Bio-monitoring the environment with honey bee colonies

How to set up, execute and finalise an apiculturist citizen science study for bio-monitoring the environment with honey bee colonies

By the INSIGNIA consortium

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Content

1 Introduction – The INSIGNIA study

The INSIGNIA study is a grant-funded proposal submitted to the call of the European Commission (EC) on "*Environmental monitoring of pesticides use through honey bees*". The study aimed 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 study has been completed in 2021.

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 and availability of diverse pollen sources;
- To make a list of applied non-legislated pesticides detected in this study.

This paper is the abbreviated edition of the "*Guideline for Apicultural citizen science to apply the honey bee colony for bio-monitoring of the environment*". This guideline is the result of the INSIGNIA study and is a best practice guide for Apicultural citizen science studies for bio-monitoring with honey bee colonies. It comprises an extensive description of the application of honey bee colonies for these studies in all its aspects. This guideline and the supporting INSIGNIA studies, reports, and publications are available on the INSIGNIA website (https://www.insignia-bee.eu). This website will be maintained for seven (7) years after the end of the INSIGNIA study 2018-2021. The "Guideline for Apicultural citizen science to apply the honey bee colony for bio-monitoring of the environment" is also available on the EC websites EU Pollinator Information Hive (https://wikis.ec.europa.eu/display/EUPKH/EU+Pollinator+Information+Hive) and on EU Food Safety Protection of bees (https://ec.europa.eu/food/plants/pesticides/protection-bees_en).

2 Why bio-monitoring

The honey bee colony has a comprehensive interaction with the environment on account of its food. Due to its foraging behaviour on flowers, contaminants, both solid in small and ultra-small particles, and as a liquid, are inadvertently collected and brought into the hive. In the colony, about a quarter of the colony forages approximately ten times a day, visiting numerous flowers. The large number of foragers and the accumulation of foraged material and any associated contaminants in the hive is the real power of the honey bee colony as a bio-monitoring tool. As a result, the colony reflects both the food availability as well as the environmental pollution status of the foraging area. The pollutants collected are not restricted to those in nectar, pollen, water and propolis taken from the soil by the plants themselves. They also include pollutants deposited on the flowers, leaves, buds, and surface water by airborne deposition, and direct and indirect deposition

of plant protection products, biocides and veterinary medicinal products. Inside the hive nectar, pollen, pollutants and plant pathogens are exchanged intensively among the in-hive bees via trophallaxis, auto- and allogrooming and physical contact. The incoming food follows either the nectar route or the pollen route for storage, consumption and application. The contaminants attached to or dissolved in the food follow the same routes. The non-food routes by which contaminants enter the colony are the wax-, water-, propolis- or loose particles routes. Loose particles follow a maximal 2-week route on the bees in the hive. Each route has its typical circulation and final storage. For detecting contaminants, a customised planning and sampling strategy is required.

Part of the freshly collected 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 cells. As a result, freshly collected nectar circulates within hours in the colony. In the hive, the nectar with contaminants is quickly diluted with non-contaminated nectars. The mixing of the nectars during honey processing, and filtering in the honey stomach of the bees, makes honey an inconsistent matrix for contaminants. Pollen is stored by the pollen foragers as beebread. Within two weeks the major part is consumed during the reproduction periods of the colony. During pollen collection, besides pollen, other particles as pollutants are brushed into the corbicula (pollen basket of the bee) as well. This makes pollen and beebread good contaminant matrices. Practical issues of pollen and beebread collection must be taken into account. There is the aforementioned short in-hive period of beebread, its scattered storage by the bees, and the short pollen collection period in the pollen trap. Honey bee colonies collect water from ditches, puddles, mud, rivers and lakes. Water is not stored but is used directly for temperature regulation and dilution of food. Although not stored, any pesticides in the water will be among the xenobiotic molecules circulating in the colony. Wax binds non-polar lipophilic molecules which enter the colony via nectar, pollen or directly via contaminated bees. The physicochemical properties and chemical stability of contaminants influence to what extent contamination accumulates in the wax. This selective binding makes wax a less reliable contaminant collector. Because of the long in-hive residence time of months to years, it forms an archive. Therefore customised methods are required to collect wax. Propolis is a lipophilic, resin-like material that bees collect from plant exudate and is used mainly for sealing the beehive. Due to its lipophilic capacity and physical stickiness it accumulates contaminants. Theoretically, propolis is a contaminant collector. However, because of low and strongly colony- and region-dependent variation in the amounts of propolis collected, it is by definition an inconsistent contaminant matrix. Another group of contaminants and micro-organisms, not bound to the food, finds 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. The residence time of pollen on the bees in the hive is at least 10 days. This 10-day period is an indication of the in-hive residence time of loose particles. Because of this, the sampling frequency in a bee-monitoring program must therefore be at least bi-weekly (fortnightly).

In fact, applying the honey bee colony for bio-monitoring means having the forager bees do the collection outside and letting the colony do the accumulation of the contaminants to detectable amounts in the hive, as will happen naturally. It is up to the researcher to choose the best matrix for sampling of the colony. This sampling of the colony is actually subsampling but we will speak of "sampling" for practical reasons. A matrix is a substance that carries e.g. xenobiotics or plant pathogens and is not altered by these contaminants or micro-organisms.

2.1 Honey bee colony monitoring matrices for contaminants

The execution and nature of sampling matrices differs significantly. Wax, honey, propolis and water are non-consistent, variable matrices. The honey bee itself is a matrix of pollen grains, plant pathogens and contaminants that are stuck to the branched hairs and mouth and feet parts. Sampling of hive-entering bees is by definition sampling a mixture of age cohorts of bees with different exposure rates. An in-hive bee sample is a mixture of age cohorts. The in-hive bee samples can be a rather reliable matrix because of the in-hive exchange and trophallaxis and homogeneous mixture of the age cohorts. Nevertheless, this sampling of honey bees for bio-monitoring is not recommended. It is an invasive sampling, that impacts the bio-monitoring tool "honey bee colony". This leaves pollen as the main, most reliable bee-matrix for contaminants and also for pollen diversity. In the INSIGNIA study, we developed a non-biological in-hive passive sampler, the APIStrip. It is a polystyrene strip covered with Tenax (a porous absorbent polymer), which is placed between two combs in the center 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".
In summary, the APIStrip is the best available in-hive non-biological sampler for pesticide contaminants, and pollen is the best matrix for pollen diversity. Bio-monitoring for other contaminants like micro-plastics and plant pathogens requires other non-biological in-hive passive samplers which are currently being tested.

2.2 Apiary sampling, colony sampling, sample pooling

Colonies in an apiary divide themselves over the surroundings with only partly overlapping foraging areas. Consequently, it takes more than one colony to cover an area, and the pooled apiary samples are thereby, by definition, a spatial sample. It takes minimally two (2) colonies per apiary for a pooled apiary sample. Two colonies result in comparable results compared to three (3) colonies. For bio-monitoring with honey bee colonies, apiary pooling is recommended.

2.3 Global application of the honey bee colony as a bio-monitoring tool

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. In large-scale studies, the land use determines the "region" and bio-monitoring colony density.

2.4 Apicultural citizen science

Citizen science is research, conducted partly or completely by citizens and non-professional scientists, in collaboration with and under the direction of professional scientists and scientific institutions. 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 for citizen science studies. Commitment of the beekeeper and support for the beekeeper are both essential.

3 The study set-up

A research study starts with a question or hypothesis, formulated in a study proposal. The key process is to define the exact question or hypothesis, and next, to translate this study question into a study plan. In practice, it is an interplay between client and executor. This results in the definite study question and corresponding study plan. In this process, the practice, scientific standards, finance and manpower are weighed up, in order to come to a balanced but appropriate plan including timelines, responsibilities, and processes. The study plan comprises the layout of the study, study course, responsibilities of participants, and instructions, communication, recordings, reporting, data storage, statistical analysis of the data, and modeling procedures. The research integrity and data integrity are crucial in a study plan.
INSIGNIA developed a successful format. This format describes tasks and responsibilities of a sponsor/commissioning company, one (1) study director (SD), multiple Apiculturalist coordinators (AC), one (1) Data curator (DC), one (1) Communications curator (CC) and of course many beekeepers/Apiculturalist citizen scientists (CS).

3.1 Study director SD

The SD has the responsibility for the study in all its aspects. The SD coordinates and participates in writing the study plan with the AC, the laboratory specialists, statisticians and modellers involved, according to scientific standards. He/she is the pivot between the sponsor and possibly stakeholders.
The AC is responsible for the practical implementation and the course of the citizen science study in a country or region. He/she is the pivot between the SD and the multiple CSs.
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 a DC and CC to control the process.

The study plan process is depicted in figure 1.

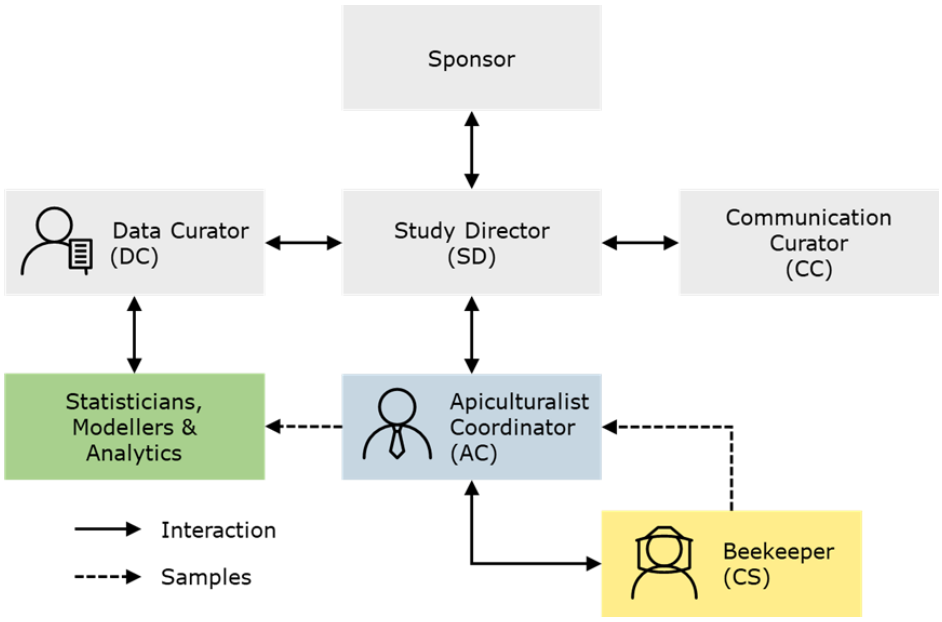


Figure 1: Interactions of key participants in the study plan

3.2 Apiculturalist coordinator AC

The AC is responsible for the practical implementation of the study plan by organising the local part of the study, performing the actual sampling in a particular area/region/country, and taking care of all requirements for adequate sampling. This comprises the recruitment of the beekeepers; assigning a CS number to each beekeeper; providing the beekeepers with all information of the "why, what and how" of the study, its specific goals, and instruction about the study protocol with all appropriate tools, as well as providing the beekeepers with the toolbox to conduct the sampling and recordings.

3.3 Apiculturalist citizen scientist CS, the beekeeper

The basic conditions for an Apiculturalist citizen scientist (CS) to participate are practical, namely having internet access, an email address and minimally 2 non-migrating honey bee colonies close to their home address. Commitment to the pre-determined sampling rounds to do the appointed pre-, post-, and actual sampling activities is a prerequisite. Pre-conditions for the beekeeper are an awareness of the environmental surroundings; being reliable in sticking to the agreements and appointments and being a skilled beekeeper with an affinity to science. The CS is the key player in these studies and must therefore be well informed about the study, its specific goals, the study progress, and study results. This is all about communication. All communication channels available can be made use of, such as AMA ("ask me anything") meetings, WhatsApp groups, Signal groups, Facebook groups, or whatever is applicable to keep in touch with the CS.

3.4 Instruction, toolbox, sample, storage, and transport

The quality and reliability of the data/results concerning pesticides, pollen or anything else sampled depends on the quality of the sampling. Non-reliable samples result, by definition, in a non-reliable study result. Therefore correct sampling and sample storage constitute the basis of every monitoring study. Equally, all efforts in the recruitment of CS, sampling, analytics, statistics, modelling, and study reports are useless if there is not a solid, clear and unambiguous sample label for each collected sample, that cannot be misinterpreted.

3.5 Instruction

Instruction tools consist of an instruction picture manual; links to tutorial and instruction videos/movies; demonstrations, or whatever is required for good instruction under the local conditions, ideally on-site education near to the beekeeper. The instructions comprise an instruction for sample labelling/completion of pre-printed labels that cannot be misinterpreted; strict procedures for the actual sampling; strict instructions for sample storage at the beekeeper's premises and clear instructions plus adequate mailing boxes/envelopes for sample shipping to the AC. 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.

3.6 Toolbox

In order to be able to do the job, the CS must be provided with a toolbox. Depending on the study objective, this toolbox contains all tools for sampling, sample labelling, sample storage, and sample transport from CS to AC. The toolbox is assembled and sent to the CS by the AC.

3.7 Transport

During and after the sampling period, the AC will organise the transport from the CS to the AC. 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)). Once the samples are at the AC, they are checked for damage and missing labels, and if needed completed/corrected in communication with the beekeeper. The AC stores all samples according to the instruction in the study plan until shipment of the samples to the analytical laboratories.

4 Data integrity

Data integrity is the responsibility of the SD and the practical execution can be assigned or delegated to a participant, the Data curator (DC). The SD assigns the data management task to the DC. Data integrity comprises correct data transfer, data safety, and integrity of the data warehouse. This may comprise, depending on the study set-up, sending email alerts to the beekeepers before sampling; sending questionnaires via online questionnaire programs; uploading the results of the lab analyses, and compiling data sets for statistical analysis and modelling.

5 Online survey

The online survey questionnaire serves two purposes. Firstly it confirms on 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. The online survey set-up allows the addition of other languages as needed. The AC makes sure that every participant in their country/region 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 required languages by the AC.
Information to be collected via the online survey, for data integrity, are: 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 the number of occupied bee lanes, and 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 Communication

Internal and external communication is the responsibility of the SD and the practical execution can be assigned or delegated to a participant, the Communication curator (CC). The CC makes notes for external contacts; 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; takes care of the website and website upload; takes care of/feeds the social media being used; makes, or has made, the format of the picture manuals, to be translated/adapted by the AC; makes, or has made, instruction video and other visual instruction material, and organises that participants contribute to the study blog, notes, and other output.

The texts for automated communication with CSs will be translated by the AC into the required languages, depending on the participants in the study. It is the responsibility of the DC to send out emails to all CSs for each sampling round.

7 Acknowledgement

This study was granted by the European Commission, Directorate-General for health and food safety: Number SANTE/E4/SI2.788418-SI2.788452 - INSIGNIA. The content of this guideline represents the views of the authors only and is their sole responsibility; it cannot be considered to reflect the views of the European Commission or any other body of the European Union. The European Commission does not accept any responsibility for use that may be made of the information which this guideline contains.

In the attachment are the pollen- and pesticides protocol presented as in the aforementioned "*Guideline for Apicultural citizen science to apply the honey bee colony for bio-monitoring of the environment*".

8 Attachments

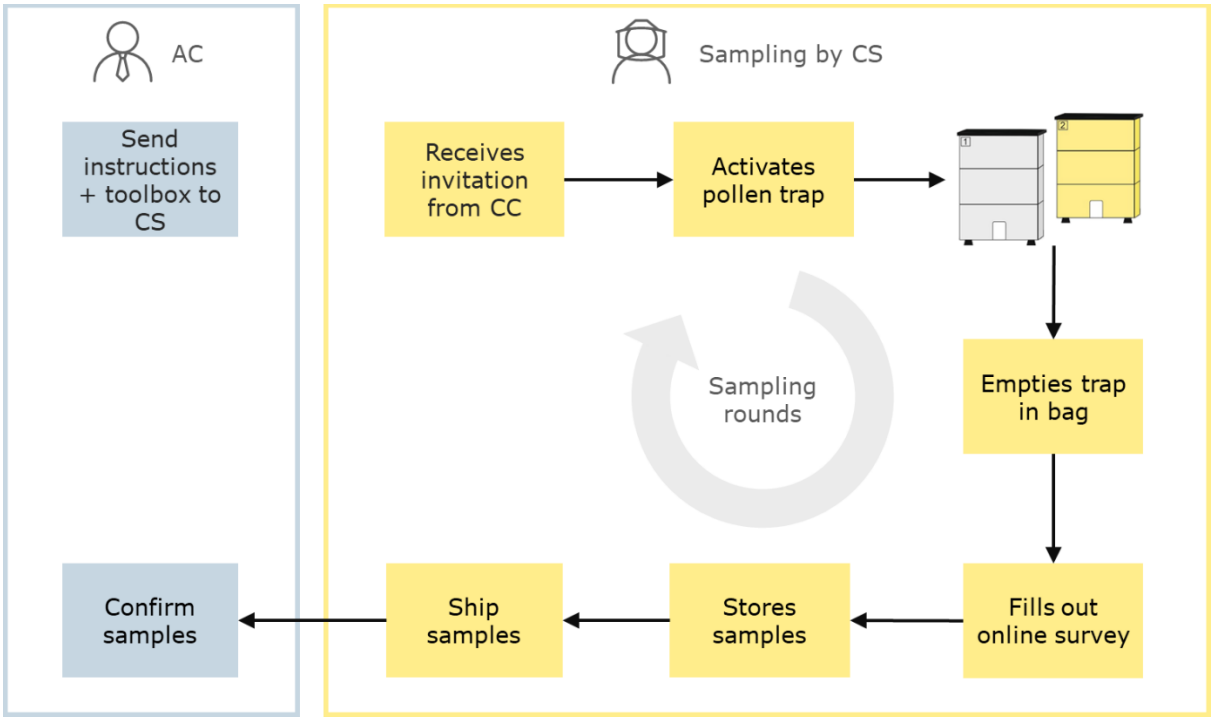
A protocol is by definition a step-by-step description of a procedure from the very start to the very end. Here we provide protocols for bio-monitoring of pesticides by honey bee colonies and bio-monitoring of pollen for pollen origin/diversity. Each monitoring subject demands its specific toolbox, sampling techniques, recording, storage, and shipping, and therefore its own protocol. That is why 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 bio-monitoring for more than one subject.

8.1 Protocol for honey bee colony monitoring for pollen origin and diversity

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

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.

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
      - Boxes for shipping of samples to the AC
  - Contact during the study

*Preferably the beekeeper's 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 sub-sample 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.

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 the collection, trapping can be extended to a maximum of one day of effective trapping.

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 email alert. Additionally, this can be combined with a questionnaire for collecting relevant information concerning the sampling, such as location, the number of colonies in the apiary, varroa control strategy, or other bee parasites/pathogen treatments.

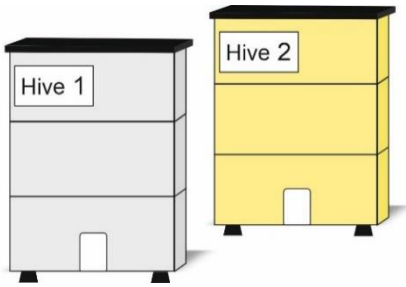
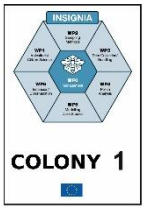
The toolbox

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	☐
Graphite pencils	1	☐
Larger bag (1 per sampling round, 1l zip lock bag)	10	☐
For pollen sampling and storage, two (2) options are provided **Option 1**: per hive and sampling round a 20 gram sample is taken, stored in a 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)	☐
Option 2. measurement tool 5 gram	2 (1 per hive)	☐
Option 1. sample bag: airtight plastic zip lock bags	20	☐
Option 2. sample bag: airtight plastic zip lock bags	80	☐
Option 1. pre-printed labels (one per hive)	20	☐
Option 2. pre-printed labels (one per hive)	80	☐
Option 2. storage: silica bags sachets of 5 to 6 gram	80	☐
Option 2. storage: 80 paper tea filters	80	☐
Option 1. transport: Styrofoam boxes	2	☐
Option 1. transport: cool blocks	2	☐
Option 2. transport: cardboard postal boxes	2	☐

Picture manual and other instruction tools

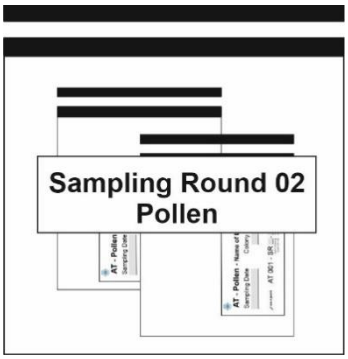
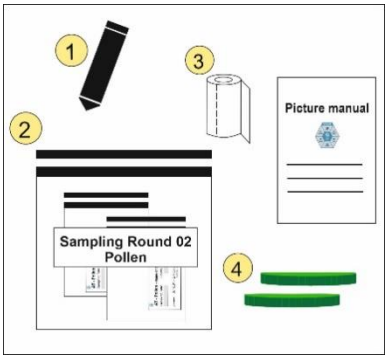
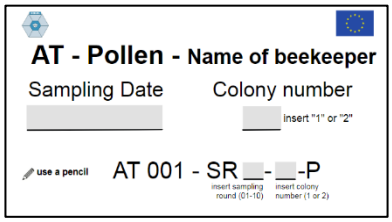
Prior to study start

	**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.	 Identification example of study colony 1: 
	**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).	

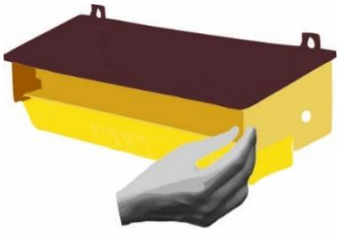
Preparations for sampling

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.

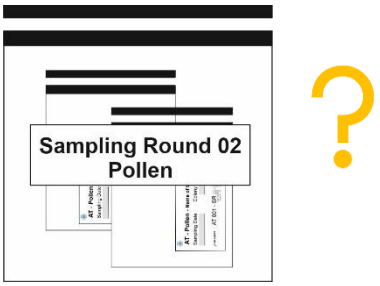
Sampling time window (example)				
Day	Thursday	Friday	Saturday	Sunday
Weather forecast & day job	working	free	free	free
Sampling	⊘	⊘	**Morning (~8 am)** ①close pollen trap **Evening (~5 pm)** ②harvest pollen ③open pollen trap ④if not enough pollen; leave trap closed	**IF NOT ENOUGH POLLEN YESTERDAY:** **Evening (~5 pm)** ⑤harvest pollen ⑥open pollen trap

	Preparation of sample bags: find the right bag for the current sampling round in your tool box.	
	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.	 

Sampling procedure

	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.	

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 the online survey). Harvest again and put the pollen in the same sample bag from the day before.

If you have more pollen, a sub-sample of the homogenised pollen harvest must be taken by

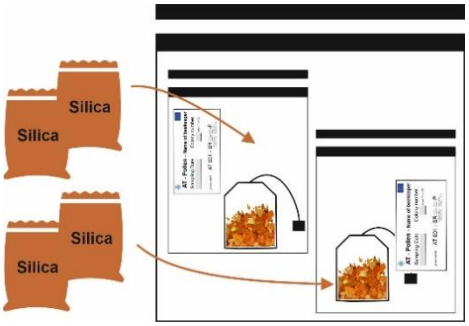
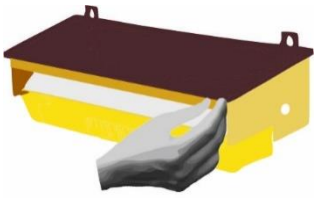
1. collecting all the pollen in a large plastic bag, shaking until the pollen harvest is well mixed and taking a full measurement tool amount from this mixture.
2. shaking the pollen pellets in the drawer until the pollen harvest is well mixed and taking a full lid amount from this mixture.

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

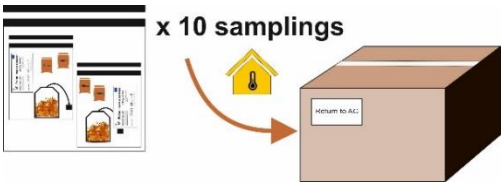
After making sure that the bag is **tightly sealed** and the corresponding **label** is **LOOSELY INSIDE THE BAG**, repeat the sample procedure with hive 2.

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.

	The remaining pollen from a more than 20 gram sample can be used according to your own preference.	
	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.	
	Option 1 Bring your 20 gram samples to your **freezer** at home shortly after the sampling procedure.	
	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.	
	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.	

Storage at the beekeeper's premises

	**Option 1** Store the pollen in its plastic bag in the freezer.	
	**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 air-tightly and the pollen is stored at room temperature in the cardboard box for sending back.	

Quality control

	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. **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. **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.

The transport to the Apiculturalist Coordinator (AC)

	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. **Option 1:** cold chain transport in a Styrofoam box (in toolbox). **Option 2:** in a cardboard box and transported at room temperature. It is up to the Apiculturalist Coordinator to organise the transport from the beekeeper to the Apiculturalist Coordinator by postal service or by courier. Regardless of the transportation mode, the costs are borne by the study and not by the beekeeper CS.

Data entry

	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.
	**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.
	**Thank you for your valuable contribution!** **<u>Apiculturalist Coordinator</u>** Email: _____ Phone number: _____ (contactable during sampling periods - Thursday to Sunday from 9 am to 5 pm) (Messenger group: _____) Website:

Support/Video Manual (example from the INSIGNIA Study)

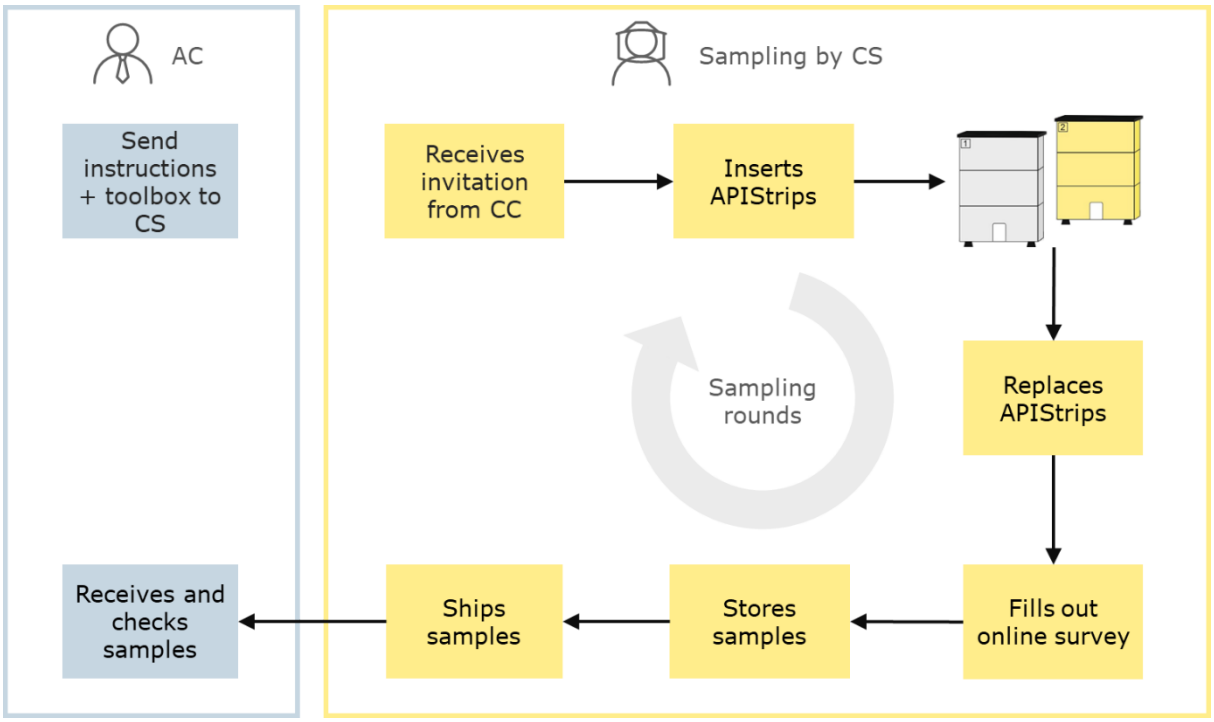
	You will find accompanying instruction videos and constantly updated blog entries on our INSIGNIA YouTube channel and on our website, respectively. During the active sampling periods, the Apiculturalist Coordinator is available via the above-mentioned phone number and social media. If you have any questions 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.
	**Video manual pollen:** www.youtube.com/watch?v=97josWrHu-A **Blog:** https://www.insignia-bee.eu/blog/

8.2 Protocol for honey bee colony monitoring for pesticides

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

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

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
      - 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.

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 in 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.

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 email alert. 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.

The toolbox

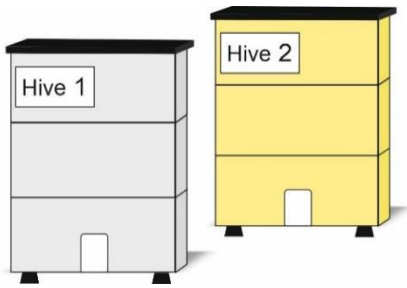
The toolbox provided by the Apiculturalist Coordinator includes the equipment needed by every CS for pesticide monitoring. It contains materials for 10 sampling rounds with 2 bee colonies. The CS should check the materials for completeness.

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

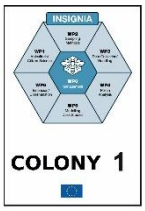
Picture manual and other instruction tools

Prior to study start

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.



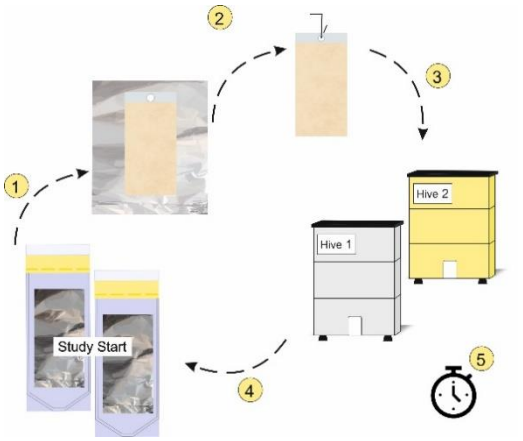
Identification example of study colony 1:



Two weeks before first sampling

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

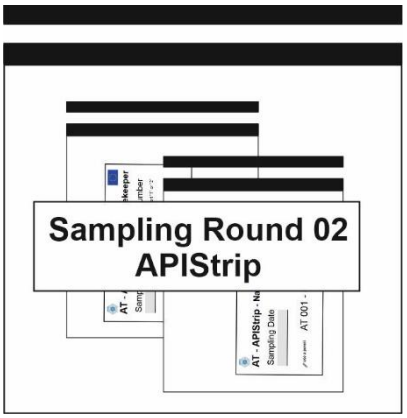
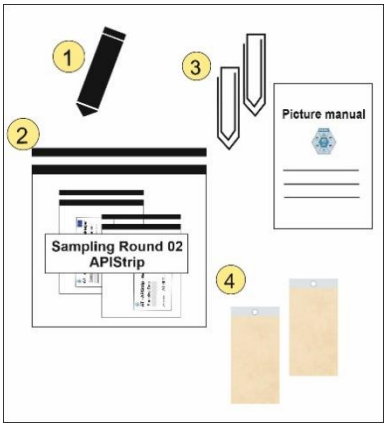
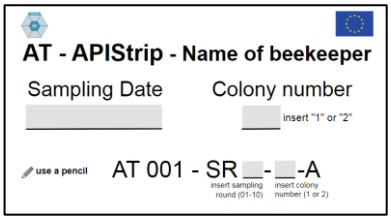
1. There are **2 plastic envelopes containing the first two APIStrips** in the toolbox (wrapped in aluminium foil; labelled "Study Start").
2. Unpack a new APIStrip and avoid touching the surface (to avoid contamination risk for lab analysis; no risk for CS or bees!). Note, that the APIStrip does not 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.



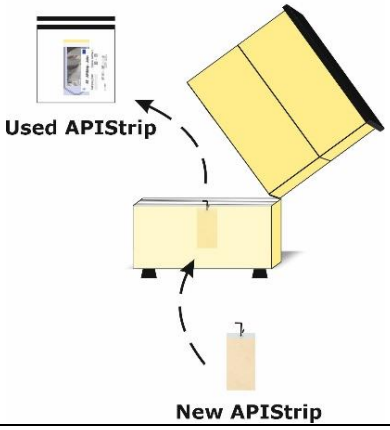
Preparations for sampling

Invitation email: prior to each sampling round an **invitation email** will be sent by the Data urator 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.

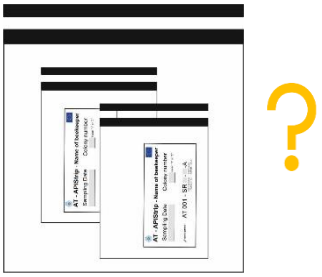
Sampling time window (example)				
Day	Thursday	Friday	Saturday	Sunday
Weather forecast & day job	working	free	free	free
Sampling			**Occasion 1 Morning (~8 am)** exchange APIStrips	**Occasion 2 Morning (~8 am)** exchange APIStrips

	Preparation of sample bags: identify the correct **bag** for the current sampling date. 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).	
	For each sampling round prepare: 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**	 

Sampling procedure

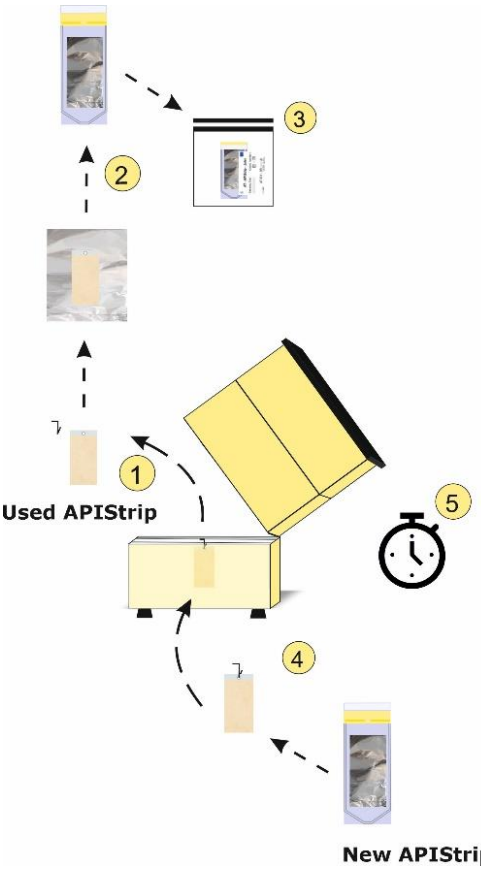
	Two weeks after the last APIStrips were placed in the hive, they must be removed and replaced by new ones.	

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.

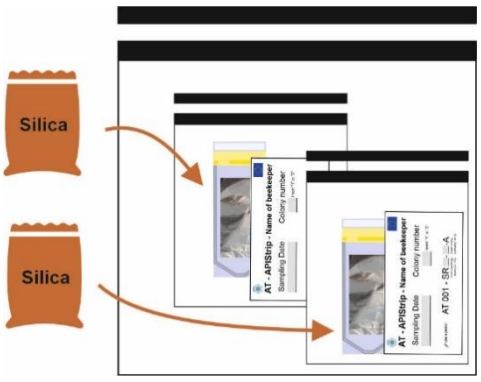


Actual sampling:

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.

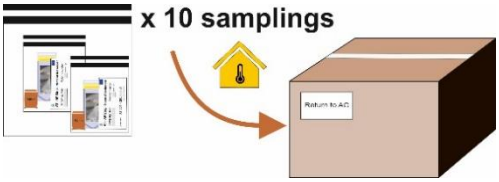


Add the 5-6 gram silica gel sachet to the APIStrip envelope with the label 2



	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.	

Storage at the beekeeper's premises

	Store the exposed APIStrips in the sample box, provided in the toolbox, at room temperature.	

Quality control

	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. For quality control, two (2) extra APIStrips are provided in the toolbox. These APIStrips will remain unopened 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.

The transport to the Apiculturalist Coordinator (AC)

	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. It is up to the Apiculturalist Coordinator to organise the transport from the beekeeper to the Apiculturalist Coordinator by postal service or by courier. Regardless of the transportation mode, the costs are borne by the study and not by the beekeeper CS.

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	**Video manual APIStrip:** www.youtube.com/watch?v=97josWrHu-A **Blog:** https://www.insignia-bee.eu/blog/