flow

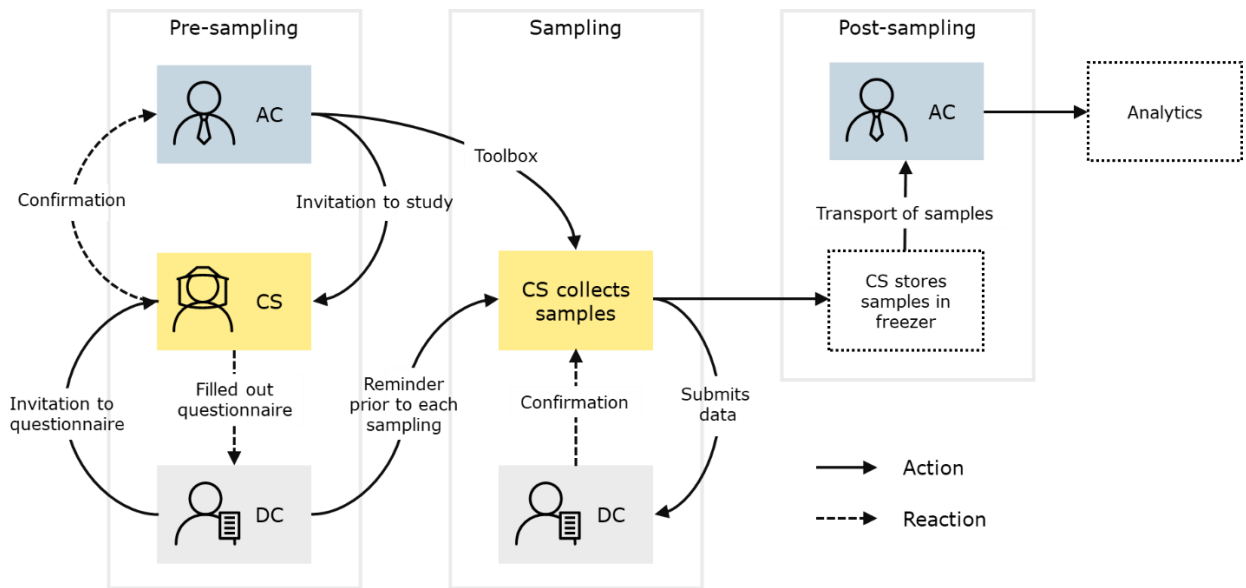


Figure 2.6. Workflow describing crucial interaction steps of CSs with AC and DC, and showing their responsibilities

6.1.4 Flowchart of communication with the Apiculturalist Citizen Scientist (CS)

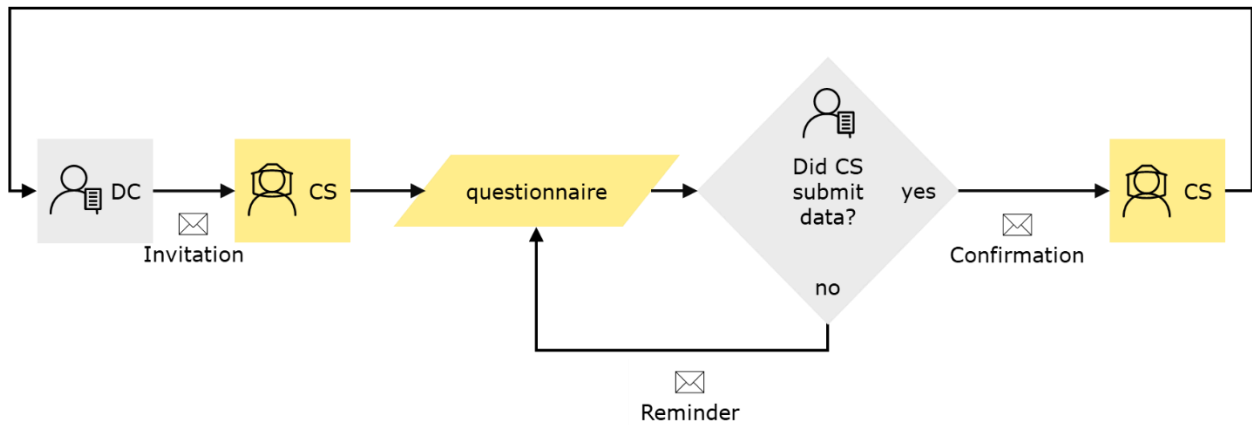


Figure 2.7. Typical communication routine with the citizen scientist



3 - Protocols

Introduction

This chapter consists of protocols. Each protocol is about a specific subject such as pesticides or pollen. The rationale behind this design is that each monitoring subject demands its specific toolbox, sampling techniques, recording, storage and shipping. The format of the protocols is identical in order to facilitate the combination of protocols if the honey bee colony is to be used for bee-monitoring with passive samplers for more than one subject. Because of having identical formats, there is some textual overlap in each protocol. We consider this a minor issue in relation to the complexity of combined protocols, with the chances of inherent errors. In practice, the protocol for specific monitoring subjects can be combined in one study plan – the study-specific Apicultural citizen science protocol.

3.1

Protocol for the Apiculturalist Citizen Scientist for honey bee colony monitoring for pollen origin and diversity





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1 Study design

Environmental monitoring with honey bee colonies for pollen origin and diversity by Apiculturalist Citizen Scientists.

1.1 Objective

A scientifically substantiated protocol to quantitatively record pollen origin and diversity available for honey bee colonies, by using honey bee colonies as the bio-monitoring tool.

1.2 Lay-out of the study

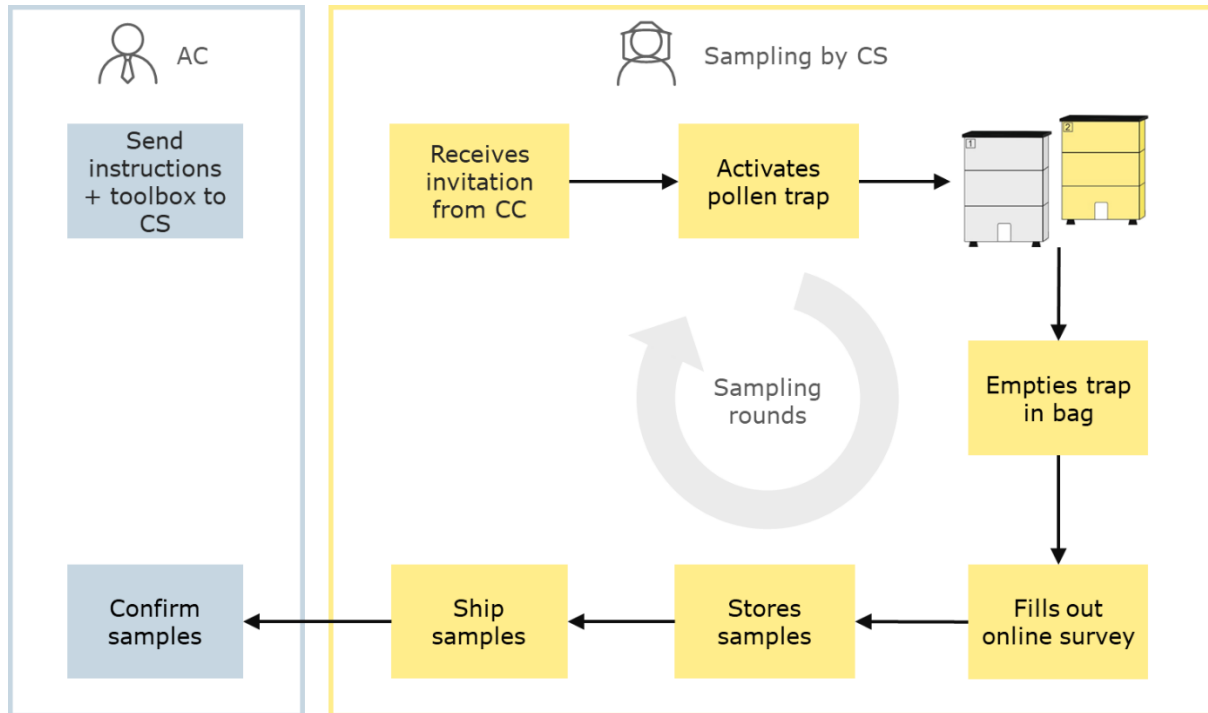


Figure 3.1.1. Lay-out of the study

- The Apiculturalist Coordinator (AC) provides the beekeeper with:
 - Instructions on how to sample, how to label, how to store and how to ship samples using
 - a picture manual and /or
 - an instruction video / tutorials and /or
 - physical meetings and /or virtual instructions
 - a sampling scheme
 - sampling detail
 - Toolbox
 - All relevant tools
 - Pollen trap (optional)*
 - Storage materials plus labels
 - Storage materials
 - Boxes for shipping of samples to the AC
 - Contact during the study

*Preferably the beekeeper's own pollen traps should be used, but if not available the pollen trap can be part of the toolbox.



- The CS is the pivot in this sampling process.
 - The CS activates the pollen trap for defined periods according to instructions;
 - The CS de-activates the pollen trap according to instructions;
 - The CS empties the pollen trap according to instructions;
 - The CS homogenises the pollen and takes a subsample according to instructions;
 - The CS labels the pollen bag containers according to instructions;
 - The CS stores the pollen bags according to instructions;
 - The CS sends the pollen bags to the AC according to instructions;
 - The CS fills out the online questionnaire after each sampling.

2 Sampling scheme

Sampling of pollen is actually a snapshot sampling of a one-day pollen influx every 14 days*.

Sample scheme for bi-weekly sampling with the pollen trap

Sample round (SR)	Month	Sampling day**			
		Thursday	Friday	Saturday	Sunday
SR1	April/May				
SR2	May				
SR3	May				
SR4	June				
SR5	June				
SR6	July				
SR7	July				
SR8	August				
SR9	August				
SR10	September				

* The reason for the once-every-14- days-sampling-scheme is that the average flowering period of a crop is about 14 days.

** The window of sampling days runs from Thursday till Sunday. This offers the possibility to choose the best day for pollen trapping and to avoid bad weather days. If a trapping day was unsuitable for collection, trapping can be extended to a maximum of one day of effective trapping.

3 Alerts/ questionnaires for the data warehouse

The CS receives an invitation email and an invitation to complete an online questionnaire from the CC. The questionnaire needs to be completed with care and following the instructions to finish the data submission process. The quality of the data provided is extremely important because it will become part of the data warehouse in which all data are stored and which is the basis for the evaluation of the results by statisticians and modellers. A few days prior to the pollen trapping day(s), the CS receives an alert email. Additionally, this can be combined with a questionnaire for collecting relevant information concerning the sampling, such as location, number of colonies in the apiary, varroa control strategy or other bee parasite / pathogen treatments.



4 The toolbox


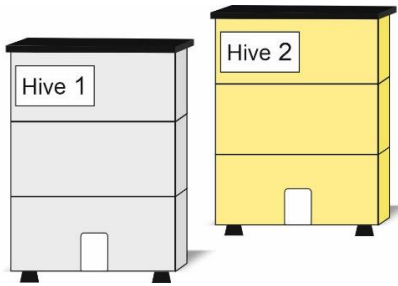
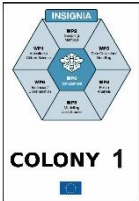


The toolbox provided by the AC includes all equipment needed by every CS for pollen monitoring. It contains materials for 10 sampling rounds with 2 bee colonies. The CS should check the materials for completeness.

Materials	Amount	Checklist
Picture manual (including sample scheme)	1	<input type="checkbox"/>
Graphite pencils	1	<input type="checkbox"/>
Larger bag (1 per sampling round, 1l zip lock bag)	10	<input type="checkbox"/>
<i>For pollen sampling and storage, two (2) options are provided</i>		
Option 1: per hive and sampling round a 20 gram sample is taken, stored in freezer and transported in cold chain transport		
Option 2: per hive and sampling round 4 x 5 gram pollen is taken, per 5 gram samplers, dried with silica gel at room temperature - room temperature storage and room temperature transport.		
Option 1. measurement tool 20 gram	2 (1 per hive)	<input type="checkbox"/>
Option 2. measurement tool 5 gram	2 (1 per hive)	<input type="checkbox"/>
Option 1. sample bag: airtight plastic zip lock bags	20	<input type="checkbox"/>
Option 2. sample bag: airtight plastic zip lock bags	80	<input type="checkbox"/>
Option 1. pre-printed labels (one per hive)	20	<input type="checkbox"/>
Option 2. pre-printed labels (one per hive)	80	<input type="checkbox"/>
Option 2. storage: silica bags sachets of 5 to 6 gram	80	<input type="checkbox"/>
Option 2. storage: 80 paper tea filters	80	<input type="checkbox"/>
Option 1. transport: Styrofoam boxes	2	<input type="checkbox"/>
Option 1. transport: cool blocks	2	<input type="checkbox"/>
Option 2. transport: cardboard postal boxes	2	<input type="checkbox"/>




5 Picture manual and other instruction tools

5.1 Prior to study start

	<p>Choose two (2) bee colonies prior to the study start: The best choice will be well-overwintered, non-migrating colonies of average strength (queenright, all stages of brood present, no symptoms of diseases), in the same apiary and near to your home. Assign the two colonies the numbers 1 or 2 to clearly identify them during the whole sampling season.</p>	 <p>Identification example of study colony 1:</p> 
	<p>Install pollen traps: Each hive will be equipped with a pollen trap. It is your choice which pollen trap model you want to use for pollen sampling. Follow the specific instructions of the manufacturer for installing the trap. The pollen trap must be installed without additional gaps between the trap and the hive (bee tight).</p>	

5.2 Preparations for sampling

	<p>Invitation email: prior to each sampling round you will receive an invitation email. Please choose the one day during the specified 4-day sampling period with the best weather forecast and that also fits best for you to collect the samples (table below). If you do not receive the email, please check your spam box or contact the Apiculturalist Coordinator.</p> <table border="1" data-bbox="424 1563 1326 1962"> <thead> <tr> <th colspan="5">Sampling time window (example)</th> </tr> <tr> <th>Day</th> <th>Thursday</th> <th>Friday</th> <th>Saturday</th> <th>Sunday</th> </tr> </thead> <tbody> <tr> <td>Weather forecast & day job</td> <td> working</td> <td> free</td> <td> free</td> <td> free</td> </tr> <tr> <td>Sampling</td> <td></td> <td></td> <td> Morning (~8 am) ① close pollen trap Evening (~5 pm) ② harvest pollen ③ open pollen trap ④ if not enough pollen; leave trap closed </td> <td> IF NOT ENOUGH POLLEN YESTERDAY: Evening (~5 pm) ⑤ harvest pollen ⑥ open pollen trap </td> </tr> </tbody> </table>	Sampling time window (example)					Day	Thursday	Friday	Saturday	Sunday	Weather forecast & day job	working	free	free	free	Sampling			Morning (~8 am) ① close pollen trap Evening (~5 pm) ② harvest pollen ③ open pollen trap ④ if not enough pollen; leave trap closed	IF NOT ENOUGH POLLEN YESTERDAY: Evening (~5 pm) ⑤ harvest pollen ⑥ open pollen trap
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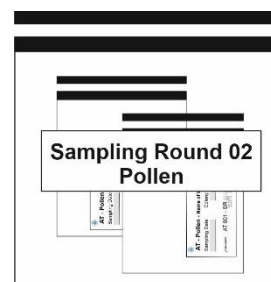
	<p>Preparation of sample bags: find the right bag for the current sampling round in your tool box.</p>	
	<ol style="list-style-type: none"> 1 A graphite pencil, otherwise the lettering could become blurred and unrecognisable. 2 Two pre-labelled sample bags (one for hive 1, one for hive 2): write the sampling date and the colony number on the labels in the corresponding lines. Complete the blanks at the bottom by writing the sampling round (SR) and colony number. This is the actual sampling ID. Option 1. Two sample bags (one for hive 1, one for hive 2) Option 2. Two x 4 sample bags (4 for hive 1, 4 for hive 2) 3 A piece of kitchen paper towel to avoid contamination. 4 Option 1: the 20 gram measure tool Option 2: the 5 gram measure tool 	<div style="border: 1px solid black; padding: 5px; margin-top: 10px;"> <p>AT - Pollen - Name of beekeeper</p> <p>Sampling Date Colony number</p> <p>_____ _____ insert "1" or "2"</p> <p><small>use a pencil</small> AT 001 - SR _____ - _____ - P</p> <p style="font-size: small; margin-left: 100px;"> <small>insert sampling round (01-16)</small> <small>insert colony number (1 or 2)</small> </p> </div>

5.3 Sampling procedure

	<p>Activation of the pollen trap: activate the pollen trap in the morning of the sampling day by closing it/installing the screens (depending on the chosen design). Before activation, line the collection tray of the pollen trap with kitchen towel to avoid cross contamination between sampling rounds. The trap needs to be closed for one complete day (morning to evening) to collect enough material (if there is not enough material, let it be closed up to 3 days, but empty out the tray every evening)- if problems occur, contact the Apiculturalist Coordinator.</p>	
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**Have you prepared the right sample bags?
Have you prepared the right labels?**



Emptying the pollen trap/actual sampling: in the evening, empty the pollen trap of hive 1 and transfer it to the right sample bag (for hive 1).

For analysing the samples, we need a **minimum amount** of 20 gram pollen, Use the provided measurement tool to estimate it (see figure). If the measurement tool cannot be fully filled, harvest the pollen, but leave the trap closed until the next evening (make a note in online survey). Harvest again and put the pollen in the same sample bag from the day before.

If you have more pollen, a subsample of the homogenised pollen harvest must be taken by

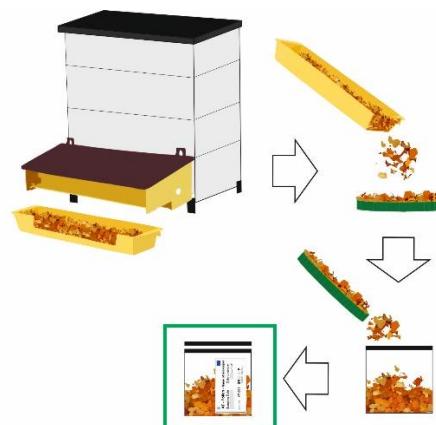
1. collecting all the pollen in a large plastic bag, shake until the pollen harvest is well mixed and take a full measurement tool amount from this mixture.
2. shaking the pollen pellets in the drawer until the pollen harvest is well mixed and take a full lid amount from this mixture.

This is the pollen sample of this colony and sampling round.

After making sure that the bag is **tightly sealed** and the corresponding **label** is **LOOSELY INSIDE THE BAG**, repeat the sample procedure with hive 2.

In case you have not enough material (less than the measurement tool), transfer the pollen to the corresponding sample bag anyway and freeze/store it with the other samples. This should also be done if no pollen at all could be collected (freeze empty and labelled sample bags).






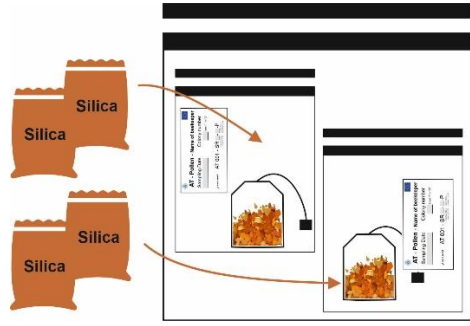

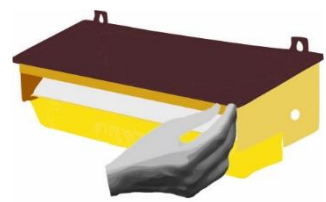
Pollen is identified by its DNA. Any cross-contamination impacts the results. Therefore, it is mandatory to apply one measurement tool per colony.





3 - Protocols



	<p>The remaining pollen from the more than 20 gram sample can be used according to your own preference.</p>	
	<p>Make a picture of the sample in a way that the label is clearly visible, with your cell phone and send this to the Apiculturalist Coordinator.</p>	
	<p>Option 1 Bring your 20 gram samples to your freezer at home shortly after the sampling procedure.</p>	
	<p>Option 2 Transfer the collected pollen sample into the provided paper tea filter. Place this in the plastic bag and add into this plastic bag (not in the tea filter) 2 silica gel sachets of 5-6 gram.</p>	
	<p>De-activation of the pollen trap: after sample collection, open (=de-activate) the pollen trap to provide sufficient forage for your bees. Discard the kitchen towel lining the trap and clean the drawer and lids to avoid cross-contamination between sampling rounds.</p>	

5.4 Storage at the beekeeper's premises

	<p>Option 1 Store the pollen in its plastic bag in the freezer.</p>	
	<p>Option 2 Per 5 gram pollen in the paper tea filter, 10 – 12 gram silica gel in sachets were already added to the zip lock plastic bag. The zip lock plastic bag is closed airtightly and the pollen is stored at room temperature in the cardboard box for return sending.</p>	

5.5 Quality control




	<p>From the moment the samples are transferred to the sample bags, they must be permanently protected from light and kept frozen (option 1) or dry (option 2) to avoid mould formation. Mould may destroy the valuable sample for the purpose of further DNA analysis.</p> <p>Option 1: Make sure to transport the samples as soon as possible to your freezer and store them at minus 20 °C until transport to the Apiculturalist Coordinator is decided.</p> <p>Option 2: Make sure to store the samples as soon as possible under the required dark (cardboard box) and dry (silica gel) conditions until the transport to the Apiculturalist Coordinator is decided.</p>
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5.6 The transport to the Apiculturalist Coordinator (AC)



	<p>The process for transporting the samples from the beekeeper to the Apiculturalist Coordinator will be decided on an individual basis and local conditions. The Apiculturalist Coordinator will give instructions.</p> <p>Option 1: cold chain transport in a Styrofoam box (in toolbox). Option 2: in cardboard box and transport at room temperature.</p> <p>It is up to the Apiculturalist Coordinator to organise the transport from the beekeeper to the Apiculturalist Coordinator by postal service or by courier.</p> <p>Regardless of the transportation mode, the costs are borne by the study and not by the beekeeper CS.</p>
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5.7 Data entry

	<p>Last but not least, sample information is crucial. Therefore, it is necessary to answer the online questionnaire after each sampling round. Prior to each sampling round, you will receive a unique invitation email with a personalised link to your online questionnaire. The link allows the automatic recording of your answers/data entries from the present sampling round. If you have difficulties reaching the website or if you do not receive the invitation email, please check your spam box or contact the Apiculturalist Coordinator. Note any derogations of the sampling scheme or irregularities, using the comment section of the questionnaire.</p>
	<p>Successful data transmission If you miss the data entry, you will receive a reminder email. If you have successfully submitted your data, within a few minutes you will receive a confirmation email. After data submission, the link will be de-activated and cannot be used again. If you made some mistakes during data entry, no problem - please immediately contact your Apiculturalist Coordinator.</p>
	<p>Thank you for your valuable contribution!</p> <p><u>Apiculturalist Coordinator</u> Email: _____ Phone number: _____ (contactable during sampling periods - Thursday to Sunday from 9 am to 5 pm) (Messenger group: _____) Website: _____</p>

5.8 Support/Video Manual (example from the INSIGNIA Study)

	<p>You will find accompanying instruction videos and constantly updated blog entries on our INSIGNIA YouTube channel and on our website, respectively. During the active sampling periods, the Apiculturalist Coordinator is available via the above-mentioned phone number and social media. If you have any questions or other requests outside of the sampling periods, please write an email to the Apiculturalist Coordinator. Further, you are also invited to the national messenger group to exchange your experiences with the other citizen scientists of your country.</p>
	<p>Video manual pollen: www.youtube.com/watch?v=97josWrHu-A</p> <p>Blog: https://www.insignia-bee.eu/blog/</p>

6 Background information for ITS2 metabarcoding

The genetics lab performs “metabarcoding of ITS2” to identify the botanical origins of the pollen samples. Following specific protocols, the DNA of the pollen will be extracted, amplified for the ITS2 genomic region in a two-stage Polymerase Chain Reaction (PCR). In stage one, amplification is performed with an extended oligo that includes MiSeq adapters and the ITS2 specific primers. In stage two, unique sequence indexes are incorporated in the amplicon, allowing sample pooling. Further library preparation for sequencing encompasses multiple steps, including purification, normalisation, pooling, and determination of pool size and concentration. The final library is then sequenced in a high-throughput Illumina MiSeq platform. In the final step, the generated sequences will be compared to a reference ITS2 database of the European flora for taxonomic assignment of the mixed pollen samples.

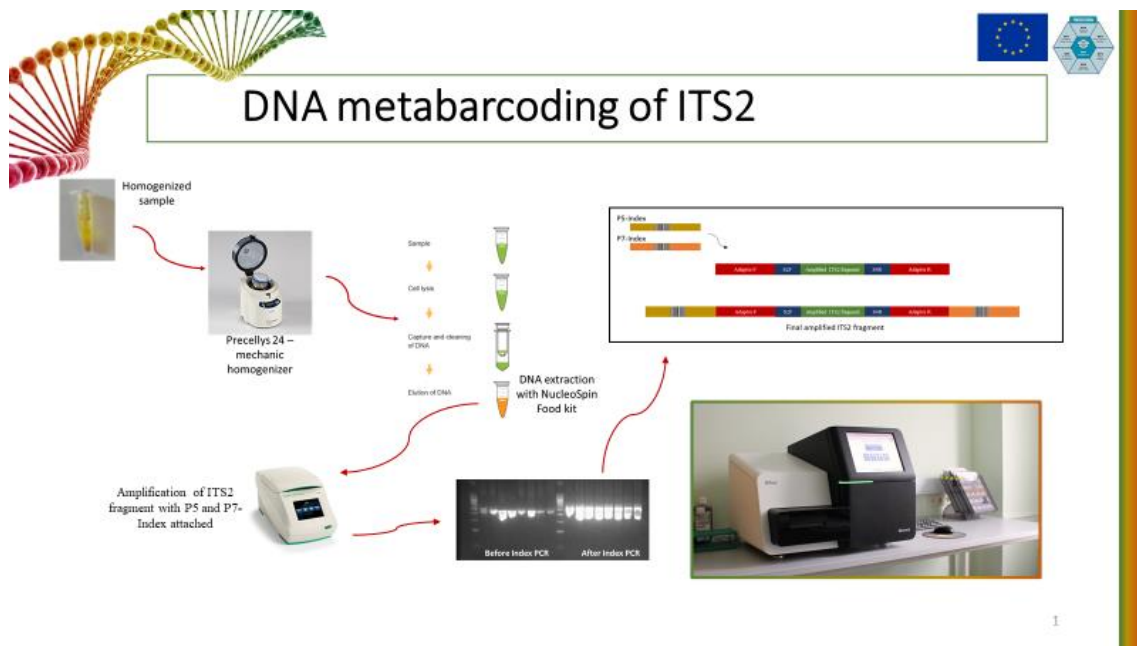


Figure 3.1.2. Metabarcoding process flowchart



3.2

Protocol for the Apiculturalist Citizen Scientist for honey bee colony monitoring for pesticides





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1 Study design

Environmental monitoring with honey bee colonies for pesticides in the environment by Apiculturalist Citizen Scientists (CS).

1.1 Objective

A scientifically substantiated protocol to qualitatively record the pesticides in the environment by using honey bee colonies as the bio-monitoring tool.

1.2 Lay-out of the study

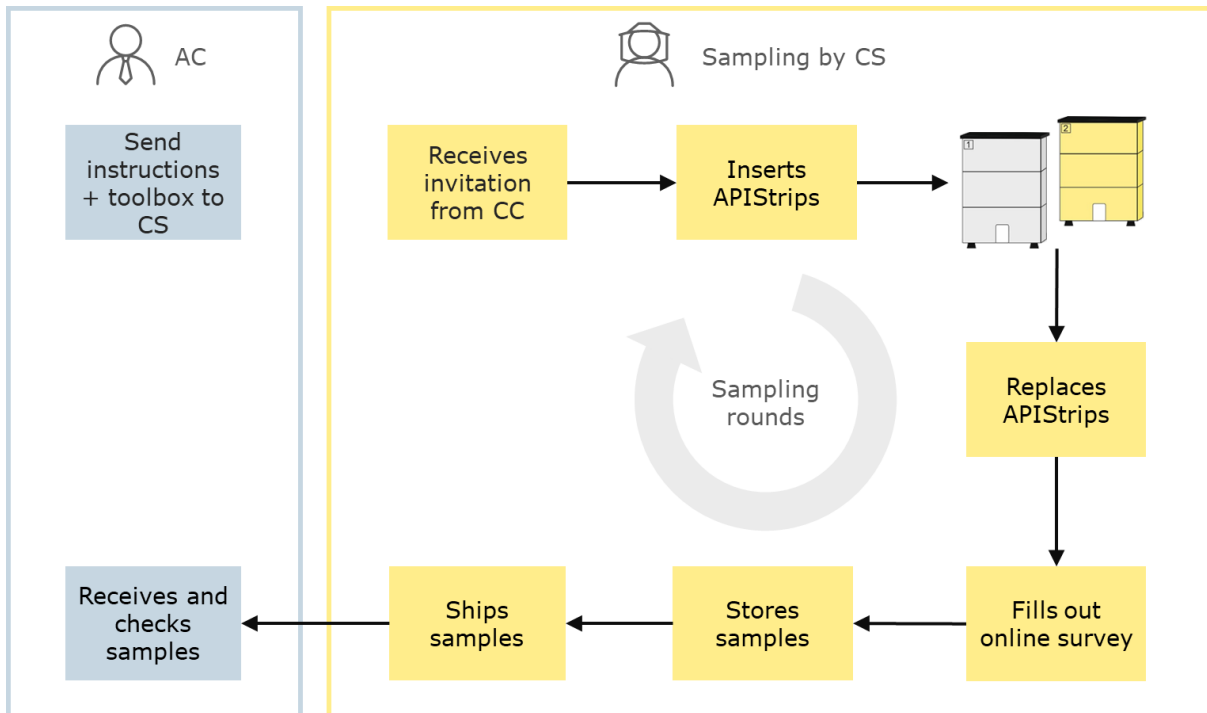


Figure 3.2.1. Lay-out of the study

- The Apiculturalist Coordinator (AC) provides the beekeeper with:
 - Instructions on how to sample, how to label, how to store and how to ship samples using
 - a picture manual and / or
 - an instruction video / tutorials and / or
 - physical meetings and/or virtual instructions
 - a sampling scheme
 - sampling detail
 - Toolbox
 - All relevant tools
 - APIStrips (non-biological in-hive passive sampler)
 - Storage materials plus labels
 - Storage materials
 - Boxes for shipping of samples to the AC
 - Contact during the study



- The beekeeper CS is the pivot in this sampling process.
 - The CS inserts the APIStrips;
 - The CS replaces the APIStrips according to instructions;
 - The CS labels the APIStrips containers according to instructions;
 - The CS stores the APIStrips according to instructions;
 - The CS sends the APIStrips to the Apiculturalist Coordinator (AC) according to instructions;
 - The CS fills out an online questionnaire after each sampling.

2 Sampling scheme

Sampling with the APIStrip is actually replacing an exposed strip after about 14 days with a new one.

Sample scheme for bi-weekly sampling with the APIStrip

Sample round (SR)	Month	Sampling day*			
		Thursday	Friday	Saturday	Sunday
Start = insert first APIStrip	Mid-April				
SR1**	April/May				
SR2	May				
SR3	May				
SR4	June				
SR5	June				
SR6	July				
SR7	July				
SR8	August				
SR9	August				
SR10**	September				

* Start on one of these days and try to be consistent in replacing the strip on the same day of the week, e.g. start on a Sunday and try to stick to Sundays. The actual replacement date must also be filled on the labels and in the online questionnaire.

** SR 1 = the first replacement by taking out the exposed strip and inserting a new one. This is repeated each SR. SR10 is the last take-out day and there is no inserting of a new APIStrip.

3 Alerts/ questionnaires for the data warehouse

The CS receives an invitation email and an invitation to complete an online questionnaire from the CC. It is important that the questionnaire is filled in carefully and following the instructions to finish the data submission process. The quality of the data provided is extremely important, because it will become part of the data warehouse in which all data are stored and which is the basis for the evaluation of the results by statisticians and modellers. Some days prior to the insert and subsequent APIStrip replacement days, the beekeeper receives an alert email. Additionally, this can be combined with a questionnaire for collecting relevant information concerning the sampling, such as location, number of colonies in the apiary, varroa control strategy or other bee parasite/pathogen treatments.




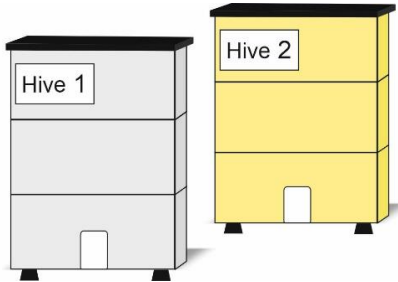
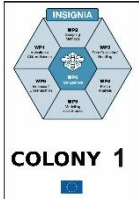
4 The toolbox

The toolbox provided by the Apiculturalist Coordinator includes the equipment needed by every CS for pesticide monitoring. It contains materials for 10 sampling rounds with 2 bee colonies. The CS should check the materials for completeness.

Materials	Amount	Checklist
Picture manual (including sample scheme)	1	<input type="checkbox"/>
Graphite pencils	1	<input type="checkbox"/>
Pre-printed labels (one per hive)	20	<input type="checkbox"/>
Bigger bags (1 per sampling round; e.g. 1l zip lock bag)	10	<input type="checkbox"/>
Sample bags for "APIStrip" (2 per sampling round; airtight zip lock bags)	20 + (2 extra)	<input type="checkbox"/>
APIStrips (in aluminium foil and plastic envelope)	20	<input type="checkbox"/>
Blank APIStrips (in aluminium foil and plastic envelope)	2	<input type="checkbox"/>
Paperclips or wire for strips inserts in bee-lane	20	<input type="checkbox"/>
Silica bags sachets of 5-6 gram	20	<input type="checkbox"/>
Cardboard postal boxes	2	<input type="checkbox"/>

5 Picture manual and other instruction tools

5.1 Prior to study start

	<p>Choose two (2) bee colonies prior to the study start: The best choice will be well-overwintered, non-migrating colonies of average strength (queenright, all stages of brood present, no symptoms of diseases), in the same apiary and near to your home. Assign the two colonies the numbers 1 or 2 to clearly identify them during the whole sampling season.</p>	 <p>Identification example of study colony 1:</p> 
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5.2 Two weeks before first sampling

Put the first two APIStrips in the hives (one per hive):

- 1 There are **2 plastic envelopes containing the first two APIStrips** in the toolbox (wrapped in aluminium foil; labelled "Study Start").
- 2 Unpack a new APIStrip and avoid touching the surface (to avoid contamination risk for lab analysis; no risk for CS or bees!). Note, that the APIStrip does not have Tenax at the top, where no contamination can happen. Attach a metal wire/rod with a T-construction at the end or simply use a paperclip and bend it. Use a thin branch to support the construction if necessary.
- 3 Insert the APIStrip in the middle bee-lane of the lowest box of hive 1. The T-construction squares over the bee-lane and prevents the strip from falling down.
- 4 Repeat the steps for **hive 2**.
- 5 Leave the APIStrip in the hive for a total of **two weeks**. Note. It can happen that the strip slides down on the frame, so if you cannot find the strip, check in the middle of the frame.

5.3 Preparations for sampling

Invitation email: prior to each sampling round an **invitation email** will be sent by the Data Curator to the beekeeper. Please choose the one day during the specified 4-day sampling period that fits the best in your daily practice. If you do not receive the email, please check your spam box or contact your Apiculturalist Coordinator.

Sampling time window (example)				
Day	Thursday	Friday	Saturday	Sunday
Weather forecast & day job	working	free	free	free
Sampling			Occasion 1 Morning (~8 am) exchange APIStrips	Occasion 2 Morning (~8 am) exchange APIStrips

	<p>Preparation of sample bags: identify the correct bag for the current sampling date.</p> <p>The bag includes two small labelled bags with new APIStrips for the current sampling round in your tool box (figure: example for sampling round 2).</p>	
	<p>For each sampling round prepare:</p> <ol style="list-style-type: none"> 1 A graphite pencil, for writing, otherwise the lettering could become blurred and unrecognisable 2 Two pre-labelled sample bags (one for hive 1, one for hive 2): write the sampling date and the colony number on the labels in the corresponding lines. Complete the blanks at the bottom by writing the sampling round (SR) and colony number. This is the actual sampling ID. 3 Paperclips or wire 4 APIStrips 	<div style="border: 1px solid black; padding: 5px; margin-top: 10px;"> <p>AT - APIStrip - Name of beekeeper</p> <p>Sampling Date Colony number</p> <p>_____ _____ insert "1" or "2"</p> <p><small>use a pencil</small> AT 001 - SR _____ -A</p> <p style="font-size: 8px; text-align: right;"> <small>insert sampling round (01-10)</small> <small>insert colony number (1 or 2)</small> </p> </div>

5.4 Sampling procedure



	<p>Two weeks after the last APIStrips were placed in the hive, they must be removed and replaced by new ones.</p>	
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	<p>Have you prepared the right sample bags? Open the bags for the present sample round, take out the two plastic envelopes containing the fresh strips (wrapped in aluminium foil) and put them near you. You will need the new APIStrips soon. The bags are now empty. Write the sampling date and the hive number on the bags (today's date!!). Use a pencil. The empty bags are used for the APIStrips that stayed two weeks in the hives and will be sampled today.</p>	
	<p>Actual sampling:</p> <ol style="list-style-type: none"> 1 Open the hive and lift the APIStrip. Remove the wire/branch. 2 Wrap the strip with aluminium foil and put it back into the plastic envelope. Put the APIStrip envelope together with the correct label into the right sample bag (be aware of the hive number). 3 Make sure that the sample bag is tightly sealed and the corresponding label is in the bag. 4 Do not forget to hang in a fresh APIStrip into the hive and repeat the steps for hive 2. 5 Leave the APIStrip in the hive for a total of two weeks. Note. It can happen that the strip slides down on the frame, so if you cannot find the strip, check in the middle of the frame. 	<p>Used APIStrip</p> <p>New APIStrip</p>
	<p>Add the 5-6 gram silica gel sachet to the APIStrip envelope with the label 2</p>	


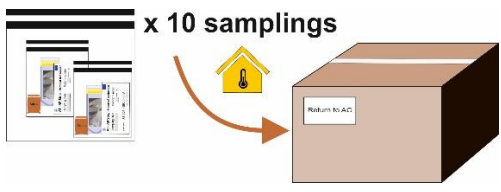


3 - Protocols




	<p>Make a photograph of the sample in a way that the label is clearly visible, with your cell phone and send this to the Apiculturalist Coordinator.</p>	
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5.5 Storage at the beekeeper's premises

	<p>Store the exposed APIStrips in the sample box, provided in the toolbox, at room temperature.</p>	
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5.6 Quality control




	<p>From the moment the samples are transferred to the sample bags, they must be permanently protected from light and under dry conditions to avoid mould formation and degradation of pesticide residues. The aluminium foil keeps the light out and the silica gel sachet in the airtight zip lock plastic bag ensures dry conditions.</p> <p>For quality control, two (2) extra APIStrips are provided in the toolbox. These APIStrips will remain <u>unopened</u> by the beekeeper and Apiculturalist Coordinator and are stored and transported together with the exposed APIStrips. In the laboratory these strips will be analysed together with the exposed strips to detect any possible contamination in the storage and transport process.</p>
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5.7 The transport to the Apiculturalist Coordinator (AC)



	<p>The process for transporting the samples from the beekeeper to the Apiculturalist Coordinator will be decided on an individual basis and local conditions. The Apiculturalist Coordinator will give instructions.</p> <p>It is up to the Apiculturalist Coordinator to organise the transport from the beekeeper to the Apiculturalist Coordinator by postal service or by courier.</p> <p>Regardless of the transportation mode, the costs are borne by the study and not by the beekeeper CS.</p>
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5.8 Data entry

	<p>Last but not least, sample information is crucial. Therefore, it is necessary to answer the online questionnaire after each sampling round. Prior to each sampling round, you will receive a unique invitation email with a personalised link to your online questionnaire. The link allows the automatic recording of your answers/data entries from the present sampling round. If you have difficulties reaching the website or if you do not receive the invitation email, please check your spam box or contact the Apiculturalist Coordinator. Note any derogations of the sampling scheme or irregularities, using the comment section of the questionnaire.</p>
	<p>Successful data transmission If you miss the data entry, you will receive a reminder email. If you have successfully submitted your data, within a few minutes you will receive a confirmation email. After data submission, the link will be de-activated and cannot be used again. If you made some mistakes during data entry, no problem - please immediately contact your Apiculturalist Coordinator.</p>
	<p>Thank you for your valuable contribution!</p> <p><u>Apiculturalist Coordinator</u> Email: _____ Phone number: _____ (contactable during sampling periods - Thursday to Sunday from 9 am to 5 pm) (Messenger group: _____) Website: _____</p>

5.9 Support/Video Manual (example from the INSIGNIA Study)

	<p>You will find accompanying instruction videos and constantly updated blog entries on our INSIGNIA YouTube channel and on our website, respectively. During the active sampling periods, the Apiculturalist Coordinator is available via the above-mentioned phone number and social media. If you have any questions or other requests outside of the sampling periods, please write an email to the Apiculturalist Coordinator. Further, you are also invited to the national messenger group to exchange your experiences with the other citizen scientists of your country.</p>
	<p>Video manual APIStrip: www.youtube.com/watch?v=97josWrHu-A</p> <p>Blog: https://www.insignia-bee.eu/blog/</p>



6 Background information on the APIStrip

The APIStrip is a passive, non-invasive sampling tool that accumulates pesticides circulating in a bee colony. In comparison to the one-day sampling of the pollen trap, the strip can stay in the hive for a longer period to accumulate more material. The strip is made of a thin plastic sheet that is impregnated with Tenax, a substance that adsorbs molecules onto its surface which can be analysed by gas- and liquid chromatography in the laboratory. The coating was tested and proved as **bee-friendly**, which means that no unwanted substances will be released into the hives. To protect the matrix from light, it comes in an aluminium envelope / foil. The APIStrip is inserted in the bee lane in the middle of the lowest box (centre of the brood nest) of the hive. Any simple wire /rod with a T-construction (e.g. bent paper clips) will keep the APIStrip in its place. You can also use a thin and short branch to secure the wire. It is not recommended to use matches, as the strip may fall down and as the bees may remove the coating of the match head. To avoid any contamination between the sampling rounds, use a new paper clip / rod for each APIStrip.





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1 APIStrip extraction procedure

The desorption procedure of the pesticides from the Tenax surface is carried out as follows: first, the APIStrips are cut in small pieces and placed inside 50 mL PTFE centrifuge tubes. Then, 10 mL of acetonitrile are added and the samples are automatically shaken at 1250 rpm (Geno/Grinder 2010, SPEX) for 3.5 minutes and centrifuged at 4000 rpm for 5 minutes.

Procedural internal standards are used as surrogate standards to control the extraction performance: dichlorvos-D6, malathion-D10, carbendazim-D3 and triphenyl phosphate (TPP). The recovery of dichlorvos-D6, malathion-D10 and carbendazim-D3 is checked by LC and the recovery of malathion-D10 and triphenyl phosphate is tested by GC.

For the preparation of the injection vials, different procedures are followed according to the analytical technique. For liquid chromatography vials, 1 mL of extract is evaporated under a gentle nitrogen stream and reconstituted with 100 μ L of acetonitrile; afterwards, 400 μ L of ultrapure water are added. For gas chromatography, 500 μ L of extract are evaporated and reconstituted with 50 μ L of ethyl acetate. In both cases, the vial preparation entails a 10-fold concentration. Injection internal standards (dimethoate-d6 for LC, lindane-d6 for GC) are employed to check the variations in the injection volume.

Calibration curves are prepared as follows: a blank APIStrip extract (500 μ L for GC-MS/MS, 1 mL for LC-MS/MS) is evaporated and reconstituted with an organic solvent (50 μ L ethyl acetate for GC-MS/MS, 100 μ L acetonitrile for LC-MS/MS) containing a mixture of pesticides at 0.5, 1, 5, 10, 50, 100 or 200 μ g/L. For the LC vials, 400 μ L of ultrapure water are added.

1.1 GC-QqQ-MS/MS analysis

The analyses by gas chromatography is performed in an Agilent Intuvo 9000 GC system equipped with an Agilent 7693 autosampler and an Agilent 7010 GC-MS/MS triple quadrupole. Data acquisition and processing is developed by Agilent MassHunter QQQ Acquisition and Quantitative Analysis software version 10.0. The samples are injected using a multimode injector inlet in splitless mode, through an Agilent ultra-inert inlet liner with a glass wool frit. The injection volume is 1 μ L. The injector temperature is kept at 80 °C during the solvent evaporation stage (0.1 min) and then ramped up to 300 °C at 600 °C/min for 5 min and up to 250 °C at 100 °C/min. Two planar columns (Agilent), HP-5MS UI 15 m long \times 0.25 mm i.d. \times 0.25 μ m film thickness are used.

The oven temperature program is as follows: 60 °C for 0.5 min, up to 170 °C at 80 °C/min and finally up to 310 °C at 20 °C/min. The total run time is 12.4 min with 2.1 additional min for backflushing at 310 °C. The instrument works at a constant flow (1.28 mL/min column 1, 1.48 mL/min column 2). The system works in dynamic MRM, acquiring the transition in a \pm 0.2 min window from the retention time of the analyte. Helium (99.999% purity) is used as the carrier and quenching gas, and nitrogen (99.999% purity) as the collision gas. The collision and quenching gas flows are 1.5 mL/min and 2.25 mL/min, respectively. Both the transfer line and the ion source - operated in electron ionisation- are maintained at 280 °C. The quadrupole analyzer temperature is fixed at 150 °C. The solvent delay is 2.6 min.



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For the optimisation of the MS parameters, all pesticides are monitored in full-scan mode in the 50–550 m/z range. The first step is the selection of the precursor/s ion/s for each analyte and the retention time, injecting individual solutions for each pesticide at 1 mg kg^{-1} in full-scan mode. The ion with the highest intensity and m/z relationship is selected as the precursor ion. Precursor ion fragmentation is performed by collision-induced dissociation with nitrogen, from which the best fragment ions are chosen. Once the fragment ions are selected, the adequate CE for each transition is assayed in the 3–40 eV range.

1.2 LC-QqQ-MS/MS analysis

An Agilent UPLC 1290 Series (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent Technologies 6490 Triple Quad LC/MS is used for this study. Data acquisition and processing is developed by Agilent MassHunter QQQ Acquisition and Quantitative Analysis software version 10.0, using dynamic MRM software features with a retention time window of 0.8 min. The injection volume is 5 μL , and the chromatographic separation is carried out with a Zorbax Eclipse Plus C8 column (Agilent), 2.1 mm \times 100 mm \times 1.8 μm . The system employs 0.1 % formic acid in milliQ water (mobile phase A), and 0.1 % formic acid and 5 % water in acetonitrile (mobile phase B) with the following gradient: 20 % of B for 2 min, a linear gradient up to 100 % of B in 13 min and finally an isocratic mode at 100 % of B for 2 min. Subsequently, an equilibration step coming back to 20% of B (2.5 min) is performed. The system is provided with a JetStream electrospray ion source, employing nitrogen as the nebulizer gas. This ion source is configured as follows: 120 °C for the drying gas temperature, 13 L/min for the drying gas flow rate, 45 psi for the nebulizer pressure, 375 °C for the sheath gas temperature and 10 L/min for the sheath gas flow rate. The MS uses nitrogen as the collision gas (99.999 % purity), 380 V for the fragmentor and 3,000 V for the capillary voltage, both in positive and negative mode.

For the optimisation of the MS parameters, all pesticides at 0.1 mg/L (acetonitrile:water, 1:1, v/v) are injected directly into the MS system in full-scan mode within the 50–800 m/z range. From this injection, the precursor ion is selected. Then, one more injection in product ion mode is needed to choose two fragment ions and the optimum collision energy (CE) for each transition. The most intense transition is selected as the quantifier transition (SRM1), while the second most intense is chosen as the qualifier transition (SRM2).



2 Metabarcoding procedure

All steps, from pollen homogenisation, DNA extraction, to sample preparation for PCR, should be carried out in a safety cabinet to prevent any contamination from airborne pollen. Additionally, before starting, all equipment and materials (e.g. SC, micropipettes, magnetic stirrer, centrifuge) should be carefully cleaned with 10% bleach and 70% ethanol and autoclaved (e.g. beakers, magnets, racks). The UV light of the safety cabinet (and of the room, if exists) should be turned on for 1 hour. Negative controls should be included in every batch of DNA extraction and PCR plate, for monitoring potential contamination.

2.1 Pollen homogenisation

Pollen homogenisation is carried out by weighing 2 g of pollen pellets into a 50 mL beaker, followed by adding 4 mL of sterile ultrapure water. This solution is homogenised using a magnetic stirrer until the pollen pellets are totally dissolved. A volume of 200 μ L of the homogenised solution (\sim 50 mg of pollen) is placed in a 1.5 mL microcentrifuge tube and then centrifuged at maximum speed for 3 minutes. After centrifugation, the supernatant is discarded and 1 mL of absolute ethanol is added to the tube, which is then stored at -20°C until DNA extraction.

2.2 DNA extraction

DNA is extracted from the \sim 50 mg pollen using the NucleoSpin Food Kit (Macherey-Nagel, Düren, Germany), according to manufacturer's instructions, with minor modifications as follows:

- a) The ethanol-stored pollen samples are centrifuged at maximum speed for 3 minutes, and the supernatant (ethanol) is discarded;
- b) The pollen is transferred to a 2.0 mL screwcap tube containing a mix of zirconia beads of varying sizes (100 μm , 200 μm , 400 μm , 800 μm , 3 mm) to target all pollen grains;
- c) A volume of 550 μL of the NucleoSpin lysis buffer is added to the 2.0 mL tube and the mixture is ground in a powerful tissue homogeniser (e.g. Precellys, Bertin) to assure exine rupture of all pollen grains;
- d) The next DNA extraction steps strictly follow manufacturer's instructions.

After extraction, the quality and yield are measured in a SPECTROstar® Nano (BMG Labtech, Ortenberg, Germany) and the DNA extracts are diluted to 10 ng/ μL before PCR.

2.3 Library preparation

ITS2 library preparation follows a dual indexing strategy (Kozich et al., 2013) and starts with a two-stage PCR: amplicon and indexing. PCR products are sequenced in the Illumina MiSeq platform using a paired-end protocol to generate millions of overlapping reads.

a) Amplicon PCR

In stage-one PCR, the ITS2 nuclear region is amplified using the universal primers ITS-S2F (Chen et al., 2010) and ITS-S4R (White et al., 1990), which have been expanded to include the Illumina sequence adaptors for the stage-two PCR (Figure 4.1; Table 4.1).

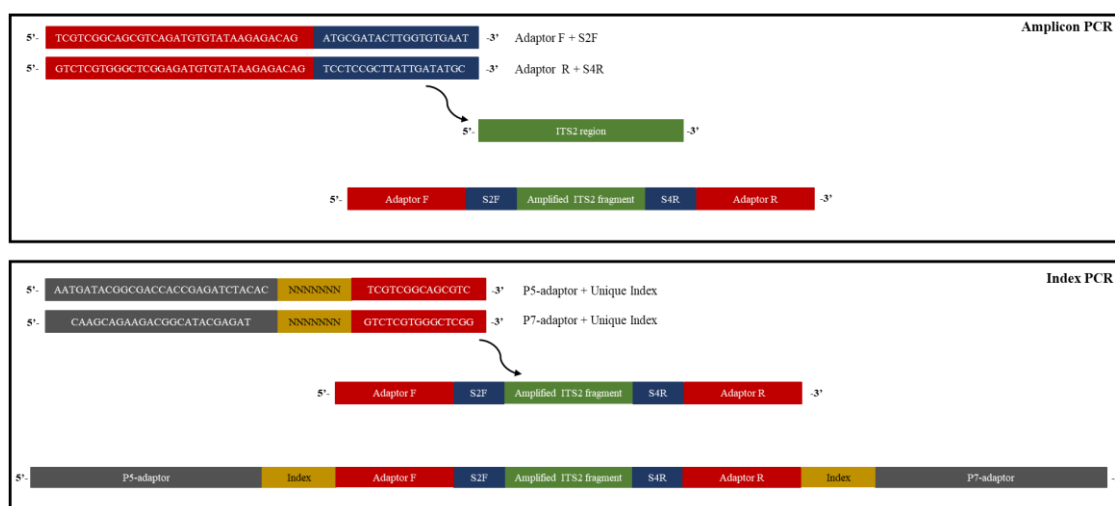


Figure 4.1. Structure of the Illumina library

To overcome the problem of low base diversity, typical of amplicon libraries, and therefore improving sequence read base quality, a pool of seven different forward and reverse oligos are used in the PCR (Wu et al., 2015). The difference between each oligo is the addition of any nucleotide "N" between the adaptor and both primers (Table 4.1).

Table 4.1. Oligos with variable number of Ns (underlined). The 33-nt adapters are marked in red. The ITS2-specific forward (ITS-S2F) and reverse (ITS-S4R) primers are boldface. A 10 μ M pool of the seven different oligos is used in stage-one PCR.

Adaptor-ITS-S2F (forward)	Adaptor-ITS-S4R (reverse)
5' TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAGATGCGATACTTGGTGTGAAT 3'	5' GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAGTCCTCCGCTTATTGATATGC 3'
5'TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAG <u>N</u> ATGCGATACTTGGTGTGAAT 3'	5'GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAG <u>N</u> TCCTCCGCTTATTGATATGC 3'
5'TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAG <u>NN</u> ATGCGATACTTGGTGTGAAT 3'	5'GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAG <u>NN</u> TCCTCCGCTTATTGATATGC 3'
5'TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAG <u>NNN</u> ATGCGATACTTGGTGTGAAT 3'	5'GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAG <u>NNN</u> TCCTCCGCTTATTGATATGC 3'
5'TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAG <u>NNNN</u> ATGCGATACTTGGTGTGAAT 3'	5'GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAG <u>NNNN</u> TCCTCCGCTTATTGATATGC 3'
5'TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAG <u>NNNNN</u> ATGCGATACTTGGTGTGAAT 3'	5'GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAG <u>NNNNN</u> TCCTCCGCTTATTGATATGC 3'
5'TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAG <u>NNNNNN</u> ATGCGATACTTGGTGTGAAT 3'	5'GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAG <u>NNNNNN</u> TCCTCCGCTTATTGATATGC 3'

PCR reactions are carried out in a T100TM Thermal Cycler (BioRad, California, USA) using a 10 μ L total volume containing 10 ng/ μ L of DNA, 5 μ L of Q5[®] High-Fidelity 2X Master Mix (NEB, MA, USA), and 0.5 μ L of the extended ITS-S2F and ITS-S4R oligo pools at 10 μ M (Table1). Each pollen sample is amplified in triplicate (e.g. three PCR plates for 96 samples, including controls). Thermal cycling conditions are 98°C for 3 min, 35 cycles of 98° C for 10 sec, 52° C for 30 sec and 72° C for 40 sec, and a final extension of 72°C for 2 min.



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For checking amplification success, 2 μL per sample of PCR products are loaded on a 1% agarose gel and run at 95V. The remaining $\sim 8 \mu\text{L}$ of the amplicons are purified from unincorporated reaction components by using reversible immobilisation (SPRI) paramagnetic beads (Agencourt AMPure XP, Beckman Coulter, CA, USA) with 0.8X of beads per μL of PCR product.

b) Indexing PCR

In the stage-two indexing PCR, unique 7-nt sequence indexes are incorporated into the amplicons. These unique sequences allow pooling of hundreds of samples in the sequencing library. The indexing PCR is prepared individually for each replicate using 2.0 μL of 1:10 dilution of the previously purified amplicon, 5 μL of KAPA HiFi HotStart ReadyMixPCR Kit (Kapa Biosystems, MA, USA), 0.5 μL of 1 μM of forward and reverse oligos, in a total volume of 10 μL . The oligo scaffold includes the MiSeq-specific sequence (forward: P5, reverse: P7), to attach to the sequencing flow cell, a 7-nt unique index, to allow multiplexing, and the 14-15 nt adapters to attach to the stage-one amplicon (Figure 4.1, Table 4.2). Thermal cycling conditions are 95°C for 3 min, followed by 10 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final extension of 72 °C for 5 min. Products are purified with the paramagnetic bead protocol used before and then run on a 1% agarose, for quality checking of the indexing reaction.

Table 4.2. Oligos used in the indexing PCR. The sequences in yellow and blue are the MiSeq-specific adapters P5 and P7, respectively, read by the MiSeq flow cell. The 7-nt underlined sequences are examples of unique indexes, which allow sample pooling. The size of the pool depends on the number of unique indexes used. The remaining 14-15 nt adaptors will attach to the amplicon of stage-one PCR.

P5-index (forward)	P7-index (reverse)
5' <u>AATGATACGGCGACCACCGAGATCTACAC</u> AC CAACT TCGTCGGCAGCGTC 3'	5'CAAGCAGAAGACGGGCATACGAGAT <u>AGG</u> TATT <u>GTCTCGTGGGCTCGG</u> 3'
5'AATGATACGGCGACCACCGAGATCTACAC <u>CG</u> AATGC TCGTCGGCAGCGTC 3'	5'CAAGCAGAAGACGGGCATACGAGAT <u>CCT</u> ATCT <u>GTCTCGTGGGCTCGG</u> 3'
5'AATGATACGGCGACCACCGAGATCTACAC <u>G</u> A TCTCG TCGTCGGCAGCGTC 3'	5'CAAGCAGAAGACGGGCATACGAGAT <u>CCT</u> GCGA <u>GTCTCGTGGGCTCGG</u> 3'



2.4 Library normalisation, pool size and quantification

Purified indexed amplicons are quantified on an Epoch Spectrophotometer and then normalised to a final concentration of 10 nM. After normalisation, all the samples from each PCR plate are combined into one pool. For further confirmation that the correct ITS2 fragment has been amplified, the size distribution of the amplicons is determined for each pool on a TapeStation 2200 using the HS D1000 kit (Agilent Technologies, CA, USA). Next, each pool is quantified by a SYBR green quantitative PCR (qPCR) assay using the KAPA Library Quantification kit (Kapa Biosystems, MA, USA) and the BIORAD C1000 Real Time Thermo cycler (BioRad, California, USA). After quantification, pools are combined equimolarly into one final pool for sequencing (sequencing library).

2.5 Library sequencing

The sequencing library is diluted to 2 nM, spiked with 10% Illumina-generated PhiX control library and then sequenced on MiSeq (Illumina, CA, USA) using the MiSeq Reagent Kit v2 (500 cycles) with paired-end reads, according to manufacturer's instructions. Once the sequencing run is completed (after ~39 hours), the pools are de-multiplexed into the original samples, based on the unique 7-nt index, and the primers are trimmed using the BaseSpace™ platform. The sequence reads generated for each sample for the ITS2 fragment are imported into a bioinformatics pipeline (modified from Sickel et al., 2015) for analysis. Briefly, the reads are merged, quality filtered and then classified to the species level using an updated ITS2 reference database that includes sequences representing bee plants collected by the INSIGNIA consortium. The entire work flow for ITS2 metabarcoding of mixed pollen samples is shown in Figure 4.2.

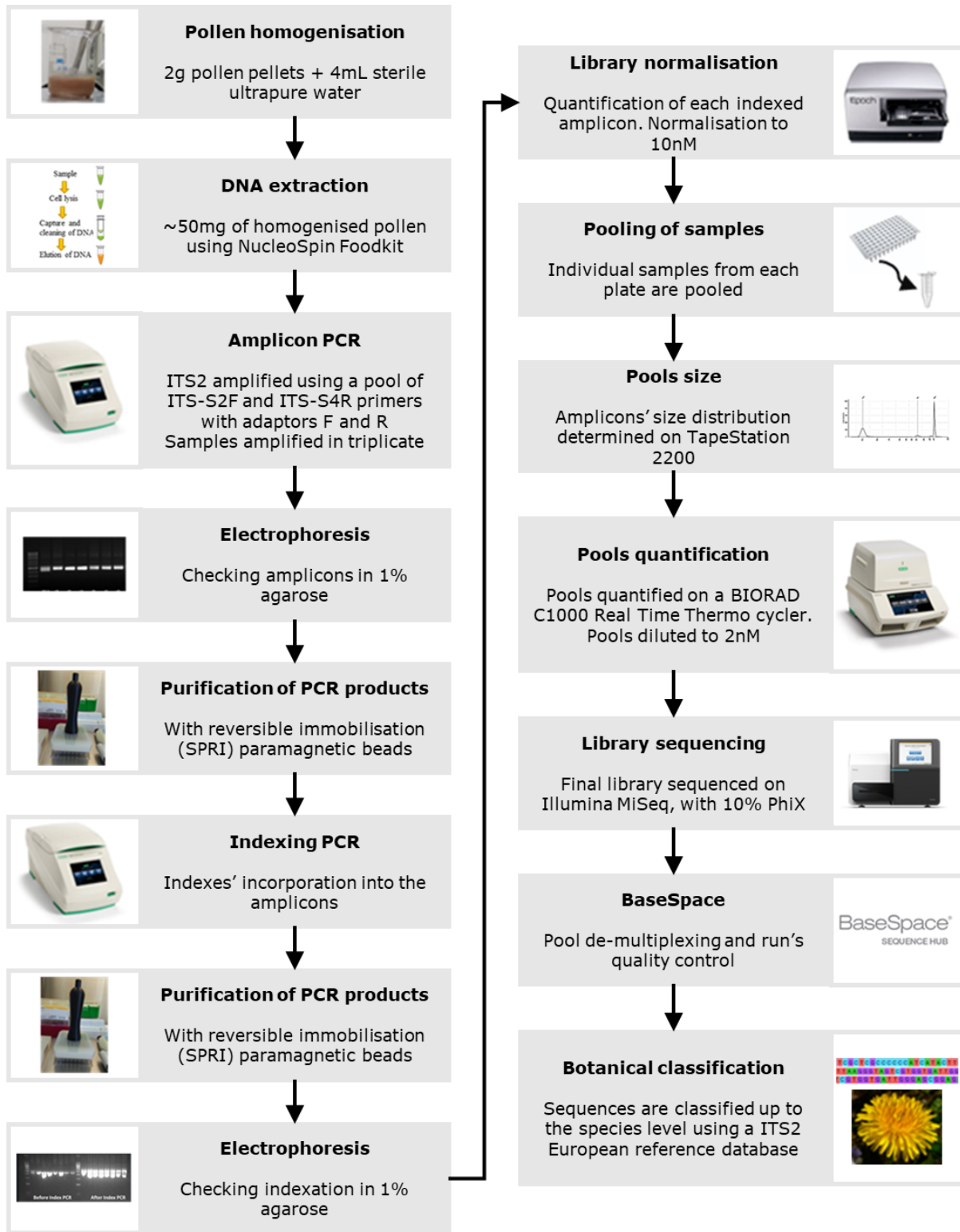


Figure 4.2. Work flow for ITS2 metabarcoding of mixed pollen samples



3 Statistical procedures for analysis of numbers of pesticides

3.1 The aim

The main aim is to assess differences between the numbers of pesticides recorded by sampling date, by the apiary within a country, and by country (if relevant). If a large number of apiaries are participating within any one country, apiaries might be categorised by geography or landscape type and this could be used instead of apiary as an explanatory factor.

This is qualitative analysis based on the number of pesticides only, rather than actual concentrations of any one pesticide residue detected.

3.2 Data processing

The data source is a spreadsheet recording the assembled data from the laboratory analysis of the APIStrips. If this does not directly contain numbers of pesticides detected for each colony in each apiary per sampling date for each participating beekeeper (Apiculturalist Citizen Scientist) in each participating country, the data will need to be processed accordingly. Data will be protected according to the General Data Protection Regulation (GDPR). Pesticide concentration data may be processed by setting any blanks / not detected codes to 0 and any recorded concentrations to 1, to record absence / presence data, then summing across the columns recording absence / presence of each pesticide for a given row. Each row corresponds to a colony / apiary / sampling date combination within any given country. The number of pesticides detected will then be available for each row, for use in further analysis as below.

In the INSIGNIA study all detected pesticide residues were treated as indicating presence of the pesticide, regardless of the recorded concentration i.e. any recordings of <LOQ or < 0.5ppb were also included, as advised by the consortium's analytical chemists.

3.3 Descriptive statistics

Summary statistics (including, for example, number of observations, minimum, mean, median, maximum, lower and upper quartiles) should be used to form an informal impression of the data overall for each country, and of any differences between sampling dates or apiaries within a country. Separate analyses for each country are appropriate.

Histograms or barcharts, and / or boxplots, are useful for visualising the distribution of the data. Barcharts are suitable if the maximum number of pesticides recorded for any one sampling occasion is not large, otherwise histograms may be preferred. Boxplots are useful for identification of any outliers (extreme or unusual observations) as well as assessing level and spread.

Parallel boxplots of the number of pesticides are recommended for indication of unusual observations and comparisons of the level and spread of the data between apiaries or between sampling dates. Each individual boxplot will correspond to all the data recorded for one apiary or one sampling date for a given country.

Two-way frequency tables and image plots (frequency plots) displaying colour-coded frequencies of the number of pesticides are also recommended to show differences between apiaries or sampling dates, for a given country. Greater depth of colour in one cell of the plot indicates a greater frequency of occurrence of that number of pesticides at the apiary or date indicated by that cell. The rows will correspond either to apiaries or sampling dates and the columns will correspond to number of pesticides, or vice versa.



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The image plots make visual identification of any patterns straightforward and also allow comparison of the corresponding plots for each country.

For a given country, the number of times or proportion of times any pesticides were detected at all across the sampling dates may be compared between the apiaries. If a large number of apiaries are participating, the number of times or proportion of times any pesticides were detected in any apiaries could be compared between sampling dates.

3.4 Statistical tests

Tests will be used to formally assess for differences in recorded numbers of pesticides between apiaries or sampling dates for a given country. As the number of pesticides is likely to have a skewed distribution and is count data, likely to have a limited range, a Kruskal-Wallis test of difference in distribution in number of pesticides for different sampling dates or different apiaries is appropriate. If this indicates significant differences, this is followed by multiple comparisons of apiaries or sampling dates, for example using pairwise Wilcoxon rank sum tests corrected for multiple testing. Many options are available for such correction. In the INSIGNIA study, this approach was used with a Benjamini-Hochberg correction for multiple testing.

For a given country, to test the significance of any differences in the proportions of times pesticides were detected at all for different apiaries or for different sampling dates, a Pearson's Chi-squared test can be used on the frequencies of detection and non-detection, or alternatively, if the observed frequencies of detection are low, a Fisher's exact test is appropriate.

3.5 Statistical modelling

As the aim is to relate the number of pesticides to sampling date and apiary for any one country, study of the statistical significance of the effects on number of pesticides of sampling date and apiary, individually or simultaneously, can be accomplished through fitting generalised linear models (GLMs). Poisson, quasi-Poisson or Negative Binomial GLMs are suitable, using a log link function, depending on the variability present in the data. Sampling date and apiary should be treated as factors. Generalised linear mixed models (GLMMs) are also appropriate, including fixed effects for sampling date and apiary, with colony (within apiary) as a random effect.

4 Modelling

The authors of the WUR models are well aware of the EFSA Scientific Opinion on good modelling practice. The Random Forest model used in INSIGNIA is a statistical model. It does not fall under the umbrella of the EFSA Scientific Opinion which focusses on mechanistic models. The pollen foraging model is a variant of the model published by Baveco et al. (2016), and is mechanistic. This model has been developed prior to the EFSA SO. The current version of the pollen foraging model is under development. Future evolutions of the model may lead to applicability in the risk assessment of PPPs, at which point the model, model description and documentation will be assessed against the EFSA SO. Suggestions made therein, as well as following the rationale of the TRACE framework by Schmolke et al. (2010) and updated by Grimm et al. (2014) will be taken into consideration.

4.1 Occurrence of pollen and pesticides in the INSIGNIA landscape

In order to visualise the occurrence of pollen and pesticides in the landscape, pollen and pesticides entering the hive that were sampled during the monitoring campaign were used in two different model approaches. These approaches comprised a 'mechanistic pollen foraging model' and a 'pollen and pesticide classification model'. The mechanistic model (Figure 4.3) simulates bees foraging on pollen in a realistic landscape. It has its roots in energetics, which means that bees will forage in fields where the energetic gain from pollen is maximised. This could mean, for example, that bees forage on a pollen resource with a lower energy content if the net energy gain is higher compared to a pollen resource with a higher energy content but which may be further away from the hive. Ultimately, the types of pollen and pesticides (in the pollen) that are returned to the hive are emergent properties of the model. Detailed information on the landscape, land-use, crop-specific pesticide usage, application times and rates and residues around a hive location are needed, as well as data on plant phenology to infer pollen availability and assumptions on the linkage between landscape and pollen sources (i.e., which pollen originate from which types of landscape elements).

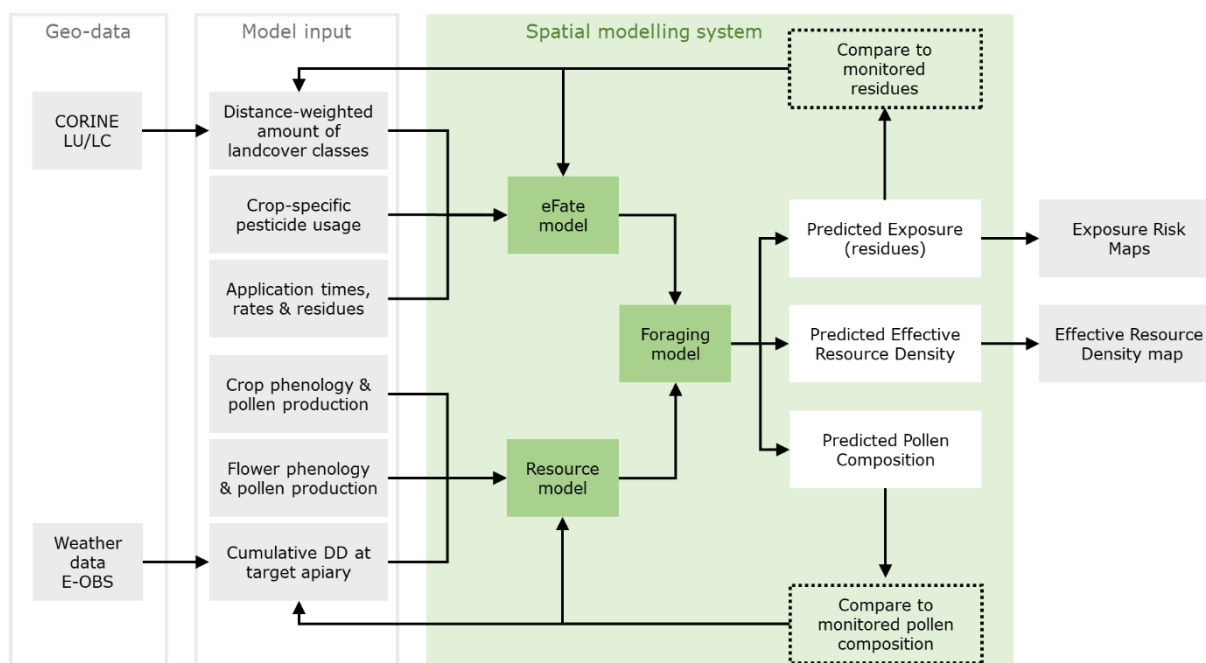


Figure 4.3. Overview of the modelling approach. The different modules deal with (1) the geodata sets used to define the landscape; (2) the definition of the (pollen) resource landscape; (3) the definition of the exposure

landscape; (4) the pollen foraging modelling from which the foraging locations and the pesticide residues in the collected pollen can be obtained.

The classification models (Figure 4.4) are machine learning models (random forest models) that allow for predictions of the number and type of pollen and pesticides that are most likely to be present at a given location and at large spatial scales (e.g., at the scale of a whole country). Although, in contrast to the mechanistic model, it is challenging to gain insights in the driving processes, the classification models have a

lower data requirement. Moreover, they are free from assumptions on the links between the landscape, land-use, and available pollen or presence of pesticides, and do not need information on pesticide usage, application times and rates, and residues that may be available for local areas, but are difficult to obtain at large spatial scales (e.g., at the scale of a whole country).

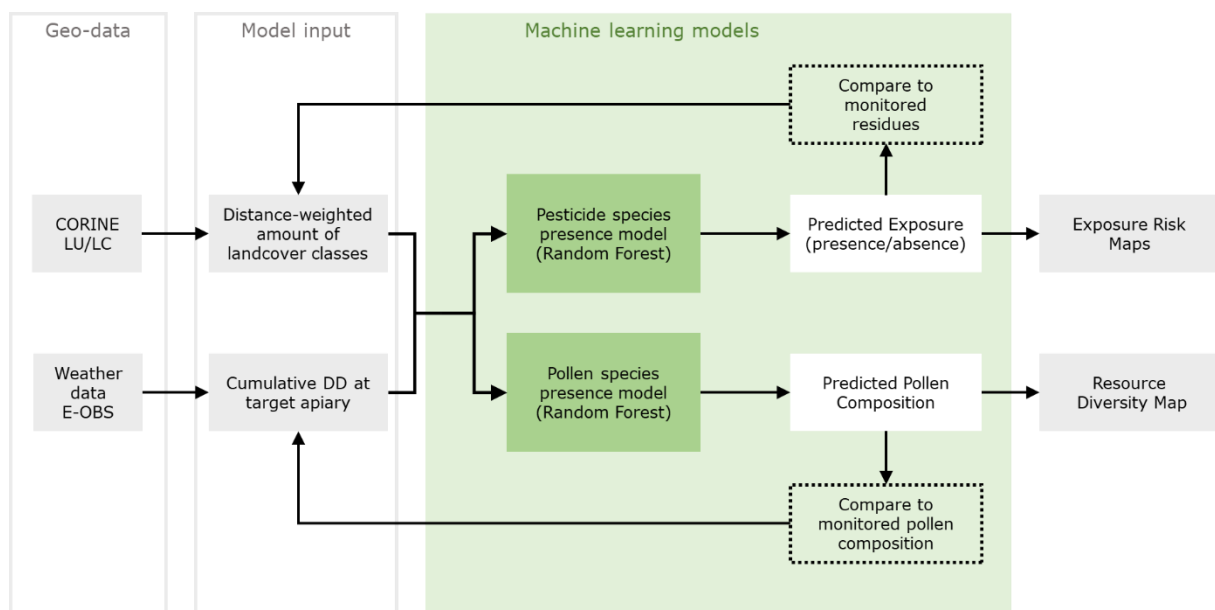


Figure 4.4. Overview of the machine-learning based modelling of the pesticide and pollen species expected in pollen

To apply either type of model, two main types of input geo-data need to be collected and prepared (see Figure 4.3 and Figure 4.4): monitoring data for pollen and pesticides collected in the INSIGNIA study; and two sources of spatial and temporal geodata, i.e. the 'CORINE Land Use and Land Cover' data and the 'pan-European weather' data set. They form the heart of the mechanistic and statistical models, the technical details of which are described in "Mechanistic pollen foraging model" and "Pollen and pesticide classification model" respectively. Before describing these models, first the underlying geo-data sets are discussed.



4.2 Spatial and temporal geodata

4.2.1 CORINE Land Use and Land Cover data

The first step in preparing the necessary data for the mechanistic and classification models is the geo-data (see box 'Geo-data' in Figure 4.3 and Figure 4.4). After the first processing step, which is described in the section "Kernel model", the data can be used as input to the models (see box 'Input data' in Figure 4.3 and Figure 4.4). The data describe the landscape around the locations of hives used in this study and are used to form a link between the physical landscape and the presence of pollen and pesticides. The data set used is the CORINE Land Cover (CLC) dataset, which is based on satellite imagery. It was first developed in the 1980s with the aim to standardise data collection on land and land-use in Europe and to support environmental research and policy. The dataset contains information on the land use and land cover over the whole of Europe at a relatively coarse 100m resolution. In other words, the whole of Europe is divided into 1 hectare grids, and each grid cell is placed into a category describing the land-use. In total 44 different land-use classes are recognised. They are sub-divided into five main categories: artificial surfaces (e.g., roads, built up areas), agricultural areas (e.g., pastures, arable fields), forests and semi-natural areas (e.g., rough pastures), wetlands (e.g., bogs, marshes), and waterbodies (e.g., rivers and lakes). The most recent CORINE raster version (coordinate reference system EPSG:3035 - ETRS89-extended / LAEA Europe – Projected; 2018) was downloaded from <https://land.copernicus.eu/pan-european/corine-land-cover/clc2018?tab=download> and used for this study. Obviously, there is no need to use the entire data set for the whole of Europe for each hive. Therefore, in order to extract the relevant information from the CORINE database (i.e., within the vicinity of a hive) Python scripts are used to obtain so-called clipped maps that contain information on the type of CORINE CLCs in the local area. Pre-processing steps of the models expect maps in ascii-grid format. In the INSIGNIA study, two spatial scales are used to extract CLC data. Firstly, bee foraging is assumed to be limited to the landscape within a 3.25 km radius of the hive. With this distance in mind, for pollen resources a hive location is defined by a clip of 65 by 65 cells with the hive coordinates in the centre. In other words, the hive is at the centre of a 'patch' with an area of 6.5 by 6.5km. Secondly, the foraging landscape that is used by bees can be influenced by pesticides coming from within this area but also from outside this area by, for example, drift deposition. Therefore, for the landscape that determines the potential sources of exposure to pesticides, a larger clip area is used, i.e., 513 by 513 cells.

4.2.2 Pan-European weather data

The second primary source of input geo-data required by both models is on temperature patterns (see Weather data E-OBS in the box 'Geo-data' in Figure 4.3 and Figure 4.4). The reason is that the phenology of plant species determines when that species starts flowering and thus when pollen is available. It is, however, impossible to obtain information on plant phenology for individual plant species from geo-data directly. The number of cumulative degree days (CDD10) above a threshold value (10 °C in this report) is generally considered to be an important species-specific requirement. Indeed, species-specific thresholds in CDD10 for first bloom and / or full bloom can be found in phenology calendars such as: <https://www.oardc.ohio-state.edu/CDD10/>. For maximum generality, for each pollen resource the flowering period is defined as a distribution over CDD10 values with a base temperature of 10 °C. To apply the models for specific location and time of the year, the CDD10 up to this date for this location will be needed. The CDD10 can be calculated from temperature time-series by summing the difference between the daily average temperature (geometric mean between minimum and maximum) and the threshold temperature.

See Figure 4.5 for an example of accumulated CDD10s for INSIGNIA apiaries in 2019. To avoid the accumulated CDD10 from decreasing after cold days (i.e., incorrectly reverse plant development), if the minimum or maximum temperature are below the threshold temperature they get the same value as the threshold. This is the so-called Modified Average Method (for more information see <http://cues.cfans.umn.edu/old/IPM-Tactics/IPM-tactics.html> chapter 11: Using Degree-Days and Plant Phenology to Predict Pest Activity).

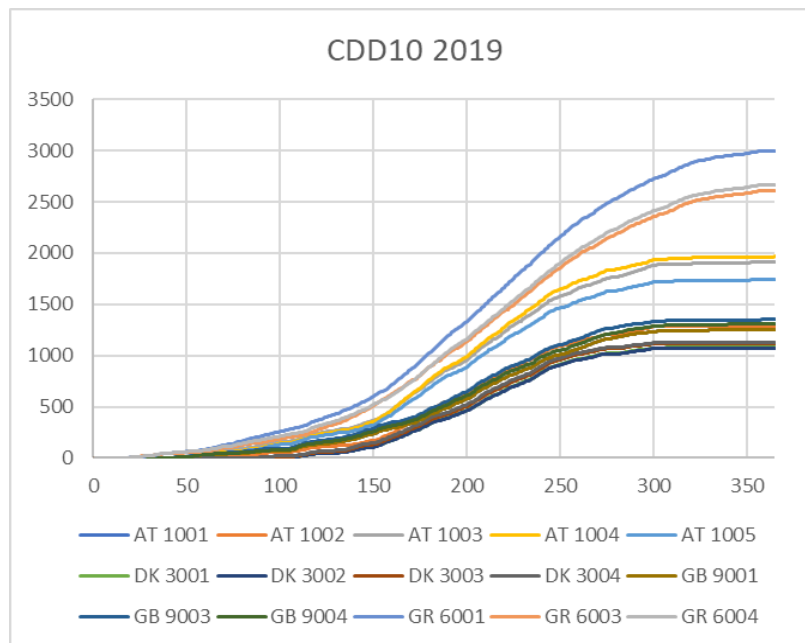


Figure 4.5. Cumulative degree days with base temperature 10 °C for the INSIGNIA apiaries in 2019

For any location in Europe, and for a large number of years (from 1950 onwards), time-series of daily temperatures were obtained from the daily gridded observational dataset for precipitation, temperature and sea level pressure in Europe (E-OBS), provided by the Copernicus Institute, on a 0.1 degree regular grid (<https://www.ecad.eu/download/ensembles/download.php>). For the calculations, version 21e was used. Note that for a probabilistic approach, one can download ensemble spread data (difference between the 5th and 95th percentile indicating the 90% uncertainty range). However, here a deterministic approach was followed, based on the median values (i.e., the median values of time-series of minimum and maximum temperatures of an ensemble of models). Additionally, the ensemble median values can be downloaded in smaller chunks from the website: http://surfobs.climate.copernicus.eu/dataaccess/access_eobs_chunks.php. Finally, for recent periods (July 2020 – January 2021 at the time of writing: 4 March 2021) monthly datasets are available from: http://surfobs.climate.copernicus.eu/dataaccess/access_eobs_months.php). An example of the contents of such an E-OBS dataset, for 31 December 2019, is shown with the apiaries in Denmark, Austria, UK and Greece, in Figure 4.6. Using the R packages *ncdf4*, *raster*, *rgdal* and *ggplot2*, the timeseries for given hive coordinates and year can be extracted for use in the model (R-script available upon request to the authors). With the temperature data pre-processed, the required input geo-data are complete and can be used as the first set of input data (see Figure 4.3 and Figure 4.4).

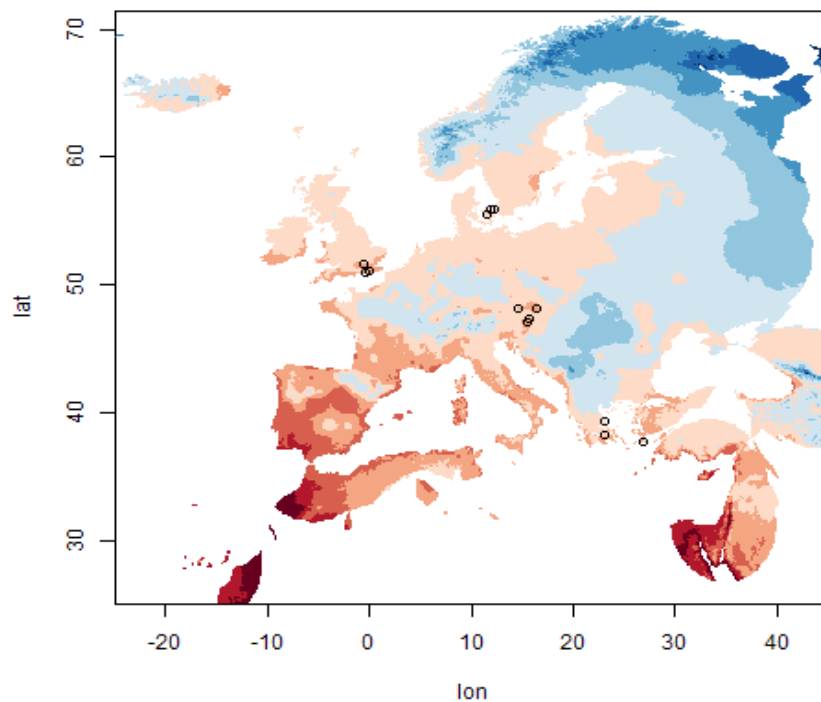


Figure 4.6. Location of the apiaries plotted on the map with temperatures on 31 December 2019. Indicative colours range from warm (dark red) to cold (dark blue).

4.3 Mechanistic pollen foraging model

4.3.1 Kernel model

For a given hive location, the processed CORINE CLC data represents the landscape around the hive. It is assumed that within this local landscape, areas closer to the hive will have a stronger influence on pollen and pesticides than areas that are further away. To account for this, a so-called kernel model is used to analyse the landscape around a hive location. The model calculates a distance-weighted impact for each grid cell, where grid cells closer to the hive “weigh heavier” than grid cells further away. The impact of the CLC that is at the hive location has an impact value of 1 and the further away a cell is, the smaller its influence will be. At this stage it is assumed that the influence of a land cover class at the hive location is representative of the experienced foraging landscape used by bees belonging to the hive in the centre. In other words, bees are assumed to use all of their surroundings at the same distance equally. Next, all calculated values are summed for each of the CORINE CLCs which gives a total “impact” of a certain land-use category at the location of the hive. As mentioned, the relevant landscape for pollen is defined as a 65x65 grid (cell size 100m) and for pesticides we take into account an eight times larger landscape on a 512x512 grid. Furthermore, the influence of the surrounding area is assumed to decrease exponentially. Four different spatial kernels were tested to “distribute” the impact of CLCs around the source cells such that impact is halved at distances of 69, 347, 693 and 3466 m.

Principal component analysis and hierarchical clustering of kernel data

Depending on their location, apiaries may “experience” a similar landscape. To investigate relationships between apiary sites in the INSIGNIA study and using the different kernel distances, a Principal Component Analysis (PCA) was performed followed by a clustering analysis. The clustering analysis gives insight into how apiaries are associated to each other in increasingly larger “experienced” landscapes. The analyses



were done using the *FactoMineR* package in R (script available from authors upon request). In the PCA analysis, the number of dimensions (i.e., the number of axes fitted to the data) was reduced so that a minimum of 85% of variance is explained. For all four kernel distances, this resulted in retaining the first seven PC-axes. The principal component scores from the PC-axes were then used as input for a hierarchical clustering analysis.

4.3.2 Plant phenology

Crop and flower phenology are input requirements into the mechanistic model (see Resources box in Figure 4.3). Plant phenology dictates when and which pollen resources are available to bees. In the foraging model, for a given location and date, a CDD10 is calculated (see Pan-European weather data for details). Subsequently, using the CDD10 value, plant phenology is implemented as a filter for scaling the basic resource availability. In the INSIGNIA study, phenology is defined with a Gaussian distribution around the mean optimal flowering CDD10 for a plant species, and in case of an optional second flowering period, with a second Gaussian distribution around the mean CDD10 for the second flowering period. To make the distribution more discrete, values smaller than 0.01 fraction flowering are set to zero. The function is normalised, dividing it by the maximum value (at the mean), resulting in the function for the fraction of blooming plants depending on the CDD10 (x) for a single flowering period:

$$\text{Equation 1} \quad \mathbf{F}(x) = e^{-\frac{1(x-\mu)^2}{2\sigma^2}}$$

with m representing the mean optimal flowering CDD10 and s the standard deviation in CDD10 days.

4.3.3 Pollen resources

Utilising the phenology information, the landscape in terms of land-cover classes is converted into the 'raw' resource landscape by setting the presence of the main (pollen) resources (flowering plant species) in each of the land-cover classes (see Resource model balloon in Figure 4.3). The following section provides a more detailed description of the resource model.

Presence is defined in terms of the amount of pollen available when the species is in full bloom, as pollen density in g per m². As spatial units are 1 ha cells, this density applies to the whole cell. For pollen-providing trees, it is more conveniently calculated as the number of individuals per ha divided by 10,000. The absolute amount of resources available in the 1 ha cell is then equal to the tree density * 10,000 (g ha⁻¹). For resources that cannot be assumed to be uniformly distributed within the 1 ha grid cell, a coverage fraction smaller than 1.0 should be set. This will be the case for composite land cover classes that represent a combination of landscape elements, e.g., a mosaic of small agricultural fields and semi-natural elements. The effective pollen density in such an area, for a flower species that occurs in e.g., the semi-natural elements, will then be 1 / coverage * density. Note that for foraging choices made by the bees, this pollen density may be the relevant unit, while for estimating the amount of resources provided by the landscape the absolute amount (for the whole grid cell) may play a role. To define the basic resource landscape, a table with pollen density per plant species against (relevant) land-cover classes is required and a similar table with coverage fractions. These tables thus define $N_{i,j}$ and $C_{i,j}$ as the pollen provider density (ind m⁻²) and coverage fraction (-) of resource i in land-cover class j , respectively.

The actual effective resource availability R (in g pollen per m²) provided by species i in a cell with landcover class j at time t is defined as:



Equation 2 $R_{i,j,t} = F_{i,t} \cdot 1/c_{i,j} \cdot P_{i,j}$

The phenology of the species acts as a filter (F , between 0 and 1) scaling the pollen resource availability. The absolute amount of resource G in kg per ha offered by the resource over the whole grid cell is then given by:

Equation 3 $G_{i,j,t} = F_{i,t} \cdot P_{i,j} \cdot \frac{10000}{1000}$

The pollen density while optimally flowering, $P_{i,j}$, is the product of the pollen provider density, $N_{i,j}$, and characteristics of the pollen provider that do not depend on habitat nor time, S_i (g pollen ind⁻¹):

Equation 4 $P_{i,j} = N_{i,j} \cdot S_i$

This S_i is defined as the product:

Equation 5 $S_i = \text{flower(head)s/individual} \cdot \text{pollen/flower(head)s}$

As input for the model, the abundance of the specific pollen provider, the coverage fraction of the pollen provider’s habitat and the invariant pollen provider characteristics thus need to be defined (Table 4.3).

Table 4.3. Abundance N_{ij} [ind m⁻²] of plant species i in each CLC j , fraction coverage of the pollen provider habitat within the grid cell c_{ij} and the invariant pollen-provider properties (amount of pollen per individual plant) S_i .

Species i	$N_{i,1}$	$c_{i,1}$...	$N_{i,j}$	$c_{i,j}$	S_i	

4.3.4 Exposure landscape

The exposure to pesticides in the mechanistic model (see “eFate model” balloon in Figure 4.3) is based on 11 CORINE CLCs that refer to agricultural use (Table 4.4). For six of these 11, the actual crop grown is at least broadly defined. For the other classes, additional (national, regional) data would be needed to assign a crop to them. Even when a (dominant) crop would be known, additional data would be needed on the use of pesticides (e.g., active substances, application rates, number of applications) on this crop. Given the lack of such data, we do not attempt to define an environmental fate model. Rather, we take a simplified approach based on the kernel calculations described in the section “Kernel model” and using only agricultural CORINE CLCs. With additional information on the application rate of a pesticide in a given CLC and of the fraction of the applied substance that will be distributed outside the area of direct application and of the relationship between deposition and residue in pollen (see box “Exposure” in Figure 4.3), the summed impact can be converted into expected residues at the hive.



Table 4.4. Land-cover classes in the CORINE database referring to agricultural use

<i>Land-cover class</i>	<i>Agricultural use</i>
211	Non-irrigated arable land
212	Permanently irrigated land
213	Rice fields
221	Vineyards
222	Fruit trees and berry plantations
223	Olive groves
231	Pastures
241	Annual crops associated with permanent crops
242	Complex cultivation patterns
243	Land principally occupied by agriculture with significant areas of natural vegetation
244	Agro-forestry areas

4.3.5 Pollen foraging

With the outputs from the “Resource” and “eFate” models, the “foraging model” can be run (see Figure 4.3). The outputs from the foraging model are: predicted exposure (residues); predicted mixture risk; predicted pollen composition; and the predicted effective resource density (Figure 4.3). The outputs can be compared against the monitoring data, and the model can be iteratively refined. Once a best-performing model has been established, the outputs can be used to produce exposure and risk maps, as well as effective resource density maps (Figure 4.3). The following provides an overview of the foraging model.

For honey bee pollen foragers, we assume it is the amount of pollen protein that can be obtained from a pollen resource that determines its attractiveness (Liolios et al., 2016). This amount is determined by both the protein content of the pollen and the presence of the resource in the surrounding landscape. Gain of the pollen foraging is the amount of protein collected. To be able to deal with it in a framework balancing gains and costs of collecting the pollen protein, gains and costs need to be expressed in a common currency, for which we use energy (in J). Energy gain is thus the amount of pollen collected, multiplied by its protein content and the conversion factor of protein to energy (17 kJ/g). Energetic costs are the transport costs (unloaded flight from hive to foraging location and loaded flight back to the hive) and the foraging itself. For the latter we apply a single coefficient, lumping the outcome of the processes of movement from flower to flower and from plant to plant, and the actual extraction of the pollen from each flower. The rate with which pollen is collected (f , in mg s^{-1}) depends also on the resource density (g m^{-2}), following a functional response function that puts an upper limit on the rate of collection:

$$\text{Equation 6} \quad f = \frac{aR*1000}{1+ahR*1000}$$



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Here pollen density (R) is converted to mg per m^2 , a represents the attack rate ($m^2 s^{-1}$), and h the handling time ($s mg^{-1}$). NB compared to the nectar foraging model of Baveco et al. (2016) here the species-specific differences in time required to handle a single flower(head) are ignored. The time required to collect a full load of pollen from the resource patch is then:

$$\text{Equation 7} \quad t_L = \frac{\gamma}{f}$$

with γ representing the full capacity (mg) of the forager. Selection of resource patches to exploit is based on the net energetic efficiency (NEE) associated with each. Net energetic efficiency is defined as:

$$\text{Equation 8} \quad NEE = \frac{EI - EE}{EE}$$

with EI representing energy intake (in J), the energetic gain, and EE energy expenditure (in J), the energetic costs. Each resource patch in the landscape that surrounds a hive will have a specific value of NEE . From all resource patches, the ones with the highest NEE will be selected. As a colony is often exploiting several pollen resources at the same time, more than 1 resource may be selected. The number of pollen foragers is divided over the resource patches, proportional to their NEE . Each hourly timestep of the model, an absolute amount of pollen is collected of a particular pollen providing species and originating from a specific location. Of the same species, pollen may be collected from different locations (grid cells). Similarly, at the same location (grid cell) pollen may be collected from different plant species. Note that in our terminology, "resource patch" refers to a species-grid cell combination.

In the resource patches, the pollen foraging in one hour lowers the available pollen density the next hour. With a single colony and grid cells of default 1 ha size, the impact of foraging on density may be small. However, when pollen densities are low, or only a fraction of the 1 ha cell is suitable habitat for the pollen provided, or an apiary with multiple colonies is considered, or a considerable background competition for pollen is assumed, foraging might well lead to severe resource depletion in the exploited patches, resulting in spatial shifts of foraging effort over the landscape.

4.4 Pollen and pesticide classification model

4.4.1 Random forests for classification

The machine learning approach used here is called a random forest. Like real forests, a random forest is made up of individual trees, or so-called classification trees. Each classification tree is slightly different owing to random selection of sample data and splitting variables. It is a basic classifier that places observations in an outcome category based on a set of splitting decisions (Figure 4.7). By combining the results of all trees in the random forest model, an ensemble output is created. This makes it a powerful and suitable model for the INSIGNIA data. Namely, without knowing or assuming anything about the precise underlying interdependencies between the monitored pollen and pesticides and the surrounding landscape from which they come, the random forest model can be used as a predictive model that improves as more data are added (see boxes around Exposure and Resources in Figure 4.4). All the required input geo-data are the same as for the mechanistic model (see boxes "Geo-data" and "Input data" in Figure 4.4.). Below follows more background information on random forests in the context of the INSIGNIA pollen and pesticide data analysis; for the random forest models the monitoring data needs to be processed and this is described in the section "Fitting models to INSIGNIA pollen and pesticide data".

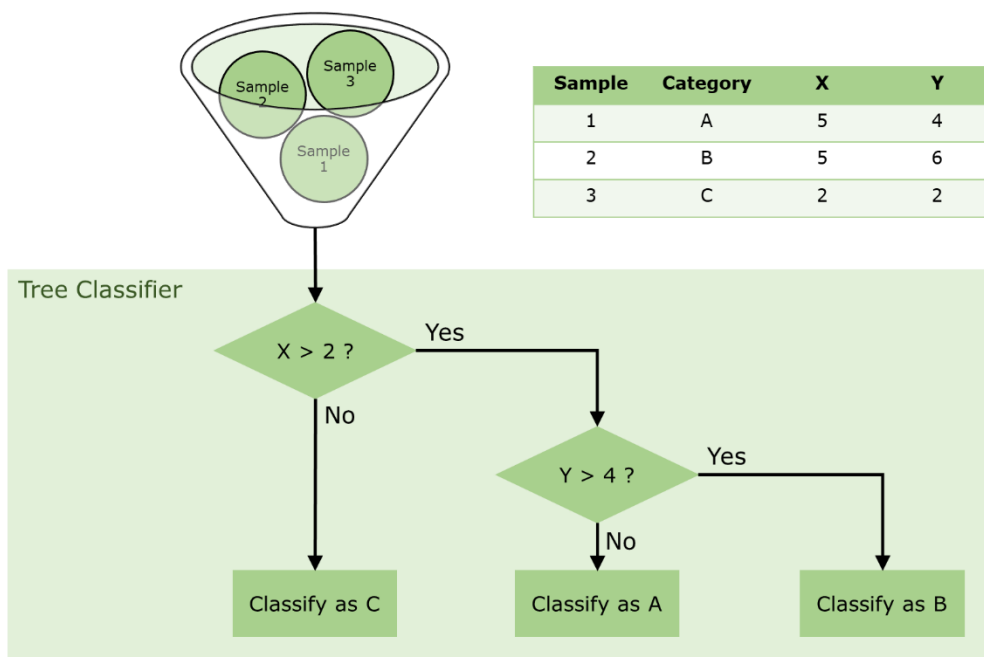


Figure 4.7. Example of a simple classification tree. Samples are associated with explanatory variables X and Y, at each split in the tree samples are divided into groups based on a splitting variable and subsequently placed in a category.

To predict the probability of a pollen family or a pesticide being present in a location, random forest classification models were fitted using the *ranger* package (Wright & Ziegler, 2017) in R (R Core Team 2020). The *ranger* package was chosen because it is fast and has a similar performance compared to other random forest models (Wright & Ziegler, 2017). For each tree in the forest the training data were bootstrapped, sampling 50% of all data to grow each tree in the forest, thus limiting between tree correlation and so improving classification performance of the random forest as an ensemble of tree classifiers. To further reduce the effect of between tree correlation, at each split in a tree a best splitting variable (based on the Gini index) is selected from a randomly selected



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subset of the predictor variables (Breiman, 2001). Each time an observation in the training data is passed through the classifier, it is placed into a certain outcome category (predicted outcome); each tree can lead to a different outcome for a sample. Next, from the ensemble of classifiers (i.e., the collection of all trees) a probability estimate is given for the membership of a sample to a certain category based on the proportion of all the trees that predict that category for the sample (observation).

There are a number of parameters that can be adapted in the tree fitting procedure (see Wright & Ziegler, 2017 for details), and the four most important parameters (and their chosen values) that were adapted here are: the sample fraction (0.5), the number of trees (2000), the number of randomly selected predictor variables (11), and the minimum size of a terminal node (1, i.e., the number of observations that reach a terminal node in a tree).

4.4.2 Fitting models to INSIGNIA pollen and pesticide data

Pollen and APIStrip samples were split to individual observations. Distance weighted CORINE CLC data and CDD10 data (see Figure 4.4) were collected and linked to individual observations, so that each observation is associated with the landscape around the respective hives, and cumulative temperature and month information for pollen and APIStrip samples, respectively. The long distance kernel (see section "Kernel model") was deemed most suitable to describe the surrounding landscape of a hive. Based on the long-distance kernel, CORINE CLC impact was calculated for each apiary (see section "Kernel model"). Note that, in contrast to the mechanistic model, no assumptions on the likely sources of pollen and/or pesticides are made with respect to the different landcover classes, and therefore here all 44 CORINE CLCs are used. CDD10 information was extracted for the observation's location and date (see section "Pan-European weather data") for pollen and month information was assigned as temporal information for pesticides.

Owing to the fact that most pollen and APIStrip samples had more than one pollen family or pesticide present, the classifier for individual samples is not accurate (i.e., the out-of-bag error is large). Instead, using the probability estimates of class membership for each sample and applying a cut-off value, we obtain a most likely set of pollen or pesticides in the same sample. A range of cut-off values was tested from 0.01 to 0.1. If the probability of class membership is equal or larger than the cut-off value, it is accepted in the set of most likely pollen or pesticides. Lower cut-off values thus result in a less strict classifier. Separate models were created for Austria and Denmark, for both pollen and pesticides (i.e., four classification models).

From all random forest models created, the performance was checked against 30 random subsets of 20% of the available pollen or pesticide data. For each sample, all classes with a probability above the cut-off value were accepted as likely to be present and were stored. Each predicted sample set was compared against the observed pollen or pesticides in the sample (see Figure 4.4). Next, two ratios were calculated: 1) the ratio of accepted classes present in the sample versus actual classes in the sample (true positives, range: [0,1]); 2) the ratio of accepted classes *not* present in the sample versus the actual classes in the sample (false positives, there are 61 unique pesticides in the data used for the classification model, in the most extreme case there is only one pesticide in a sample and 61 (i.e., all) pesticides are accepted in the prediction giving the following range: [0,60]).



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Mean true positive and false positive ratios ($n = 30$) were computed and used to select the cut-off value that maximises the true positives and minimises the false positives. Note here that the implication of having false positives is not the same for the pollen and pesticide models. For the pollen, a false positive would falsely suggest a more diverse foraging landscape. For the pesticides, a false positive would falsely suggest a landscape with a higher presence of pesticides. However, from an environmental risk assessment point of view, which aims to be conservative, this means a false positive for pollen is less conservative, whereas a false positive for pesticides would be more conservative. Hence, for the pollen model, we may accept a slightly lower ratio of true positives if this results in a substantially lower ratio of false positives. Conversely, for the pesticide model, we may accept a slightly higher ratio of false positives if this comes with a substantially higher ratio of true positives.

4.4.3 Test of variable importance

A final classification model was fitted to the pollen and pesticide data for each country. These models are ready to be used for prediction at large spatial scales. It would still be of interest to get some insights into the role of the different explanatory variables. Although it is difficult to directly relate model responses to input variables (as one would normally do with, for example, a generalised linear model), it is possible to infer how important a variable is. To test the influence of each of the variables on the classification results of the model, predictions were made by iteratively permuting one predictor variable and logging the change in the ratio of true positives and false positives, based on 50 permutations. For important variables, a large change in both ratios is expected, whereas for variables that carry less information, smaller changes are expected.

4.4.4 Country-scale prediction of pollen and pesticides to produce maps

The objective of the final model objects is to make pesticide predictions for five months of the year (i.e. May – September) and for Austria and Denmark. For pollen, the same was done but using CDD10 values, calculated for the middle of the month for each date/location combination. For this the required data (see Figure 4.4) was collected based on a 10km spacing of points for which CORINE CLC kernel impacts were extracted using the long distance kernel.

For every 10km grid cell the model predicts the pollen families and pesticide types present. For simplicity and visualisation the results are reported as the number of predicted pesticides and pollen, though for each prediction the actual accepted pollen families and pesticides are also produced.



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