SYLVA Training Package 1: Producing reference datasets for training bioaerosol identification algorithms

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SYLVA
Observing life in air

Introduction

This training package was developed as part of the SYLVA Horizon Europe project which aims to radically improve the temporal and spatial coverage, timeliness, and availability of information about bioaerosols across Europe.

This training package is focused on producing training datasets for developing bioaerosol identification algorithms. It outlines the techniques and methods that can be used for producing datasets for various purposes and for the range of instruments used in the SYLVA project (from real-time devices through to eDNA monitoring). This include the Hund Wetzlar BAA500, Plair Rapid-E+, and Swisens Poleno real-time devices as well as how to prepare and store material for eDNA monitoring.

The purpose of this training package is to provide protocols for how to:

- Collect a pollen sample from the field
- Cultivate certain fungal spores and bacterial strains
- Store the various collected or grown samples
- Introduce pollen, fungal spores, and bacteria into an automatic bioaerosol monitor

Collection and growing processes

Collecting pollen in the field:

Pollen should ideally be collected in areas free from potential anthropogenic pollution, e.g., rather in a forest than on a roadside. The timing of biological pollen release depends on the taxa, so you may need to go out early in the morning or later in the afternoon. Collection should preferably take place on a sunny and dry day.

Material required:

- Sampling tube (e.g., falcon-50) or container for larger flowers/branches (e.g., Tupperware)
- Waterproof marker, pen, and paper
- Mobile phone (with a camera, the possibility to take GPS coordinates or with a map application, and ideally, also with an application that provides access to real-time weather measurements)
- Gloves and face mask (to avoid sample contamination and allergic sensitisation/reactions)
- Bag or box to transport the sample(s) protected from light

Procedure:

- Identify a flowering plant from which you want to collect pollen
- Take photos of (1) the full plant including the ground so that the soil can be seen, and (2) a close-up of the flowers and the leaves
- Note down the time and lat/lon coordinates, since this may be useful in case you want to collect samples another year.
- Note down the weather conditions, including wind. If there is a weather station within a few kilometres, note down the measured temperature, relative humidity, and wind speed and direction.
- Put on your gloves and mask.

- Place the catkins into the falcon-50 tube and gently shake the branch so that the pollen falls out of the catkin and into the tube. This may need to be repeated on a large number of catkins to obtain enough pollen (see note below).
- In certain circumstances, collecting enough pollen in the field is not possible. In this case, it may be necessary to bring flowers/inflorescences/catkins into the laboratory where their stems can be placed in water until antithesis (pollen shedding) takes place. The pollen can then be collected directly in tubes or on a flat surface (e.g., white paper) from which the pollen can subsequently be gathered. This may be after just a few hours or after a few days. Ideally, the pollen should not be left for too long on the paper so that it does not dry out too much.
- Before storage, the collected pollen should be sieved to remove other particles (e.g., pieces of anthers, small insects, etc.). Sieves with a 50-micron mesh should work well for most pollen types.

Figure 1: If it is not possible to collect fresh pollen from mature catkins or flowers it is possible to bring immature ones into the laboratory where they can be kept until they mature and release pollen. Left: pollen collection directly into a falcon-50 tube. Right: pollen collection on a clean piece of paper which can later be put into tubes.

Note:
Training datasets should typically have at least 10’000 particles and since aerosolization is not always very efficient, approximately 100’000 pollen grains should be available to obtain the minimum number of particles for each training dataset. This typically corresponds to a sample weight of 80 mg.

Selecting datasets from monitoring data:

In certain cases, it may be relevant to select datasets from monitoring data, i.e., from an instrument that is measuring in the field. This may be if, for example, if a particularly interesting event is noticed

either from directly looking at the data, which is possible for the Hund BAA500 and Swisens Poleno, or from a manual Hirst-type trap if available at the same location. In this case, data from the specific period needs to be manually selected by visual inspection to select a training dataset for a specific bioaerosol type – if this can be determined either using the data from the real-time or the manual device.

Cultivating fungal spores:

Here a general protocol using Potato Dextrose Agar (PDA) is proposed to get sporulating plates, but specific steps may be necessary to grow or induce sporulation of certain spores (see notes below). Many species also grow on malt agar and in some cases, where species require certain proteins for spore development, agar Sabouraud can be used. It is important to note that certain species do not develop spores in rich media, in which case it is necessary to use selective media (again, see the notes below).

Because some species are strictly biotrophic, spores can be collected directly from the symptomatic plant tissues. In this case, it is recommended to use freshly-affected leaves/fruit/shoots since old lesions are typically colonized by secondary microorganisms and thus a single spore-type sample is unlikely to be obtained.

It is also important to note that most fungi grow well between 20-23°C, however, certain taxa prefer higher (e.g., Aspergillus 27-29°C) or cooler temperatures (Venturia spp. 14-16°C).

Required equipment:

- Inoculating loop/needle
- Sterile petri dishes with PDA
- Sterile pipettes and tips
- Laminar flow hood or clean bench
- Incubator

Protocol:

1. Dissolve 24g of PDA in 1L of distilled water.
2. Autoclave this mixture at 121°C for 15 minutes.
3. Pour the autoclaved media into sterile petri dishes.
4. Sterilise the inoculating loop/needle.
5. Perform the inoculation under a laminar flow hood or at a clean bench to prevent contamination.
6. Take a small amount of your spore/mycelium sample from a source culture (either from a natural or a stored sample).
7. Streak the spores/mycelium across the PDA plate in a zigzag.
8. Invert the petri diches (i.e., lid down) and place them in the incubator. The choice of incubator temperature depends on the species (see notes below), as do the light conditions.
9. Monitor growth on a daily basis. Mycelial growth should become apparent after a few days.
10. Once sporulation is evident, you can scrape the surface of the culture with a sterilised spatula or loop to collect the spores.
11. To store the spores, suspend them in sterile distilled water or 0.01% Tween 20 solution (this helps to disperse the spores uniformly). Spores in suspension can be stored at 4°C for short-

term use. For long-term storage, consider creating a glycerol stock (typically 15-20% glycerol) and storing the sample at -80°C.

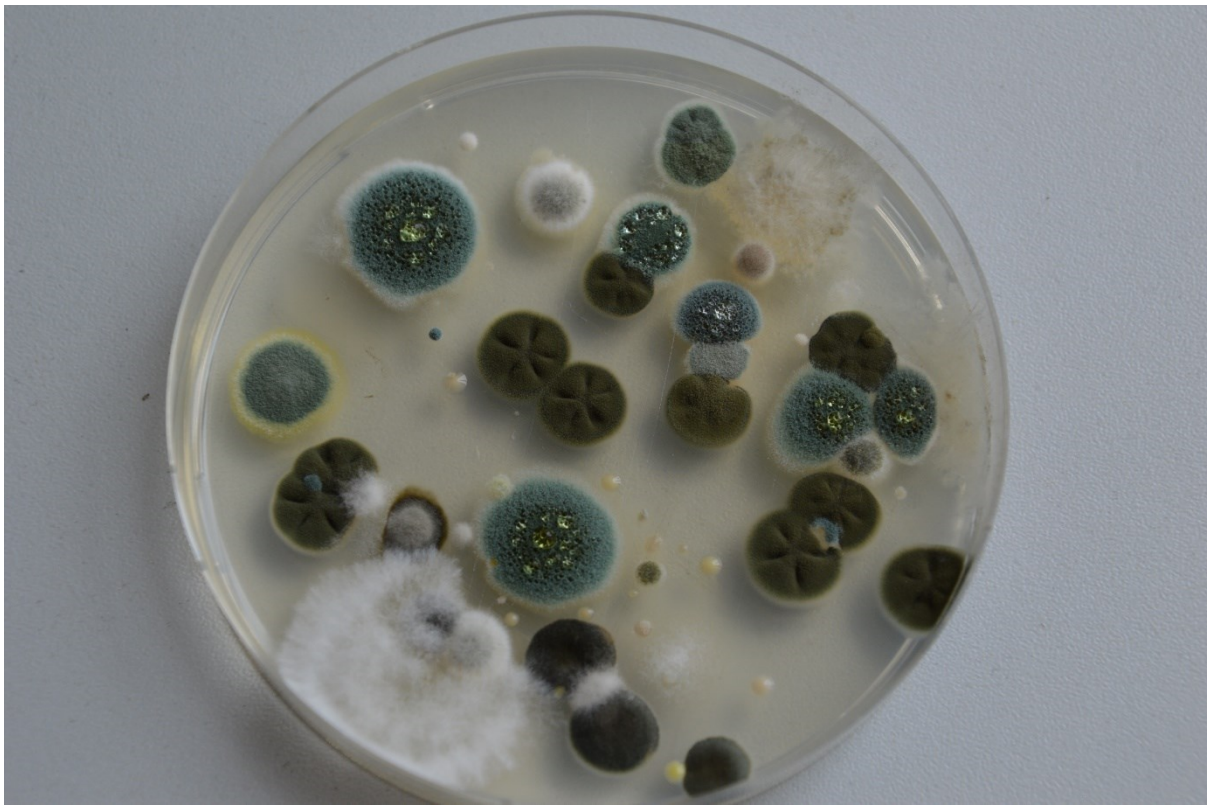

Figure 2: Fungi growing on an agar plate.

Many fungi naturally produce spores as they mature. If the fungi do not produce spores after 7-14 days, it may be necessary to additionally stimulate or change the conditions so that sporulation occurs. This may include exposure to light if incubating in the dark, moving the sample to a location with a light/dark cycle, or changing the humidity – often dryer conditions can stimulate sporulation. Depleting nutrients can also cause fungi to sporulate so if using a rich medium, transferring the fungi to a minimal medium may induce sporulation.

Notes:
Specifications for certain typical airborne fungal spores:

- Alternaria spp.: characteristic dictospores
- Aspergillus spp.: produce small hyaline spores, typically 2-4 microns in size. In general, it is necessary to grow isolates on specific media and sequence specific DNA regions to ensure correct identification. It is important to note that certain Aspergillus species are human pathogens, particularly to immunodeficient people.
- Blumeria: spores need to be collected from affected plant tissue.
- Botrytis cinerea: easy to grow and can contaminate other petri dishes if care is not taken.
- Cladosporium: easy to grow, with many species (approx. 200) that have similar spores. Characteristically present distinct hilum.
- Erysiphe necator: spores need to be collected from affected tissue.

- Fusarium: easy to grow, with many species (approx.. 180) with various types of spores (macro- and micronidia).
- Hymenoscyphus fraxineus: isolates grow well in Hagem agar media.
- Penicillium spp: usually produce abundant small spores.
- Phytophthore: depending on the species, this oomycete needs specific media for inoculum production. Check what requirements are needed for the specific species in references.
- Plasmopara viticola: spores need to be collected from the affected tissue. Media with leaf discs can be used for inoculum production.
- Puccinia: spores need to be collected from the affected tissue.
- Trichoderma: easy to culture genus on carbon-rich media
- Venturia: although some species can grow on media, it is recommended to harvest spores from affected plant tissue as spores grown on media are frequently morphologically different.

Cultivating bacteria:

The following species were selected for description in this document: Pseudomonas luteola, Sphinomonas spp., Pantoea spp., Pseudomonas spp.

Protocol:

1. Obtain agar plates or prepare them as following:
    a. Mix the below-listed components until the solution becomes clear:
        - LB agar
        - 5g yeast extract
        - 10g peptone from casein
        - 10g sodium chloride
        - 12g agar-agar
        - 1L sterile water
    b. Autoclave the solution using the liquid program
    c. Use a water bath to cool the solution down to 55°C
    d. Pour the cooled agar media into sterile petri dishes
    e. Let cool for several hours/overnight
2. Obtain or prepare the liquid media:
    a. Mix the following components until the solution is clear:
        - 4g NaCl
        - 4g Tryptone
        - 2g yeast abstract
        - 400ml MQ
    b. Autoclave the mixture on the liquid program
3. Obtain the appropriate bacterial material: from a stab or glycerol stock (or similar).
4. Using a sterile loop, pipette top or toothpick, touch the bacteria and spread it in the streak plate method shown in Figure 3 to form discrete colonies and verify sample purity.
5. Grow the colonies overnight at room temperature (~22°C)
6. Pour the desired volume of liquid media into a flask/tube.
7. Using a sterile loop, pipette tip or toothpick, select a single colony from the agar plate
8. Swirl the bacterial mass into the liquid media and cover the flask/tube loosely with aluminium foil or a cap.

9. Incubate the bacterial culture in a shaking incubator at 150-220 rpm/min at room temperature (~22°C) until the desired cell density is reached.
10. Wash the bacteria with a buffer:
    a. Collect the cells via centrifugation at 8000rcf for 3 minutes.
    b. Resuspend the cells in an appropriate buffer (e.g., 1ml of 10mM Tris-HCl).

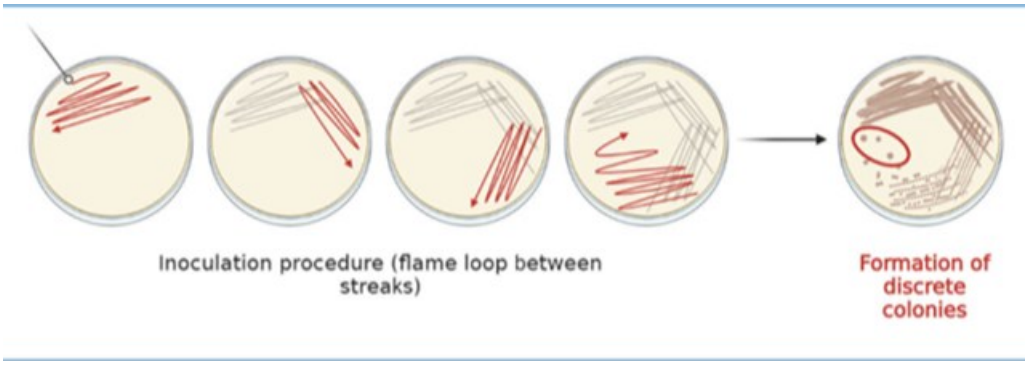

Figure 3: Procedure to inoculate sterile ager plates with bacteria.

Bacterial cells suspended in the buffer can be aerosolised with a nebulizer or a Swisens Atomizer for introduction into an automatic monitoring instrument. For eDNA analyses, a volume of 2mL is required after step 9.

Sample storage

Storing dry bioaerosol:

Pollen:

For short-term use, pollen samples can be stored at room temperature in a dark place. For long-term storage, keep the sample in a -80°C freezer.

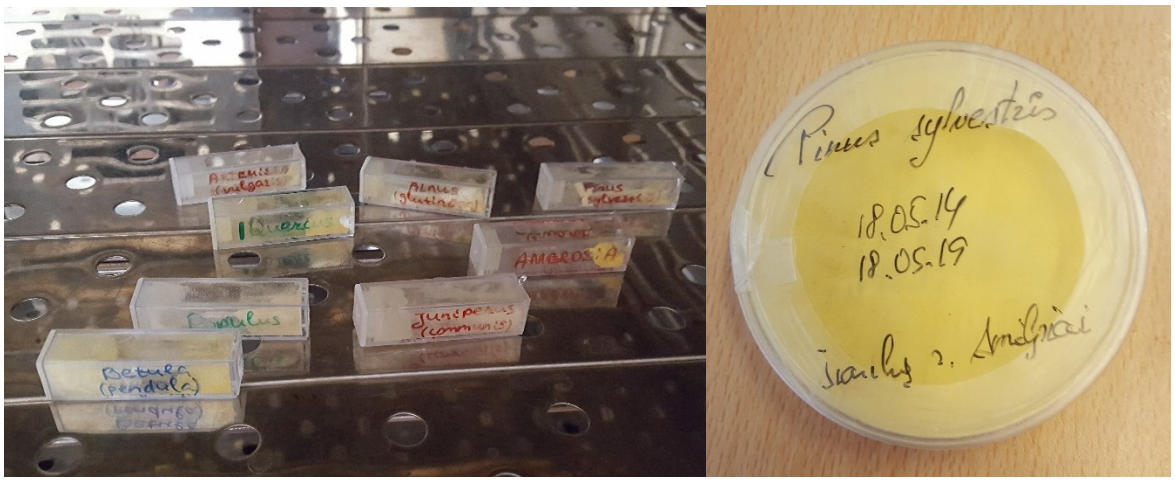

Figure 4: Examples of pollen samples for storage

Fungal spores:

Fungal spore suspensions can be stored at 4°C for short-term use. For long-term storage, consider creating a glycerol stock (typically 15-20% glycerol) and storing it at -80°C.

Storing bioaerosol for eDNA analyses:

Samples can be stored at -20°C for <1 year. For long-term storage, keep the sample in a -80°C freezer.

Introducing bioaerosol into the instrument

Pollen:

Plair Rapid-E+

A critical aspect in the process is to allow a sufficient number of bioaerosol entre the Plair Rapid-E+ without there being too many or too few particles. Sampling of particles other than those of interest should be avoided since filtering out unwanted particles from the Rapid-E+ measurements requires precisely knowing their characteristics and this is, to date, not possible. To ensure a clean airflow, a HEPA air filter can be sued (one that does not slow the airflow, e.g., https://etafilters.com/products/hepa-capsule-filter-inline-filter-1).

Figure 5: Introduction of pollen samples into the Rapid-E+ device in the laboratory

A system has been developed to aerosolise pollen for the Rapid-E+. This system consists of a plastic hose, low resistance HEPA filter, cyclone made from an Eppendorf tube and pipette tips, and aquarium pump, and sterilisation filter (see Figure 6). All components that come into contact with the pollen are only used once. Essentially, pollen are made airborne by the airflow created by the cyclone

(clean air from the aquarium pump is sucked into the device). The quantity of pollen sampled can be increased by shaking the cyclone.

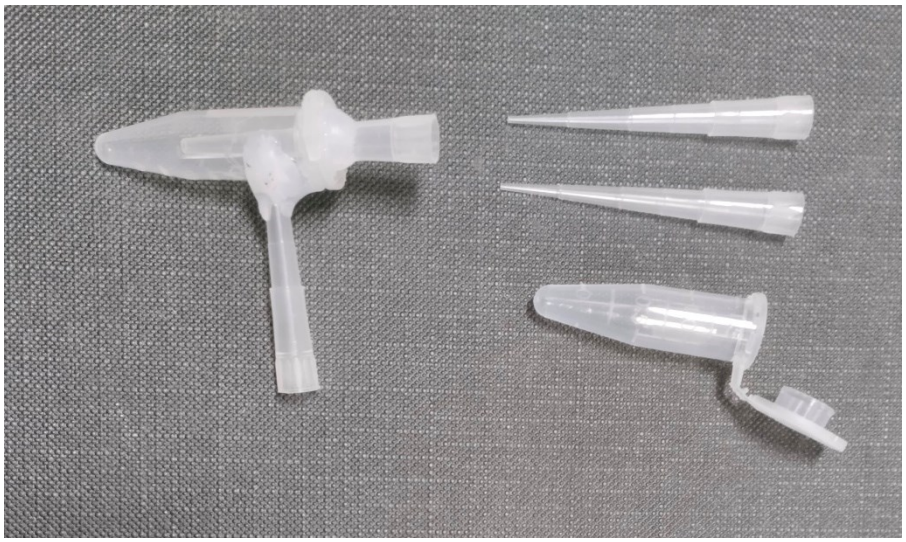

Figure 6: Detailed view of the system developed to introduce pollen into the Rapid-E+ device

After exposing the Rapid-E+ to each pollen type, the sampling orifice needs to be thoroughly vacuum cleaned to ensure there no pollen grains remain in the device before the next sample is introduced.

Swisens Poleno:

Swisens have developed the Atomizer which is specifically designed to aerosolise pollen and fungal spores.

Protocol:

1. Remove any debris (e.g., pieces of leaves, etc.) from the sample
2. Prepare ~1mm of pollen in a cuvette and start the reference data generation protocol:
    a. Note which Poleno (e.g., P12) and which Atomizer (e.g., MCH1) you are using.
    b. For each pollen sample, note what range of values you use to obtain a stable particle rate (vibration frequency Hz – e.g., 64, vibration amplitude % - e.g., 17-21, blower speed % - e.g., 17-22).
    c. Sparsely place a sub-sample of the pollen grains on a dry clean slide and fix the slide simply with adhesive tape. Label the slide with the sample name (see reference data generation protocol).
    d. Look at the slide under the microscope and note any particular observations, e.g., 10% of the pollen grains are broken. Take a few pictures of the pollen grains and note the magnification used, e.g., 60x.

Figure 7: It is important to verify all samples under the microscope to ensure the identification of the target species and to note any particularities (e.g., broken pollen grains)

3. Once you have completed the reference dataset generation, put any pollen remaining in the cuvette back into the Falcon 50 tube with the rest of the sample.
4. Let the pollen dry naturally in the tube fore 2-3 days at room temperature by placing a coffee filter/tissue with an elastic band over the top instead of the cap. Regularly agitate the tube over the 2-3 days so that the pollen do not aggregate. Keep the sample protected from light, e.g., in a cupboard.
5. Once the pollen is dry, put the original cap back onto the Falcon 50 tube and store the sample in a freezer (or in a fridge if no freezer is available).

Figure 8: The Swisens Atomizer attached to the inlet of the Swisens Poleno Mars device

Note, the Swisens Atomizer is not completely airtight. Usually this does not influence measurements, however, it is important to note that it is a feature of the device. When using the Atomizer outdoors, windy conditions can affect the rate at which particles are made airborne, with gusty wind creating irregular sampling rates.

Hund Wetzlar BAA500

Particular pollen samples may be introduced into the BAA500 either with a Swisens Atomizer or manually. Images then need to be selected manually using the validation software (which displays images of all particles): https://validation.pollenscience.eu.

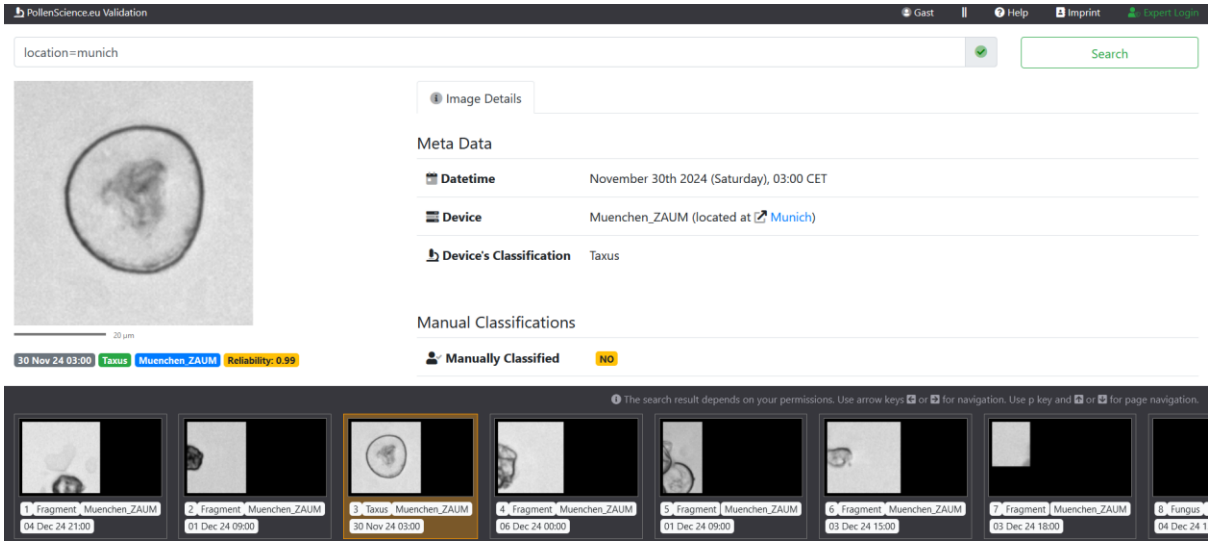

Figure 10: Screenshot of the PollenScience validation website that has been specifically developed for the BAA500 device

In general, however, reference training datasets have typically been manually compiled from images taken during regular operation over the pollen season.

Fungal spores and bacteria:

Plair Rapid-E+

A critical aspect in the process is to allow a sufficient number of bioaerosol entre the Plair Rapid-E+ without there being too many or too few particles. Sampling of particles other than those of interest should be avoided since filtering out unwanted particles from the Rapid-E+ measurements requires precisely knowing their characteristics and this is, to date, not possible. To ensure a clean airflow, a HEPA air filter can be sued (one that does not slow the airflow, e.g., https://etafilters.com/products/hepa-capsule-filter-inline-filter-1).

A system has been developed to aerosolise pollen for the Rapid-E+. This system consists of a plastic hose, low resistance HEPA filter, cyclone made from an Eppendorf tube and pipette tips, and aquarium pump, and sterilisation filter (see Figure 2). All components that come into contact with the pollen are only used once. Essentially, pollen are made airborne by the airflow created by the cyclone (clean air from the aquarium pump is sucked into the device). The quantity of pollen sampled can be increased by shaking the cyclone.

After exposing the Rapid-E+ to each pollen type, the sampling orifice needs to be thoroughly vacuum cleaned to ensure there no pollen grains remain in the device before the next sample is introduced.

Swisens Poleno:

Usage of the Swisens Atomizer with a self-made cyclone (description from Branko Sikoparija):

- Lower the Poleno trigger settings according to the size of the fungal pores (usually better to set the trigger size somewhat lower, to measure all possible spore morphologies).
- Loosen the fungal spores inside the cyclone by shaking the cyclone vigorously

- Place the cyclone with the collected spores in the Swisens Atomizer instead of a cuvette
- Attach the airflow tube (without the reducer) to the cyclone harvesting opening
- For certain fungal spores (e.g., A. tenuissima, A. alternata, A. chartarum, C. herbarum, C. sphaerospermum, Stemphylium), the addition of a small metal piece to the cyclone can dramatically improve aerosolization. This was not necessary for P. chartarum, Curvularia, E. nigrum, E. rostratum, A botrytis, Chaetonmium, B. cinerea, F. culmorum, F. oxysporum.
- Place the Swisens Atomizer onto the inlet of the Poleno and conduct a cleaning cycle to remove any particles from the device.
- Set a measurement campaign label identifying the sample
- Turn on the Poleno and turn on the Atomizer
- You may need to change the Atomizer settings depending on the fungal spore.
- Place a sparse sample of the fungal spores on a dry and clean glass slide. Place some adhesive tape over the sample and check the fungal spores under the microscope. Note any particularities about the sample, label the slide with the sample name, and take some photos, noting the magnification. This should help determine if the particles being sampled are debris, parts of the mycelium, or just spores.

General procedure when using the Swisens Atomizer:

- The vibration amplitude is used to control the particle rate. Typically, you want around 200-300 particles/minute.
- Aim for about 10'000 particles for each reference generation dataset. Given that the dataset will need to be cleaned, it is advisable to have a dataset with about 15'000 particles initially (a certain number will be removed since the images are not present, etc.)
- Use the Swisens DataExplorer to create a reference dataset. Use the DataExplorer to take a precursory look at the images from the dataset to understand if there are images of single spores, agglomerates, mycelium fragments, debris, etc.

Hund Wetzlar BAA500

Samples of fungal spores can be introduced into the BAA500 either with a Swisens Atomizer or manually. Images then need to be selected manually using the validation software (which displays images of all particles): https://validation.pollenscience.eu.

In general, however, reference training datasets have typically been manually compiled from images taken during regular operation, see for example, Gonzalez-Alonso et al., 2023.