Ref. Ares(2024)5618477 - 02/08/2024

Deliverable D4.2

Guidance document for the implementation of a standardized pre-analytic workflow in selected fields

Work package number and title	*WP4 Standardization and Quality Control*
Work package leader	*MUG*
Relevant Task	*T4.2*
Lead contributor to Deliverable	*MUG*
Other contributors to Deliverable	*AIT, CABI, INRAE, HMGU, DMSZ, EMBL, SU*
Dissemination level	*PU*
Due date (month)	*M18*
Version	*1*

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1. Introduction

1.1 Aim of MICROBE

The project MICROBE aims:

- to deliver innovative, validated technological approaches for optimal collection and preservation of microbiome samples (maintaining their taxonomic and functional biodiversity), as well as for the targeted isolation of microbiome members and assembly of synthetic consortia;
- and to provide a comprehensive operational blueprint for the establishment of microbiome biobanking infrastructure, including technological requirements, methodological workflows, data pipelines, legal and ethical guidelines, training plans and business opportunities.

MICROBE also addresses important issues related to standardization and quality control (QC) by developing guidelines for the implementation of standardized workflows and validating developed workflows in ring trials.

1.2 Microbiome research field, standardization and sample pre-analytics

The microbiome research field is a fast-evolving area with currently only a few widely accepted (ISO and CEN) quality standards. In contrast to industry, such standards are generally not yet well established in research communities. However, the rapid developments and the growing spectrum of analytical technologies have raised awareness of the importance of quality controls and standardization in microbiome research. Particularly for biobanks (including microbiome biobanks), acting at the interface between research and industry and aiming to provide access to samples and data of a high and defined quality, it is crucial to follow standards relevant to the industry. This also applies to research projects that focus on the development of novel applications and technologies. The European Commission recently published the COMMISSION RECOMMENDATION (EU) 2023/498 that advised applying ISO and/or CEN standards also for research, particularly for EU projects and 'to make standards a tangible component of the project' (European Commission, 2023).

Along the entire microbiome (analytical) workflow (pre-analytical, analytical and post-analytical phase) numerous factors can artificially modify the final analytical result.

The pre-analytical phase has been shown to be a main, but still often underestimated, contributor to inaccurate or even false and irreproducible analysis. It consists of multiple steps or processes that often do not lie within one hand (e.g., the biobank's hand) but are performed by multiple involved parties. This increases the need for a coordinated and standardized process and the recording of relevant metadata.

Over the past years, international ISO and European CEN standards have been developed for the human microbiome field. These standards are relevant for manufacturers of *in vitro* diagnostic devices (IVDs) and diagnostic laboratories, but also biobanks and research laboratories. One of these standards is the pre-analytical sample quality standard **CEN/TS 17626:2021 for human specimens intended for isolated microbiome DNA analysis** (*Molecular* *in vitro diagnostic examinations. Specifications for pre-examination processes for human specimen. Isolated microbiome DNA*) (CEN/TS 17626:2021; Stumptner et al., 2022).

As the human microbiome research field is more advanced, it was chosen to serve as an example of best practices for other (i.e., environmental) microbiome research fields. The existing European standard CEN/TS 17626:2021 was used as a template for the development of pre-analytic guidance documents for other sample types relevant to the MICROBE project and this deliverable document.

1.3 Description of Deliverable D4.2 and objective of Task T4.2

Deliverable D4.2 belongs to Work Package WP4, "*Standardization and Quality Control (QC)*", and is the result of Task T4.2 *"Development of a guidance document for sample pre-analytics for selected microbiome fields where no standards exist".*

This deliverable report (D4.2 "*Guidance document for the standardised pre-analytic workflow")* presents requirements for the implementation of a standardized pre-analytic workflow in the plant microbiome field.

Plant microbiome was selected as a relevant field for several reasons:

- Plants and plant seeds are one of the case study sample types defined in the MICROBE project (in addition to soil and marine water).
- The importance of pre-analytical standardization has been recognized by the plant microbiome community and highlighted in the scientific literature (e.g., Sare, 2020; Pollock, 2018; Lucaciu, 2019; Berg, 2020; Bullington, 2021; Prakash, 2020; Wassermann, 2021; Dundore-Arias, 2020; and references therein).
- There are currently no ISO and CEN standards regulating the pre-analytical phase of plant samples intended for microbiome analysis (as revealed in the D4.1 report *"Inventory of currently available standards/guidelines/best practices for microbiome sample pre-analytics"*).
- The European Plant Science Organisation's (EPSO) Working Group 'Plants and Microbiome'[1] shares an interest in pre-analytical and metadata standards. MICROBE WP6 initiated a collaboration with EPSO (as part of T6.3 activities) to jointly work on this topic and bring experts from the plant microbiome community on board.

1.4 Scope of this D4.2 guidance document

The guidance document specifies requirements/recommendations for the pre-analytical phase of plant samples intended for microbiome analysis.

It is applicable to the plant microbiome research community, including researchers and biobanks. This document, as well as the approach used for creating it, shall serve as an example for other microbiome fields and sample types such as soil and marine water.

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[1] European Plant Science Organisation's (EPSO), https://epsoweb.org/workinggroups/plants-and-microbiomes/

The document could even provide the basis for a future CEN or ISO standard for plant microbiome samples or as input for ISO standards on biobanking.

2. Method

In order to create the **D4.2 *"Guidance document for the standardised pre-analytic workflow",*** two virtual workshops with plant microbiome experts from MICROBE and EPSO were held (6 March 2024; 5 June 2024). The workshop outcomes were supplemented with evidence from scientific literature, ISO and CEN standards, and best practices/guidelines. For this, MUG performed extensive desk research considering relevant scientific literature and documents identified in D4.1. A Milestone report (*Ms4 Standardization WS held)* about these two expert workshops was also prepared.

In brief,

- In preparation for the workshops:
    - a pre-analytical workflow scheme for plant microbiome studies was drafted by MUG;
    - the major pre-analytical workflow steps along this workflow were listed by MUG using the human standard as a template;
- The **first moderated online MICROBE – EPSO Workshop** was organized and held by AIT and MUG (6 March 2024**). The aim was to identify potential pre-analytical variables for plant samples intended for microbiome analysis.**
- The workshop outcome on pre-analytical variables was **supplemented with evidence from scientific literature, ISO and CEN standards,** and other sources resulting from an extensive desktop search performed by MUG.
- The aggregated pre-analytical variable list was circulated among MICROBE and EPSO experts for review and served then as a basis for the second workshop.
- The **second moderated online MICROBE – EPSO Workshop** organized by AIT and moderated by MUG was held on 5 June 2024. **The aim was to work out requirements/recommendations for the pre-analytical variables identified in the first workshop.**
- Again, the workshop outcome on requirements/recommendations was **supplemented with relevant evidence from scientific literature, ISO and CEN standards,** and other sources resulting from an extensive desktop search by MUG.

This report describes the results from workshops with plant microbiome experts supplemented with scientific evidence.

3. Results

Results reported in this D4.2 report include:

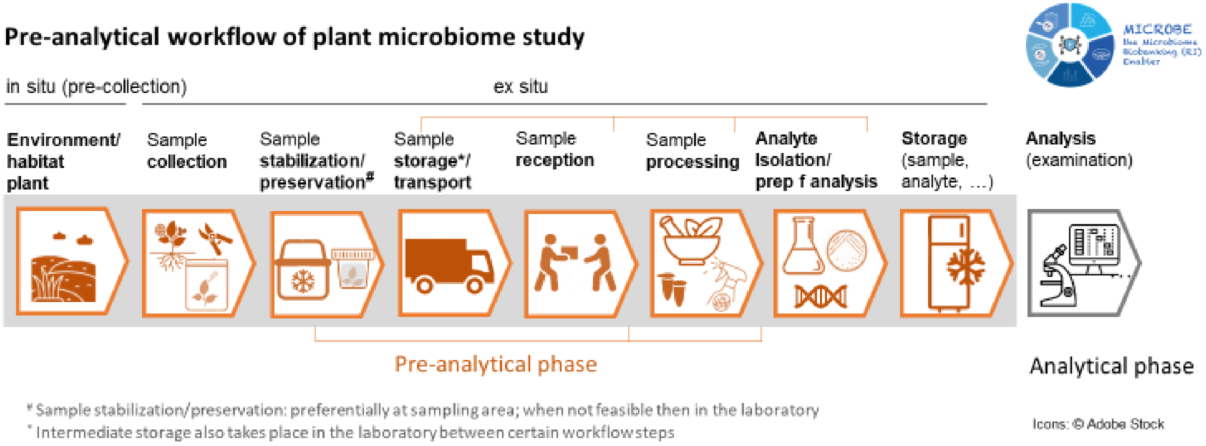
- An outline of a typical pre-analytical workflow for plant microbiome studies;
- An overview of potential pre-analytical variables that should be considered when planning and performing plant microbiome studies;
- A list of requirements, recommendations, and options on how to handle the plant samples during the pre-analytical phase and how to deal with variables to avoid pre-analytical errors and alterations of the microbiome and the microbiome analyte (e.g., DNA, RNA) profiles.

3.1 Pre-analytical workflow of plant microbiome studies

The pre-analytical workflow of plant microbiome study is a multistep process (Figure 1).

It starts with the environment, habitat and plant and includes the sample collection (e.g., in the field or glasshouse), typically followed by sample stabilization/preservation (e.g., freezing, or fixation in stabilizer solutions) and intermediate storage and transport to the laboratory (or biobank). After reception in the laboratory/biobank there is often a sample processing step (e.g., aliquoting or pooling of samples, sterilization, and/or homogenization of the sample). As the last steps, analyte isolation (e.g., DNA or RNA extraction and assessment of their yield and quality) or other types of preparation for analysis (such as cultivation/propagation) can take place.

Figure 1: Typical pre-analytical workflow of plant microbiome study



3.2 Pre-analytical variables in plant microbiome studies

Several pre-analytical parameters/variables exist at each workflow step. They can drastically change the plant microbiome composition and biomolecule profile (e.g., DNA or RNA profiles) in the sample and thus also the resulting data and interpretation.

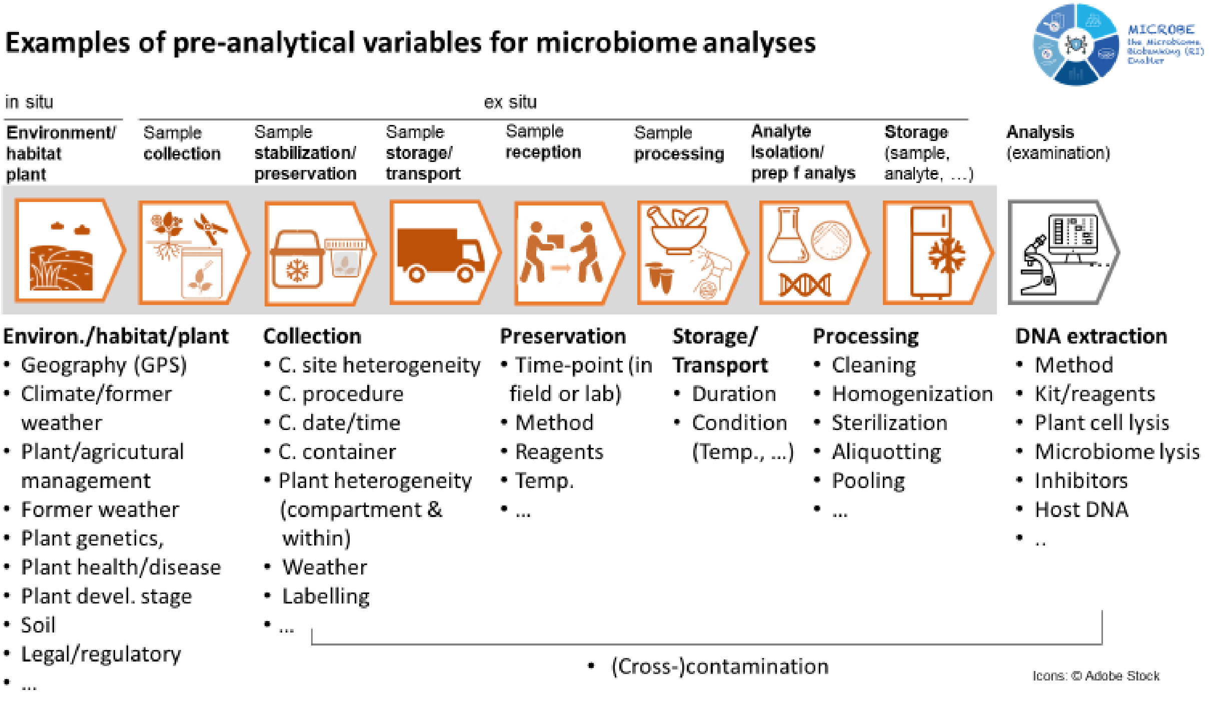
Major examples of pre-analytical variables are described below and shown in Figure 2.

Microbiome composition and density are influenced by environmental and habitat conditions, including the geographics of the location, ecosystem, climate (season, weather), and plant/agricultural management practices. (Bintarti, 2022; Bullington, 2021; Attia, 2022; Dundore-Arias, 2020; Newman, 2022; Kumar, 2024) Microbiomes differ depending on the plant species, age/developmental stage, health/disease status, type and exact part of the plant compartment, and on the exact sampling site because of an inherent variability of the plant material and the heterogeneity of the collection sites/area. (Lucaciu, 2019; Kumar, 2024; Wasserman, 2021; Newman, 2022; Fleishman, 2022; Naylor, 2017; Compant, 2019) Also, the soil as a breeding ground has a major effect on the plant microbiome. (Fleishman, 2022; Lucaciu, 2019; + references therein)

Moreover, changes in microbiome and biomolecule profiles can occur during and after sample collection. Upon removal of a plant sample from its habitat, undesirable growth and/or demise of certain microbial species, as well as induction and/or degradation/fragmentation of analytes (i.e., biomolecules such as DNA and RNA) can occur when samples are not rapidly preserved or used for further processing or analyte isolation. Intermediate storage and transport duration and conditions, as well as preservation method and timepoint, can, therefore, have an impact. (Bullington, 2022; Lucaciu, 2019; Prakash, 2020; Pollock, 2018; Diskin, 2017)

Plant sample processing steps and methods can also influence the microbiome and analyte profile. These steps include, for example, cleaning (e.g., rinsing, washing, vortexing, or sonicating), separation of plant compartments, homogenization (e.g., grinding), surface treatments with sterilizing agents, and subsampling, aliquoting or pooling of samples. (Sare, 2020; Fleishman, 2022; Dundore-Arias, 2020; Lucaciu, 2019)

Figure 2: Examples of pre-analytical variables for microbiome analyses



Examples of pre-analytical variables for microbiome analyses

in situ | ex situ

Environ./habitat/plant
- Geography (GPS)
- Climate/former weather
- Plant/agricutural management
- Former weather
- Plant genetics,
- Plant health/disease
- Plant devel. stage
- Soil
- Legal/regulatory
- …

Collection
- C. site heterogeneity
- C. procedure
- C. date/time
- C. container
- Plant heterogeneity (compartment & within)
- Weather
- Labelling
- …

Preservation
- Time-point (in field or lab)
- Method
- Reagents
- Temp.
- …

Storage/ Transport
- Duration
- Condition (Temp., …)

Processing
- Cleaning
- Homogenization
- Sterilization
- Aliquotting
- Pooling
- …

DNA extraction
- Method
- Kit/reagents
- Plant cell lysis
- Microbiome lysis
- Inhibitors
- Host DNA
- ..

- (Cross-)contamination

Icons: © Adobe Stock

Also, different analyte isolation methods, particularly the homogenization and lysis steps, can alter the analyte profile and quantity isolated because different plant compartment cells and microbial species have different lysis requirements to disrupt their cell walls and liberate the within contained biomolecules (e.g., Gram-negative versus Gram-positive bacteria, or versus fungi). (Salehi, 2023; Abdel-Latif, 2017; Broberg, 2019; Giangacomo, 2020; Haro, 2021; Simmons, 2018)

In addition, impurities and inhibitory compounds (such as polysaccharides, phenolic compounds, and lignin) in the plant samples can disturb analyte isolation and interfere with subsequent analysis steps. The presence of high amounts of plant host DNA (Haro, 2021; Wassermann, 2021), in addition to microbial DNA introduced by reagents such as remnant plasmid DNA from the generation of recombinant enzymes and/or DNA isolation kits, can further impact the analysis result.

During all workflow steps, contamination with microbiome and analytes from other sources than the respective plant compartment can modify the analysis result.

Consequently, these can make the research outcome unreliable or even result in failure because the subsequent microbiome analysis might not examine the real situation in the plant (compartment) but a profile altered by the pre-analytical process.

Moreover, missing metadata in terms of information about the pre-analytical phase can render analysis non-reproducible and non-comparable. (Cernava, 2022; Dickie, 2018; Dundore-Arias, 2020)

Therefore, standardization of the entire pre-analytical workflow and recording of relevant metadata is essential.

3.3 Requirements/recommendations for the pre-analytical phase and (meta)data

To support the standardization of the entire pre-analytical workflow, special measures have to be taken. These shall secure the stability of the microbiome profile and the availability of relevant metadata.

Respective requirements and recommendations for such measures have been elaborated as described above and are based on outcomes from the MICROBE – EPSO expert workshops, scientific literature and various ISO/CEN standards, particularly CEN/TS 17626 serving as best practice examples.

Table 1 provides a summary of the major requirements/recommendations for plant samples intended for microbiome analysis. It includes requirements/recommendations for DNA isolation, quality and quantity assessment as an example analyte.

Table 1 Major requirements/recommendations for plant samples intended for the microbiome analysis

Outside the laboratory – Pre-collection

- **Select representative location(s) suitable for the type/aim of study/analysis**
- **Ensure legality and conformation with regulations** (e.g., for sample collection, acquisition, transport, export/import, sharing, etc.) by considering laws/regulations and getting relevant permissions and certificates.
- **Consider and identify** the following **for the study protocol to be prepared and the selection of the location and document respective metadata**
  - geographics of the location (i.e., latitude, longitude, altitude e.g., using GPS)
  - type of environment and ecosystem (acc. to existing classifications, ontologies and maps, e.g., natural or controlled/artificial environment (incl. conditions); e.g., NASA biome map or EnvO ontology)
  - type of biodiversity (acc. to existing classifications, ontologies and maps such as the Global Restoration Index, the vegetation cover acc. to LUCAS; e.g., using photos for documentation)
  - plant/agricultural management practice (history)
  - former weather conditions (e.g., mean annual temperature or precipitation, special weather events such as droughts)
  - plant species (use Latin name)
  - plant age/developmental stage (e.g., acc. to BASF, Bayer, Ciba-Geigy, Hoechst (BBCH) scale)
  - plant phytosanitary status (incl. phytosanitary certificate)
  - properties of soil breeding ground (e.g., physical properties such as soil type, texture class, and structure, and, where required, chemical properties such as pH) (acc. to existing soil maps and classifications)
- **Prepare a study protocol where** all steps of the entire **pre-analytical workflow**
  - are in accordance with the study purpose
  - address the requirements of the microbiome analysis
  - are compatible with the other pre-analytical workflow steps (e.g., preservation compatible with DNA isolation kit/method)
  - and which ensures the microbiome (analyte) profile in the sample is not compromised during the pre-analytical process but preserved as present *in vivo/in situ.*
- **The entire pre-analytical workflow shall be specified, verified and validated for the intended analysis**

Outside the laboratory – Sample collection

- **Prepare and follow a sampling plan/strategy** which
  - is compatible with the study purpose,
  - addresses the requirements of the microbiome analyte isolation and analysis,

- and ensures the microbiome (analyte) profile in the sample is not compromised during the pre-analytical process but preserved as present *in vivo/in situ.*

- **The sampling plan/strategy describes,** but is not limited to the
  - project name, study type and aim
  - involved parties
  - basics about location, habitat and host (i.e., plant)
  - sampling area (e.g., size, geographics)
  - collection/sampling method, procedure, equipment used
  - sampling pattern and sampling order
  - sample types/species and compartments to be collected
  - number of samples, replicates, and controls to appropriately consider the heterogeneity of the sampling site/area
  - and the amount/size per sample to be collected;

- **Have procedures for collection, processing, and stabilization** to be applied at the collection site. **Follow them and link them to the sample** (e.g., via sample ID). The procedures include, but are not limited to,
  - methods (e.g., for collection, separation of plant compartments, cleaning of samples, etc.)
  - corresponding equipment, devices, and reagents (with company name, cat.no.).

- **The procedures are verified and validated in the context of the entire workflow required for the specific analysis.**

- **Use a unique sample ID** (e.g. code)

- **Use a dedicated collection method, equipment and devices for sample collection**

- For sample collection, **use appropriate (i.e., robust, clean, tight (where needed dry and sterile) sample containers of suitable size and material** to avoid, e.g., leakage, breakage of the container, evaporation, loss and contamination of the sample, and maintenance of sample integrity.

- **Use appropriate labelling materials and procedures** (e.g. suitable for the sample containers, and withstanding conditions at the collection site, as well as during storage and transport)

- **Identify/document the name of the organization** (and, when required, the **name or ID of the person(s)) collecting the sample.**

- **For sample collection, follow the sampling plan** and **document the exact sampling position** of each sample.

- **Collect sufficient numbers of samples, replicates, controls, and amounts of samples** (in terms of size or weight)

- **Document the sample type/plant compartment collected and the respective methods** (particularly of plant compartment separation)

- **Document date (and time) of sample collection**

- **Document if samples are processed at the sampling site/area** (e.g., compartments separated, samples cleaned, subsampled, and pooled, etc.)

- **Avoid contamination** of samples with microbiome from other sources (e.g., soil, humans) than on/in the respective plant, e.g., by using gloves, proper equipment and procedures)

- **Document any unintended deviations from the procedures**

- **Document weather conditions at the time of sample collection**

- It is advisable to **have different sizes and types of containers available on-site** so that if unexpected materials are encountered, they can be properly sampled.

Outside the laboratory – Preservation/stabilization

- **Have a procedure for sample preservation** to be applied at the collection site. **Follow it and link it to the sample** (e.g. via sample ID). The procedures include, e.g., methods, equipment, conditions, and reagents (with company name, cat.no.).

- **Possible preservations include,** e.g.,
  - snap-freeze in liquid nitrogen (considered the gold standard for nucleic acid-based analysis),
  - preserve in liquid stabilizers (e.g., dedicated commercial microbiome or microbiome DNA stabilizers, ethanol, or RNA stabilizers);
  - or other verified and validated preservation procedures

- **Ideally, preserve samples without delay after collection at the sampling site/area** (e.g., by freezing or chemical stabilization using liquid stabilizers)

- **If liquid stabilizers are used** for sample preservation, **apply correct volumes and mixing/homogenization techniques**; follow the instructions of the manufacturer (if available).

- **Where preservation immediately after collection** at the sampling site/area **is not possible/feasible, specify, verify and validate an alternative procedure** (e.g. keeping samples cooled (at e.g. 4-8°C) or at ambient temperature. This is done **in the context of the entire workflow** required **for the intended analysis**.

  (In order to test if/how intermediate storage and transport affect the intended microbiome analysis, time-course and condition experiments for intermediate storage and transport can be performed. These can, for example, be done by storing the sample for different storage duration(s) and temperature(s) or by performing a transport simulation study and then examining the effect on the analysis outcome).

- **Document the preservation method, devices, reagents and conditions (e.g. temperature)** used

- **Document the preservation date and time** (to allow for calculation of the total storage time between collection and preservation)

- **Document any unintended deviations from the procedures**

Outside the laboratory – Intermediate storage/transport

- **Have procedures for intermediate storage and transport**. **Follow them and link them to the sample (e.g. via sample ID)**. The procedures include e.g. methods, equipment, devices, and conditions.

- Where samples are stabilized/preserved at the sampling site/area
  - **store and transport samples stabilized by freezing ≤ −20 °C frozen ≤ −20 °C**
  - **store and transport samples in (liquid) stabilizers at conditions as specified by the stabilizer manufacturer**

- For all samples (native, and preserved)
  - **take measures to keep the required temperature** during intermediate storage and transport
  - and **avoid thawing, freeze-thaw cycles, accidental freezing, and repeated temperature fluctuations**

- **Ideally monitor intermediate** storage **and transport conditions** in a suitable manner (e.g., temperature logger)

- **Document intermediate storage and transport duration and conditions** (e.g., **temperature**)

- **Document any unintended deviations** from the procedures

Inside the laboratory – Sample reception

- **Have instructions with a checklist for sample reception in place**

- **Have criteria for sample rejection/not acceptance in place** (e.g., thawed sample)

- **Check the identity of the sample, type of sample, and sender**

- **Register sample and attribute a unique internal ID** (number/code), if required (e.g., in biobank)

- **Document the date and time of the sample reception**

- **Check and document the legality of the sample** (e.g., the correctness and completeness of legal and regulatory documents such as ABS-/Nagoya-relevant documents, permits for collection, transportation, export and import, phytosanitary certificate, customs declaration, etc.)

- **Document ID of rejected/not accepted sample and reason why**
- **Document any obvious deviations from procedures** (e.g., transport issues such as temperature, broken container)
- **Document the name or ID of the person receiving/taking over the sample**

Storage of samples (between reception and processing)

… same as above, particularly:

- **Document storage place/position**
- **Document storage temperature and duration**
- **Document the time interval between reception and sample processing**

Sample processing

- **Sample processing is suitable for the sample type and compatible with the entire pre-analytical workflow** (e.g., preservation) **and intended analysis**
- **Have procedures for collection, processing, and stabilization** to be applied. **Follow them and link them to the sample**. The procedures include, e.g., methods, equipment, settings, and reagents (with company name, cat.no.)
- **Document applied sample processing methods and conditions** (e.g., temperature, duration and rpm of vortexing, Watt and pulses of sonication)
- **Document the date** (and ideally start time) **of sample processing**
- **Use clean** (and, where required, sterile) **devices**
- **Keep the sample at the appropriate temperature during processing**, particularly during homogenization of frozen samples and sonication
- **Consider to collect (and freeze) also cleaning liquid for microbiome analysis**
- **When aliquoting, pooling and re-packing, ensure sample traceability,** e.g., by **using unique IDs and documentation** that allows for identification of mother, sister, and/or original sample)
- **When aliquoting, pooling and re-packing, use appropriate containers and labelling materials** suitable for long-term storage and to avoid contamination, evaporation or spillage

- **Prepare aliquots to avoid multiple retrieval and thawing** of a sample

Storage of samples (until microbiome analyte isolation)

… same as above, particularly:

- **Document storage place/position**
- **Document storage temperature and duration**
- **Document the time interval between reception and sample processing**
- **Document every retrieval of an aliquot**
- **Use storage systems (i.e., facilities, equipment) to monitor the storage conditions** (e.g., temperature, and, where needed, humidity)
- **Have backup storage systems in place** (particularly as a biobank)

Microbiome analyte isolation

- When there is no plant microbiome analyte isolation kit:
  - **use or modify another commercial analyte isolation kit** (e.g., for soil) and **protocol any deviations**
  - or develop a customized procedure
  - **verify and validate the procedure with the intended analysis in the context of the entire workflow.**
- **The isolation procedure**
  - **fulfils the requirements and specifications of the intended analysis**
  - **is compatible with the type of sample** (i.e., plant/plant compartment) and **its microbial biomass** (high or low)
  - and **is compatible with the type of sample stabilization/preservation** applied (e.g., stabilizer reagent and/or freezing).
- **Have written procedures in place, follow them and link them to the sample** (e.g., via sample ID)
- **Document the kit/reagent/method/procedure applied** for microbiome analyte isolation.
- **Document the date** of microbiome analyte isolation
- **Document the name or ID of the person isolating** the microbiome analyte (for internal purposes)

- **Use an appropriate homogenization method** that effectively releases microbial cells from the respective plant sample

- **Use a lysis step that is sufficiently effective to lyse plant cells and microbial cells** (e.g., by bead-beating)

- **Document** (at least in the written procedure)
  - **type of homogenization applied** (incl. equipment, conditions (such as temperature, mechanical forces) and duration)
  - **type of lysis applied** (incl. equipment, conditions (such temperature, mechanical forces,) and duration)
  - **if and which method of inhibitory compound removal was applied**
  - **if and which method of plant DNA removal was applied**

- **Specify and verify the maximum tolerable amount of plant analyte** (in the isolated microbiome analyte sample) **for the intended analysis**

- **Where needed** (e.g., because the contamination with plant analyte is above the tolerable threshold), **enrich microbiome analyte and document this**

- **Include negative and positive controls*** to ensure the extraction efficiency of each analyte extraction run
  (*i.e. negative control: where all extraction steps are carried out without sample. Positive control: mixture of microbial isolates (e.g. Gram-positive, Gram-negative, fungi) or plant sample spiked with known organisms)

- **Wear gloves and appropriate clothing** when isolating microbiome analyte

- **Perform isolation on clean and sterilized surfaces** (e.g., with ethanol)

Assessment of analyte quantity and quality – on the example of microbiome DNA

- **Specify and verify appropriate methods for assessing the microbiome analyte quantity (yield, concentration) and quality (purity and integrity (degradation/fragmentation)).**

- **The assessment of analyte quantity/quality is done in a buffered solution** (for spectrophotometric methods)

- **Quantity assessment may include**
  a. absorbance measurement (spectrophotometry) (A260);
  b. fluorescence spectroscopy

- **Quality assessment may include**
  a. test for purity by absorbance measurements (spectrophotometry) (e.g. wavelength scan, A260/A280 ratio, A260/A230 ratio);
  b. test for analyte/DNA integrity (by e.g. electrophoresis, capillary electrophoresis, chromatography);
  c. test for presence of interfering substances (using exogenous controls (spiked DNA controls) or inspecting qPCR response curves for anomalies) (Tichopad, 2010) or using an endogenous RNA/DNA sequence for a PCR inhibition test by introducing increasing eluate volumes into the examination;
  d. test for high amounts of plant DNA presence in the isolated microbiome DNA, e.g. by qPCR.

- **Document analyte quantity and quality and assessment method used**

- **Test microbiome analyte isolation performance in a microbiome analyte proficiency test program**, where available.

- **Prepare aliquots to avoid multiple retrievals (and thawing)** of a sample

- **Ensure traceability of isolated microbiome analyte aliquots** (in a way that allows for identification of mother, sister, and original microbiome plant sample)

- **Use appropriate containers and labelling material suitable for long-term microbiome analyte storage** and to avoid contamination, evaporation or spillage

Storage of isolated microbiome analyte

- **Have procedures for storage of isolated microbiome analyte, follow them and link them to the sample** (e.g., via sample ID).

- **Document storage place/position**

- **Document storage temperature and duration**

- **Document time interval between microbiome analyte isolation and analysis**

- **Document every retrieval/thawing of an analyte aliquot**

- **Use monitored freezers/nitrogen tanks** (for frozen sample storage)

4. Conclusion & outlook

The pre-analytical phase is a very important, but still often underestimated, contributor to inaccurate or even false and irreproducible analysis in the microbiome field – also in plant microbiome research. Therefore, raising awareness about the pre-analytical phase with its multiple steps and pre-analytical variables is important. It is the first step towards standardization of this phase, which needs - as the second step - the formulation of specific requirements and recommendations. The human microbiome field is rather advanced. This is due to laws such as the In Vitro Diagnostic Regulation (IVDR) and quality management established in (diagnostic) laboratories and biobanks for accreditation according to international ISO quality standards (e.g. ISO 15189 for diagnostic laboratories, ISO 20387 for biobanks). The pre-analytical sample quality standard CEN/TS 17626 for human samples used or microbiome DNA analysis, further supports standardization of the pre-analytical phase in the human field. CEN/TS 17626 is currently developed to ISO standard ISO/TS 18701,

The content presented in the D4.2 report shall contribute to this endeavour.

The visualization of the pre-analytical workflow (Figure 1) with the listed major pre-analytical variables helps to raise awareness about the pre-analytical phase as a multistep process.

The overview of potential pre-analytical variables (Annex) makes conscious of the fact that along all steps of the pre-analytical phase, numerous factors exist which i) can modify sample quality in terms of alteration of the microbiome community and biomolecule profiles and ii) can in turn lead to unreliable or even false research data. It also makes aware that there are numerous (meta)data arising along this phase and describing the pre-analytical conditions, the sample itself and its handling.

The requirements/recommendations for pre-analytical processes and metadata (Table 1) shall help to avoid or at least minimize pre-analytical errors and alterations of the microbiome community and analyte profiles during the pre-analytical phase. They shall contribute to a more complete documentation of metadata which are relevant for reusing samples and data, for determining the fitness-for-purpose of samples, and for comparing research data.

This list will also support the MICROBE WP3 in the establishment of appropriate microbiome data infrastructure that enables the implementation of recommended standards and metadata harmonization tools (T3.2).

Involving experts in the process of identifying preanalytical variables and phrasing recommendations was important for several reasons: i) to make use of the expert knowhow related to plant microbiome, ii) to transfer the human standards to the plant microbiome situation, but also to iii) increase the acceptance in the plant microbiome research community thanks to the joint elaboration.

In order to achieve a broader outreach to the plant microbiome research community, experts from MICROBE and EPSO plan to write a joint paper for a scientific journal.

In the following steps, we will refine the current requirements/recommendations (e.g., by exploring examples for possible classifications and sources for metadata, and by defining terminology). Since not all requirements/recommendations are equally important, a rating will be performed by MICROBE and EPSO experts in a Delphi-like approach. The wording in the text will be adjusted accordingly.

Both, the pre-analytical workflow scheme for plant microbiome studies (Figure 1) and the respective pre-analytical variable and requirement/recommendation list (Annex and Table 1, respectively) can be used to elaborate comparable documents for soil and marine water samples intended for microbiome analysis.

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*Further sources not referenced in the text but used for preparing the requirements/recommendations and searched for retrieval of metadata, metadata classifications and ontologies and integrated in Table 1,

Deliverable D4.2

Guidance document for the implementation of a standardized pre-analytic workflow in selected fields

Annex

Work package number and title	*WP4 Standardization and Quality Control*
Work package leader	*MUG*
Relevant Task	*T4.2*
Lead contributor to Deliverable	*MUG*
Other contributors to Deliverable	*AIT, CABI, INRAE, HMGU, DMSZ, EMBL, SU*
Dissemination level	*PU*
Due date (month)	*M18*
Version	*1*

Pre-analytical variables and metadata relevant for plant microbiome studies

Pre-Analytic Phase - Key Steps	Key Pre-Analytical Variables & Details	
IN SITU - pre-analytical effects on specimens/samples		
Pre-Collection	**ENVIRONMENT / HABITAT / PLANT**	
	• **Location / site / habitat**	
	○ **Geographic**: e.g. latitude, longitude & altitude	
	○ **Type of environment** e.g. natural (e.g. managed field, native environment/nature) vs artificial (greenhouse, laboratory, seed bank)	
	○ **Type of ecosystem** (e.g. grassland, desert, forest, wetland, mountain)	
	○ **Biodiversity on sampling site** (e.g. richness of plant community)	
	○ **Agricultural & plant management practices** e.g. - fertilization/nutrient addition - pesticide application - watering - management history (e.g. cropping history, agricultural management before current plant rotation) - mowing/grazing (machines/animals)	 H H
	• **External condition history (before collection)**	
	○ **Climate** (e.g. alpine, Mediterranean, tropical	
	○ **Former weather conditions** (e.g. precipitation - amount and the type of rainfall) temperature; drought)	
	• **Plant**	
	○ **Plant species / genotype**	
	○ **Mother plant / genotype**	
	○ **Plant age and/or developmental stage (**life cycle such as seed storage, emergence, growth, flowering, fruit development, maturity, ripening, senescence, and decomposition)	
	○ **Phytosanitary status** (plant health/disease status) e.g. healthy diseased, type of disease, severity (area/volume of plant tissue relative to total plant)	H
	○ **Root extrudate**	
	• **Legally and regulatory relevant documents** (e.g. Nagoya-relevant documents, phytosanitary permits, customs declaration, export/import documents)	H
	• **Soil (breeding ground):** Soil parameters e.g.:	

	○ **Physical properties** (e.g. soil type, texture class, structure)	
	○ **Chemical properties** (e.g. pH, N, P, K; organic carbon and other matter; dry matter, micronutrients, other (electrical conductivity, cation exchange capacity)	H
	○ **Biological properties** (e.g. microbial biomass, potential enzyme activities (if available))	H
EX SITU pre-analytical effects on specimens/samples		
Sampling/ collection	**SAMPLING / COLLECTION**	
	• **Sampling site/area/design (incl. habitat & sample heterogenicity)**	
	○ **Degree of heterogenicity** at sampling area, **size of sampling area,** **design/distribution of sampling** (e.g. stratified sampling e.g. in quadrants or random sampling)	H
	○ **Soil** at plant collection site (particularly for rhizosphere microbiome analysis), e.g. physical properties (such as texture/density, structure/porosity/aggregate size)	H
	• **Sampling method/procedure**	
	○ **Sampling method/procedure**	H
	○ **Sampling equipment/instrument(s)** (e.g. for associated soil: soil core collectors, sterilized sieve, for plants: shovels [e.g. for digging up plants], scissors and/or shears [e.g. for cutting plant roots, leaves], trays, cutting board, knives, bags, containers; gloves;	
	○ **Sample/plant compartment** (e.g. root, leaf, seed, …) and the exact site/part within a compartment (e.g. different root parts near surface or root tip)	H
	○ **Separation of compartments** (in the field versus in the lab)	
	○ **Cleaning** of the sample (if relevant) in the field vs in the lab and by which cleaning methods (e.g. for removal of soil and/or rhizosphere): – Dry methods (e.g. scraping off with disposable spatulas, shaking roots, or removed with gloved hand); – Wet methods (e.g. washing of soil e.g. by soft methods like agitating root in sterile liquid to remove adherent soil and microbes, or shaking/agitating, or by harsher methods: vortexing or sonication in buffers with/without detergents); – Mechanical separation methods (e.g. removal of the coating from the seeds for endophyte microbiome analysis)	
	○ **Sample fractionation, subsampling vs pooling** e.g. full root vs root tip, multiple leaves or entire single leave or parts of a single leave (is it destructive method e.g. cutting a root which release endophytes or be contaminated with rhizosphere, or not)	H

	○ **Sampling date/time (schedule)** (e.g. time important when sampling last the entire day and for circadian rhythm; data: indicator of season)	
	○ **Weather conditions** at time of collection (e.g. precipitation - amount and the type of rainfall; temperature)	
	○ **Control samples** (i.e. sampling of environmental controls like soil, rhizosphere, root extrudate where needed)	
	○ **Sample container(s) type, condition, label(ling)** – Container type and condition: (e.g., closure integrity, material resistance to temp./chemicals size (for holding sample & preservation solution) evaporation or spillage, breakage, leaking; – Labelling (unique ID linked to information about the specimen and associated (meta)data) e.g. label or tag, material: tag fading or falling off leading to loss of biological material identity)	
Preser-vation /stabili-zation	**PRESERVATION / STABLIZATION**	
	• **Preservation/stabilization method/procedure** (e.g. method, reagents, specimen size, volume ratio of specimen- to-preservative/stabilizer solution; temperature, duration)	H
	• **Stabilization date/time** (time interval to collection)	H
Inter-mediate storage/	**STORAGE & TRANSPORT**	
	• **Intermediate storage/transport condition and duration** (e.g. method, duration, temperature, unintended temp. fluctuations, thawing or freezing; breaking/leakage; humidity/moisture; air)	H
	• **Sample container(s) type** ... *same as above* (e.g., closure integrity, composition; material resistance to temp./chemicals, size (for holding sample & preservation solution) evaporation or spillage, breakage, leaking)	☐
Reception	**RECEPTION**	
	• **Reception procedure, date/time, person** e.g. legal/regulatory and specimen quality requirements	H
Sample processing	**SAMPLE PROCESSING**	
	• **Sample processing method/procedure & condition** e.g. cleaning, sterilization, cutting, grinding or other homogenization etc., methods incl. reagents and equipment used, durations and temperature applied, specimens (single, pooled); aliquoting, labelling; time-point	H
Storage of samples	**STORAGE OF SAMPLES** (long-term or short-term until e.g. analyte isolation or analysis).	
	• **Sample storage container type and labelling** (e.g. suitability for storage, information on label, robustness of label)	

	• **Storage method, condition and duration** (e.g. temperature (RT, +4°, -80° or liquid nitrogen liquid or gas phase); temperature variations, humidity, light, time until further processing or analysis)	
Analyte isolation or other pre-processing for analysis	**ANALYTE ISOLATION (e.g. RNA, DNA, protein)**	
	• **Analyte isolation method** e.g. – date/time, – type of kit/method or own protocol, deviations from instructions, – plant cell and microbial cell lysis technique, – plant analyte content (e.g. DNA), – inhibitory compounds & impurities (e.g. in plants like tree bark: polysaccharides, phenolic compounds, lignin, that disturb DNA, RNA, and protein extraction; e.g. in soil: polysaccharide, heavy metals, or humic substances, that can interfere with downstream applications such as PCR and RT-PCR. – contamination from kits/reagents/personnel/air;	H
	• **Contamination** (e.g. with microbiome cells or DNA (e.g. from other samples, by lab personnel, environment, equipment, reagents)	
	• **Analyte quantification and quality control method** (e.g. type of method, buffers and equipment used)	
	• **Analyte storage method, condition and duration** (e.g. temperature (RT, +4 °C, -80 °C or liquid nitrogen liquid or gas phase); temperature variations, humidity, light, time until further processing or analysis)	
Legend:	H = highly relevant	