



DIVERFARMING

Crop diversification and low-input farming across Europe: from practitioners' engagement and ecosystems services to increased revenues and value chain organisation



HANDBOOK ON WP4 PROTOCOLS FOR SAMPLING, SAMPLE PROCEDURE AND ANALYSIS

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Editors: Sören Thiele-Bruhn and Raul Zornoza





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Executive summary

With the long-term view of increasing diversification and biodiversity in Europe (CAP objective) and fostering sustainable development of bioeconomy, the Diverfarming consortium has come together to develop and deploy innovative farming and agribusiness models. Diverfarming will increase the long-term resilience, sustainability and economic revenues of agriculture across the EU by assessing the real benefits and minimising the limitations, barriers and drawbacks of diversified cropping systems using low-input agricultural practices that are tailor-made to fit the unique characteristics of six EU pedoclimatic regions (Mediterranean South and North, Atlantic Central, Continental, Pannonian and Boreal) and by adapting and optimising the downstream value chains organization through executing 14 field case studies and 8 additional long-term experimental plots.

Within Diverfarming, WP4 aims to provide scientific understanding of the link between diversified cropping systems, above- and belowground biodiversity, and the resulting ecosystem services provided by soil microorganisms, soil invertebrates and vegetation in agro-ecosystems. Soil organisms contribute to all biogeochemical cycles, soil organic matter mineralization and stabilization, shape soil structure and have associations with plant species promoting growth and development. Diverfarming will assess: i) the capability of diversified cropping systems with low-input practices to increase biodiversity; ii) how this is controlled by the climate, soil type, crop and cropping system; iii) the functional correlations between soil microbial diversity and activity within different crop associations, agricultural practices, climatic regions, soil factors, nutrient inputs and nutrients cycling; iv) effects of crop diversification, agricultural practices and strategies on invertebrate abundance and diversity and on the occurrence of soil-borne diseases and pests (fungi, insects, nematodes); and v) the interrelationship between microbial diversity and functioning, invertebrate diversity, vegetation diversity and soil quality within a multidisciplinary approach by development of accurate models.

For this purpose, the first step is to develop a handbook to provide standardization of protocols among partners for field and laboratory investigations, so that by applying the same methodology we can effectively compare the results.



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1. Introduction. Microbial community structure and soil-borne diseases/pests

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Importance and applications

Aboveground biodiversity of plants and belowground biodiversity of the soil biome are strongly interdependent. Agricultural soil use affects not only soil chemical and physical properties but also the soil biome even more with its structural diversity and ecosystem functions, such as C storage and turnover, nutrient cycling, biotransformation of organic pollutants and thus water purification, or the modulation of soil structure (Creamer et al., 2016). It can thus be expected that changes in soil use will affect the soil biome and previous studies have shown that especially shifts in the communities' structural diversity are an early indicator of reactions towards such impacts (Schloter et al., 2018).

Diverfarming aims to investigate changes in agricultural soil use through the insertion of additional, valuable plant species in the cultivation of annual and permanent crops, respectively. The thereby improved agrobiodiversity will affect (i) soil microorganisms and (ii) faunal species. To identify such effects the aim is to investigate the soil microbial community structure and functions; earthworms, as a key group of soil faunal species; and the naturally occurring plant vegetation.

Hence, ideal parameters to identify positive effects of increased agrobiodiversity are what is tested within Diverfarming. Today, methods in soil biology enable an in-depth analysis of microbiomes and other key species living in the soil. However, it is indispensable to exactly define common methods and protocols in order to come up with comparable and combinable findings between the individual projects in order to achieve an even higher mutual output of the project. Consequently, it is the objective of this handbook to provide detailed information and instructions on parameters and endpoints that will be investigated by all the individual projects within Diverfarming. In soil biology these parameters are:

- DNA and RNA extraction, storage, shipping and handling
- Next generation sequencing of DNA for qualitative metagenomics using an Ion Torrent and MiSeq sequencer, respectively.
- Bioinformatics for metagenomics data evaluation.
- qPCR of functional genes, i.e. *amoA*, *nirK*, *narG*.



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- qPCR of selected pathogens and pests.
- Microbial enzyme activities (fluorogenic or colorimetric determination):
 - dehydrogenase
 - β -glucosidase
 - leucine-aminopeptidase
 - acid phosphatase (cut-off pH 7) or in high pH soil (pH ≥ 7) alkaline phosphatase
 - arylsulfatase
 - potential nitrification
- Earthworm hand-sorting and chemical extraction; identification of total density and total mass and – if possible – of density and mass, respectively, by ecological groups, and species.
- Plant species: Determination using the quadrant technique; data evaluation by determination of richness, vegetation cover and Sorensen's similarity index (SI).

Soil sampling

Soil samples destined for soil biological testing must be subsamples of the same bulk used to measure all different parameters (i.e. DNA and RNA extraction, enzymatic activities, total carbon, total nitrogen, clays etc.). The sampling depth for soil samples in Diverfarming is set at 0-10 cm, although soil biological analyses are supposed to focus solely on the lower part (5-10 cm) of these first 10 cm of soils. Mulch layers, litter layers, stone layers and crusts at the soil surface should be excluded from soil biological analysis (unless purposely targeted). Furthermore, rhizosphere and areas with entangled roots (e.g. typical for permanent grassland) should be avoided during sampling, unless it is a specific research question of the project.

Stones (particle > 2 mm) and plant roots and litter are eliminated from soil samples before analytical testing. However, the content of stones, roots and particulate organic material might be valuable information. It is suggested to record these contents on a gravimetric basis or based on a volumetric estimation.

Soil samples destined for soil biological analysis will contemplate replicates: Five samples per treatment (the same used for the measurement of other biological parameters). The five samples should belong to the total set of nine soil samples taken from one treatment (see e.g. chapters on soil chemical analyses). The five samples should have soil chemical and physical properties well within the average of all nine samples. They must not have extreme properties in order to avoid outliers.

Soil samples should be taken and handled with care to avoid unrepresentative or inhomogeneous samples and to save from subsequent decay of the soil biome. (See section 3.0.2 "Sampling and handling of soil prior to DNA and RNA extraction").



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The concept of barcoding in Diverfarming and for sequencing

Within Diverfarming, a barcoding is to be generated by the coordinator for each sample. This barcode traces each sample to its origin, including treatments etc.

When DNA is extracted, due to procedural aspects described below, it receives an additional DNA barcode. For instance, sample 1 from Cartagena long-term experiment 1 will be AGAA and sample 2 AGAT. Thus, we refer to the sample Diverfarming barcode and the DNA barcode that is used only during the sequencing and analysis process. DNA extractions have a Diverfarming barcode. Sequencing reactions have an internal barcode to allow multiplexing of sequencing reactions.

Soil preparation

Soils are sampled and further pre-processed for the field moisture status (see also further sections 3.0.1 – 3.0.3 for sample transport, storage and shipping). However, soils must not be excessively wet so that free water drips off. This will inhibit exact mass determination of the soil sample. In the event that they are too wet, they should be gently dried at air temperature in the laboratory or in an oven with recirculating air (set air recirculation at maximum) at $<35^{\circ}\text{C}$. Drying can be further improved by spreading the soil sample in thin layers onto moisture absorbing paper. Check soil moisture repeatedly to not overdry the soil. Subsequently, soils are sieved to ≤ 2 mm and carefully homogenised. Visible roots and stones should be manually removed at any steps of soil sampling and preparation. If freezing is necessary for soil storage, the sample should be split into several subsamples in order to avoid repeated cycles of freezing – thawing and freezing again. Homogenise samples again after thawing to avoid inhomogeneous distribution of moisture within the sample.

Subsequently, representative aliquots are used to determine soil dry mass and soil moisture, respectively, as well as soil water holding capacity (WHC). This should be done after storage, using the thawed samples because freezing and storage can substantially alter soil moisture and WHC.

Soil Dry Mass and Water Content (Wilke, 2005)

Principle. Soil samples are dried at $105 \pm 5^{\circ}\text{C}$ until mass constancy is reached. The mass difference between moist and dried soil is the measure of the water content. The water content is calculated on a gravimetric basis (g water /g soil), following ISO 11465 (1993). Calculation based on volumes is also possible but not preferred within Diverfarming (cm^3 water/ cm^3 soil). Finally, the water content of soil is given as a percentage by weight of oven-dried soil. The optimum water content for microbial processes is in the range of 40–60% of the maximum water-holding capacity (WHC), which corresponds to water suction pressures of -0.01 to -0.031 MPa.

Equipment. Drying oven, thermostatically controlled with forced air ventilation and capable of maintaining a temperature of $105 \pm 5^{\circ}\text{C}$; desiccator with an active drying agent; analytical balance, accuracy 1 mg; glass or porcelain jars with lid (25–100 mL volume).



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Procedure. Label the jars and lids with a temperature resistant marker. Dry jars with lid at $105 \pm 5^\circ\text{C}$ and subsequently cool, with the lid closed, in a desiccator for at least 45 min. Determine the mass (m_0) of the closed container with an accuracy of ± 1 mg. Weigh ~ 10 g of soil into the jar. Note the exact mass of the soil plus the jar with lid with an accuracy of ± 1 mg (m_1). Place soil and jar in an oven at 105°C and dry until constant mass is achieved (Typically 12 to 48 h). To this end, the jar is opened but the lid must be dried as well and must not be interchanged. Cool the jar with the lid closed in a desiccator for at least 45 min. Determine the mass (m_2) of the closed jar containing the oven-dried soil with an accuracy of ± 10 mg immediately after removal from the desiccator.

Calculation. Calculate the dry mass content (w_{dm}) or water content (w_{H_2O}) on a dry mass basis expressed as percentages by mass with an accuracy of 0.1% (m/m) using the following equations:

$$w_{dm} (\%) = (m_2 - m_0) / (m_1 - m_0) \times 100$$

$$w_{H_2O} (\%) = (m_1 - m_2) / (m_2 - m_0) \times 100$$

with m_0 being the mass of the empty container with lid (g); m_1 the mass of the container with air-dried soil or field-moist soil (g); and m_2 the mass of the container plus oven-dried soil (g).

Remarks. In general, decomposition of organic material can be neglected at temperatures up to 105°C . However, for soil samples with a high organic matter content ($> 10\%$ m/m) the method of drying should be adapted by drying to a constant mass at 60°C . Some minerals similar to gypsum lose crystal water at a temperature of 105°C .

Soil Water Holding Capacity (WHC)

The water holding capacity (WHC) of structured field soil is substantially different from a sieved and homogenised sample of the same soil. Hence, information on field capacity etc. of that field soil does not represent useful information for laboratory testing.

Principle. The WHC is determined by repeatedly adding excess water to an exact mass of soil. The mass of water leaching from the soil and/or the water retained by the soil is determined.

Equipment. Funnel, glass containers, e.g. measuring cylinders, filter paper (Schleicher-Schuell, 595 $\frac{1}{2}$ or similar), analytical balance, accuracy 10 mg.

Procedure. Prepare a funnel with a filter (e.g. folded filter Schleicher-Schuell, 595 $\frac{1}{2}$). Moisten the filter paper with water (but avoid excess of water that drips off) and record the mass of the filter and funnel with an accuracy of ± 10 mg (m_0). Weigh 10 to 20 g of soil into the funnel with filter. Use approximately similar weights for the different samples (for example do not weigh in 10 g of one sample and 20 g of the replicate sample) and record the exact mass with an accuracy of ± 10 mg (m_1). If moist soil is used the exact amount of contained water must be known (m_2) (see above the method on "Soil Dry Mass and Water Content"). Place the funnel into a container in order to collect leaching water. If the amount of leaching water is to be determined on a mass basis, the mass of the empty container must be determined beforehand with an accuracy of ± 10 mg (m_3). Add an excess of water using a defined volume



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(e.g. 50 ml = m_4), and wait until no more water drips off (typical waiting time 2 hours but this depends on the soil texture). Place a lid (e.g. a Petri dish) on top of the funnel to avoid losses due to evaporation. Pour the leached water again onto the soil in the funnel. Repeat this step once more (in total three times). Finally, determine the mass of the leached water in the container (m_5) and/or the mass of the funnel with the filter paper and the moist soil (m_6).

Calculation. Calculate WHC (ml/g) using the following equations (a – e). The mass (or volume) of retained water can be either determined from the mass of water saturated soil (a and b) or the mass of leached water (c and d). For simplicity, the mass and volume of water are considered as 1 g = 1 ml.

a) $m_6 - m_0 = \text{soil}_{\text{saturated}} \text{ [g]}$

b) $\text{soil}_{\text{saturated}} - m_1 + m_2 = \text{water}_{\text{retained}} \text{ [g]}$

c) $m_5 - m_3 = \text{water}_{\text{leached}} \text{ [g]}$

d) $m_4 + m_2 - \text{water}_{\text{leached}} = \text{water}_{\text{retained}} \text{ [g]}$

e) $\text{water}_{\text{retained}} / (m_1 - m_2) = \text{WHC [g/g]} \sim [\text{ml/g}]$

1.1 Storage of soil samples prior to subsequent biological analyses

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Importance and applications

Biological parameters of soil samples should be determined as quickly as possible to avoid any changes in the structural community composition and activities of soil biota. However, this might not always be possible, especially when large numbers of samples need to be taken on one date and subsequently analysed. In this case it is advisable to store all samples under the same, controlled conditions after proceeding with soil preparation (sieving etc.). In any case, moist soil should be used for storage and subsequent determination of parameters.

Table 1 lists storage conditions that are recommended in existing ISO standard test methods, or which have been recently under discussion. This is in order to prepare ISO/DIS 18400-206 Soil quality — Sampling —Part 206: Guidance on the collection, handling and storage of soil for the assessment of biological functional and structural endpoints in the laboratory.



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It must be noted that for the determination of enzyme activities, short-term storage of 1 to 2 days at room temperature or 3 to 4 days at 15°C is preferable compared to any cooling or freezing of samples that will considerably affect the measured endpoint.

! Generally, within a study, storage conditions that are used for a test method should not be changed between samples. Consequently, if not all the samples can be analysed within 3 to 4 days (which is rather likely), it is recommended to freeze (-20°C) and thaw all samples following the same protocol as described below. See also chapter 3.0.2 for specific conditions for DNA and RNA.

Before a prepared and stored soil is used for a biological laboratory test, it should be pre-incubated. Pre-incubation allows germination and removal of seeds, and the re-establishment of an equilibrium of biological activity following the change from sampling or storage conditions to incubation conditions. Pre-incubation conditions vary with the purpose of the test method but should approach test conditions as far as is practicable. The pre-incubation period depends on the purpose of the study, the soil composition and the storage/pre-incubation conditions. For tests on biological activities, a period of between 2 d and 28 d is generally adequate. When marker compounds such as DNA, microbial biomass carbon etc. must be extracted, no acclimation period is required.

Thawing of samples that were previously frozen must be done with special care. For the analyses of microbial activity (e.g., soil respiration), a thawing period of one week at 4°C and another three days at 20°C are recommended (If necessary, a shortened thawing period of one day at 20°C may also be suitable). For DNA and RNA analyses, the thawing period should be as short as possible to avoid degradation processes.

Freezing the samples can change the water-holding capacity. Therefore, water-holding capacity should be determined after thawing.



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Table 3.0.1. Storage conditions and duration for the assessment of biological endpoints when analysis cannot be performed immediately

Test objective	Moist soil 4°C days/months	Moist soil -20°C years	Moist soil -80°C or -180°C (liquid nitrogen) years
Invertebrates	3 months	—	—
DNA	—	2	10
RNA	—	—	10
Microbial biomass			
— substrate-induced respiration	7 d	1	—
— fumigation-extraction	7 d	1	—
Potential ammonium oxidation	7 d	1	—
Nitrogen mineralisation	7 d	1	—
Microbial soil respiration	7 d	1	—
Dehydrogenase activity	7 d	1	—
Enzyme activity patterns	7 d	1	—
Denitrifying enzyme activity	7 d	1	—



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1.2 Sampling and handling of soil prior to DNA and RNA extraction

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Importance and applications

High-throughput DNA sequencing technology is used in Diverfarming to characterise fungal and bacterial diversity in agricultural soil samples. Soil is highly heterogeneous, especially at microscale, and it is therefore essential that samples from different soil types, regions and crops are taken and handled in a similar way to prevent the introduction of unwanted variables and biases. In fact, given the sensitivity of modern amplicon sequencing approaches even small differences in sample preparation can affect the outputs in terms of recovered species. The reliability of the representation of microbial communities, and the efficacy of all the downstream analyses (i.e. pathogens quantification, and other qPCR applications) will depend on the handling and preservation techniques that will be applied to Diverfarming's soil samples. Storage time and temperature (at sampling, during shipping to the laboratory, and at the laboratory) can substantially alter the soil community's structure, influencing the recovery of DNA or RNA of certain taxa more than others (Rubin et al., 2013 and references therein). In one study performed on more than 500 soil types, frozen soil samples maintained the highest alpha diversity and differed least in beta diversity compared to other storage systems, suggesting the utility of cold storage for maintaining consistent communities. However, responses to storage of microbial communities are strongly soil dependent and seem to become more critical with increasing organic matter content (Bainard et al., 2010; Plassart et al., 2012; Terrat et al., 2015).

Principle

Soil aliquots destined to DNA and RNA extraction must be sieved (<2 mm) to homogenise the sample and reduce potential contamination with plant and animal material. High clay and/or moisture content, however, can inhibit effective sieving. In this case the removal of visible organic debris and sample homogenisation must be performed manually. Once homogenised, soil samples need to be stored until further processing; the storage conditions must be chosen carefully. The homogenisation, sieving and collection in dedicated containers can be carried out in the field when possible, or in the laboratory. In any case the storage and eventual shipping need to be done according to the same procedures, which are described below.



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Storage of soil samples for DNA extraction. Procedure

The DNA extraction procedure can start from either fresh soil or (more feasibly) from frozen soil. The best option is to put samples immediately at -20°C or lower. Since most microbial cells burst during the freeze-thaw cycle that occurs when samples are extracted, a single freeze-thaw cycle is desirable in order to obtain reproducible amounts of DNA. Therefore, soil samples for DNA extraction should be stored at -20°C , already sieved, homogenised and weighed in sterile DNase free vials (example: 2ml screw-cap, cryogenic tubes, sterile, DNase-free).

As most labs may not have the facilities to freeze soil in the field, we propose a method where **samples will be taken, put on ice in the field (i.e. in a thermo-stable shipping box, e.g. made from Styrofoam, with enough wet ice packs to keep the temperature around 4°C during transport) sieved or hand homogenised upon arrival, aliquoted to 500mg in individual tubes and frozen to -20°C .** In this way we avoid the destruction of DNA inside the soil after thawing, and we can directly proceed to add the C1 buffer to the whole sample. We recommend that up to five (or more) vials for each sample (technical replicates) are stored at -20°C . Each vial must be univocally labelled with cold-resistant writing/stickers. **The weight of subsamples that will be extracted needs to be very accurate and must be recorded to be used in the following calculations.**

A larger subsample of the same frozen soil must be kept along with vials, to be used in the measurement of soil water content at the moment of the extraction, if not already measured before freezing. This value is needed in calculations that will refer the extracted DNA to each gram of dry soil.

In the event that you need to prepare the aliquots and weight soil for DNA or RNA extraction starting from frozen larger soil samples, the thawing period shall be as short as possible to avoid nucleic acids degradation processes.

Collection/storage of soil samples for RNA extraction. Procedure

For collection, transport and storage of soils needed for total RNA extraction, it is recommended to use the LifeGuard™ Soil Preservation Solution which is commercialised by Quiagen. This product efficiently protects nucleic acids from degradation in soil samples preventing RNase and DNase activity.

1. Weigh 2.5 g of soil in a 15 ml RNase and DNase free Tube (i.e. a 15ml screw-cap cryogenic tube) and add 6 ml of LifeGuard™ Soil Preservation Solution (1 g of soil requires 2.5 ml of solution – the solution can be added to the tubes in sterility, before going to the field). If the soil cannot be weighed in the field use a volume of soil as a reference (a 5 ml tube, or equivalent).
2. Vortex or gently mix the soil and the solution by hand to obtain a mixture.
3. Store the soil in the LifeGuard™ Soil Preservation for one month at -20°C (2 weeks at 4°C or 1 week at room temperature).
4. Shipping can be performed at this stage; at 4°C , using boxes with ice packs as in eluted DNA shipping (see section 3.0.3).



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5. When you are ready for the total RNA extraction, the samples can be slowly thawed at 4°C, if kept at -20°C, then centrifuged at 2500 x g for 5 min at 4°C to remove the solution and collect the soil to be further processed.

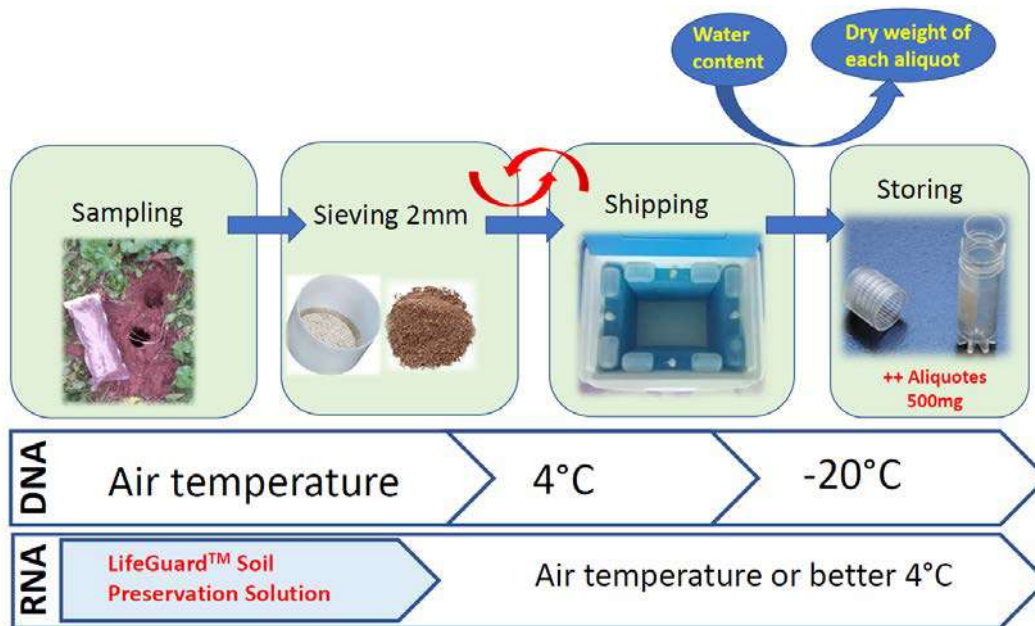


Figure 3.0.1 Diagram of the main steps needed for sampling and handling soil prior to DNA and RNA extraction

Reagents

LifeGuard Soil Preservation (from Qiagen. 100 ml bottle costs about 180 Euro), for the ambient temperature stabilisation of microbial RNA in soil. (<https://www.qiagen.com/us/shop/sample-technologies/protein/stabilization-and-fixation/lifeguard-soil-preservation/#orderinginformation>)

Materials and equipment

- 2ml screw-cap, sterile, RNase-free, DNase-free cryogenic tubes (i.e. from BRAND, code 114841, or Sigma-Aldrich screw-cap TPP® 2.0 ml cryotubes, code Z760951)
- Cold resistant small labels and/or adhesive tapes
- Permanent-ink pen or labels with cold resistant glue (try before use)
- Sterile 15 ml RNase-free, DNase-free screw-cap tubes (if used for shipping be sure the closure is safe for liquids).
- Box container for 2ml vials (and for 15 ml vials in case you ship soil for RNA extraction)
- Styrofoam boxes
- wet ice packs
- Parafilm



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- -20°C freezer
- Precision scale (4 decimals)
- A field scale (to weigh soil for RNA extraction, 2.5 g/6 ml LifeGuard solution)
- Gloves suitable for RNase and DNase free lab.



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1.3 Storage and shipping of eluted DNA

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Importance and applications

The procedures used for DNA extraction and its purity at the time of storage determine the stability of the stored eluted sample. In fact, DNA is sensitive to nucleases and chemical hydrolysis (possible degradation processes are: depurination, depyrimidination, deamination). DNA is sensitive also to oxidation reactions due to the presence of trace amounts of metals (Roder et al., 2010; Ivanova & Kuzmina, 2013).

Genomic DNA can be stored at 4°C or even at room temperature without degradation, for short periods of time (1-2 days), however if this is the case, samples should be monitored for DNA concentration and evaporation. Storage of DNA for the medium term is done at -20°C or -80°C.

Acidic conditions cause hydrolysis of DNA, therefore DNA in the aqueous phase is stored under slightly basic conditions. Samples of DNA destined to multiple downstream analyses should be stored in dosed aliquots to avoid repeated freeze-thaw cycles (Roder et al., 2010; Ivanova & Kuzmina, 2013).

Procedure

Once extracted, DNA from Diverfarming case studies can be stored frozen at -20°C in 10 mM Tris pH 8.0 or in double-distilled water (DNase free) (but avoid the use of TRIS:EDTA buffer). It is important that each sample is univocally labelled according to Diverfarming samples' barcoding and is supported by basic information on dilution and quality (the weight of fresh soil used to extract it, the initial concentration and eventual dilution as ng/μl, the 260/280, the 260/230 ratio as quality value – see chapter 3.1 for details).

DNA shipping can be done at room temperature if the DNA has just been extracted and it is already stored at 4°C (not frozen) or it can be shipped at about 4°C on ice packs (blue ice). If the DNA is stored frozen, multiple freezing-thawing events should be avoided, thus DNA should be shipped on dry ice.

Remember to always include a shipping information sheet that contains detailed sample information, indicating the person/s receiving the parcel and the sending laboratory.



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After quantitation and quality checks, eluted DNA must be split into 3 tubes.

Tube 1 must be sent to:

Instituto de Biotecnología Vegetal Genética Molecular,
Edificio I+D+i
Plaza del Hospital s/n
Universidad Politécnica de Cartagena
30202 Cartagena, Spain

Tube 2 to:

Laboratorio di Biologia Molecolare e Microbiologia
Centro di ricerca Agricoltura e Ambiente
Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA)
Via della Navicella, 2-4
00184 Roma, Italia

Tube 3 must be kept at -20°C as a reference by the laboratory for possible further downstream analyses.

Use glued paper labels to identify your samples. If you write with a marker, it can fade away with ice/freezing.

Place the DNA sample in a 1.5 or 2 mL screw cap microcentrifuge tube and use Parafilm to seal the top of the centrifuge tube to ensure that it will not open during transit. Pack the tube/s in a freezer box, or in larger plastic tubes (i.e. 50 mL Falcon-like conical vial) or using other feasible methods to protect it/them from breaking (if there is space between the top of the box/vial and the lid, fill with paper to prevent tubes from freely shifting during transit), seal the box and/or the large vials into a plastic envelope to keep them clean and dry. Place all into a thermo-stable shipping box (i.e. Styrofoam, filled with ice packs) and if there is space add other clean packing materials to ensure that the ice packs and the box containing your DNA are not shaken.

Use the fastest available courier, e.g. FedEx, UPS, DHL or USPS. Label your contents as “non-hazardous research sample”. Label the box as “temperature sensitive”. Possibly try to ship on Monday or Tuesday to avoid any delay associated with delivering on a weekend. Determine whether the country you are shipping to has a holiday. It is important that the people receiving the samples are informed in due time about the shipping schedule. If a tracking code is delivered, share it with the receiving laboratory/person.

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2. Deoxyribonucleic acid (DNA) extraction

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Importance and applications

Soil microbial communities play a key role in the maintenance of soil functions and in ecosystems homeostasis. The major bottleneck to the study of soil microbial communities is their very limited culturability: it is possible to cultivate only 1% of microorganisms using traditional culture techniques. Molecular biology methods, based primarily on DNA amplification, allowed to obtain a deeper insight into the structure, composition and richness of soil microbial communities.

Several protocols have been proposed to extract total microbial community DNA from soil (Krsek & Wellington, 1999). Comparative studies on the efficiency of DNA extraction and purification from different soils showed a great variation in the efficiency of lysis, yield and purity of the DNA that can be obtained from different protocols. The type of procedure can affect the success of downstream analytical techniques such as PCR (Picard et al., 1992) and sequencing (Zielińska et al., 2017). Biases can strongly affect the outcome of microbial community analysis (Zielińska et al., 2017).

Bacteria, fungi and archaea exist in several forms in soil, including dead mycelia, sclerotia, spores, vegetative cells, dwarf cells and cysts. In order to obtain DNA from these structures, it is necessary to lyse them. To obtain DNA from these varied structures, in all the protocols so far proposed, samples are treated with a buffer and cells lysed either mechanically (bead-beating, sonication, freezing–thawing) or chemically (with chemicals such as SDS, phenol, various detergents or the enzymes lysozyme, proteinase K), or more frequently by a combination of these treatments (Krsek & Wellington, 1999). After the lysis, different purification steps follow, with a series of precipitations obtained generally using saline or alcoholic solutions (potassium acetate, ethanol, isopropanol, polyethylene glycol, spermine HCl), and purification steps (phenol/chloroform, spin columns, gradient centrifugations, hydroxyapatite chromatography). Commercial purification kits usually combine these steps to provide an easy-to-use combination of passages to gain a high reproducibility that minimises possible biases, due to bottleneck passages. Quality and quantity of extracted DNA relates strongly to the characteristics of the soil used: humic substances, clays, metals, organic xenobiotics, organic matter content influences the yields and the efficacy of the different lysis, separation and purification steps (Lombard et al., 2011; Soliman et al., 2017). Variation in results can also be attributable to skill level differences among technicians/operators (Lombard et al., 2011; Philippot et al., 2012).



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Standardisation in the sense of shared sample handling and analysis protocols, based on strictly defined procedures as defined for example by the International Organization of Standardization (ISO), are needed when microbial communities from different soils must be compared based on analysis performed by different laboratories. Philippot et al. (2012) developed and validated a protocol for direct extraction of total DNA from soil samples (Philippot et al., 2012), which was formally acknowledged as the ISO-11063 method. This standard has been further modified on the basis of subsequent studies (Plassart et al., 2012; Terrat et al., 2015) aimed at improving its efficiency towards fungal DNA (Terrat et al., 2015). Diverfarming, although recognising that these strictly defined standards are the most promising way to achieve inter-laboratory comparability of data, decided to adopt a commercial DNA extraction Kit, the DNeasy PowerSoil® DNA Isolation Kit (Qiagen), and to provide the partners with an additional detailed protocol, based on the instructions provided by the company, but with particular attention in the description of the most ambiguous passages, especially with reference to the extraction of particularly difficult samples.

Principle

DNA (Deoxyribonucleic acid) extraction is the process by which DNA is separated from proteins, membranes and other cellular material contained in the cell from which it is extracted, and purified. During the procedure, additional materials of the matrix are removed to ensure an extraction that ideally should represent the biological DNA present in the subject of study.

There are different soil DNA extraction protocols and the selection of the right protocol or extraction kit is the “key” to obtaining the right results from our soil studies. The DNA extraction requires the right amount of soil to obtain enough DNA, careful handling to avoid contamination, and ensuring a successful nucleic acid extraction and purification for downstream analyses. Different steps are crucial for a successful soil nucleic acid extraction: effective disruption of cells and tissue; denaturation of nucleoprotein complexes; inactivation of nucleases (DNase and RNase) and away from contamination. In fact, yield, quality, and integrity enable the good results in the downstream processes.

Different DNA extraction methodologies that are widely used include DNA purification using: CTAB (N-acetyl-N,N,N-trimethyl ammonium)/NaCl; phenol/chloroform; silica bead to capture DNA; solid-phase purification or column-based protocols. The current tendency is to use commercial kits that allow an easier and fast extraction process. Except for the original QIAGEN columns, most kits are based on the original protocol of Vogelstein and Gillespie whereby DNA is bound to a silica matrix with the aid of NaCl (Vogelstein & Gillespie, 1979). The purified DNA is eluted with a low salinity buffer such as 10 mM Tris pH 8-8.5 or even water.

One effective kit for isolating total DNA is the DNeasy PowerSoil® DNA Isolation Kit (Qiagen). In principle it is a standard kit that includes three previous steps to disrupt the microbial cells in the soil, precipitate proteins and clean the sample from humic compounds that inhibit further downstream processing such as PCR or ligation. The procedure is very effective for isolating DNA molecules of all



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types of soil sample with high or low microbial load. This kit uses patented inhibitor removal technology to remove PCR inhibiting compounds, including humic substances associated with soil DNA.

The DNeasy PowerSoil® DNA Isolation Kit and other kits work with rather small soil samples of 250 mg to 500 mg. For very heterogeneous soil samples this might not be sufficient to obtain replicate analyses with acceptable standard deviation among replicates. In that case, the extraction and analysis of larger soil samples may be advantageous. For that purpose, the DNeasy PowerMax Soil® DNA Isolation Kit (Qiagen) can be used, which works with large (10 g) soil samples. The DNA extraction protocol for this DNA isolation kit is also described in this chapter.

Soil DNA extraction by PowerSoil® DNA Isolation Kit (Qiagen) – regular (small) 250 mg soil samples

Soil sampling and pre-processing

Soil samples will be taken from the field as specified in Chapter 3.0 and elsewhere in this handbook, stored in sterile falcon or plastic bags and placed on ice (i.e. using containers) directly in the field. Sieving, determination of soil moisture and water holding capacity as well as storage should be performed according to the methods described in chapter 3.0 and section 3.0.2 therein. Samples should be aliquoted to 500 mg in individual 2 ml Eppendorf tubes, ideally before freezing. In this way the destruction of DNA after thawing inside the soil is avoided, and we can directly proceed to add the C1 buffer to the sample.

The amount of sample to be initially weighed is one of the “key” steps in soil DNA extraction, which determine the amount of DNA available for downstream analyses. The DNeasy PowerSoil® DNA Isolation Kit is designed to process 0.250 g of soil, although it gives better results with 0.500 g.

Soil DNA extraction: living and dead biota

The DNeasy PowerSoil® DNA Isolation Kit (Qiagen) proved to be more efficient with some recommendations: cell disruption/homogenisation is a critical step for complete homogenisation and cell lysis, thus a vortex at maximum speed for more time than that suggested by the kit’s protocol optimises this step.

Take care to heat the lysis solution (referred as C1 in the suggested kit) to 70°C for 5 minutes, to dissolve the salts. Once dissolved it must be cooled down, in order to avoid DNA degradation.

Following step by step, the description of soil DNA extraction by **DNeasy PowerSoil® DNA Isolation Kit (Qiagen)**.

1. **Add 0.5 grams of the soil sample to PowerBead Tubes and gently vortex for 30 min.** After the sample has been loaded into the PowerBead Tube, begin dissolution of humic acids, and proteic acid degradation. This is the first critical step to complement the following cell lysis step in which the sample disperses in the PowerBead Solution).



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2. Check Solution C1, pre-heated to 70°C for 5 min to dissolve the precipitate. **Add 60 µl of Solution C1 to step 1 and briefly vortex to mix.** Solution C1 contains SDS, thus this is an important step in which the addition of Solution C1 allows the cell lysis to form a white precipitate in the Eppendorf within a few minutes of reaction.
3. **Vortex PowerBead Tubes using a vortex or a flat-bed vortex pad with tape and vortex at maximum speed for 10 min when we use less than 12 preps.** If we use more than 12 preps we extend the vortex up to 20 min.
4. **Centrifuge at room temperature for 2 min at 10 000 x g.** The supernatant may still contain some particles. While centrifuging, **add 250 µl of Solution C2** into each clean tube. **Incubate at 4°C for 15 min** (the incubation can be extended to overnight).
5. **Remove the tube of Step '4' from the centrifuge and carefully transfer the supernatant (between 400 and 500 µl) to step '5'.** Discard the pellet.
6. **While centrifuging, aliquot 200 µl of Solution C3 into each clean tube.**
7. **Remove the tube of Step '6' from the centrifuge and carefully transfer the supernatant to step '6'.** Discard the pellet. Incubate at 4°C for 15 min.
8. **Centrifuge at room temperature for 2 min at 10 000 x g.**
9. **After centrifugation, carefully remove the tube and transfer the entire volume (up to 750 µl) into a clean tube, avoiding the pellet which must be discarded. Add 1.2 ml of Solution C4, and mix gently.** The pellet at this step contains additional non-DNA organic and non-organic material including humic acid, cell debris, and proteins. The volume at this step is 1850 µl (1200 µl of C4 + 750 µl of sample by step 8).
Take care to shake solution C4 before use; this is a high concentration salt solution allowing binding of DNA to the Spin filters provided by the kit.
10. **Load 675 µl of step '9' onto a Spin Filter and centrifuge at 10 000 x g for 1 min at room temperature. Load the remaining supernatant onto the spin filter and centrifuge at 10 000 x g for 1 min at room temperature (~ 3 loading).** At this step, DNA is selectively bound to the silica membrane in the spin filter device in high salt solution. Discard the flow through at the end of each centrifugation.
11. **Add 500 µl of Solution C5 to each tube of the spin tube. Apply centrifuge to the tube at room temperature for 5 min at 10 000 x g**
12. **Carefully discard the flow through and repeat centrifugation for 1 min to avoid residual Solution C5.**
13. **Carefully transfer the spin filter to a clean tube and add 100 µl of Solution C6 (preheat to 60°C) to the centre of the white filter membrane of the spin filter.** (it is advisable to clean the outer part of the filter from any droplet left using clean paper. Solution C6 is a sterile elution buffer. Let C6 sit on the filter for 5' at room temperature before the final centrifugation step.
14. **Centrifuge at room temperature for 3 min at 10 000 x g. Discard the Spin Filter. The DNA is now ready for any downstream analysis.**
15. **Keep DNA frozen (-20°C to -80°C)** for medium- to long-term storage. Shipping should take less than one week (see chapter xxx for storing and shipping DNA). Split the eluted DNA into three tubes. Tube 1 remains stored in your lab, tube 2 is for analysis of bacteria and tube 3 is for fungi.



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DNA yield and quality check

The quantity and quality of DNA can vary depending on the extraction. The measurement process consists in the quantification of double-strand DNA (dsDNA) and the assessment of its suitability for downstream applications, such as PCR, Next Generation Sequencing or quantitative PCR.

The “golden standard” is the Bioanalyzer that measures quantity, quality and size of the fragments in a chromatography. However due to the costs, the most frequently used and recommended approaches to evaluate the quality and the quantity of DNA are: NanoDrop or similar, based on UV spectroscopy, and Qubit 2.0, (or similar) based on fluorophores specifically binding dsDNA.

- Nanodrop is a low-cost effective, fast and easy instrument but accurate only for the quality, while it underestimates the quantity, so we recommend using the Nanodrop or similar exclusively to evaluate the DNA quality. Thus, the use of Nanodrop is recommended mainly for estimating the 260/280 ratio. DNA quality is measured by reading the whole absorption spectrum (220-750 nm) with NanoDrop or similar and calculating DNA concentration at both 260/280 and 260/230 nm.
- Qubit fluorometer or similar, measures the nucleic acid concentration indirectly, allowing to measure very small quantities of DNA (NanoDrop cannot measure picogram quantities). It is a stand-alone instrument that does not require a computer connection. Qubit requires a calibration consisting in the preparation of the appropriate standard solutions provided with a kit. It is suggested to carefully follow the manufacturer's instructions.

DNA should have a A260/A280 ratio >1.7

A260/A230 > 1.8, and yields >12 ng/μl.

It means that “good quality extracted DNA” must have: A260/A280= 1.8-1.9, and A260/A230= 1.9

DNA concentration and purification

DNA extraction from soil often yields low levels of DNA, compromising downstream analysis, such as next generation sequencing. Efficient recovery of DNA is thus vital for providing a DNA starting sample that can successfully maximise results. The goal of extraction is to lyse the cells, allowing the release of DNA, separating the DNA from other cellular components, and to purify the DNA for further analysis, eliminating compounds that can inhibit downstream analysis. If the DNA quality absorbance ratio resulted in a crude DNA extract that is not suitable for the further downstream analyses, we recommend carrying out a purification.

The Amicon Ultra 0.5 mL Centrifugal Filters (EMD Millipore Corporation, Billerica, MA) for DNA Purification and Concentration is the most efficient device used in the extraction process to remove potential PCR inhibitors and concentrate DNA, obtaining optimal downstream analysis results. The Amicon Ultra-0.5 device is supplied with two microcentrifuge tubes. The manufacturer's recommendations for purification and concentration of nucleic acids indicate that the 30K NMWL device is optimal for recovering nucleic acids. After extraction, the total amount of DNA can be added to the microcentrifuge tube provided by the kit. Following the manufacturer's instructions, place the filter device into the centrifuge rotor, and spin for 15' at room temperature, 7.500 g to recover the concentrated solute,



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invert the filter and concentrate the collection tube. Spin for 2' at 1.000 g to transfer the concentrated and purified sample from the device to the tube.

Dilution of DNA template

The crude DNA extracts must be diluted to minimise the inhibitors in Real Time quantitative PCR reaction, such as substances co-extracted with DNA and a potential inhibitor of the quantitative PCR assay. The extracted DNA is diluted to 10 ng μl^{-1} (in water DNase free) and stored for the downstream analysis.

Amplification and inhibition test

The DNA amplifiability test is important to ensure the best results in the further biological analyses. PCR analysis with appropriate bacteria 16S rDNA primers may be used to assess the quality of the genomic DNA extracted with the described procedure. The purity of DNA obtained after purification may be tested using up to 60 ng of DNA and its serial 10-fold dilution. Bacteria DNA is amplified using the primer 63f and 1087 r (as reported by Liu et al., 1997; Canfora et al., 2015; Canfora et al., 2017). PCR reactions are performed in 30- μl final volumes containing 3 μl 10-fold PCR buffer, 10 mM of dNTP mix, 1.3 mM of each primer, 50 mM of MgCl_2 , 0.2 U of hot start Taq DNA Polymerase, and five 10-fold dilution of 60 ng isolated DNA. The PCR conditions to use are as follows: starting denaturation at 95°C for 5 min, 34 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min, following final extension of 8 min at 72°C.

The reagents and components required are provided by the kit and should be carefully stored at room temperature, or, where necessary (as in the case of Qubit 2.0 kit standard) at 4°C. The DNeasy PowerSoil DNA Isolation Kit similar to the Qubit 2.0, does not require additional reagents.

Materials and equipment

The required equipment is as follows: centrifuge, pipettors from 10 to 1000 μl , filter tips, 2ml and 1.5ml Eppendorfs, vortex suitable for the homogenisation step, fluorimeter, spectrophotometer.

Calculations

DNA concentration is estimated automatically both by NanoDrop software and by Qubit 2.0, using the following formula:

Concentration ($\mu\text{g}/\text{ml}$) = (A260 reading – A320 reading) \times dilution factor \times 50 $\mu\text{g}/\text{ml}$.

Soil DNA extraction by DNeasy PowerMax Soil® DNA Isolation Kit (Qiagen) – large 10 g soil samples

Following step by step, the description of soil DNA extraction by **DNeasy PowerMax Soil® DNA Isolation Kit (Qiagen)** - (starting material: 10g of soil)



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3. Add 15 ml of PowerBead Solution to a PowerBead Tube, and after that add up to **10 grams of soil sample to PowerMax Bead Tubes. Gently vortex for 1'**. After the sample has been loaded into the PowerMax Bead Tube, begin the dissolution of humic acids, and proteic acid degradation. This is the first critical step to complement the following cell lysis step in which the sample disperses in the PowerMax Bead Solution).
4. Check Solution C1, pre-heated to 70°C for 5 min to dissolve the precipitate. **Add 1.2 ml of Solution C1 to step 1 and vigorously vortex to mix.** Solution C1 contains SDS, thus this is an important step in which the addition of Solution C1 allows the cell lysis to form a white precipitate in the Eppendorf within a few minutes of reaction.
3. **Vortex PowerMax Bead Tubes using a vortex or a flat-bed vortex pad with tape and vortex at maximum speed for 10 min.** *As an alternative, to optimise the lysis, we can place the tubes in a shaking bath set at 65°C and shake at maximum speed up to 40 min.*
4. **Centrifuge at room temperature for 5' minutes at 2500 x g.** The supernatant may still contain some particles. While centrifuging, **add 5 ml of Solution C2** into each clean tube. **Incubate at 4°C for 15 min** (the incubation can be extended to overnight).
5. **Remove the tube of Step '4' from the centrifuge and carefully transfer the supernatant to step '6'.** Discard the pellet.
6. **While centrifuging, aliquot 4 ml of Solution C3 into each clean tube. Add step'5' and incubate at 4°C for 15 min.**
7. **Centrifuge at room temperature for 4' minutes at 2500 x g.**
8. **After centrifugation, carefully remove the tube and transfer the entire volume into a clean tube, avoiding the pellet which must be discarded. Add 30 ml of Solution C4, and mix gently.** The pellet at this step contains additional non-DNA organic and non-organic material including humic acid, cell debris, and proteins. This step requires three independent centrifugations.
Take care to shake solution C4 before use; this is a high concentration salt solution allowing binding of DNA to the Spin filters provided by the kit.
9. **Load step '8' onto a Spin Filter and centrifuge at 2500 x g for 3 min at room temperature. Load the remaining supernatant onto the spin filter and centrifuge at 2500 x g for 3 min at room temperature (~ 3 loading).** At this step, DNA is selectively bound to the silica membrane in the spin filter device in high salt solution. Discard the flow through at the end of each centrifugation.
10. **Add 10 ml of Solution C5 to each tube of the spin tube. Apply centrifuge to the tube at room temperature for 5 min at 2500 x g**
11. **Carefully discard the flow through and repeat centrifugation for 5 min to avoid residual Solution C5.**
12. **Carefully transfer the spin filter to a clean tube and add 5 ml of Solution C6 (preheat to 60°C) to the centre of the white filter membrane of the spin filter.** (it is advisable to clean the outer part of the filter from any droplet left using clean paper. Solution C6 is a sterile elution buffer. Let C6 sit on the filter for 5 min at room temperature before the final centrifugation step.
13. **Centrifuge at room temperature for 3' minutes at 2500 x g. Discard the Spin Filter. The DNA is now ready for any downstream analysis.**



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14. **Keep DNA frozen** (-20°C to -80°C) for medium- to long-term storage. Shipping should take less than one week (see section 3.0.3 for storing and shipping DNA). Split the eluted DNA into three tubes. Tube 1 remains stored in your lab, tube 2 is for analysis of bacteria and tube 3 is for fungi.

Remarks

- Take care to use clean pipettors.
- Use appropriate Molecular grade reagent, as well as carrying out a good homogenisation to allow the cell lysis.
- It is advisable to avoid cross-contamination, so the extraction of DNA from the soil should be done in different places to the preparation of the PCR.
- Pipette carefully and filter tips to avoid contaminating the reagents.
- Check for the expected quantitative yield and quality of DNA before giving it for sequencing or qPCR (see Tables 3.1.1 and 3.1.2 at the end of this chapter).

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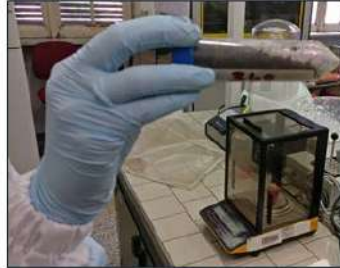
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Images on DNA extraction

1. Add 0.5 grams of soil sample to *PowerBead Tubes* and gently vortex for 30''



2. Check Solution C1, pre-heated to 70°C for 5' to dissolve precipitate. Add 60 μ l of Solution C1 to step 1 and briefly vortex to mix



3. Vortex PowerBead Tubes using a vortex or a flat-bed vortex pad with tape and vortex at maximum speed up to 10 min,



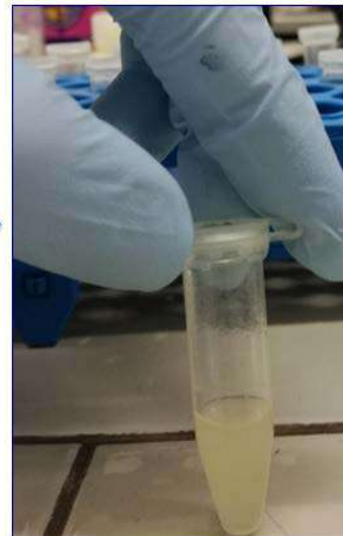
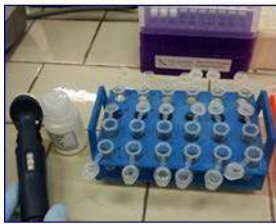
4. Centrifuge at room temperature for 2' minutes at 10000 x g.



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5. Remove tube of Step '4' from centrifuge and carefully transfer the supernatant (between 400 and 500 μ l) to step '5'. Add 250 μ l of Solution C2 into each clean tube. Incubate at 4°C for 15 min (it's possible extend the incubation to overnight).

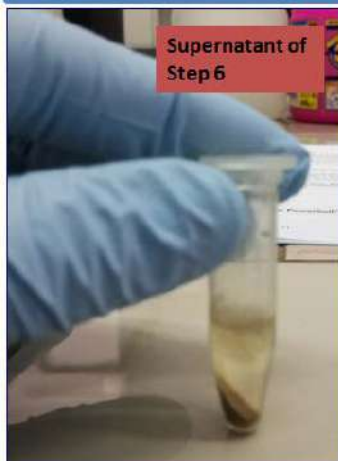


6. While centrifuging, aliquot 200 μ l of Solution C3 into each clean tube.



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7.. Discard the pellet. Add the C3. Incubate at 4°C for 15 min.



8. Centrifuge at room temperature for 2' minutes at 10000 x g.

Discard the pellet.

9. After centrifugation, carefully remove the tube and transfer the entire volume (up to 750 µl) in a clean tube, avoiding the pellet which must be discarded. Add 1.2 ml of Solution C4, and mix gently.



10. Load 675 µl of step '9' onto a Spin Filter and centrifuge at 10000 x g for 1' at room temperature. Load the remaining supernatant onto the spin filter and centrifuge at 10000 x g for 1' at room temperature (~ 3 loading).





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11. Add 500 μ l of Solution C5 to each tube of the spin tube. Apply centrifuge to the tube at room temperature for 5' minutes at 10000 x g.

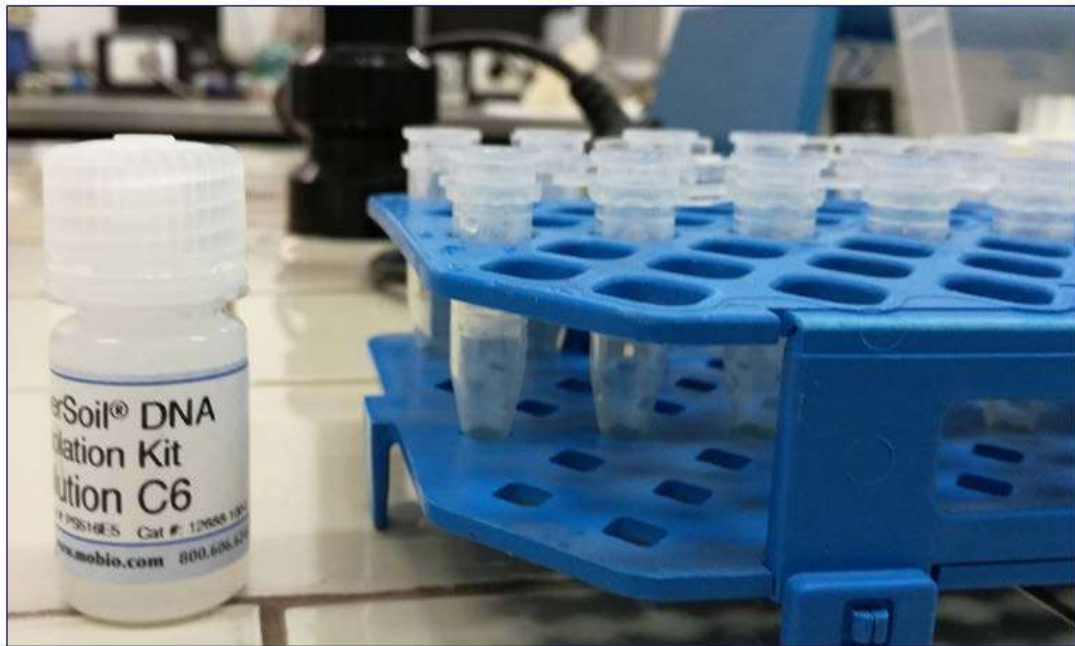


12. Add 500 μ l of Solution C5 to each tube of the spin tube. Apply centrifuge to the tube at room temperature for 5' minutes at 10000 x g.



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13. Carefully transfer the spin filter to a clean tube and add 100 μ l of Solution C6 (preheat to 60°C) to the centre of white filter membrane of spin filter.



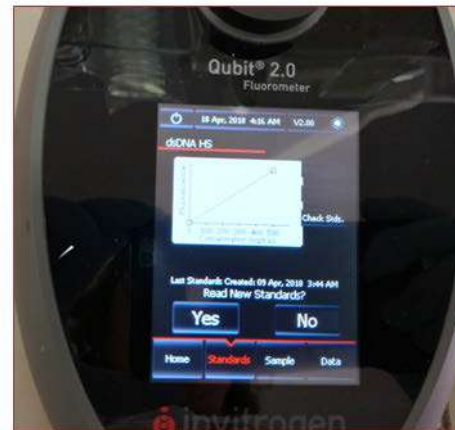
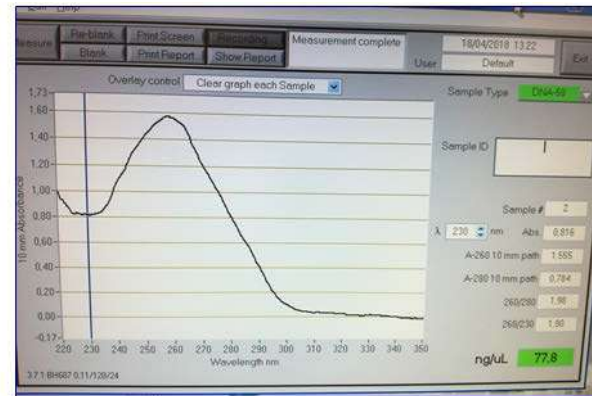
14. Centrifuge at room temperature for 3' minutes at 10000 x g. Discard the Spin Filter. The DNA is now ready for any downstream analysis.

Keep DNA



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DNA yield and quality check It is necessary to give reference numbers (ranges) for both quality and quantity





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Table 3.1.1 Major requirements for and expected outcome from DNA extraction

Subsequent DNA analyses	Technical replicates	Extracted DNA samples	Concentration		Initial volume	Final volume	A260/A280
			Ci ng/μl	C ng/μl	Vi μl	Vf μl per reaction	
NGS ILLUMINA - fungi	1	1	minimum 10 ng/μl	10	12.5	12.5	>1.8
NGS Ion Torrent - bacteria	1	1	1.5 ng/μl		18	18	>1.8
qPCR Functional genes	3	3	minimum 10 ng/μl	10	10	90	>1.8
qPCR Pathogens	3	3	minimum 10 ng/μl	10	3	27	>1.8
						total 147.5	

Table 3.1.2 Expected DNA yield from extraction

	extracted DNA Volume	yield Range ^b
250 mg soil ^a	100 μl	2 - 20 ng
10 g soil ^a	5 ml	50 - 400 ng

^a Extraction of different soil masses (250 mg vs. 10 g) requires the use of different extraction kits.

^b In the event of insufficient DNA yield and/or quality, extraction must be repeated, eventually by using the 10g-kit (DNeasy PowerMax Soil® DNA Isolation Kit, Qiagen) instead of the 250 mg-kit (DNeasy PowerSoil® DNA Isolation Kit, Qiagen).



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3. RNA extraction and reverse transcription

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Importance and applications

This chapter provides a protocol for total soil microbial RNA extraction. It is intended to be applied in investigations on the functional state of microbial communities. Changes in the relative abundance of soil microorganisms (i.e. of key functional guilds), through time, space or in response to stresses or treatments can be monitored and documented. The majority of taxa, in fact, tend to be inactive and respond only upon stimulus, or stress or change of state that induce them to react. While the detection of organisms' DNA indicates the presence of all species, active, inactive or dead, the isolation of their RNA indicates their viability and functioning. RNA, in fact, is a labile molecule that lasts in soil for very short periods of time. Diverfarming will use soil microbial RNA investigations in some case studies to gain a better understanding of the links between structure and function of microbial communities. To this end, ribosomal RNA will be used as the template in the analysis of metabolically active microbial communities. The protocol described here is based on a commercial kit but suggests modifications in the procedure based on the author's experience with different soil types.

Principle

The RNA (Ribo Nucleic Acid) extraction is the process by which RNA is separated from DNA and proteins. The RNA is converted to DNA with a Retro Transcription (RT reaction). RT is a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) by using total cellular RNA or poly(A) RNA, a reverse transcriptase enzyme, a primer, dNTPs and an RNase inhibitor. The resulting cDNA can be used for some downstream molecular biological applications, similarly to the DNA directly extracted from soil.

Reagents

The reagents and components required are provided by the kit and should be carefully stored at room temperature, or according to the manufacturer's instructions. The RNeasy PowerSoil Total RNA Kit as well as the Qubit 2.0, NanoDrop and SuperScript™ II Reverse Transcriptase, do not require additional reagents. Total RNA extraction requires CTAB and phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.5 – 8.0).

Materials and equipment



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The required equipment is as follows: centrifuge, pipettors from 10 to 1000 µl, vortex suitable for the homogenisation step, NanoDrop or spectrophotometer, fluorimeter.

Procedure

Soil RNA preparation: sample amount to process

Soil is sampled, pre-processed, stored and/or transported and shipped, respectively, following the common protocol (see chapter 3.0 and subsections). Also take note of the remarks at the end of this chapter. All this and a uniform RNA extraction procedure are important, since soil is a very complex environment, and the relative abundance of species in soil microbial communities can be biased by the yield and quality of extracted RNA. The **RNeasy PowerSoil Total RNA Kit** is designed to process up to 2.0 g of soil, however it can be scaled up easily to accommodate 2.5 g of soil.

Soil RNA extraction: use of commercial kits integrated with different adjustments

The RNeasy PowerSoil Total RNA Kit requires additional steps to be more efficient and be able to remove PCR inhibitors for the highest RNA yield. The procedure described here is an improvement on the manufacturer's protocol.

Important Recommendation: For the collection, transport and storage of soils needed for total RNA extraction, it is recommended to use the LifeGuard™ Soil Preservation Solution. This product puts microbial RNA in soil samples into stasis immediately upon contact, preserving gene expression profiles and microbial community structure information. This is an effective product to maintain RNA integrity and for isolating RNA starting with fresh soil. After adding the solution, RNA is maintained stabilised for up to one month at -20°C, 1 week at room temperature, or 2 weeks at 4°C.

1. Weigh 2.5 g of soil into the 15 ml RNase and DNase free Tube and add 6 ml of LifeGuard™ Soil Preservation Solution (1 g of soil requires 2.5 ml of solution).
2. Vortex or gently mix the soil and the solution by hand to obtain a mixture.
3. Store the soil in the LifeGuard™ Soil Preservation Solution following the manufacturer's instructions.
4. When you are ready for the total RNA extraction, samples can be centrifuged at 2500 x g for 5 min at 4°C to remove the solution and collect the soil.
5. Add up to 2.5 g of soil to the provided Bead Tube and resuspend it in 2.5 ml of Bead Solution followed by 0.25 ml of Solution SR1 and 0.8 ml of Solution SR2. After the addition of these reagents, the dissolution of soil begins, followed by cell lysis. SR1 is an SDS-based product, and aids the cell lysis, while SR2 is a precipitation reagent, removing non-DNA organic and inorganic material including proteins and extracellular materials.

*Solution SR1 contains SDS and requires heating at 60°C to dissolve the white precipitate.

6. *The following step is an additional step, so it is not included in the manufacturer's protocol.
7. Vortex at maximum speed for 5 min up to 10 min: this step is needed to ensure complete homogenisation and dissociation of nucleoprotein complexes. As an alternative to vortexing, shaking could be introduced using a horizontal shaker set at 25°C for 30 min, attaching the tubes horizontally to the platform.
8. Centrifuge at 2500 x g for 15 min and transfer the suspension obtained to a new tube. Discard the pellet.
9. *The following step is an additional step, so it is not included in the manufacturer's protocol. This step has been introduced to maximise the lysis and optimise the RNA yield. The CTAB reagent improves RNA extraction, giving consistently more RNA yield.



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10. Add 2 ml of CTAB 2% (W/V), followed by 3.5 ml of phenol:chloroform:isoamyl alcohol. Mix well to enhance the mixture. Remove the tube from the shaker and centrifuge the resulting mixture at 2500 x g for 15 min and transfer the aqueous phase containing RNA into a clean collection tube. Centrifugation separates the mixture into 3 phases: a red organic phase (containing proteins), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA). We strongly recommend this step to gain a more complete lysis. Phenol:chloroform:isoamyl alcohol is added to the CTAB to maximise lysing efficiency and yield.
11. Vortex at maximum speed for 15 min. Remove the tube from the vortex and centrifuge at 2500 x g for 10 min at 4°C.

Warning: Discard the phenol:chloroform:isoamyl alcohol in an appropriate waste container.

12. Remove the tube from the centrifuge and gently transfer the aqueous phase to a clean collection tube. Subsequently, add 2 ml of Solution SR3 to the aqueous phase and allow the sample to stand for 10 min at 4°C.

After adding SR3, a further precipitation occurs. It is needed to ensure complete dissociation of proteins and cell debris.

13. Centrifuge the resulting mixture at 2500 x g for 10 min at 4°C.
14. Transfer the supernatant in the new clean collection tube and take care to not disturb the pellet.
15. Add 5.5 ml of Solution SR4 and allow the sample to stand overnight at -20°C.
16. SR4 is isopropanol solution and allows the nucleic acid precipitation.
17. Centrifuge the resulting mixture at 2500 x g for 30 min at 4°C.
18. While centrifuging, aliquot 1 ml of Solution SR5 to the clean collection tube: take care to shake Solution SR5 to mix.

Remove the supernatant by a last centrifugation and resuspend the pellet by pipetting or vortexing to allow a better dispersion. Finally, prepare the **RNA Capture Column** for each sample.

19. Remove the cap of a new collection tube, placing the RNA Capture Column inside the 15 ml Collection Tube.

Prepare the RNA Capture Column, adding 2 ml of solution SR5 and allow it to gravity flow completely through the column.

20. Add the RNA isolation sample of step 14 to the **RNA Capture column** and allow it to gravity flow through the column.
21. After collecting flow through, wash the column adding 1 ml of solution SR5 and allow it to gravity flow through the column. During this step, the nucleic acids are bound to the column matrix, and the second washing with SR5 ensures the cleaning of unbound contaminants, avoiding the contamination of the eluted RNA.
22. Carefully transfer the RNA Capture Column to a new collection tube. Shake and add 1 ml of Solution SR6: in this step RNA is eluted through the column by gravity flow.
23. Carefully transfer the eluted RNA in a new **2.2 ml collection tube** and add 1 ml of solution SR4. Invert to mix and allow it to stand for 10 min at -20°C.
24. Centrifuge the resulting mixture at 13000 x g for 15 min at 4°C.
25. Discard the supernatant and air dry the pellet decanting the supernatant onto paper (by turning the opened tubes and keeping them upside-down on clean absorbing paper).
26. Resuspend the RNA pellet adding 100 µl of Solution SR7. This product is an RNase/DNase-free water without EDTA.

RNA yield and quality check

The quantification of RNA is essential to guarantee the suitability for downstream molecular biological applications such as PCR amplification, reverse transcription, and sequencing. The quality of RNA can vary depending on the extraction efficiency. RNA integrity and its concentration clearly affect all the



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downstream processes. There are several approaches used to evaluate RNA yield, purity and integrity. One main quantification approach is based on fluorescence or ultraviolet absorbance of RNA at a specific absorption peak at 260 nm. The intensity of the peak is proportional to the concentration of nucleic acid. The fluorescence method requires a fluorometer and an RNA-binding fluorescent dye that binds specifically to single-strand RNA. The intensity of fluorescence is directly proportional to the amount of binding RNA.

For the measurement of RNA concentration in extracts, the most frequently used and recommended devices are NanoDrop™, based on UV spectroscopy, or Qubit® 2.0 (both Thermo Fisher Scientific), based on fluorophores specifically binding ssRNA. Nanodrop is a low-cost effective, fast and easy instrument but accurate only for the quality, while it underestimates the quantity. Thus, the use of Nanodrop is recommended mainly for estimating the 260/280 ratio. NanoDrop is a spectrophotometer that uses two optical fibres installed in the pedestal, emitting light from a Xenon lamp, and a sample arm (spectrophotometer).

RNA quality is measured by reading the whole absorption spectrum (220-750 nm) with NanoDrop and calculating RNA concentration at both 260/280 and 260/230 nm. Nanodrop is more convenient but provides only an approximation of the RNA concentration.

The Qubit fluorometer measures the nucleic acid concentration indirectly, allowing to measure very small quantities of RNA (with NanoDrop it is impossible to measure picogram quantities). The stand-alone instrument does not require a computer connection. Qubit requires a calibration consisting in the preparation of the appropriate standard solutions provided by the kit (following the manufacturer's instructions). It is suggested to carefully follow the manufacturer's instructions. The QUBIT™ RNA HS Assay Kit enables an accurate quantification of RNA, allowing the evaluation of RNA up to very low quantities.

RNA concentration and purification

The RNeasy Mini kit (QIAGEN) allows the purification and the clean-up of total RNA. However, after the extraction it is also recommended to allow the immediate stabilisation of RNA. We also suggest the Amicon Ultra 0.5 mL Centrifugal Filters (EMD Millipore Corporation, Billerica, MA), usually used for DNA Purification and Concentration.

Follow the manufacturer's instructions of RNeasy Mini Kit.

Removal of genomic DNA

For the removal of genomic DNA contamination, RTS DNase kit uses DNase I max enzyme that efficiently removes DNA. It is based on a resin that binds to the enzyme forming a combined enzyme-resin complex without the need for EDTA. This enzyme-resin complex removes even very high DNA contaminations within 20 min. Refer to the manufacturer's instructions when using the suggested kit.

RNA reverse transcription in cDNA

RT-PCR is performed using **SuperScript™ II Reverse Transcriptase** according to the manufacturer's instructions. Reactions are performed as reported in the following:



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- First-strand cDNA synthesis in a final volume of 20 μL :

Oligo(dT)12-18 (500 $\mu\text{g}/\text{mL}$) or 50–250 ng random primers or 2 pmole gene-specific primer (GSP)	1 μL
1 ng to 5 μg total RNA or 1–500 ng of mRNA	x μL
dNTP Mix (10 mM each)	1 μL
RNase free Water, molecular grade	up to 12 μL

- Reverse transcription is carried out at **65°C** for **5 min**. At the end of incubation, allow it to stand immediately at -20°C or on ice. Briefly centrifuge for 1 min at 1000 \times g and proceed to add the following:

5 \times First-Strand Buffer	4 μL
0.1 M DTT	2 μL
RNaseOUT™ (40 units/ μL) (optional) ^a	1 μL

^a Use *RNaseOUT™ only in the case of <50 ng RNA.

- Mix gently and allow to stand at **42°C** for **2 min** (if you are using **oligo(dT)12-18 or GSP**) or at **25°C** for **2 min** in the case of **random primers**.
- Add **1 μL (200 U)** of **SuperScript™ II RT**, gently mix by pipetting according to the manufacturer's protocol.
- Incubate at **25°C** for **10 min**, if using **random primers**, or at **42°C** for **50 min**
- Inactivate the reaction by incubating at **70°C** for **15 min**.

The cDNA is now ready for downstream molecular biological applications. To remove RNA complementary to the cDNA, add 1 μL of RNase H heating at 37°C for 20 min.

Refer to chapter 3.1 'DNA extraction', for cDNA estimation of concentration.

Calculations

RNA concentration is estimated automatically both by NanoDrop software and by Qubit 2.0, using the following equation:

$$\text{Concentration } (\mu\text{g}/\text{ml}) = (\text{A}_{260} \text{ reading} - \text{A}_{320} \text{ reading}) \times \text{dilution factor} \times 40 \mu\text{g}/\text{ml}.$$

Remarks

The RNA is an unstable molecule and it is well known that its crude extract has a very short lifetime. Extraction protocols thus requires careful handling to ensure a successful nucleic acid purification. According to what has been mentioned, it is strongly recommended to use a lab cleaner product to avoid RNase contamination.

To protect from the decay of RNA, it is recommended to use **LifeGuard™ Soil Preservation Solution** for the collection and transportation of soil samples. This product is a specially formulated proprietary fluid developed to protect RNA from degradation. It puts microbial RNA in soil samples into stasis



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immediately upon contact, preserving gene expression profiles and microbial community structure information. After adding the solution, soil microorganisms are maintained in stasis, and are immediately stabilised. According to the manufacturer's instructions, it is recommended to store nucleic acids for up to 30 days at -20°C, 1 week at 4°C, or 3 days at room temperature.

Wear RNase-free gloves and clean the laboratory work area using a cleaning product specific for RNase removal.

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4. Determination of selected microbial, functional genes by qPCR from soil extracted DNA

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Importance and applications

The method described here is largely based on the ISO standard 17601 (ISO, 2016). It is the procedure used to set up and perform quantitative PCR (qPCR) to quantify the abundance of functional groups from soil extracted DNA. The quantification of functional groups by qPCR assays can be successfully used to determine not only potential enzyme activities (compare chapter 3.8) but also to determine the actual activity status from the quantity of relevant genes. In Diverfarming the *amoA*, *nirK* and *narG* gene of the nitrogen cycle will be investigated. The *amoA* gene, encoding the α -subunit of the AMO enzyme, is widely used to investigate nitrification (Levy-Booth et al., 2014; Schauss et al., 2009). It is suitable as a marker gene for molecular studies of AOA and of AOB communities, due to its strongly conserved nucleotide sequence and due to the essential role of *amoA* in the energy generating metabolism (Norton et al., 2002). The *narG* gene (and the *napA* gene) is typically determined in studies on NO_3^- reduction, while nitrite conversion to NO or N_2O by nitrite reductase is well represented by the *nirK* gene (Cu-containing) and *nirS* gene (cytochrome cd1) (Levy-Booth et al., 2014).

Sample preparation and storage

Soils are sampled, transported, pre-processed, stored and shipped as described in chapter 3.0 and subsections therein. The DNA extraction is done following the method in chapter 3.1. For convenience, before starting PCR and qPCR dilute template DNA to 10 ng/ μl and 1 ng/ μl .

Principle

The objective of the method is to determine the abundance of selected microbial gene sequences from soil DNA extract. The method comprises four tasks and eight steps (see below).

Task 1 qPCR standard preparation and calibration of the qPCR assay



Step 1: Primers for qPCR – Step 2: qPCR standard preparation – Step 3: Calibration of the qPCR

Task 2 Preparation of soil DNA template and inhibition test

Step 4: Preparation of soil DNA – Step 5: Inhibition test

Task 3 qPCR assay

Step 6: qPCR assay

Task 4 Validation and analysis of the qPCR assay

Step 7: Checking qPCR efficiency and dissociation curves – Step 8: Calculation of the copy number of the gene of interest in the soil DNA extract

It is necessary to validate three critical steps for each qPCR assay, i.e. calibration of qPCR assay (step 3), validation of quality of DNA extracts for qPCR assay (step 5) and validation of the qPCR assay (step 7). This is in accordance with the guidelines on “Minimum Information for Publication of Quantitative Real-Time qPCR Experiments” (MIQE; Bustin et al., 2009)

The standard describes SYBR Green® (Molecular Probes, Eugene, Oregon, USA) qPCR assay which has been validated by an international ring test (ISO, 2016). However, other dsDNA intercalating fluorescence dyes and TaqMan® (Roche Molecular Systems, Inc., Pleasanton, CA, USA) qPCR assays can also be used.

A general scheme of the workflow is shown in Fig. 3.3.1 at the end of this chapter.

Reagents

All required reagents should be freshly prepared.

- A) Soil DNA is extracted following the method described in chapter 3.1 of this Handbook.
- B) DNA ladder with known lengths and concentrations of fragments.
- C) Oligonucleotides used as PCR primers purified with standard desalting (vendor not specified).
- D) Competent bacteria: *Escherichia coli* strain, usually used for cloning of PCR product.
- E) Plasmid: Cloning vector replicating in *E. coli* containing annealing sites for SP6 and T7 primers flanking the cloning site. Commercially-available cloning kits can also be used if they meet the requirements described here.
- F) Enzyme: T4 DNA ligase. Not required if a commercial cloning kit is used.
- G) Taq polymerase (and a commercial qPCR kit if used).
- H) T4 gene T32.
- I) Bovine serum albumin (CAS No. 9048-46-8).
- J) Ampicilline sodium, $C_{16}H_{18}N_3NaO_4S$ (CAS No. 69-52-3). (Alternative: Kanamycin sulphate, $C_{18}H_{36}N_4O_{11} \times H_2SO_4$ (CAS No. 25389-94-0)).



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- K) Boric acid, BH_3O_3 (CAS No. 10043-35-3).
- L) Deoxynucleotide solution, dNTPs.
- M) Ethidium bromide (CAS No. 1239-45-8). **Note: Ethidium bromide is a highly toxic chemical. Although listed in the ISO standard, its use is not recommended, but should be replaced by less harmless alternatives such as SYBR Green®!**
- N) Ethylenediaminetetraacetic acid disodium salt (EDTA), $\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_8\text{Na}_2 \cdot x \cdot 2 \text{H}_2\text{O}$ (CAS No. 6381-92 6).
- O) Glucose, $\text{C}_6\text{H}_{12}\text{O}_6$ (CAS No. 50-99-7).
- P) Hydrochloric acid, HCl (CAS No. 7647-01-0).
- Q) IPTG, isopropyl-beta-D-thiogalactopyranoside (CAS No. 367-93-1).
- R) Magnesium chloride, MgCl_2 (CAS No. 7786-30-3).
- S) Magnesium sulphate, MgSO_4 (CAS No. 7487-88-9).
- T) Molecular-biology-grade water, H_2O .
- U) Potassium chloride, KCl (CAS No. 7447-40-7).
- V) Sodium chloride, NaCl (CAS No. 7647-14-5).
- W) Tris[hydroxymethyl]aminomethane, $\text{C}_4\text{H}_{11}\text{NO}_3$ (CAS No. 77-86-1).
- X) X-Gal, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (CAS No. 7240-90-6).
- Y) 5.6 Product for bacterial culture medium: 5.6.1 Bacto™ tryptone, enzymatic digest of casein.
- Z) 5.6.2 Yeast extract powder (CAS No. 8013-01-2).
- AA) Ampicilline solution: 2 g of ampicilline sodium in 4 ml of 0.22 μm filter sterilised H_2O . Adjust to 20 ml with sterilised H_2O , prepare 1 ml aliquots and store at -20°C .
- BB) EDTA, 0.5 mol/l, 186.10 g of EDTA in 1 000 ml of H_2O , adjusting with NaOH (10 mol/l) to pH 8.0.
- CC) SYBR Green®.
- DD) IPTG stock solution: 1 g of IPTG in 8 ml of H_2O . After careful mixing, the solution is adjusted to 10 ml and sterilised under security microbiology post. Prepare 1 ml aliquot of IPTG and store at -20°C .
- EE) Solid LB medium, 10 g of bacto™ tryptone, 5 g of yeast extract, 5 g of sodium chloride, 15 g of agar, in 1 000 ml of H_2O . After autoclaving for 20 min at 120°C , 1 ml of ampicilline stock solution at 100 $\text{mg} \cdot \text{ml}^{-1}$ is added to LB medium and plated in Petri dishes (20 ml) under a security microbiology post. 100 μl of IPTG solution is plated on solid LB-amp medium. When the IPTG solution is entered in LB-ampicilline medium, 20 μl of X-Gal solution is plated on solid LB-amp medium. Solid LB medium is stored at 4°C until its use.
- FF) SOC medium, 20 g of bacto™ tryptone, 5 g of yeast extract, 0.58 g of NaCl, 0.95 g of MgCl_2 , 2.46 g of MgSO_4 , 3.60 g of glucose in 1 l H_2O . Sterilise by 20 min autoclaving at 120°C . Prepare 950 ml aliquots and store at -20°C .
- GG) Tris-HCl, 1 mol/l, 121.14 g of tris in 1 000 ml of H_2O , adjusting with 4 mol/l HCl to pH 8.0.
- HH) TBE buffer $\times 10$, pH 8.0, 108 g of tris base, 55 g of boric acid, 40 ml of 0.5 mol/l EDTA (pH 8.0) in 1 000 ml of H_2O .



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- II) TBE buffer × 1: 100 ml of TBE buffer × 10 in 900 ml of H₂O.
- JJ) TE buffer × 10: pH 8.0, 100 ml of 1 mol/l Tris-HCl pH 8.0, 20 ml of 50 mmol/l EDTA pH 8.0 in 880 ml of molecular grade water.
- KK) TE buffer × 1, 100 ml of TE buffer × 10 in 900 ml of H₂O.
- LL) X-gal solution, 250 mg of X-Gal in 5 ml of dimethylformamide 5 ml. After careful mixing, prepare 0.5 ml aliquot and store at -20°C.

Materials and equipment

Pipettes, pipette tips, appropriate PE test tubes, pH-meter, scales, incubator (with agitation), autoclave, centrifuge, fume hood cabinet, laminar flow cabinet, horizontal electrophoresis system, quantitative PCR, allowing the real time quantification of amplicons from various DNA templates with detection limit of one copy of a sequence target per sample analysed. Fluorometer able to quantify double-strand DNA or spectrophotometer (not recommended), able to quantify double-strand DNA at 260 nm.

Procedure

qPCR standard preparation and calibration of qPCR assay (task 1)

In the SYBR Green® qPCR assay amplicons are quantified at the end of each PCR cycle. This is done with SYBR Green® that fluoresces when intercalated in the double helix of the amplicon. The purpose of this task is to describe the definition of the appropriate amplicon to settle down a qPCR assay (step one), the preparation of qPCR standard (step two) and the calibration of the qPCR assay (step three).

Primers for qPCR (task1, step 1)

It was agreed that three functional genes are to be investigated within the Diverfarming project, i.e. *amoA*, *nirK* and *narG*. Suitable primer pairs as reported by the literature are listed in Table 3.3.1. General information on the amplicon design and the main parameters to be considered to design oligonucleotide primer pairs is given in the ISO standard 17601 (ISO, 2016).



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Table 3.3.1. Primers for amplicons of *amoA*, *nirK*, *narG*, and cloning site.

Amplicon	Forward	reverse	Reference
<i>amoA</i>	amoA-1F GGGGTTTCTACTGGTGG T	amoA-2R CCCCTCKGSAAAGCCTTCTC	Okano et al., (2004)
<i>nirK</i>	nirK876 ATYGGCGGVCAYGGCGA	nirK1040 GCCTCGATCAGRTTTRTG GTT	Henry et al., (2004)
<i>narG</i>	narG1960m2F TAYGTSGGGCAGGARAA ACTG	narG2050m2R CGTAGAAGAAGCTGGTG CTGTT	Lopez-Gutierrez et al., (2004)
Cloning site	SP6 ATTTAGGTGACACTATAG	T7 TAATACGACTCACTATAG GG	

Two different approaches can be used for qPCR standard preparation and calibration in order to quantify functional genes. See A) and B) in the following.

A) qPCR standard preparation (task 1, step 2)

Suitable standards must be used for qPCR. For *amoA*, Schauss et al. (2009) used a serial dilution of the fosmid clone 54d9 as standard that was previously described by Leininger et al. (2006).

The procedure used to generate qPCR standards targeting a sequence of the microbial gene of interest (GOI) from different DNA templates (pure bacterial or fungal isolate, environmental DNA or artificial DNA) is described in step 2 of task 1. It also reports the procedure used to insert the qPCR standard in a cloning vector, transform *Escherichia coli* and purify recombinant plasmids harbouring qPCR standard for further use for qPCR assays. The quality of the DNA template used for amplifying the qPCR standard by PCR shall be verified by electrophoresis on 1% agarose gel (AGE) in TBE buffer stained with appropriate staining (e.g. SYBR Green®). The concentration of DNA is measured with fluorometer, comparing to known DNA standard in AGE, or spectrophotometer. The DNA template is diluted to 10 ng/μl in a final volume of 20 μl and stored at -20°C.

The amplification reaction using the specific primer pair (Table 3.3.1) is carried out in a final 25 μl volume containing 2.5 μl of 10 × Taq polymerase buffer, 200 μmol/l of each dNTP, 1.5 mmol/l of MgCl₂, 0.5 μmol/l of each primer and 0.625 U of Taq polymerase. A volume of 2.5 μl of DNA (e.g. 2.5 or 25 ng of DNA) is used as template for the PCR reactions. Commercially-available PCR kits can also be used as described by the manufacturer. The PCR thermocycler cycling programs for different amplicons are as follows:

amoA

Initial denaturation: 95°C, 10 min



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39x 95°C 15 s, 58°C 30 s, 72°C 45 s

1x 95°C 15 s, 58°C 30 s, 72°C 45 s

Final extension: 72°C, 10 min

Expected PCR product length 491 bp

nirK

Initial denaturation: 95°C, 10 min

6x 95°C 15 s, 63 to 58°C 30 s (-1°C by cycle), 72°C 30 s, 80°C 15 s

40x 95°C 15 s, 60°C 30 s, 72°C 30 s, 80°C 15 s

Final extension: 72°C, 10 min

Expected PCR product length 165 bp

narG

Initial denaturation: 95°C, 10 min

6x 95°C 15 s, 65 to 60°C 30 s (-1°C by cycle), 72°C 30 s, 80°C 15 s

40x 95°C 15 s, 60°C 30 s, 72°C 30 s, 80°C 15 s

Final extension: 72°C, 10 min

Expected PCR product length 110 bp

Cloning site

Initial denaturation: 95°C, 10 min

40x 95°C 15 s, 65°C 30 s, 72°C 90 s

Final extension: 72°C, 10 min

The expected PCR product length for the cloning site depends on the plasmid used for cloning. For example, for pCRII and pGem-T the expected lengths of PCR product of insert free (self-ligated) plasmids are 187 bp and 225 bp, respectively.

The expected length of the amplicon is verified by electrophoresis on 2 % agarose gel in TBE buffer stained with appropriate staining. Amplicons are purified either from the gel using appropriate methods or by using exclusion chromatography columns to remove primers. Purified amplicons are then quantified as described earlier in this chapter.

Cloning and dilution preparation of qPCR standard

Ligation of amplicon of qPCR standard.

An optimal ligation of amplicon into the cloning vector should be done at a 3:1 molar ratio of the mass of PCR product to be used for ligation:



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$$\text{Mass of PCR product (ng)} = \frac{\text{mass of plasmid DNA (ng)} \times \text{length of the insert (bp)}}{\text{length of plasmid (bp)}} \times 3$$

The ligation reaction is made of the required mass of purified amplicon, 50 ng of plasmid DNA, 5 µl of 2 × ligation buffer, 3 U of T4 DNA ligase and molecular grade water to reach a final volume of 10 µl. The vector is provided with the cloning kit, for which two options are suggested: (1) pCRII (Invitrogen) or pGEM.-T easy (Promega). The ligation reaction is incubated overnight at 4°C or for adequate T4 DNA ligase, one hour at ambient temperature. A commercially-available PCR product cloning kit containing annealing sites for SP6 and T7 primers flanking the cloning site can also be used as described by the manufacturer.

The efficiency of the ligation reaction can be verified by electrophoresis by loading 1 µl ligated plasmid and open plasmid (i.e. 5 ng of plasmid) on 1 % agarose gel in TBE buffer stained with appropriate staining. Ligated plasmid due to its super-coiled structure migrates faster in AGE than a linearised one.

Transformation of competent *Escherichia coli*

Thaw competent cells (10⁸ cfu/µg of DNA) on ice. Add 1 µl of the ligation reaction mixture to cells, mix smoothly (not pipetting up and down) and incubate on ice for 20 min. Heat shock cells by incubating at 42°C for 30 s and immediately place cells on ice and incubate for an additional 2 min. Add 950 µl of SOC medium and incubate at 37°C under agitation at 150 min⁻¹ for one hour. Plate 100 µl aliquots of cells suspension onto LB/Amp/IPTG/X-Gal solid medium. Petri dishes are incubated at 37°C overnight in the dark.

Screening for recombinant clone

Incubate plates at 4°C for several hours to enhance colouration of bacterial colonies. White (containing inserted PCR product) and few blue (self-ligated) colonies are picked and streaked onto LB/Amp/IPTG/X-Gal solid medium and incubated overnight at 37°C. Several white and few blue colonies are picked and placed in 100 µl molecular grade water. PCR is carried out using SP6 and T7 primers listed in table 3.3.1 with the parameter described in **task 1 step 2** by using 2.5 µl of bacterial suspension as template for the PCR reactions to confirm the presence of the insert in the recombinant clone. The size of the expected qPCR amplicon is verified by electrophoresis on 2 % agarose gel in TBE buffer stained with appropriate staining.

Purification and quantitation of plasmid

Recombinant and one self-ligated clones, confirmed by PCR and AGE, are inoculated to 10 ml LB/Amp liquid medium and incubated at 37°C under agitation (150 min⁻¹) overnight. Plasmid is purified from 2 ml cell suspension using conventional mini-preparation. Plasmid DNA is quantified by fluorometer or by comparing to the known DNA standard in AGE or by spectrophotometry. Store purified plasmids at -20°C or -80°C until used.



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The concentration of undiluted plasmid is measured exactly before diluting by fluorometer or comparing the known DNA standard in AGE. Do not use the spectrophotometer at this point. Plasmid copy number can be facilitated by using an online calculator such as oligo calc (<http://www.basic.northwestern.edu/biotools/oligocalc.html>) or using the following formula:

$$\text{copies} / \mu\text{l} = \frac{6.022 \times 10^{23} \left(\frac{\text{molecules}}{\text{mole}} \right) \times \text{DNA concentration} \left(\frac{\text{g}}{\mu\text{l}} \right)}{\text{Number of bases} \times 660 \text{ daltons}}$$

where 6.022×10^{23} (molecules/mole) is Avogadro's number and 660 Da is the average weight of a single base pair.

Prepare an initial solution (100 μl) containing 0.5×10^8 copies / μl in molecular grade water. Prepare tenfold serial dilutions until a concentration of 0.5×10^1 copies / μl is reached. Store dilutions at -80°C until used.

Calibration of the qPCR (task 1, step 3)

SYBR Green® qPCR assay

The qPCR calibration assay is performed on serial dilution of the linearised standard plasmid ranging from 10^8 to 10^1 copies per reaction. GOI is amplified by using the specific primers pair listed in Table 3.3.1. The amplification is carried out in a final 15 μl volume containing 2 μl of dedicated plasmid standard, 1 $\mu\text{mol/l}$ of each primer, 7,5 μl of 2 \times Taq master mix or 1,5 μl of 10 \times Taq master mix containing SYBR Green®, dNTPs, MgCl_2 and Taq polymerase. Molecular grade water is added to reach the final reaction volume. qPCR reaction is performed in a real-time thermocycler according to the cycling program described in **qPCR standard preparation** for the amplicon of interest. The final extension in the program is replaced by a dissociation stage (melt analysis). The fluorescence is collected at the end each cycle and in melt analysis where the temperature is gradually raised from 60°C to 95°C . If a commercial qPCR-reaction kit is used, follow the manufacturer's instructions to set up reactions. qPCR calibration is performed in duplicate and two non-template controls (NTC) are also included.

Establishment of the calibration curve and calculation of qPCR efficiency

At the end of the assay, the results are analysed using the automatic option. qPCR is validated with four observations. They are: (1) no amplification in NTC reactions, (2) a single dissociation peak for each dilution of qPCR standard (3) a single qPCR product of correct size in AGE and (4) a linear calibration curve (standard curve) with r^2 equal or superior to 98 %. The qPCR calibration curve gives the number of cycle threshold (Ct) as a function of the amount of the log of the number of copy of standard sequences. For example, in linear regression $y = -3.3386 + 39.574 \text{ Ct}$ at copy number 1 represents a Ct value of 39.574 and is the end point forming a line with slope -3.3386 when Ct versus the log standard



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copy number is plotted. The r^2 is the percentage of the data which matches the hypothesis that the given standards form a standard curve.

Ct can also be represented as an equation $Ct = axq + c$ where,

q is the copy number of qPCR standard;

a is the slope of the calibration curve;

c is the ordinate at the origin (Ct for 1 copy of qPCR standard);

The efficiency (E) of the qPCR assay is estimated in equation $E = 10^{(-1/\alpha)} - 1$ where,

α is the slope of the calibration curve.

For quick reference a calibration slope -3.32 is equal to 100 % efficiency. A 100 % efficient qPCR reaction in 2-fold- or a 10-fold-dilution of a given DNA template gives a Ct difference of 1 or 3.3, respectively.

B) Alternative qPCR standard preparation and calibration of qPCR assay (task 1)

A bulk soil sample (samples involved in the experiment) is used to extract a DNA mixture and amplify the target gene. In this way, the calibration is performed starting by the amplification of a known amount of DNA. This amplicon is then used to construct the standard curve.

- DNA of a soil mixture (different sub-samples representative of each experimental thesis) is extracted following the method described in chapter 3.1 of this Handbook. The quality of the DNA template used for amplifying the qPCR standard by PCR shall be verified by electrophoresis on 1% agarose gel (AGE) in TBE buffer stained with an appropriate staining (e.g. SYBR Green®; see Note). The concentration of DNA is measured with a fluorometer, comparing to the known DNA standard in AGE, or with a spectrophotometer.
- The extracted DNA is diluted to $10 \text{ ng } \mu\text{l}^{-1}$ in a final volume of $100 \mu\text{l}$ and stored at -20°C .
- DNA is amplified in duplicate for each target gene, following the SYBR green chemistry protocol. The amplification is carried out in a final volume of $25 \mu\text{l}$ volume containing $10 \mu\text{l}$ of starting DNA, $12.5 \mu\text{l}$ of SYBR Green®, $1.2 \mu\text{M}$ of each primer, and PCR-grade water up to $15 \mu\text{l}$.
- The amplified product is then purified after qPCR reaction, using a commercially-available kit. During this step, qPCR amplicon is validated following that reported here in **'Establishment of the calibration curve and calculation of qPCR efficiency- points 1, 2, 3**. If two PCR products are observed, a first purification by AGE shall be carried out to cut and purify the expected PCR product. A second purification shall be done to obtain a suitable amplicon for qPCR with a good quality.



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- The concentration is measured with a fluorometer and the amplicon can be used to calculate the target gene of interest (GOI) copy number, following that described in '**Purification and linearization of plasmid**'.
- Prepare an initial dilution (100 μ l) a known amount of copies in molecular grade water (10^8) and prepare a 10-fold dilution series, covering until 10^1 dilution.

Preparation of soil DNA template and inhibition test (task 2)

Preparation of soil DNA (task 2, step 4)

As described in **Sample preparation and storage** soil DNA used for qPCR is diluted to 10 ng/ μ l and 1 ng/ μ l. The Diverfarming project applies considerably to qPCR and other analyses based on soil DNA. It is therefore practical in the long run to target the amount of template DNA in qPCR for the lowest practical concentration, such as 10^2 - 10^4 copies of GOI per reaction, as the project continues. In theory, qPCR does not have a detection limit as a single copy can be detected. In practice, 10 copies per reaction can be considered as the lowest reliable detectable concentration.

Inhibition test (task 2, step 5)

Inhibition in qPCR occurs when components used in qPCR hinder the activity of the (Taq) polymerase. Such a component is the intercalating fluorescent dye SYBR Green® itself used in qPCR. However, inhibition in qPCR usually refers to impurities, such as humic acid substances, co-purified with sample nucleic acids. These impurities may have an impact on PCR efficiency, thus delaying the amplification and therefore decreasing the samples copy number in absolute quantification. To follow MIQE guidelines, the inhibition should be tested systematically at the beginning of each qPCR work. Two inhibition tests are described below.

Spiking of exogenic DNA in soil DNA extract

The presence of inhibitors in sample DNA can be quantified by spiking a known amount of exogenic DNA to sample DNA. The protocol below describes usage of purified self-ligated plasmid detected in **Screening for recombinant clone**. This plasmid contains annealing sites for primers T7 and SP6 but no inserted DNA and serves as exogenic DNA. Prepare duplicate reactions as described in **SYBR Green® qPCR assay** and spike approximately 10^4 copies of exogenic DNA per reaction. Add the intended dilutions (mass in ng) of each tested soil DNA as the template. A good starting point is to use a 1 ng/ μ l template DNA concentration. Also prepare reference reactions containing only the self-ligated plasmid and reactions without any template DNA (NTC) in duplicate. Perform the qPCR reaction described for primers T7 and SP6 with 30 s extension time and dissociation stage. Analyse the results using manual Ct settings.



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The inhibition test is validated by observing:

- a) no amplification for NTC,
- b) similar Ct values in qPCR performed from spiked soil DNA extract and plasmid only DNA.

Soil DNA dilution showing no inhibition is chosen as the template to perform the qPCR assay. If a full (no amplicon) or partial inhibition (delayed Ct value) is observed, then the soil DNA extract should be further diluted to remove the inhibition effect and submitted again to a new inhibition test. If this does not remove the inhibition issue the DNA extracts must be further purified as recommended in ISO 11063 and again submitted to the inhibition test. When acceptable results are obtained from the inhibition test, soil DNA samples can be used to run the qPCR assay.

In addition to sample dilution inhibition, caused by soil DNA, in qPCR carriers such as bovine serum albumin (BSA; 400 ng/μl) or T4 gene 32 protein (30 ng/μl) can be removed. Moreover, different (commercial) qPCR chemistries have different inhibition tolerance.

Dilution of DNA template

A dilution test can be performed to moderate the copy number samples (e.g. 10^4 copies/reaction). Dilute the DNA sample in 1:10 intervals (10 ng/μl, 1 ng/μl, 0.1 ng/μl). Include dedicated standard plasmid into the run with different dilutions. Set reactions up as described in **SYBR Green® qPCR assay** for GOI in duplicate. Perform the qPCR reaction described for dedicated primers and include the dissociation stage. Analyse the qPCR using the automatic option. The inhibition test is validated by observing:

- a) no amplicon in NTC control,
- b) for each test sample test, the Ct value difference should be the same as the Ct value difference of diluted standard plasmid for similar PCR efficiency.

If inhibition occurs, the samples should be treated as in **Spiking of exogenic DNA in soil DNA extract**.

qPCR assay (task 3, step 6)

Assay targets gene of interest (GOI) and is performed in duplicate on each template at the dilution showing no inhibition of Taq polymerase and on duplicate on plasmid standard DNA dilutions from 10^8 to 10 copies per reaction. Include NTC in duplicate made of molecular grade water. Primer pair (Table 3.3.1) specific for GOI is used as previously described in **SYBR Green® qPCR assay**. Once a calibration curve is established, the calibration curve can be imported from a previous run and be adjusted by using one reference standard concentration close to sample concentrations.



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Validation of qPCR assay (task 4, step 7)

At the end of the qPCR reaction, the results are analysed using the automatic option. For a validated assay the following requirements must be met:

- a) no amplification for NTC,
- b) a linear calibration curve with r^2 equal or superior to 98 %, and
- c) a dissociation curve showing a single peak at the expected melting temperature specific for each GOI. Due to the heterogeneity of degenerated primers and amplicons the dissociation curve can be smooth. In long amplicons multiple peaks may occur. In the event of anomalies in the dissociation curve an AGE is recommended for an additional validation point.

Calculations (task 4, step 8)

The calibration curve and qPCR efficiency shall be calculated for each assay and recorded with the estimated number of copies of the GOI. The copy number of GOI can be calculated to the copy number per ng of soil DNA or per g of soil with the following formulas:

Estimation of the number of sequences of the GOI per ng of soil DNA (I)

$$I = \frac{\text{GOI in assay}}{\text{volume of template in assay } (\mu\text{l}) * \text{concentration of template in assay } (\frac{\text{ng}}{\mu\text{l}})}$$

Estimation of the number of sequences of the GOI per g of soil (II)

$$\text{II (dry mass equivalent)} = \frac{I * \text{DNA extracted from soil (in ng)}}{\text{soil sample which DNA is extracted (in g of dry mass equivalent)}}$$

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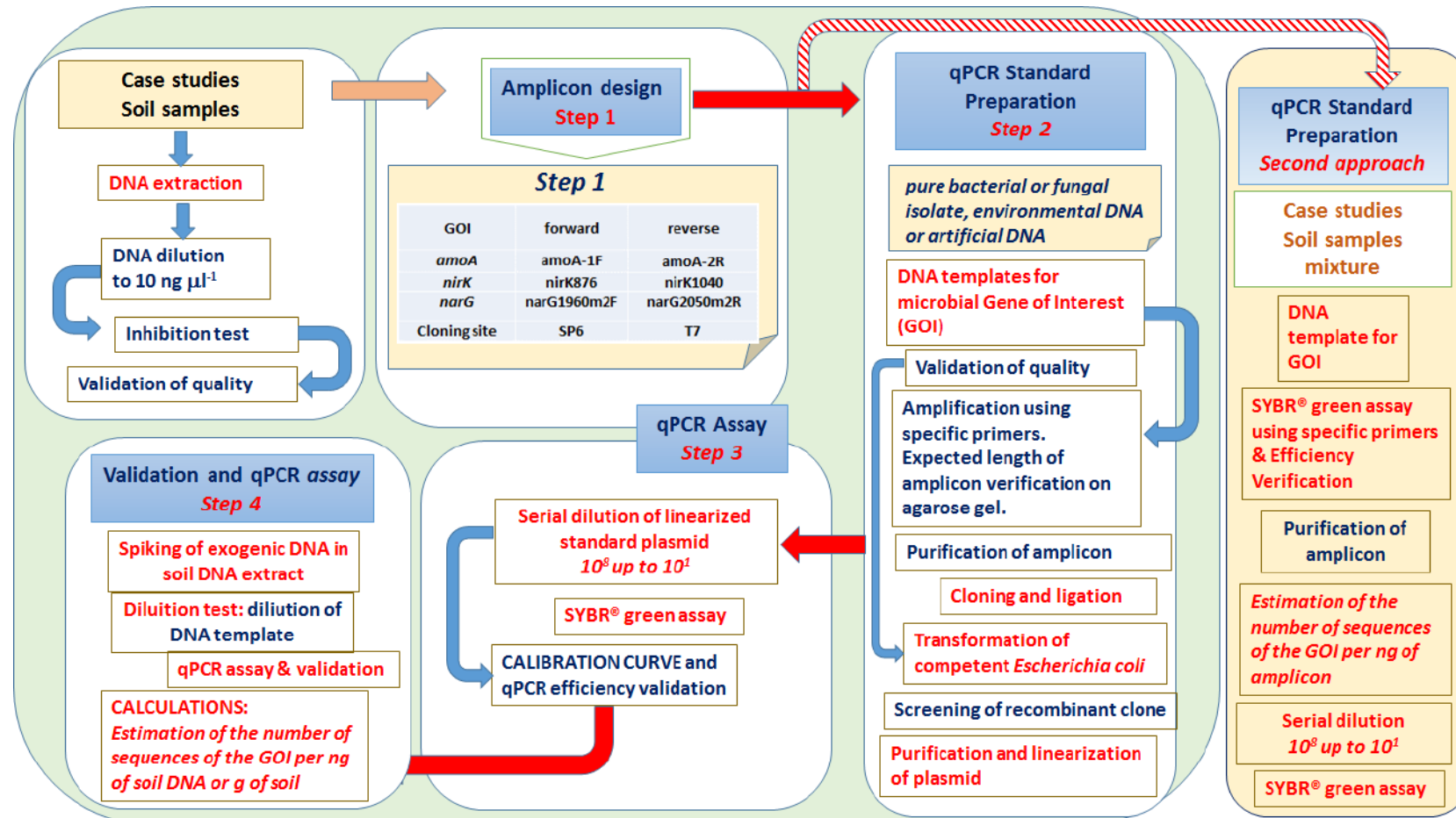


Fig. 3.3.1 General workflow of the procedure for the determination of microbial, functional genes by qPCR from soil extracted DNA



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5. Detection and quantification of soil borne diseases by qPCR

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Importance and applications

Soil-borne plant pathogens (bacteria, fungi and oomycetes) produce great losses to agricultural crops. One of the most important strategies for controlling plant diseases is an accurate identification and an early detection and monitoring of microorganisms (Lopez-Mondejar et al., 2012). The availability of fast and sensitive methods for the detection of pathogenic species in soil can strongly improve disease control and help decision making. An early detection of the pathogen even before the onset of the symptoms, is of special interest in seeds, nursery plants and plant material to avoid further spreading and introduction of new pathogens into a growing area where it is not yet present (Acero et al., 2011).

The traditional detection methods are time-consuming and require extensive knowledge on classical taxonomy (Capote et al., 2012). Quantitative real-time polymerase chain reaction (qPCR) based technology is a rapid and sensitive method that offers advantages over the traditional diagnosis reducing the time needed for diagnosis. The qPCR technology allows accurate/discriminant detection and/or quantification of pathogens that cannot be extracted or cultivated easily from soil and plant tissue, or are present at low inoculum load in samples. Nowadays a wide range of plant pathogens can be detected and quantified by real-time PCR in numerous hosts or environmental samples, e.g. *Fusarium oxysporum* in muskmelon seedlings (Lopez-Mondejar et al., 2012), *Phytophthora nicotianae* in soil and host tissues (Blaya et al., 2015), *Sclerotinia sclerotiorum* in alfalfa (Parker et al., 2014).

Diverfarming will target soil pathogenic species in a selection of case studies to be defined. The identification and monitoring of an agreed number of pathogenic species will be addressed to a comparison between differently managed soils, in order to test whether a higher diversification in agriculture could impact the diffusion and persistence of certain organisms that are noxious to crops.



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Principle

Quantitative PCR (qPCR) is based on the detection of the fluorescence produced by a reporter molecule, which increases as the PCR cycles proceed. These fluorescent reporter molecules include dyes that intercalate with any double-stranded DNA (non-specific) or sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent report, which permits detection only after hybridisation of the probe with its complementary sequence. The non-specific label method: SYBR® Green does not emit fluorescence in its free form, emitting the fluorescence signal only when binding to the dsDNA. The principal drawback to intercalation-based detection of product accumulation is that both specific and non-specific products produce a signal. So, this can lead to false positive results in the quantification (Giulietti et al., 2001). The reaction should be followed by melting curve analysis in which the melting temperature (T_m) of the generated product is determined. The shape and temperature of T_m depend on the PCR product concentration, its size and nucleotide base composition (Dreo et al., 2012).

The SYBR Green qPCR assay has been validated by an international ring test, and is widely used starting by an appropriate amplicon design optimising the qPCR assay. Specific and discriminant primer pair design can be done manually or by using appropriate *in silico* software or web-based tools and the sequence of microbial gene of interest. Since the specificity and the discriminant character are of crucial importance, several fungal SSR markers have been reported to be species specific, with a polymorphic character, thus allowing a discriminant and specific qPCR assay.

Sequence specific methods: among different probe-based detections, the most commonly-used probe is the "TaqMan". The taqMan probe is a sequence of 25-30 nucleotides in length which is labelled with a donor fluorophore (as reporter) at the 5' end, and an acceptor dye (as quencher) at the 3' end. The fluorophore does not emit fluorescence in the presence of the quencher, which dissipates the energy by proximal quenching or by fluorescent resonance energy transfer (FRET). Once the primers and the probe specifically hybridise to the DNA, the 5'-3' exonuclease activity of the Taq DNA polymerase cleaves the probe causing the liberation of the fluorophore, which therefore starts emitting fluorescence. The fluorescence detected in the qPCR cyclers is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. Probes may include fluorophores such as FAM (6-carboxyfluorescein), ROX (6-carboxyl-X-rhodamine) and quenchers such as TAMRA (6-carboxyltetramethylrhodamine) or MGB (minor groove binder).

Reagents

- Specific primers and probes according to the target.
- Premix Ex Taq (Probe qPCR) Master Mix + Rox reference dye
- Quanti Fast or Quanti Nova SYBR Green PCR Master mix, (Qiagen)
- Water - molecular biology grade (DNase, RNase, Protease, free)
- Bovine serum albumin (BSA) which enhances PCR amplifications from low purity DNA samples and prevents enzymes from adhering to tubes and tipped surfaces.



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- Soil to develop a standard curve
- Pure culture of each pathogen to detect/ quantify
- Internal PCR control (IPC) *Phocine Herpesvirus*

PhHV-267s:5'- GGGCGAATCACAGATTGAATC-3'

PhHV-337as: 5'-GCGGTTCCAAACGTACCAA-3'

PhHv-305tq: CY5-5'-TTTTTATGTGTCCGCCACCATCTGGAT-3'-BHQ

- *Escherichia coli* DH5a cells (Invitrogen, USA)
- QIAprep Miniprep Kit (Qiagen, Germany).

Materials and equipment

- Real Time PCR system (qPCR)
- Pipettes from 10 to 1000 µl, filter tips, epis or plates according to the qPCR, vortex.

Procedure

The detection and/or quantification of a specific bacterial or fungal pathogenic organism in soil samples will be performed starting from:

1. **Soil DNA extraction.** DNA extraction is a critical pre-step analysis; the quality of the final results can be significantly affected by the purity and final yield of DNA (for the DNA extraction method, see chapter 3.1).
2. **Selection or design of specific set of primers or primers and probe.** According to the method used to detect and quantify the target microorganisms we have to select or design specific primers and probe.
3. **qPCR assay.** The qPCR assay can be used with any qPCR platform or be anyhow adapted to the specific instrument that each laboratory has available (i.e. 7500 Fast Real-time PCR system (Applied Biosystems, Waltham, MA, USA). The qPCR assay has two steps, qPCR mixture and qPCR conditions.
4. **qPCR analysis and standard curve preparation.** To quantify the amount of pathogen on soil samples a standard curve for each pathogen is made.

Selection or design of specific set of primers or primers and probe.

Target gene selection is a crucial step in real-time PCR assay; sequences of the primer must be unique to identify sequences of the target in the sample of interest with high specificity and efficiency. The ribosomal DNA genes (rDNA) and the internal transcribed spacer (ITS) provide nice targets because they have conserved and variable regions that allow highly sensitive detection. Typically, 16rRNA genes



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from bacteria and ITS regions of the fungal RNA regions have been used most commonly for PCR-based identification of plant pathogens. Other sequences that are used for identification and monitoring at the species level are for example the β -tubulin gene, the elongation factor 1 alpha (EF1- α), and random amplified polymorphic DNA/sequence-characterised amplified region (RAPD/SCAR)-based targets (Okubara et al., 2005).

Non-specific label method: An optimal amplicon length ranges between 100 and 250 bp, and a primer length of 18-25 bp with a GC content of 50% and a melting temperature ranging between 58°C and 65°C. Moreover, the five nucleotides at the 3' end of each primer should have no more than two G and/or C bases.

Specific method: Primer and probe design is also one of the first important steps due to the balance between efficiency and specificity of amplification. Primers must bind to the target site efficiently under PCR conditions. Specificity can generally be defined as the tendency of the primer to hybridise to its specific target and not to non-specific targets and amplify one product. Different rules must be taken into account when primers and probes are designed. The minimum requirements to design a probe are the following: length 18-30bp, T_m (68-70°C) and %GC (40-70), absence of hairpin loops, (dG<-3) and dimers (dG<-12), moreover the sequence cannot begin with G. To design primers, the minimum requirements are also the following: length 18-30bp, T_m (68-70°C) and %GC (40-60). The distance between primers and probe must be 1 nucleotide, total length primer and probe 75-150 bp; on 3' no more than 2 T or G, absent of hairpin loops, (dG<-3) and dimers (dG<-12). Nowadays, these can be designed by different programs Primer Express, Primer 3 or Clustal X. Their specificity must be checked by BLAST tool in GenBank and afterwards with the DNA of the target microorganisms and relatives. PCR inhibitions are very frequent when soil samples are assessed. For detecting inhibitor effects, causing false negative results, an internal positive control of a conserved DNA segment or amplification of a housekeeping gene can be included in the assay (Schena et al., 2013).

qPCR assay

For the **non-specific label method**, the amplification reaction is carried out in 25 μ l reactions containing 10 μ l of template DNA (5-10 ng/ μ l), 12.5 μ l of Quanti Fast SYBR Green PCR Master Mix (Qiagen), 1.2 μ M of primer, and up to 15 μ l of PCR-grade water. In order to protect soil DNA and microbial DNA extracts from potentially present PCR-inhibitory substances, bovine serum albumin (BSA) should be added to the SYBR Green mix (Quanti Fast or Quanti Nova SYBR Green PCR Master mix, Qiagen). Experiments should be performed in duplicate or, better, in triplicate.

Specific method: The real-time PCR mixture is developed three times for each soil in a final volume of 15 μ l containing 0.9 μ l of BSA (5 mg ml⁻¹), 0.3 μ l of each specific primer for each pathogen (15 μ M), 0.3 μ l of each respective probe (5 μ M), 7.5 μ l of Premix Ex Taq (Probe qPCR) Master mix (2X) and Rox reference Dye II (50X), 1.7 μ l of water molecular biology grade (WMB) and 3 μ l of DNA sample. An internal positive control (IPC) to detect inhibition is included in each reaction (0.1 μ l of DNA from IPC, 0.3 μ l of each specific primer (15 μ M) and 0.3 μ l of probe (5 μ M)).



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The Real-Time PCR conditions for each pathogen will depend on the primers and probes used. Typical conditions are: 95°C for 1 min, followed by 40 cycles of 95°C for 10 s and 60°C for 40 s and a final step 50°C 2 min.

qPCR analysis and standard curve preparation

Two different approaches can be applied to quantify the selected pathogens.

- 1) For each selected microorganism, a specific fragment selected for a target gene is cloned into vector PCR 2.1 (Invitrogen, USA). The plasmid is used to transform *Escherichia coli* DH5a cells (Invitrogen, USA) and purify with a QIAprep Miniprep Kit (Qiagen, Germany). The presence of inserts is used with restriction enzymes following the manufacturer's protocol. The DNA concentration of the plasmid standard solution is measured using Infinite®200 PRO (Tecan Trading AG, Männedorf, Switzerland), after Picogreen reagent staining according to the manufacturer's instructions (Molecular Probes, Inc., Eugene, OR, USA) and is related to the known molecular weight of a single plasmid molecule to calculate the number of copies according to the following equation:

$$\text{Number of copies} = (a \times 6.022 \times 10^{23}) (b \times (1 \times 10^{12}) \times 650) ,$$

where **a** is the DNA concentration of the plasmid (ng), 6.02×10^{23} is Avogadro's number indicating the number of molecules/mol; **b** is the length of the plasmid containing the insert (bp), 1×10^{12} is used to convert g to pg; and 650 is the average molecular weight of one base pair. The concentration is adjusted to the number of 10^{10} gene copies, and the standard is diluted in 10-fold steps to obtain the standard curve. All qPCR reactions are performed in triplicate (Lopez-Mondejar et al., 2012; Blaya et al., 2015).

Estimation of the number of sequences of the gene of interest per g of dry soil is developed:

$$\text{copy numbers (g dry soil}^{-1}\text{)} = \text{copies } \mu\text{l}^{-1} \times \text{DNA elution volume } \mu\text{l} \times \text{g dry soil}^{-1}$$

- 2) The first step of qPCR standard preparation relies on the extraction of DNA templates. This must be done from:
 - i) Pre-bacterial or fungal cultures of interest to harbour the gene of interest (thus pathogens) by the DNA extraction method reported in chapter 3.1;
 - ii) Soil DNA (a soil used as control in the experimental design) by the DNA extraction method.

A calibration is performed by carrying out microcosm incubation with a known amount of soil DNA (used as control in the experimental design) added to a known amount of pure bacterial/fungal liquid suspension. DNA is extracted within 48h after the addition of the targeted microbes from the microcosm, amplified for each targeted gene following the SYBR green chemistry protocol (Canfora et al., 2016).



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Amplicons are purified from the gel using the conventional approach (cut of the bands) to remove primers. The purified amplicons are then quantified and the gene copy number is calculated.

The gene copy number is calculated using the following equation (<http://scienceprimer.com/copy-number-calculator-for-realtime-pcr>):

$$\text{gene copy number} = (\text{ng} _ \text{number} _ \text{mol}^{-1}) / (\text{base pairs} _ \text{ng} _ \text{g}^{-1} _ \text{g mol base pairs}).$$

The standard curve is created using dilution covering up to 6-7 orders of magnitude from 10² to 10⁸ gene copies per qPCR reaction. All qPCR reactions are performed in triplicate and three NTC are also included. Estimation of the number of sequences of the gene of interest per g of dry soil:

$$\text{Gene copy numbers (g soil)}^{-1} = (n [\text{gene of interest}]_1 \times y) z$$

$n [\text{gene of interest}]_1$ number of sequences of the gene of interest per ng of soil DNA; y is the amount of soil DNA extracted from z gram of soil sample

z is the amount of soil sample from which DNA is extracted (in g of dry mass equivalent).

Pathogens to measure

Typical bacterial and fungal pathogens of the different cultivable plants growing and investigated at the different research sites are compiled in the attached Table 3.4.1.

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Table 3.4.1 Bacterial and fungal pathogens to be investigated at the individual case studies, using molecular biological techniques.

Case Study: Country (Crop)	Bacteria	Fungi
1. Spain (almonds)	<i>Xanthomonas arboricola</i> , <i>Agrobacterium tumefaciens</i> , <i>Xylella fastidiosa</i>	<i>Polystigma amygdalinum</i> , <i>Phomopsis amygdali</i> = <i>Fusicoccum amygdali</i> , <i>Monilia laxa</i>
2. Spain (fruit trees - citrus)	<i>Candidatus Liberibacter asiaticus</i> , <i>Xanthomonas campestris</i> , <i>Spiroplasma citri</i> , <i>Xylella fastidiosa</i>	<i>Phytophthora</i> spp, <i>Alternaria alternata</i> , <i>Colletotrichum gloeosporioides</i> , <i>Botrytis cinerea</i> ,
3. Spain (cereals)		
4. Spain (olives)		
5. Italy (horticulture/cereal)	<i>Ralstonia solanacearum</i>	<i>Fusarium oxysporum</i> , <i>Rhizoctonia solani</i> , <i>Alternaria solani</i>
6. Italy (horticulture/cereal)	<i>Ralstonia solanacearum</i>	<i>Fusarium oxysporum</i> , <i>Rhizoctonia solani</i> , <i>Alternaria solani</i>
7. Italy (horticulture/cereal)	<i>Ralstonia solanacearum</i>	<i>Fusarium oxysporum</i> , <i>Rhizoctonia solani</i> , <i>Alternaria solani</i>
8. Netherlands (feed)		<i>Fusarium oxysporum</i> , <i>Rhizoctonia solani</i>
9. Germany (vineyards)		<i>Plasmopara viticola</i> , <i>Botrytis</i> ?
10. Hungary (fruit and horticulture)		
11. Hungary (vineyards)	<i>Pseudomonas</i> sp.	<i>Rosellinia necatrix</i> , <i>Roesleria pallida</i> , <i>Botrytis</i>
12. Finland (feed)		<i>Rhizoctonia solani</i> , <i>Cochliobolus sativus</i> (<i>Bipolaris sorokiniana</i>), <i>Fusarium culmorum</i> , <i>Fusarium</i> sp.,
13. Finland (feed)		<i>Rhizoctonia solani</i> , <i>Cochliobolus sativus</i> (<i>Bipolaris sorokiniana</i>), <i>Fusarium</i> sp., <i>Hymenula serealis</i>
14. Spain (machinery testing)		
Long-term study: Country (Crop)		
1. Spain (multiple horticulture)	<i>Erwinia carotovora</i> sb. <i>Carotovora</i> , <i>Xanthomonas fragariae</i>	<i>Alternaria</i> spp., <i>Botrytis cinerea</i> , <i>Dydymella brioniae</i> , <i>Fusarium oxysporum</i> , <i>Leveillula Taurica</i> , <i>Phytophthora capsica</i> , <i>Rhizoctonia solani</i> , <i>Sclerotinia sclerotiorum</i> , <i>Verticillium dahlia</i> ,



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2. Italy (rotation cereal/legumes)		
3. Spain (conservation almonds)		
4. Spain (cereal monoculture)		
5. Germany (organic vineyards)		<i>Plasmopara viticola</i> , <i>Botrytis</i> ?
7. Finland (organic and conventional feed)		<i>Rhizoctonia solani</i> , <i>Cochliobolus sativus</i> (<i>Bipolaris sorokiniana</i>), <i>Fusarium sp.</i> , <i>Hymenula serealis</i>
8. Netherlands (feed)		<i>Fusarium oxysporum</i> , <i>Rhizoctonia solani</i>
9. Hungary	<i>Agrobacterium spp.</i>	<i>Erysiphe necator</i> , <i>Botrytis cinerea</i> , <i>Plasmopara viticola</i> , <i>Guignardia bidwellii</i> , <i>Pseudopezicula tracheiphila</i> , <i>Coniella diplodia</i> , <i>Botryosphaeriaceae</i> , <i>Eutypa lata</i> , <i>Phomopsis viticola</i> , <i>Phaeomoniella chlamydospora</i> , <i>Phaeoacremonium aleophilum</i> , <i>Fomitiporia spp.</i> , <i>Cylindrocarpon spp.</i> etc.



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6. Sequencing soil samples for qualitative metagenomics – Ion Torrent Sequencing Protocol

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Importance and applications

Given the crucial importance of diversity and the abundance of soil microbiota, there are several techniques to assess community structure profile in soil (Zhou et al., 2015). One of the most effective techniques relies on DNA sequencing technology where specific amplicons are selected within the bacterial genome and sequenced through high-throughput next-generation sequencing to identify and quantify bacterial community in metagenomic samples (Pace, 1997; Kim et al., 2013). Sequencing 16S ribosomal DNA of metagenomic soil samples is considered as the ultimate assay to obtain the community structure profile in soil samples (Vasileiadis et al., 2012; Kim et al., 2013). Different DNA fragments within 16S ribosomal DNA of bacteria are hypervariable regions meaning that the specific sequences vary greatly through species and it allows the differentiation between different species in soil and their abundancy through next-generation sequencing and further bioinformatics analysis of data (Morozova & Marra, 2008; Lakshmanan, Selvaraj & Bais, 2014; Finley, Benbow & Javan, 2015)

It is well established that agricultural practices alter the composition and diversity of soil microbial communities (Luise et al., 2014). It is also proven that the land-use in the manners of diversified crop systems in agricultural areas affects the corresponding soil microbial biodiversity and community structure (Stoate et al., 2009; Szoboszlay et al., 2017). Assessment of the soil microbial community structure in the fields with different long-term agricultural practices and separately in fields with diversified aboveground crops will provide a vast amount of information about the soil microbial community as a strong indicator of soil quality in relation with land use.

Principle

The Ion Torrent system, licensed from DNA Electronics in London, detects H⁺ ions during DNA polymerisation. The technology combines semiconductor sequencing technology and biochemical properties, enabling the direct translation of chemical information into digital data. The features of the system eliminate the need for expensive optics, lasers, and complex sequencing chemistries with fluorescently labelled nucleotides (Whiteley et al., 2012; Life Technologies, 2013b, 2013a). Ion PGM is one of the most preferred systems for sequencing amplicons as it is the sequencing system, which allows sequencing of the longest fragments among equivalent systems (Lahens et al., 2017).



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Sending samples for sequencing

- Samples should be sent with the barcode given by the project coordinator (see chapter 1). Samples that are not barcoded in that way will not be sequenced.
- DNA should be sent by courier service, and does not need ice as long as the samples have not been frozen and thawed several times.
- DNA samples should be sent in clearly labelled screw-cup tubes.

Requirements of DNA quantity for sequencing

The minimum amount to be provided for sequencing is 18 μ L with a minimum DNA concentration of 1.5 ng/ μ L. DNA must also satisfy quality standards with ratios of absorbance $A_{260}/A_{280} = 1.8$ -1.9, and $A_{260}/A_{230} = 1.9$.

Target selection (Amplicon preparation)

The amplicons to be sequenced are prepared using Ion 16S™ Metagenomics Kit (Cat. no. A26216) from Thermo Fisher Scientific®.

The commercial kit includes two sets of primers already prepared and mixed and both specifically amplify a part of 16S ribosomal DNA. The first set of primers amplifies hypervariable regions of V2-4-8 and the second set of primers amplifies the regions V3-6 and 7-9. The combination of primer pools allows for sequence-based identification of a broad range of bacteria within mixed populations. As a beginning, we will perform a ring test among the primer sets to determine the best option and that option will be used for further experiments.

Purification of amplicons

The amplified 16S regions are further purified in Eppendorf® tubes following the manufacturer's instructions (Ion 16S™ Metagenomics Kit) and using a DynaMag™ magnetic rack.

Calculation of DNA input for library preparation

The analysis of size and concentration of the prepared and purified amplicons is done by using Agilent® 2100 Bioanalyzer® instrument with Agilent® software and the Agilent® High Sensitivity DNA Kit (Cat. No. 5067-4626). The system allows us to identify the exact concentration and size distribution (50-7000 bp) of the DNA samples.



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Quality and quantity requirements of DNA samples to be sequenced

High-quality RNA-free DNA is required. The required input for the library preparation (end-repair) step is **10–100 ng in 79 µL** volume (as close as possible to 100 ng).

Library Preparation

Preparation of the sequencing library is conducted by Ion Plus Fragment Library Kit (Cat. no. 4471252).

An Ion Xpress™ Barcode Adapters 1–16 (Cat. No. 4471250) kit is used to barcode samples so that several samples can be sequenced by pooling them together in the same chip. Barcodes can be further identified by bioinformatics to distinguish between the different samples following the sequencing. There are other Ion Xpress™ Barcode Adapters Kits available as grouped as 16 specific barcodes for each kit up to 96 specific barcodes in total. Other barcoding kits will be used as needed in future (from 16 to 96).

The overview of the library preparation procedure is:

- End repairing and purification of pooled amplicons
- Ligation of sequencing adapters and barcodes
- Purification of the adapter-ligated and nick-repaired DNA (using DynaMag™)
- Determination of the library concentration on Bioanalyzer (Agilent® High Sensitivity DNA Kit)

Template preparation

The libraries are diluted and arranged for suitable concentrations. The diluted and barcoded libraries are pooled together (equal amounts) to be sequenced, i.e. 16 DNA samples coming from 16 soil samples are brought to 10 ng each and pooled to obtain 160ng for sequencing. Then the pooled sample is diluted to 25ng/µL.

Templates are prepared from the libraries using Ion OneTouch™ 2 System and the Ion PGM™ Hi-Q™ OT2 Kit. Follow the instructions in the Ion PGM™ Hi-Q™ OT2 Kit User Guide (Pub. no. MAN0010902) Ion PGM™Hi-Q™ OT2 Kit (Cat. no. A27739).

Sequencing the library

The sequencing process is performed using the Ion Personal Genome Machine™ (PGM™) System and the Ion PGM™ Hi-Q™ Sequencing Kit following the protocol in the Ion PGM™ Hi-Q™ Sequencing Kit User Guide (Pub. no. MAN0009816).

We seek to obtain 20 000-40 000 reads per sample. The overall experimental setup for sequencing will be assessed considering the targeted number of reads. This means that the number of samples processed in a chip will be adjusted in order to obtain at least 20 000 reads to do the bioinformatics analysis.



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The available chips for Ion PGM are Ion 314™ Chip v2 (400-550 thousand read/run), Ion 316™ Chip v2 (1-3 million reads/run), or Ion 318™ Chip v2 (4-5.5 million reads/run) depending on the number of barcoded libraries pooled for run, initial sample complexity and/or desired sequencing depth.

The preference of the chip will be a manner of expectations and the lowest capacity chip has a potential of sequencing up to eight samples (according to the desired 20 000-40 000 reads/sample and average 80% efficiency of the chip).

In our hands, the worst sequencing reaction we obtained had 222 800 reads and the best was 457 362 reads for the 314 chip. **If a sample gives less than 20 000 reads we will check if there is a problem with DNA quality in the Bioanalyzer. If it fails, we must request a new DNA sample from the partner. If it passes, then we will do the sequencing again with the remaining DNA. If a DNA sample fails twice that sample will not be sequenced a third time.**

Remarks

The DNA samples, which do not satisfy the standards described in the sections “Requirements of DNA quantity for sequencing” and “Quality and quantity requirements of DNA samples to be sequenced” **will not be sequenced** and new DNA samples will be required from the partners.

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7. Sequencing soil samples for qualitative metagenomics – ITS Illumina amplicon protocol

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Overview

Microorganisms play an important role in ecosystem functions by mediating many of the biogeochemical processes that are critical to soil fertility and plant productivity. For this reason, it is important to understand how the agronomic practices impact on microbial biodiversity and the associated function. Advances in sequencing technologies have led to the development of sequencing machines with the ability to generate a large volume of sequence data. These technologies that are generally called “Next Generation Sequencing (NGS)” have profoundly changed the way we approach the studies of the microbial communities, becoming the technology of choice for metagenomics studies. The approaches based on NGS sequencing overcome the limits of the cultivation-based methods and allow to profile the entire microbiome by directly sequencing the DNA taken from environmental samples. The PCR amplicon sequencing of specific target regions is a widely used approach to study microbial biodiversity. The target regions commonly used are the ribosomal RNA genes because they are characterised by having highly conserved sequences that enable the design of primers targeting a wide range of taxa and hypervariable regions useful for taxonomic classification (Kim, 2013). The sequencing protocols for the study of the microbial diversity using the Illumina (San Diego, CA, USA) MiSeq sequencer are reported below. The protocol is designed to analyse the internal transcribed spacer (ITS) region to study fungal biodiversity.

DNA

DNA extraction is a crucial step in metagenomics studies. Low DNA yield may lead to a biased estimation of microbial diversity (Claassen, 2013; İnceoğlu, 2010). For this reason, DNA for sequencing should be at a minimum concentration of 10 ng/μl with at least 200 ng provided DNA is resuspended in water or in 10mM Tris HCl pH 8.5.

ITS Illumina amplicon protocol

The protocol described below is based on the technical note provided by Illumina for the sequencing of the bacterial V3/V4 16S rRNA gene region (Illumina Technical note number 15044223). The protocol has been modified in order to allow the analysis of the fungal ITS1 region using the ITS5 - ITS2 primers. Sequencing libraries are produced using a two-step PCR protocol. In the first PCR, the target region is amplified using locus specific primers tailed with sequences that allow, with a second PCR, the adding of the indexed adapter.



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Primers

Primers are ordered using standard desalting purification. Primers are shipped lyophilised and upon arrival they should be spun down by centrifugation before being resuspended. Resuspension is made in pure water at the concentration of 100 μ M (stock solution). Stock solution is diluted to 10 μ M (working solution) before use. The gene-specific primers used in the first PCR are:

Forward Primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
GGAAGTAAAAGTCGTAACAAGG

Reverse Primer 5'
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCGTTCTTCATCGATGC

The Illumina adapter sequences are highlighted in red, while the locus specific sequences are highlighted in green. The complementary regions with the Nextera oligo are underlined.

The second PCR makes use of the Nextera XT index primers from the Nextera XT index kit (Illumina, San Diego, CA, USA). The Nextera index kit enables the preparation of up to 96 libraries using dual 8bp indexes.

First PCR

This PCR is used to amplify the ITS1 region producing an amplicon tailed with sequences complementary with the Nextera indexed primers. The PCR reactions should include a negative control to assure the lack of contamination.

Prepare the following master mix:

Reagents for one sample	Volume
Template DNA (5ng/ml)	2.5 μ l
Forward primer 10 μ M	0.75 μ l
Reverse primer 10 μ M	0.75 μ l
2 X KAPA HiFi HotStart Ready Mix	12.5 μ l
PCR-grade water	8.5 μ l
Total	25 μ l

Gently mix the reaction and briefly centrifuge the tube



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Use the following thermocycling PCR conditions:

Step	Temperature	Time
Denaturation	95°C	3 minutes
25 cycles	95°C	30 seconds
	55°C	30 seconds
	72°C	30 seconds
Final Extension	72°C	5 minutes
Hold	4°C	

PCR products should be checked on a Bioanalyzer DNA 1000 or on agarose gel to verify the size. The expected size is ~250 bp.

The PCR products are cleaned up from primer and primer dimer using the Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) following the manufacturer's instructions. Resuspend PCR products in 50 µl of 10 mM Tris pH 8.5.

Second PCR

The second PCR attaches the sequencing adapters and the dual indices using the Nextera XT Index Kit. The consumables needed to perform the second PCR are shown below. Prepare the following master mix:

Reagents for one sample	Volume
Template DNA	5 µl
Nextera XT index primer 1	5 µl
Nextera XT index primer 2	5 µl
2 X KAPA HiFi HotStart Ready Mix	25 µl
PCR-grade water	10 µl
Total	50 µl

Use the following thermocycling PCR conditions:

Step	Temperature	Time
Denaturation	95°C	3 minutes
25 cycles	95°C	30 seconds
	55°C	30 seconds
	72°C	30 seconds
Final Extension	72°C	5 minutes
Hold	4°C	

The PCR products are cleaned up using the Agencourt AMPure XP beads following the manufacturer's instructions. Resuspend PCR products in 25 µl of 10 mM Tris pH 8.5.



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Sequencing

The MiSeq instrument allows the sequencing of multiple libraries pooled together. Because it is desirable to obtain the same sequencing coverage for each library, the libraries should be pooled in an equal amount. For this reason, an accurate library quantitation is needed prior to pooling.

Quantify the amplicons with the Qubit using the dsDNA HS Assay kit. To calculate the concentration the amplicon size needs to be determined using the 2100 Bioanalyzer.

The Illumina Technical note suggests calculating the DNA concentration with the following equation:

$$\text{concentration in nM} = \frac{\text{concentration in ng}/\mu\text{l}}{(660 \text{ g/mol} \times \text{average library size})} \times 10^6$$

Dilute the libraries using resuspension buffer or 10 mM Tris pH 8.5 to a final concentration of 4 nM. Mix 5 μl of each library for pooling libraries.

Low diversity libraries such as the amplicons libraries require the adding of 10-15% PhiX (Illumina, San Diego, CA, USA) ready-to-use control library to the run.

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8. Bioinformatics workflow for the analysis of soil microbial community by next generation sequencing

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Introduction

The emergence of massively parallel sequencing systems has revolutionised the way we approach metagenomic studies. The sequencing of target genes such as the bacterial 16S and the fungal ITS with Next Generation Sequencing (NGS) equipment is becoming a popular method to study microbial communities' diversity. At the same time, the large and complex datasets generated by these machines have posed several challenges for bioinformatics and have led to the development of bioinformatics tools which can handle the data produced by these technologies. QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso, 2010) is an open-source bioinformatics pipeline developed for the analysis of microbial communities based on sequencing data generated by NGS platforms. QIIME integrates different third-party bioinformatics tools into a single workflow.

The Diverfarming workflows for processing data from ITS MiSeq amplicon sequencing and 16S rRNA Ion Torrent Amplicon Sequencing are described in this chapter.

Remark

Bioinformatics tools and pipelines are continuously evolving and as a consequence the workflows proposed here may easily undergo changes during the project. It will be necessary to keep up with state-of-the-art approaches for data analysis.



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8.1 Bioinformatics workflow for the analysis of soil fungal communities by its MiSeq amplicon sequencing

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Reads pre-processing

The pipeline described in this chapter begins with the demultiplexed reads obtained from a MiSeq instrument and is based on the QIIME pipeline integrated by the use of other bioinformatics tools.

Quality filtering

The Illumina instruments generate sequencing files in Fastq format. This format stores both the sequences and the quality score for each base in a single file. The quality score (Q) measures the probability that a base has been identified incorrectly and is assigned to each base using the following equation:

$$Q = -10 \log_{10} P$$

where P is the probability that a base is called incorrectly (Illumina Technical note). This probability is calculated by sequencing machines using observable properties of the clusters, such as intensity profiles or the signal to noise ratios. For example, a quality score of 30 (Q30) means that the probability of an incorrect base call is 1 in 1000 times with a corresponding call accuracy of 99.9%. The filtering of sequences based on the Q allows to remove sequences with errors that can affect the quality of the downstream analyses such as OTUs clustering.

Use Trimmomatic to process the raw reads

Trimmomatic is a command line tool for read trimming and filtering (Bolger, 2014). To run Trimmomatic with paired-end data, two input files and the name of four output files need to be specified. The two input files are the two paired-end fastq files. The output files are produced by Trimmomatic and are two paired files with the filtered reads and two files in which the reads survived to the filtering but unpaired are stored. Execute the following command to run Trimmomatic

```
java -jar trimmomatic-0.33.jar PE -phred33 RawReads1.fastq.gz RawReads2.fastq.gz  
reads1_filtered.fastq.gz reads1_unpaired.fastq.gz reads2_filtered.fastq.gz  
reads2_unpaired.fastq.gz ILLUMINACLIP:NexteraPE-PE.fa:2:30:10 SLIDINGWINDOW:30:25  
MINLEN:150
```




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Arguments summary

- ✚ phred33 → Specifies the quality score version (phred33 or phred64)
- ✚ ILLUMINACLIP → This option is used to find and remove the illumina adapters. NexteraPE-PE.fa, is a file in fasta format containing the adapters used by MiSeq and HiSeq.
- ✚ SLIDINGWINDOW → Cut the reads when the average quality within a specified window drops below the specified threshold.
- ✚ MINLEN → Eliminate the reads below the specified length.

The sliding window parameter should be chosen every time by testing it on your reads.
For other options see the software manual.

Reads analysis

Assembly paired-end reads

The MiSeq instrument is able to generate 300bp reads in paired-end fashion from both ends of the DNA amplicons. To take full advantage of this feature, amplicon target sequencing should be designed in such a way that the two reads overlap. When this condition is satisfied the two reads can be merged into one, increasing the overall region sequenced. PEAR (paired-end read merger) is a software for merging paired-end reads from fragments of different lengths that exhibit a variety of overlapping length (Zhang, 2014). These properties are especially important for the assembly of reads from the fungal Internal Transcribed Spacers regions because these regions are characterised by the presence of length polymorphisms.

Execute the following command to run PEAR with your data:

```
pear-0.9.10-bin-64/pear-0.9.10-bin-64 -f 'reads1_filtered.fastq' -r 'read2_filtered.fastq' -o 'reads_joined.fastq' -j 4
```

Arguments summary

- ✚ -f → Name and path of the forward paired-end reads fastq file
- ✚ -r → Name and path of the reverse paired-end reads fastq file
- ✚ -o → Name and path of the output fastq file
- ✚ -j → Number of threads to use

For other options see the software manual.

Proceed with the QIIME pipeline

The fastq files must be converted into a fasta file using the following QIIME script

```
convert_fastaqual_fastq.py -c fastq_to_fastaqual -f reads_joined.fastq -o output_fasta
```



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Arguments summary

- 🚦 -f → Input fastq file
- 🚦 -o → Output directory

Other options are available in the documentation on the QIIME website.

A mapping file needs to be prepared at this step. This file can be made in Excel and saved as tab delimited text file. The file contains as many columns as needed to describe each sample. The first column should always be named “# Sample ID” followed by “BarcodeSequence” and “LinkerPrimerSequence”. After these three columns you can add as many columns as you need to describe the sample (with metadata headers). The last column should always be named “Description” and include information unique to each sample.

Because we are working with demultiplexed samples we leave the column *BarcodeSequence* and *LinkerPrimerSequence* empty. Now we can check if the mapping file is formatted in the proper format using the following QIIME script:

```
validate_mapping_file.py -o 'path/validation_output' -m 'mapping_file.tab' -p -b
```

Arguments summary

- 🚦 -m → Mapping file
- 🚦 -o → Output directory
- 🚦 -p → Disable primer check
- 🚦 -b → Not barcoded

All the fasta files are now combined into a single fasta file and labelled with QIIMEfasta labels using the following script

```
add_qiime_labels.py -i '/path/fastq' -m 'path/mapping_file.tab' -c SampleID -o '/path/output'
```

Arguments summary

- 🚦 -i → Directory in which the fasta files to combine are located
- 🚦 -m → Mapping file
- 🚦 -c → Indicate the column in the mapping file with the fasta file name
- 🚦 -o → Output directory

The output is a file called “combined_seqs.fna”

Chimera detection using VSEARCH

Chimeras are sequences artefacts produced during the PCR amplification and derived by joining two or more partial sequences coming from different biological sequences. They are formed when prematurely



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terminated amplicons generated by incomplete extensions act as primers and anneal to different but similar templates. We use VSEARCH (Rognes, 2016) to detect and remove chimeras. VSEARCH detects chimeras using the UCHIME algorithm. VSEARCH can perform chimera detection using or not a reference database. We suggest running the reference-based chimera detection using the UCHIME reference dataset downloaded from the UNITE database (<https://unite.ut.ee/repository.php>).

```
Vsearch -uchime_refpath/combined_seqs.fna -  
db/path/uchime_sh_refs_dynamic_original_985_03.07.2014.fasta -nonchimerasno_chimeras.fna -  
threads 7
```

Output file = no_chimeras.fna

OTU picking and taxonomy assignment for fungal ITS

In this step, the reads are clustered into OTUs based on a 97% sequence similarity and taxonomy is assigned to OTUs. The sequences similarity threshold can be set by specifying the following option

```
pick_otus:similarity 0.97
```







on the QIIME parameters file.

OTU picking is made using an open reference strategy. With this strategy the reads are initially clustered against a reference sequences database. The reads that did not find a match with the reference sequences are clustered de novo.

Pick-open-reference_otus.py by default uses the UCLUST clustering tool.

```
pick_open_reference_otus.py -ino_chimeras.fna -o '/path/output'-r  
path/sh_refs_qiime_ver7_dynamic_20.11.2016.fasta --suppress_align_and_tree -p  
'OTU_picking_params.txt' -a -O 6
```

Arguments summary

-  -i → The input file
-  -o → Output directory
-  -p → The parameter file
-  -a → Run in parallel
-  -O → Number of job to start in parallel (only with -a)
-  --suppress_align_and_tree → Apply if you are working with ITS amplicons (ITS sequences cannot be reliably aligned).

The output is a file called "otu_table_mc2_w_tax.biom"



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Box 1: QIIME parameters file for the pick_open_reference_otus.py command

```
# QIIME parameters file

pick_otus:enable_rev_strand_match True
assign_taxonomy:assignment_method blast
pick_otus:similarity 0.97
assign_taxonomy:id_to_taxonomy_fp
/home/path/sh_taxonomy_qiime_ver7_dynamic_20.11.2016.txt
assign_taxonomy:reference_seqs_fp /home/path/sh_refs_qiime_ver7_dynamic_20.11.2016.fasta
```

Remove low abundance OTUs



At this step it is recommended to discard low abundance OTUs using a conservative OTUs' threshold of 0.005%, as suggested by Bokulich (2013).

```
filter_otus_from_otu_table.py -i otu_table_mc2_w_tax.biom -o otu_table_mc2_w_tax_filtered.biom --min_count_fraction 0.00005
```

Now we should normalise the samples by rarefying to the lowest sequence count. We need to know the samples depth to perform this step. To extract this information from the biome table we use the following command

```
biom summarize-table -i/home/path/otu_table_mc2_w_tax_filtered.biom -o /home/path/table_summary.txt
```

Arguments summary

-  -i→ Input file
-  -o→Output file

Box 2: Example of summarise-table command output

```
Counts/sample summary:

Min: 29867.0
Max: 165726.0
Median: 62820.000
Mean: 68755.295
Std. dev.: 24965.318
Sample Metadata Categories: None provided
Observation Metadata Categories: taxonomy
```




The output file provides the information needed to perform rarefaction. From the output file we can see that 29867 is the number of sequences shown by the lowest coverage sample. We can rarefy all the samples to this value using the following command.



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```
single_rarefaction.py -iotu_table_mc2_w_tax_filtered.biom -o  
/path/otu_table_mc2_w_tax_rarefied.biom -d 29867
```

Arguments summary





-  -i → The input file
-  -o → Output file
-  -d → Number of sequences to subsample

Core diversity analysis

Now we can run a set of alpha and beta diversity analyses using the following command:

```
core_diversity_analyses.py -iotu_table_mc2_w_tax_rarefied.biom -m 'mapping_file.tab' -o  
/home/RESULTS/Core_diversity--nonphylogenetic_diversity -c condition, treatment
```

Arguments summary

-  -i → The input file
-  -o → Output directory
-  --nonphylogenetic_diversity → Apply if you are working with ITS amplicons
-  -c → Column headers in the mapping file reporting the metadata categories to compare



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8.2 Bioinformatics workflow for the analysis of bacterial soil communities by 16s rRNA gene Ion Torrent amplicon sequencing

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Principle

We describe a bioinformatic pipeline to analyse 16S rRNA gene amplicon sequencing data generated by an Ion Torrent PGM sequencing platform (PGM). PGM substantially differs from other sequencing technologies like Illumina or Roche 454-pyrosequencing by measuring pH rather than light to detect polymerisation events. This protocol is based on QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso, 2010) with some modifications and additions.

Denoising

Like any other sequencing platform, PGM technology leads to the generation of a characteristic “sequencing noise” in the form of insertion/deletion (indel) error types. ‘Homopolymer errors’ (a term originating from Roche 454 pyrosequencing) are the dominant error type in PGM data. Homopolymer errors are a consequence of inaccurate flow-values resulting in over- (insertion/s) or under-calling (deletion/s) the length of homopolymeric regions (Bragg et al., 2013). As with Roche 454, base-calling accuracy decreases with the length of the homopolymer. This type of error is particularly critical for amplicon-based analyses like 16S rRNA metabarcoding, because it can easily lead to a massive overestimation of the microbial diversity, and thus it must be corrected at the start of the pipeline. The noise-reduction programs installed in Qiime, intended for 454 data, are in principle a risky option to be applied for the particular case of PGM-amplicon data.

The bioinformatic tool ACACIA (Bragg et al., 2012) was also developed in order to treat the homopolymers problem in 454 sequencing data, but it has been suggested also for PGM-amplicon data analysis by the developers and other authors (e.g. Fantini et al., 2015). We will refer to this step as “denoising”. ACACIA is a Java program with both a graphic and command line interface that can be easily installed in the Qiime-Ubuntu Virtual Box. To denoise the data we can apply ACACIA, maintaining the default configuration parameters, with the execution of:

```
AVG_QUALITY_CUTOFF (=20), FASTA (=FALSE),  
FASTQ (=TRUE), REPRESENTATIVE_SEQUENCE (=Max),  
SIGNIFICANCE_LEVEL (=-4).
```



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Once the data has been denoised, we can proceed with the Qiime-pipeline. This protocol is very similar to the MiSeq pipeline explained in the previous chapter, with the modifications imposed by the gene analysed in this case (16S rRNA gene regions) and the type of data generated (not pair-end).

Demultiplex and quality filter reads

The next task is to assign the multiplexed reads to samples based on their nucleotide barcode (this is known as *demultiplexing*). As described previously for MiSeq data, Qiime needs a map file. At this point, the data will not be demultiplexed like in Miseq data, thus we need to fill the fields “# *SampleID*” followed by “*BarcodeSequence*” and “*LinkerPrimerSequence*” in the map file. Once the map file is done, we can apply the following command:

```
split_libraries.py -m map.txt -f denoised_sequences.fna -q denoised_sequences.qual -o  
split_library_output -z truncate_only -q 25
```

This script does a quality filtering, trim primers and adaptors and demultiplexes the reads by using a single command. With the option *-z truncate_only* the script will also truncate reverse primers in case that they are found among the sequences. *-q 25* indicates the threshold for the quality filtering. More options are available, check <http://qiime.org/tutorials/tutorial.html>.

The output reads are stored in the file *seqs.fna*. This is a fasta formatted file where each sequence is renamed according to the sample it came from. The header line also contains the name of the read in the input fasta file and information on any barcode errors that were corrected.

PCR chimera detection using VSEARCH

To filter putative chimeric sequences from the file *seqs.fna* we suggest the program VSEARCH (Rognes 2016) with the formatted RDP database. The output is the “chimeras free” fasta file *no_chimeras.fna*.

```
vsearch -uchime_ref path/seqs.fna -db /path/RDP_trainset16_022016.fa  
-nonchimerasno_chimeras.fna -threads 7
```

Open-reference OTU picking and taxonomy assignment for rRNA 16S

“OTU picking” is one of the most critical steps in this analysis. The output file *otu_table.txt* is where all the sequencing information will be condensed and later used as the input file for the diversity analysis. There are many different options that can be tested in order to obtain a good OTU resolution. We recommend the inclusion of a “mock community” sample in the analysis (a mix of bacterial DNA with a known composition) to help to determine the OTU threshold and algorithms finally chosen. *Open reference with subsampling* is the recommended strategy in Qiime.

The script *pick_open_reference_otus.py* executes a number of interesting steps including OTU picking, annealing of representative sequences with a reference GreenGenes alignment, phylogenetic tree construction (possible for the case of rRNA 16S gene) and taxonomy assignments, among others. The



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different options and parameters for these steps can be easily indicated in the command line and/or in a “parameter file” as in the previous ITS pipeline. For a safe start however, we recommend a threshold of 97% (default option), the combination of methods “sortmeRNA” and “sumacust”, both available in Qiime, and the SILVA database released 128 (Quast et al., 2013), recently updated (2017) in comparison with the Qiime default GreenGenes database (May 2013).

To generate the parameter file for the SILVA database (adjust the number of threads accordingly to the computer used):

```
echo "pick_otus:threads 4" >> SILVA_clustering_params.txt
echo "pick_otus:sortmerna_coverage 0.8" >> SILVA_clustering_params.txt
echo "pick_otus:enable_rev_strand_match True" >> SILVA_clustering_params.txt
echo "align_seqs.py:template_fp
/home/shared/rRNA_db/SILVA_128_QIIME_release/core_alignment/core_alignment_SILVA128.fna" >> SILVA_clustering_params.txt
echo "align_seqs.py:template_fpm_in_percent_id 0.6" >> SILVA_clustering_params.txt
echo "filter_alignment:allowed_gap_frac 0.80" >> SILVA_clustering_params.txt
echo "filter_alignment:entropy_threshold 0.10" >> SILVA_clustering_params.txt
echo "filter_alignment:suppress_lane_mask_filter True" >> SILVA_clustering_params.txt
echo "assign_taxonomy:reference_seqs_fp
/home/shared/rRNA_db/SILVA_128_QIIME_release/rep_set/rep_set_16S_only/97/97_otus_16S.fasta" >> SILVA_clustering_params.txt
echo "assign_taxonomy:id_to_taxonomy_fp
/home/shared/rRNA_db/SILVA_128_QIIME_release/taxonomy/16S_only/97/majority_taxonomy_7_levels.txt" >> SILVA_clustering_params.txt
```

Finally, the command to execute the script:

```
pick_open_reference_otus.py -ipath/no_chimeras.fna -o clustering -m sortmerna_sumacust -s 0.1 --min_otu_size 1 -p SILVA_clustering_params.txt
```

If more information is needed, check: http://qiime.org/scripts/pick_open_reference_otus.html.

Remove low-confident OTUs

Despite the efforts to reduce sequencing noise, chimeras, and other possible artefacts, it is recommended to eliminate the OTUs that are “found” at very low abundances, i.e., clusters with only one or just a few reads. Although many of these OTUs are possibly true biological sequences, the probability of finding a considerable proportion of artefacts among these low-abundant OTUs is high. It is obviously an arbitrary task to decide the threshold of the OTUs that should be removed before the diversity analysis, because many factors are potentially involved in the generation of spurious OTUs: sequencing platform, strategy of analysis, coverage and complexity of the microbial community, etc. This must be tested specifically with the dataset being analysed. Again, a mock community with a known number of bacterial species can help.

As a minimum, it is recommended to filter out the singletons from the final OTU table:

```
filter_otus_from_otu_table.py -ipath/otu_table.biom -o otu_table_no_singletons.biom --min_count2
```



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Core diversity analysis

At this point, OTU table, phylogenetic tree, and metadata (map.file) are used as inputs for the diversity analysis programs. Microbial ecological studies normally include analysis of alpha and beta diversity. There are plenty of options to generate this information, for example using R (package Phyloseq among others). Qiime also provides a number of scripts to produce a diversity index and graphics from the command line, which is an interesting option to obtain an overview of the results. The script `core_diversity_analyses.py` executes a workflow that generates alpha and beta diversity analysis (http://qiime.org/scripts/core_diversity_analyses.html).

Prior to the diversity analysis, we need to normalise the OTU table. There are different strategies for this (http://qiime.org/scripts/normalize_table.html), but simple rarefaction is the most common approach. Generating a summary of the OTU table will provide us with the necessary information, i.e. the number of reads of the sample with fewer reads. For example, suppose that we obtain 10500 reads in this example. Now we can try the final core diversity analysis:

```
core_diversity_analyses.py -ipath/otu_table_rarefied.biom -t path/rep_set.tre -m map_file.txt -o path/core_diversity -e 10500
```

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9. Soil enzyme activities

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Importance and applications

Soil enzymes are specialised proteins playing a key role in organic matter decomposition and plant nutrient cycling. Enzymes react with a specific substrate and catalyse its biochemical transformation. In agricultural soils, enzymes are involved in breaking down plant residues, processing and providing nutrients (e.g. NH_4^+ , PO_4^{3-}) to crops (Marx et al., 2001). Furthermore, enzymes respond to a wide range of agricultural practices such as the use of pesticides and fertilisers as well as tillage and crop rotation. Therefore, soil enzymes are regarded as sensitive indicators of soil fertility and soil quality (Shukla, 2011).

Sample preparation and storage

Enzyme activities of field soil samples are measured as soon as possible; unnecessary sample storage must be avoided. Enzyme activities most of all depend on soil moisture and temperature. Optimum moisture conditions are 50 to 60% of water holding capacity (WHC). For determination of water holding capacity and actual water content, see chapter 3.0.

If the soil moisture content substantially deviates, soil could be either carefully dried at room temperature (20 to 22°C) before sieving or, in the case of too dry soil, water is added after sieving. Wait 1 or 2 days for soil conditioning, before starting enzyme tests. However, if that soil moisture is not optimal, it must be weighed between an optimal test performance (e.g. no enzyme activity can be expected from fully dry soil) and the research question. For example, it might be the hypothesis of a study that additional plants grown on a field will reduce the soil water content and thus the soil microbial activity. In that case it might be advisable to proceed with the original (different) soil water content of samples.

Soil samples are sieved <2 mm, fine roots and other plant litter material is carefully removed.

Principle

Assays of soil enzyme activity are carried out in the laboratory under manipulated and controlled, and thus largely optimal, conditions. Hence, it has to be stated that the methods described below estimate a potential of soil enzyme activity in soil. In general, a certain amount of soil is mixed with a specific substrate and incubated for some hours. Depending on the enzyme being assayed and the chosen substrate, the reaction product emerging during incubation consists of a quantifiable compound such as MUF (methylumbelliferyl), AMC (aminomethylcoumarin), pNP (para-nitrophenyl) or INTF



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(iodotetrazolium chloride formazan), which can be extracted and measured against calibration standards either fluorometrically or colorimetrically. Additionally, the method for the determination of the potential nitrification is presented in this chapter.

Table 3.8.2. Commercially-available colorimetric and fluorogenic substrates for soil enzyme assays.

Enzyme (EC-IUBMB*)	Fluorogenic substrate	Colorimetric substrate
Dehydrogenase (1.1.1)	-	iodotetrazolium chloride (INT)
β -Glucosidase (3.2.1.21)	4-MUF- β -D-glucopyranoside	4-nitrophenyl- β -D-glucopyranoside
Leucine-aminopeptidase (3.4.11.1)	L-leucine-AMC	-
Alkaline Phosphatase (3.1.3.1) Acid Phosphatase (3.1.3.2)	4-MUF-phosphate	4-nitrophenylphosphate disodium salt hexahydrate
Arylsulfatase (3.1.6.1)	4-MUF-sulphate	potassium 4-nitrophenyl sulphate
N-acetylglucosaminidase (3.2.1.52)	4-MUF-N-acetyl- β -D-glucosaminide	4-nitrophenyl N-acetyl- β -D-glucosaminide

* = Enzyme commission number defined by International union of biochemistry and molecular biology.

According to the literature, both approaches implicate assets and drawbacks. Colorimetric determination of enzyme activity is well established and feasible with common laboratory equipment (Nannipieri et al., 2012). Due to their higher sensitivity, especially at low concentrations, fluorogenic substrates are increasingly used to detect enzyme activities in small samples or when low activity is assumed (Kandeler in Paul, 2015). Studies comparing both procedures reported different results. Marx et al. (2001) found comparable values for maximum activity of acid phosphatase and β -glucosidase (v_{max}), when samples were incubated at increasing substrate concentrations. On the other hand, the activity of acid phosphatase assayed in soils with varying organic C content and pH values was significantly higher when 4-MUF-phosphate was used, compared to p-nitrophenylphosphate (Drouillon & Merckx, 2005). In order to obtain comparable data sets, uniform laboratory procedures are crucial. Therefore, assays of enzyme activity are supposed to be conducted as described below. The fluorogenic approach that was adapted from Marx et al. (2001) for β -glucosidase, leucine-aminopeptidase, phosphatase, arylsulfatase, N-acetylglucosaminidase and colorimetric procedure was originally described by Benefield et al. (1977) and modified by Mersi and Schinner (1991) for dehydrogenase.

9.1 Fluorogenic approach

Reagents

All required reagents should be freshly prepared.

- MES buffer (pH adjusted to 6.1) for MUF containing substrates: dissolve amount of MES 2-(N-morpholino) ethanesulfonic acid corresponding to 0.1 M and bring volume to 1000 mL with deionised water.
- Trizma buffer (0.05 M, pH adjusted to 7.8) for AMC containing substrates: dissolve 0.985 g of Trizma base and 2.66 g of Trizma HCl and bring volume to 500 mL with deionised water.
- Substrate stock solution (0.01 M): dissolve corresponding amount of substrate in 300 μ L dimethylsulfoxide (DMSO) and bring volume up to 10 mL with autoclaved water.
- Substrate working solution (0.001 M): dilute substrate stock solution in a ratio of 1:10 with corresponding buffer (MES for MUF substrates and Trizma for AMC substrates).
- 4-Methylumbelliferone (MUF) standard stock solution (0.005 M): dissolve 0.022 g of 4-methylumbelliferone in 25 mL of dimethylsulfoxide (DMSO).
- 7-Amino-4-methylcoumarin (AMC) standard stock solution (0.005 M): dissolve 0.0219 g in 25 mL of dimethylsulfoxide (DMSO).
- Standard working solution (0.00001 M): dilute standard stock solution in a ratio of 1:500 with corresponding buffer.

Materials and equipment

- Black 96-multi-well plates
- pH electrode
- Autoclave
- Mechanical homogeniser (e.g. ultrasonic disaggregator)
- Laboratory glassware
- Automatic dispenser for reagents (elective)
- Incubator, adjustable to 30°C
- Plate-reading fluorescence spectrometer, excitation wavelength at 355 nm and emission wavelength at 460 nm

Procedure

- a. Prepare a soil suspension by dispersing 1 g of sieved, field moist soil (determine the dry mass beforehand) in 100 ml of sterilised and deionised water. In order to ensure equal dispersion, use an ultrasonic disaggregator at 50 J*s⁻¹ for 2 minutes.
- b. Combine 50 μ L of soil suspension, 50 μ L of buffer (Trizma buffer for AMC and MES buffer for MUF) and 100 μ L of substrate solution in a microplate-well. Prepare each sample at least in triplicate.



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- c. Standards are prepared by mixing 50 μL of soil suspension with the corresponding amounts of standard working solution and buffer solution in order to obtain final concentrations of 0, 200, 500, 800, 1200 and 1500 pmol/well in a resulting volume of 200 μL . Standard concentrations may be extended, depending on the activity of assayed enzymes.
- d. For the consideration of quenching, add 100 μL of buffer and 100 μL of substrate for each substrate to one well.
- e. Multi-well plates are incubated for 4 hours at 30°C.
- f. Fluorescence is measured immediately after the addition of the soil suspension to multi-well plates to obtain a start value. In order to evaluate the change of fluorescence i.e. enzyme activity, further readings in constant time intervals (every hour) are obligatory. Measure fluorescence with excitation wavelength set to 355 nm and emission wavelength set to 460 nm.

Calculations

$$\alpha = \frac{(\bar{\rho}_{t2} - \bar{\rho}_{t1}) * V}{m * c * \Delta t * DM}$$

With

α	the enzyme activity expressed as nmol of MUF/AMC formed /g dry soil /hour
$\bar{\rho}_{t1}, \bar{\rho}_{t2}$	the means of MUF/AMC concentration in pmol/well at t_1 and t_2 (depending on linearity of enzyme activity)
V	the initial suspension volume in mL
m	the soil sample mass in g
c	the aliquot of soil suspension transferred to well in μL
Δt	the difference of t_1 and t_2 in hours
DM	the dry matter content of sample as a percentage

Table 3.8.3. Range of activity values for fluorogenic substrates in agricultural soils.

Enzyme	nmol MUF (AMC) $\text{g}^{-1} \text{h}^{-1}$	Land use	Soil texture	Reference
β -Glucosidase	30 - 200	Organic farming	Sandy loam	Maharjan et al., 2017
Leucine-aminopeptidase	800 - 1200	Conventional farming	Fine Sand	Awad et al., 2012
Acid Phosphatase	50 - 400	Organic farming	Sandy loam	Maharjan et al., 2017
Arylsulfatase	22 - 30	Conventional cereal cropping	Sandy clay loam	Giacometti et al., 2014
N-acetylglucosaminidase	40 - 220	Conventional farming	Sandy loam	Awad et al., 2012



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Remarks

- Fluorogenic compounds (MUF and AMC) are light sensitive. Avoid exposure to light and do not store the solutions.
- Autoclaving of MES-buffer is not recommended.
- Produce all standard solutions at once for one sample series.
- Ensure adequate soil moisture in order to obtain favourable conditions for enzyme activity.
- For database coding, the following abbreviations may be used for the different enzymes: BG (β -glucosidase), LA (leucine-aminopeptidase), AP (acid phosphatase), AS (arylsulfatase), AG (N-acetylglucosaminidase).



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9.2 Colorimetric approach

Reagents

- 1 M HCl
- Tris buffer solution (0.1 M): dissolve 12.12 g of tris (hydroxymethyl) aminomethane in 800 ml deionised water, adjust pH with 1 M HCl to 7.6 and bring up to 1000 ml.
- Substrate solution (0.015 M): dissolve 0.38 g of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) in 50 ml of buffer solution.
- Analytical grade acetone
- INTF stock solution (0.001 M): dissolve 47 mg idonitrotetrazolium formazan (INTF) in acetone and complete with acetone to 100 ml.
- Prepare INTF calibration solutions as follows:

Table 3.8.4. Preparation of calibration solutions for INTF.

INTF [nmol/mL]	0	10	20	40	60	80
INTF stock solution [mL]	0	0.05	0.10	0.20	0.30	0.40
Tris buffer pH 7.6 [mL]	1	1	1	1	1	1
Acetone [mL]	4	3.95	3.90	3.80	3.70	3.60

Materials and equipment

- Spectrophotometer
- U-bottom tubes (35-50 ml), glass cuvettes, volumetric flasks and pipettes
- Incubator, adjustable to 25°C
- Centrifuge, adjustable to 20°C and to a centrifugal force of 2000 g.
- Orbital tube shaker

Procedure

- Prepare four tubes for each sample and weigh in 2 g of sieved, field moist soil to each of them. (Make sure the exact dry mass equivalent is known).
- Add 2 mL of substrate solution to three regular samples.
- Instead of substrate, the fourth sample (control) receives 2 mL of buffer solution.
- Use a tube shaker to homogenise samples, close tubes and incubate at 25°C for 4 hours in the dark.
- Add 8 mL of acetone to all samples and put them on an orbital shaker (250 rpm) for 1 hour in the dark.
- Centrifuge samples for 5 minutes at 2000 g and transfer supernatants to glass cuvettes.



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- g. Read absorbance within 1 hour on a spectrophotometer against the calibration curve zero at a wavelength of 485 nm.

Calculations

$$\alpha = \frac{(\bar{\rho}_{regular\ samples} - \rho_{control\ sample}) * V}{m * DM * t}$$

With

α	the dehydrogenase activity expressed as nmol INTF formed ×(g dry soil) ⁻¹ × hour ⁻¹
$\bar{\rho}_{regular\ samples}$	the mean of INTF concentration of regular samples in nmol/mL
$\rho_{control\ sample}$	the value of INTF concentration of the control sample in nmol/mL
V	the solution volume (volume of substrate/buffer + volume of extractant i.e. 10 mL)
m	the soil sample mass in g
DM	the dry matter content of the sample as a percentage
t	the incubation time in hours

Table 3.8.4 Range of dehydrogenase activity in agricultural soils.

nmol INTF g ⁻¹ h ⁻¹	Land use	Soil texture	Reference
180 - 510	Organic rice production	Clay loam	Lopes et al., 2011
74 - 283	Organic potato production	Loam	Liu et al., 2008



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9.3 Potential nitrification

Principle

The following method was published by ISO (2012) as international standard ISO 15685 (ISO, 2012). In order to determine the potential nitrification (ammonium oxidation) as an estimate of the potential activity of ammonium oxidising bacteria, soil samples are incubated for 6 hours at 25°C with ammonium sulphate as substrate. The amount of nitrite formed during incubation is determined. To this end, the oxidation of nitrite to nitrate is inhibited during the incubation time by the addition of sodium chlorate.

Reagents

- A) Distilled water (MilliQ).
- B) Potassium dihydrogen phosphate, $c(\text{KH}_2\text{PO}_4) = 0.2 \text{ mol/l}$.
- C) Dipotassium hydrogen phosphate, $c(\text{K}_2\text{HPO}_4) = 0.2 \text{ mol/l}$.
- D) Sodium chlorate, $c(\text{NaClO}_3) = 0.5 \text{ mol/l}$.
- E) Diammonium sulphate, $(\text{NH}_4)_2\text{SO}_4$.
- F) Sodium hydrogen carbonate, $c(\text{NaHCO}_3) = 5 \text{ mmol/l}$.
- G) Potassium chloride, $c(\text{KCl}) = 4 \text{ mol/l}$.
- H) Stock solution A. Prepare by combining 28 ml of KH_2PO_4 (B), 72 ml of K_2HPO_4 (C), and 100 ml of distilled water (A).
- I) Test medium. Prepare by combining 10 ml of stock solution A (H), 10 to 30 ml of NaClO_3 (D), and 0.198 g of $(\text{NH}_4)_2\text{SO}_4$ (E). Dilute to 1000 ml with distilled water (A).

The final concentrations in the test medium with pH of approximately 7.2 are 1 mmol/l of potassium phosphate buffer, 5 mmol/l to 15 mmol/l of sodium chlorate and 1.5 mmol/l of diammonium sulphate. The selected concentration of sodium chlorate should effectively inhibit biological nitrate formation, while not having negative effects on ammonium oxidation. In that case, the influence of the sodium chlorate concentration should be tested beforehand. All test chemicals to be added to the test medium must be dissolved in the phosphate buffer (H) and added before diluting to 1 l (see bullet point I).

Materials and equipment

- Orbital shaking incubator, thermostatically controlled

Procedure

All samples should be prepared in triplicate. Approximately 25 g of moist soil should be used for each individual sample. The water content of soil must be separately determined (see chapter 3.0).

Initial incubation. Weigh soil samples into 250 ml flasks and mix with test medium (I) to form slurries. The volume of the test medium plus the water volume contained in the moist soil should give a precise



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total liquid volume, e.g. 100 ml. Calculate the volume of medium to be added by subtracting the volume of water in the initial soil sample from the desired liquid volume, e.g. 100 ml. Incubate the slurries by placing the flasks upright on an orbital shaking incubator, thermostatically controlled at $25 \pm 2^\circ\text{C}$. Rotation should be sufficient to keep solids suspended (175 rpm). A liquid volume of the slurry >100 ml is required if the water-holding capacity of the soil is $>200\%$ (organic soils).

Sampling of soil slurry. Take aliquot samples (2 ml) of the soil slurry after 2 h and 6 h of incubation, provided that ammonium oxidation is known to be linear over this period*. The soil slurry should be well shaken at sampling times to ensure that the ratio of solution to soil is constant during the test. Dispense samples into test tubes and add 2 ml of KCl (G) to stop the ammonium oxidation. Then centrifuge the samples at 3 000 g for 2 min, or filter. Filter paper should be of high filtration speed, while its chemical purity may be less than the highest grade. Determine nitrite by a suitable method of chemical analysis such as flow injection analysis (FIA, reference method) or continuous flow analysis (CFA, reference method); their descriptions are presented in the standards ISO 11732 and ISO 14256-2 (ISO 2005 a, b).

*If necessary, check the linearity of the ammonium oxidation over time by sampling soil slurry a number of times during the 6 h of incubation. This is likely to be necessary if laboratories are not familiar with the soil types being used in the test. Some cases of non-linearity can be corrected by ensuring aerobic conditions or supplying a carbon source.

The solutions can be stored in a refrigerator (4°C to 8°C) in order to carry out analysis within 24 h.

Calculations

Calculate the rate of ammonium oxidation [$\text{ng NO}_2\text{-N} \times (\text{g of dry mass of soil})^{-1} \times \text{h}^{-1}$] from the difference between $\text{NO}_2\text{-N}$ concentrations at different measuring times.

Typical values may range from less than 10 to more than 500 $\text{ng NO}_2\text{-N} \times (\text{g of dry mass of soil})^{-1} \times \text{h}^{-1}$

Remarks on all methods

- Any enzyme activity is pH dependent. In very acidic soil, the activity will be very low or completely absent, respectively.
- Two different phosphatases exist, the acid and the alkaline phosphatase. One has its pH optimum at pH 4.5 to 5.5, the other at pH 9. Within Diverfarming it is suggested to determine the activity of the acid phosphatase unless the pH of the investigated soils is \geq pH 7.
- Due to their sensitivity towards light, solutions containing INT and INTF should be protected from light exposure during the analytical procedure.
- Colorimetric assays of other enzymes are described in Dick (2011).
- Ensure adequate soil moisture in order to obtain favourable conditions for enzyme activity.



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10. Soil microbial biomass

General remark

Two alternative methods can be used to determine MBC. The method according to ISO 14240-2 using the chloroform fumigation extraction is preferred in the Diverfarming project. However, the method using the substrate induced respiration based on the MicroResp™ approach might also be used. The advantage of this method is that it focuses on living and active (activable) microorganisms better than the fumigation extraction method. In any case, it is not recommended to switch between methods within one study.

10.1 Soil microbial biomass carbon and nitrogen – fumigation extraction method (based on ISO 14240-2)

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Importance and applications

The soil microflora governs major soil functions and ecosystem services such as organic matter turnover and nutrient cycling, and thus, soil fertility and overall quality. This functioning very much depends – among other factors – on the number of microorganisms present in soil. The fumigation extraction method is meant to determine the biomass of the living soil microbial community (mass of intact microbial cells in a given soil) that is assessed from its carbon (C) content. Additionally, the nitrogen (N) content can also be measured.

Results on soil microbial biomass carbon (MBC) and nitrogen (MBN) can also be used to better evaluate results from test methods on microbial functions (see chapters 3.3 and 3.7). This is done by relating microbial enzyme activities (especially of endoenzymes such as dehydrogenase) to the MBC. Furthermore, the ratio of MBC to MBN is a measure of the dominance of bacteria and fungi, respectively. A typical MBC/MBN ratio of soil microbial communities has a value of 6. Lower values indicate the dominance of bacteria, higher values the dominance of fungi (Ottow, 2011).

Principle

The method described here is largely based on the ISO standard 14240-2 (ISO 1997). Basically, intact microbial cells are lysed upon fumigation of the soil sample. For fumigation, the sample is exposed to a chloroform saturated atmosphere for 24 h. Subsequently, the microbial organic matter can be easily released by extraction using 0.5 M potassium sulphate (K₂SO₄). Alternatively, the use of 0.01 M calcium



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chloride (CaCl_2) is recommended (Joergensen, 1995) and also preferred in the Diverfarming project. The carbon content in the extract is determined in fumigated and non-fumigated samples using a TOC analyser, and the difference in extracted organic carbon is calculated. To calculate the microbial biomass carbon (MBC), the difference is divided by the correction factor k_{EC} according to Joergensen (1996) and Joergensen and Mueller (1996). Microbial biomass nitrogen (MBN) is determined in a similar way, extracting nitrogen from fumigated and non-fumigated samples, calculating the difference in contents and dividing it by the correction factor k_{EN} .

The carbon content of microorganisms in a soil sample is determined analytically and can be used to make comparisons between different soil samples. If a value for actual microbial biomass is required, then such analyses are multiplied by a conversion factor derived from experiments correlating a known cell mass to carbon analysis after fumigation-extraction. All conversion factors used are related to this initial factor.

Soils

Guidance for the collection, handling and storage of soil (ISO, 2009) shall be followed, as far as applicable. Soil samples are sieved <2 mm, which should be done at a moisture content of approximately 40% water-holding capacity (WHC). Determine the soil WHC as described in chapter 3.0. The water content of samples shall be higher than 30% WHC to ensure uniform chloroform distribution and effective fumigation. Take care to avoid smearing and compaction of wet soil in this method. Samples of waterlogged soils do not have to be dried prior to analysis.

Reagents

Reagents of recognised analytical grade shall be used, including:

- Silicone grease (medium viscosity).
- Ethanol-free chloroform (Trichloromethane; e.g., Merck Art.1.02444, contains stabilisers: 2-methyl-2-butene and methanol) - WARNING - chloroform is a hazardous and highly volatile; narcotic chemical. All work must be done under a fume hood. Waste must be properly disposed of. In the presence of light, ethanol-free chloroform degrades rapidly to form phosgene gas (COCl_2) which is odourless and highly toxic. See the respective safety instructions.
- Distilled H_2O (MilliQ)
- Calcium chloride $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ (e.g., Merck Art.1.02382), $c(\text{CaCl}_2) = 0.01 \text{ mol/l}$; 1.47 g $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ in 1 L volumetric flask; dissolve in distilled H_2O . Fill up to the mark after complete dissolution. The use of calcium chloride is recommended as an alternative to potassium sulphate solution, $c(\text{K}_2\text{SO}_4) = 0.5 \text{ mol/l}$ ($p = 87.135 \text{ gA}$) which is described in ISO 14240-2.
- Soda lime (e.g., Merck Art.6839)
- Small boiling stones ('Antibumping granules'; e.g., Merck Art.7913).



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Materials and equipment

- Room, or incubator, capable of being maintained at (25 ± 2) °C.
- Implosion-protected desiccator.
- Filter paper (Whatman No. 42, Schleicher & Schüll 595 1/2, Macherey-Nagel 261 G 1/4, or similar).
- Glass beakers.
- Petri dishes.
- 250 ml Polyethylene (PE) flasks.
- 100 ml PE flasks.
- Funnels.
- Vacuum line (e.g. electric pump).
- Horizontal or overhead shaker.
- Freezer, operation at $(-15^{\circ}\text{C}$ to $-20^{\circ}\text{C})$.

Procedure

Fumigation

Weigh from each soil sample at least two moist samples (mass equivalent to 25.00 g of oven-dry soil)*, one – for fumigation – in a 50 ml glass beaker and one – the non-fumigated control sample – in a 250 ml PE flask. Immediately extract the control sample (see below).

* NOTE: In case it is necessary to use less soil, the soil mass can be reduced to 10 g. However, increasing uncertainties due to sample inhomogeneity must be expected. It is not recommended to reduce the soil mass to less than 10 g. For the following extraction, the ratio of soil mass-to-volume of extractant must be kept the same (1:4). In soils containing more than 20 % organic material (as determined according to ISO 10694), reduce the ratio of soil-to-extract to 1:4 and less (to a minimum of 1:30 for soils containing 95% organic matter, e.g. organic layers) in order to obtain sufficient extracted matter. Record the mass of soil used.

Place moist filter paper at the bottom of the desiccator. Place a beaker with soda lime on top of the paper (serves for the uptake of CO_2). Place soil samples in glass beakers into the desiccator and an additional beaker containing 25 ml chloroform (trichloromethane) and about four boiling stones. Close the desiccator and evacuate for 10-15 min using a vacuum pump until the chloroform has boiled vigorously for approximately 2 min. Close the vacuum tap on the desiccator and incubate in the dark at (25 ± 2) °C for 22 h to 24 h.

After fumigation is completed, remove the beaker containing the chloroform and the filter paper from the desiccators. Remove the chloroform vapour from the soil by repeated evacuation (6 times for 2 min each) in the desiccator. Chloroform removal must be done with care, especially in clay-rich soils. The



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remaining chloroform will be subsequently extracted and bias the carbon determination (Alessi et al., 2011).

Extraction

Transfer the soil quantitatively to PE flasks. Extract fumigated and non-fumigated control samples in the same way. Add 100 ml 0.01 M CaCl_2 (or 0.5 M K_2SO_4) and shake immediately for ½ hour on a horizontal shaker. Subsequently, filtrate through a folded filter and a funnel into a 100 ml PE flask.

(ISO method: Add 200 ml of potassium sulphate, shake bottles on a horizontal shaker at 200 r/min for 30 min or an overhead shaker at 60 r/min for 45 min and filter the extracts through a folded filter paper.)

Store extracts in a refrigerator (not more than 24 h) until further analysis. If not analysed at once, store the extracts of fumigated and non-fumigated soil samples in the freezer at between -15°C and -20°C . Homogenise frozen extracts before use, after thawing at room temperature.

NOTES: A white precipitate occurs during the storage of K_2SO_4 soil extracts (especially if the samples are frozen) because they are usually supersaturated with calcium sulphate (CaSO_4). It is unnecessary to dissolve this excess CaSO_4 because it does not interfere with any of the analytical procedures described in this method.

Cell membranes of young, living roots are also affected by chloroform fumigation. In soils containing large amounts of living roots, the pre-extraction procedure given in annex B of ISO 12420-2 should be used.

Determination of carbon in the extracts

An instrumental analysis is highly recommended using an automatic carbon analyser (NPOC) for liquid samples or continuous-flow system with colorimetric detection, instead of the chemical determination using dichromate oxidation (see chapter 8.1 of ISO 14240-2).

Calculations

Calculate the extractable organic carbon (E_c) using the following equation.

$$E_c (\mu\text{g} / \text{g dry Soil}) = [(V \times D_v) - (B \times D_B)] \times (P_k / D_w + S_w)$$

where

E_c = (organic C extracted from fumigated samples) - (organic C extracted from non-fumigated samples);

V is the C concentration ($\mu\text{g} / \text{ml}$) of the sample;

B is the C concentration ($\mu\text{g} / \text{ml}$) of the blank;

D_v is the dilution of the sample, in ml;

D_B is the dilution of the blank, in ml;



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P_k , see equation (2);

D_w , see equation (2);

S_w , see equation (2).

Calculate the microbial biomass carbon MBC using the following equation:

$$MBC = E_C / k_{EC}$$

where

$k_{EC} = 0.45$ (Joergensen & Mueller, 1996; Joergensen, 1996).

Similarly, the microbial biomass nitrogen is calculated using $k_{EN} = 0.54$.

Table 3.9.1. Range of values in different agricultural soils.

	MBC (µg/g)	OC (%) ^a	HWEC (µg/g) _b	Reference
176 arable soils from temperate climate (Germany), various soil types				
Mean	287.6	2.05	630.6	(Vohland et al., 2016)
Median	253.1	1.84	589.1	
Minimum	66.7	0.98	228.4	
Maximum	846.0	4.46	1410	
Correlation to MBC (<i>r</i>)		0.81	0.69	
	MBC (µg/g)	MBN (µg/g)		Reference
4 soils with different tillage system (Poland)				
	71.5	15.0		(Furtak et al., 2017)
	80.3	17.1		
	98.5	19.3		
	159.6	31.3		

^a OC = total organic carbon of soil; ^b HWEC = hot-water extractable carbon

Remarks

- The content of MBC is often closely correlated with the organic carbon (OC) content and hot-water extractable carbon (HWEC) content of soil. However, in contrast to OC it shows a clear seasonal variation.
- The extraction with K_2SO_4 and $CaCl_2$, respectively, yields very similar results. However, $CaCl_2$ is preferred because the highly concentrated K_2SO_4 can lead to device error in TOC analysers.
- ISO 14240-2 contains some outdated analytical techniques. The microbial biomass carbon should not be determined using potassium dichromate oxidation. Potassium dichromate is a very hazardous chemical and should no longer be used in routine analysis.



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10.2 Soil basal respiration and substrate induced respiration (SIR) by MicroResp™

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Importance and applications

The MicroResp™ is a respirometry method that measures the CO₂ evolved from soil samples over short periods of time (4 to 6 h at 25°C), using a colour-forming reaction. The method can be used to estimate active microorganisms in soil and their growth rates at the presence of various substrates (Campbell et al., 2003). The original MicroResp™ protocol was developed to be used with 96-well microtiter plates, which can be read with conventional automated plate readers (Campbell et al., 2003). The protocol was later and more recently modified to be used both in other contexts with respect to soil (Drage et al., 2012), and with larger sample quantities (Mathew et al., 2015).

The incubation of soil samples in the MicroResp™ system with a selection of carbon sources such as sugars, carboxylic acids, amino acids, polymers, amines and amides (Anderson & Domsch, 1978) allows the measurement of substrate-induced respiration (SIR) of soil microbial biomass. The magnitude of the SIR response over 0-6 h characterises the initial microbial community in soil before the growth/selection of organisms occurs by the added substrates (Degens & Harris, 1997; Anderson & Domsch, 1978). The evaluation of SIR differences after the addition of different C-sources can be used to assess the structure and functional diversity of microbial communities in soils (Degens & Harris, 1997; Campbell et al., 2003, 2008). Soil respiration induced by the addition of glucose (Glucose-SIR) has been widely used as an estimate of microbial biomass (Anderson & Domsch, 1975, 1978; West et al., 1986). It identifies a metabolically active component of the microbial community (namely a glucose inducible, or potentially active microbial biomass) and when used with selective inhibitors allows for the separation of fungi and bacteria (or other groups) contributions to the total respiratory response (Sassi et al., 2012).

The MicroResp™ method has been developed and applied mainly on soils with pH < 7, but has been occasionally used on soils with higher pH and calcite. In this regard, the original method has been modified by considering the CO₂ of the soil solution, the effects of substrates and of CO₂ itself on pH and calcite dissolution (Renault et al., 2013 and references therein).

Principle

The MicroResp™ respiration system is based on the trapping of CO₂ from soil in a gel-based bicarbonate buffer and its quantification with an indicator dye (cresol red) that responds to the pH change



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within the same gel (Fig. 3.9.1). The colour change is read on a standard laboratory microplate reader, after a defined incubation time. The trapping system consists of two microtiter plates placed face-to-face: a deep-well plate that holds the soil samples and a plate that contains the detection gel. The deep-well plate has a capacity of 1.2 mL well⁻¹ and holds about 0.45 g well⁻¹ of soil, with or without substrate. The detection plate has a capacity of 300 µL well⁻¹, holds 150 µL well⁻¹ of gel with indicator. The two plates are sealed together with a silicone rubber gasket with interconnecting holes to allow free gas exchange between the deep well containing soil and the detection well containing the detection system (Cameron, 2007). It is possible to adapt the method to microplates with different number and size of wells (Renault et al., 2013; Swallow et al., 2015). The indicator plate is read with an absorbance microplate reader (Absorbance at 570nm = A₅₇₀) just before and after 6 h of incubation. The incubation time is defined as the best compromise between the need for short incubations to prevent microbial growth/selection and the need for long incubations to reduce the CO₂ gradient between the gel and the well headspace. Since CO₂ equilibration is a slow process, an incubation time of less than 4 h may underestimate microbial respiration.

According to the work of Rowell (1995), the CO₂ evolved from soil reacts with bicarbonate:



Then, the colour of the indicator dye changes with the change in pH (Fig. 3.9.1).

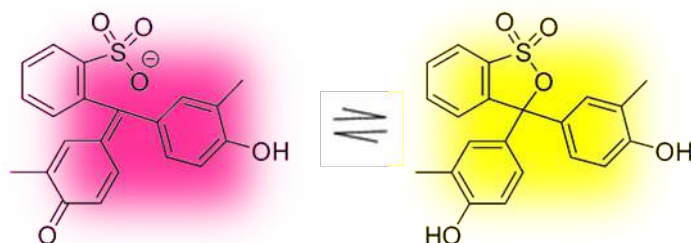


Figure 3.9.1 Reaction of cresol red (indicator dye): the H⁺ produced when CO₂ reacts with bicarbonate turns the indicator from purple to yellow (protonation).



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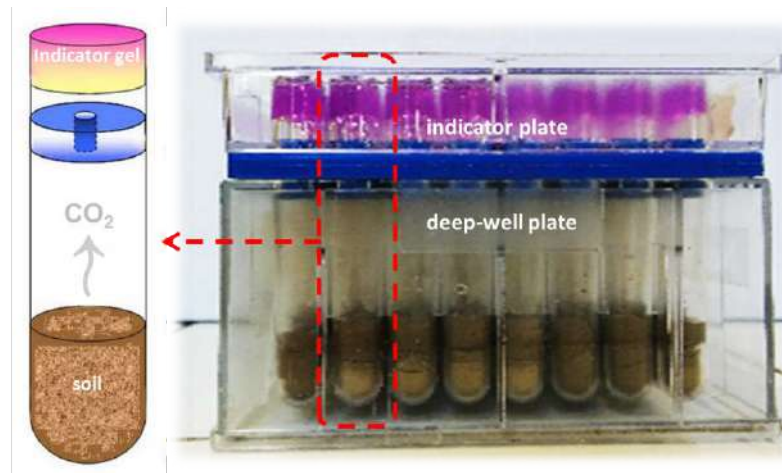


Figure 3.9.2 MicroResp™ sealed microplates and schematic diagram of the dye detection system: a deep well containing the soil sample is connected to a detection well with agar gel carrying cresol red as indicator dye. After incubation (4-6 h), the cresol red changes from pink to yellow as the pH decreases. KCl is present to reduce the effect of ionic strength on pH (from Campbell et al., 2003, modified).

Given the small amounts used, to ensure homogeneous distribution in the deep wells, the soil must be carefully sieved and mixed, and its moisture content must be pre-adjusted to the required level, i.e. $\approx 40\%$ of the water-holding capacity (WHC), so that after the carbon source in solution is added, the moisture content is (less than) 60% of the soil's WHC. Deep-well plates having larger volumes (5 ml) can be used to obtain representative samples for difficult soils.

Absorbance values are converted to CO_2 concentration [%] by construction of a calibration curve of absorbance *versus* headspace equilibrium CO_2 (Bérard et al., 2014). Campbell et al. (2003) calibrated the dye detection system by using respiration data of four different soils measured over 6 h. The best fit for the calibration curve at an absorbance of 590 nm was the exponential relationship:

$$\% \text{CO}_2 = A + B \times e^{-kx}$$

where $A = 0.222$, $B = 0.384$, $k = \ln(R)$, $x = A_{570}$, $R = 0.106$ ($P = 0.001$; $r^2 = 0.79$) (Fig. 3.9.2).

However, Cameron (2007) slightly modified the protocol by using the 570 nm optical density as the closest optimum wavelength and changing the formula used to convert the absorbance reading to $\% \text{CO}_2$. The author found a better fit for a linear-to-linear (rectangular hyperbola) curve, compared to the original formula of Campbell (2003):

$$\% \text{CO}_2 = \frac{A+B}{1+D \times A_i} \quad ,$$

where $A = -0.2265$, $B = -1.606$, $D = -6.771$.



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Reagents

- Cresol red indicator grade CAS 1733-12-6
- Potassium chloride KCl CAS 7447-40-7
- Sodium bicarbonate NaHCO_3 CAS 144-55-8
- Purified agar CAS 9002-18-0
- Indicator solution (1 L) (agar gel 10 g L⁻¹, KCl 0.15 mol L⁻¹, NaHCO_3 2.5 mmol L⁻¹ and cresol red dye 32.7 $\mu\text{mol L}^{-1}$): dissolve 18.75 mg cresol red, 16.77 g KCl, 0.315 g NaHCO_3 in 0.5 L of water in a 1 L volumetric flask over a low heat (< 50°C), bring to volume. Store at 4°C for a maximum of 2 weeks. Do not autoclave the indicator solution.
- Substrates of interest, i.e. glucose.

Materials and equipment

- 96 deep-well 1.2ml microplate (Thermo LifeSciences, Basingstoke, United Kingdom; MicroResp™).
- 96 well microplate (detection plate) (Thermo LifeSciences, Basingstoke, United Kingdom; MicroResp™).
- MicroResp™ seal
- Filling device
- Metal clamp
- Multi-channel pipettes
- Spectrophotometer Microplate Reader at 570 nm Optical Density (Absorbance = A_{570})
- Conditioning unit (thermostated dark room) for soil incubation
- Dessicator (air-tight container)
- Hot-plate stirrer for detection gel preparation
- Common laboratory equipment.

Procedure

- g. Preparation of soil samples: grind gently to disaggregate the soil and sieve through 2 mm stainless-steel sieve. Determine the soil moisture content and adjust to 40 % of the water-holding capacity (WHC) by adding deionised water. Usually, about 35-50 g of soil fresh weight is sufficient for a 96-well plate. Incubate the soil samples in a conditioning unit together with a beaker of water and a beaker of soda lime, in the dark at 25°C for 48 h (for 5 days if soil moisture has been adjusted).
- h. Preparation of detection plates: prepare the gel (3% agar) by dissolving 3g of purified agar in 100 mL of deionised water, autoclave at 121°C and allow to cool at 65°C in a water bath. Transfer 200 ml of the indicator solution to a 0.5 L beaker and warm at 65°C on a hot plate stirring constantly. When both the indicator solution and the agar gel reach about 65°C, add the purified agar (100 mL) in the indicator solution (200 mL) and mix thoroughly (agar:indicator = 1:2). Using a multi-channel pipette, dispense 150 μL aliquots per well of the indicator plate. When dispensing the agar gel into the wells of the indicator plate, take care to pipette the agar slowly into the centre of the well, omitting inclined surfaces and trapping of bubbles. Discard the first and the last dispenses and keep pipette tips warm to aid in dispensing. Store the indicator plates in the dark in a small desiccator or



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plastic box containing soda lime and a beaker of water (CO₂-free, moistened atmosphere). For longer storage cover the indicator plates with Parafilm.

- i. Preparation of substrates: 30 mg of glucose is used as C-source per gram of soil water (water contained in the soil sample). Using deionised water, prepare a stock solution of each carbon source. Deliver the stock solution in 25 µL aliquots per well of the deep-well plates, performing this rapidly for each plate because adding carbon sources increases respiration within minutes. The concentration of the stock solution must be designed to deliver 30 mg C * g H₂O_{soil}⁻¹. Calculate the amount of substrate per well (in mg) by multiplying 30 * g soil well⁻¹ * g H₂O g_{soil}⁻¹. To calculate the weight of soil in each well (g soil well⁻¹), divide the weight of the soil in one plate by the number of wells filled. Remember to record the position of each C-source on a template (the detection plate configuration will be the reverse of the deep-well plate configuration). According to the soil characteristic, the C-sources can be dispensed before or after the addition of soil to the deep-well plate, ensuring that the soil contacts the substrates at the same time for all wells.
- j. Calibration: determine the calibration curve for absorbance A₅₇₀ versus headspace equilibrium CO₂ concentration by parallel measurement of soil respiration. Equilibrate the dye solutions at different CO₂ concentrations prepared with standard gas mixtures (Rowell, 1995). Alternatively, incubate four different soils in jars for 6 h, with or without glucose (30 mg C × (g H₂O_{soil})⁻¹), and measure the headspace CO₂ concentration every 2 h. Place 4 microtiter detection wells (breakable CombiStrips of the Thermo LifeSciences) in each jar, prepared as prescribed by the MicroRespTM protocol, to be reassembled and read with the plate reader when required.
- k. Setting-up: place the soil into the deep-well plate by using the MicroRespTM filling device, which is positioned over the deep-well plate through a false bottom¹. Place 300 µL of soil into the filling device, tapping gently to ensure consistent packing². The soil moisture content must be within 30 and 60% of its maximum WHC to not affect the microbial activity and to easily manipulate the soil, it is therefore possible to adjust the aliquot of substrate stock solution delivered to the soil samples according to the soil characteristics. Record the weight of the placed soil, and then remove the false bottom, allowing the soil to fall into the deep wells and make contact with the C-source solutions, if already dispensed. The weight of the sample per well is approximate, since the method is on a volumetric basis. Add the C-sources (if not already dispensed), and immediately seal the deep-well plate with the gasket and proceed with the measurement. When using more than one soil, use tape to isolate columns of the filling device and of the plates before filling.
- l. Measurement: switch on the spectrophotometer and read the indicator plate with a microtiter plate reader at 570 nm (time 0, store electronically), then place it firmly on the MicroResp gasket and seal the system closing the metal clamps (be aware that the soil sample in slot A1 is measured by slot A12 of the indicator plate). Incubate for 6 hours at 25°C in the dark, then disassemble, peel off the gasket and repeat the absorbance measurement at 570 nm (time 6). A new indicator plate may

¹ It is recommended that samples are run at least in triplicate to ensure the best estimate of a mean absorbance per carbon source.

² The deep-well plates can be prepared the day before the MicroRespTM set-up, in this case cover the plates with Parafilm and store overnight at 4°C. Allow to warm at room temperature before use.



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be attached to continue respiration measurements over time (time i^{th}). It is advisable to estimate the initial CO_2 partial pressure (usually 0.04 - 0.1% in lab air), by reading the absorbance of empty wells.

Calculations

Export the absorbance values of time 0 and time 6 to a spreadsheet, transpose in columns and normalise data. Then, convert the absorbance values to headspace CO_2 concentrations using the calibration curve of Cameron (2007):

$$\% \text{CO}_2 = \frac{A+B}{1+D \times A_i} \quad ,$$

where $A = -0.2265$, $B = -1.606$, $D = -6.771$.

This curve fitting was calibrated over 6h on soil $\text{pH} < 7$ using a Vmax microplate reader (Molecular Devices, USA) at A_{570} . It is advisable to recalibrate the method for individual laboratories, spectrophotometers, type of environmental samples and incubation conditions.

The soil basal respiration (RESP) and the microbial biomass (MBC) are calculated according to Anderson and Domsch, 1978, after adding separately distilled water and glucose to the soil samples. The method assumes the induced respiration (SIR) to be proportional to active microbial and fungal biomasses (Anderson & Domsch, 1978; Campbell et al., 2003).

The CO_2 respiration rate per gram of dry soil per hour RESP ($\mu\text{g CO}_2\text{-C g}^{-1} \text{h}^{-1}$) is calculated as follows:

$$\text{RESP} = \frac{\left(\frac{\% \text{CO}_2}{100} \right) \times V \times \left(\frac{44}{22.4} \right) \times \left(\frac{12}{44} \right) \times \left(\frac{273}{273 + T} \right)}{Wf \times \left(\frac{Wd}{100} \right) \times h}$$

T = the incubation temperature ($^{\circ}\text{C}$);

V = the well headspace volume (μL), normally 945 μL for the standard method set-up;

Wf = the soil fresh weight per well (g);

Wd = the soil sample dry weight (%);

h = the incubation time (h);

The microbial biomass (MBC) is calculated according to Anderson and Domsch (1978):

$$\text{MBC} = 40.4 \times \text{basal respiration} + 0.37$$



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Two ecophysiological indices, i.e., the microbial coefficient MBC/OC (%) and the microbial metabolic quotient qCO_2 ($mg\ CO_2\text{-}C\ g^{-1}\ MBC^{-1}$) can be derived:

$$qCO_2 = \frac{\text{basal respiration} \times 1000}{MBC}$$

The metabolic quotient indicates the maintenance energy requirement of soil microbial communities and MBC/Corg reflects the carbon availability for the growth of soil microbes (Anderson 2003), both are used to assess the responses of soil microbial communities to changes in environmental conditions.

Remarks

- Suitable substrates: amino acids (L-arginine, γ -amino butyric acid, L-alanine, L-cysteine-HCl, L-lysine-HCl, and N-acetyl-glucosamine), aromatic carboxylic acid (3,4-dihydroxybenzoic acid), carbohydrates (D-Fructose, D-galactose, D-glucose, L-arabinose, and D-trehalose), carboxylic acids (citric acid, L-malic acid, and oxalic acid) etc.
- Indicator plates can be regenerated in a plastic box containing soda lime and a wet tissue paper, re-equilibration takes between 24 and 36 hours after which the indicator gel returns from yellow/orange to dark red/purple. However, storage affects the properties of the gel: soda lime partly extracts CO_2 supplied as $NaHCO_3$, and dries out the gel, which can skew the calibration (Renault et al., 2013). Therefore, discard all plates that at time 0 display a coefficient of variation > 5% (% CoV).
- Deep-well plates can be cleaned from soil and be reused.
- For some soils, the CO_2 evolution can be overestimated due to calcite dissolution associated with CO_2 -induced change in soil solution pH (Oren & Steinberger, 2008).
- Keep firm control over temperature, which can affect all the thermodynamic constants as well as microbial activity.

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11. Earthworm sampling

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Importance and applications

This protocol describes the method for estimating the population density and mass of earthworms. Earthworms are common macrofauna in many temperate and boreal arable soils. Their activities contribute to chemical, physical and biological aspects of soil quality (Bertrand et al., 2015). The influences are in many respects beneficial for soil conditions and earthworms may notably enhance yields, particularly in low input systems (van Groenigen et al., 2015). Earthworm species can be divided into ecological groups with characteristic soil impacts and responses to field management. While earthworm diversity in an arable field is typically relatively low, all ecological groups are often present, motivating the study of community composition. Due to their pivotal role in soils, well known taxonomy and the relative simplicity of sampling them, earthworms are a preferred indicator group in applied soil ecological studies (Griffiths et al., 2016)

Principle

Earthworms will be sampled by combined soil hand-sorting and chemical extraction following the forthcoming update of the ISO-standard for earthworm sampling (ISO 23611-1:2006) with small modifications. In the new version of the ISO-standard, mustard-oil (allyl-isothiocyanate (AITC)) will replace formalin as the extraction chemical. The procedure has already been successfully applied in studies on arable land (e.g. Epie et al., 2015).

In practice, a topsoil block is first excavated and earthworms are hand-sorted from the soil. This is preferably done in the field but when that is not possible samples can be transported to the laboratory for sorting. With hand-sorting one obtains mainly plant litter layer (*epigeic*) and topsoil (*endogeic*) dwelling earthworms. The time it takes to sort a sample varies considerably. For instance, well-structured and easily friable medium or coarse textured soils without dense root mat are many times faster to sort than compacted clayey soils with dense roots. In any case the sampling is laborious and should preferably be done by a group of people.

Chemical extraction is done simultaneously by pouring extraction solution at the bottom of the sampling pit and collecting the earthworms which emerge from the subsoil irritated by the solution. The chemical extraction is done mainly to obtain deep burrowing, *anecic* earthworms which live in vertical, deep penetrating burrows (often > 1 m). When anecic species are absent from a site and their significant increase at the site over the experiment can be ruled out, chemical extraction can be omitted. The presence of anecics can be recognised from the soil surface by the presence of middens (mixtures of collected litter and surface casting) above the openings of their burrows (\varnothing up to 7-10 mm). Anecic earthworms are negatively affected by strong and frequent tillage and in yearly mouldboard ploughed fields the species is usually absent.

Earthworms are stored in formalin in the field and later transferred to alcohol for mass measurements and identification. Despite the growing reservations against the use of formalin (because of human



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health issues and its negative effects on non-target organisms), formalin is here recommended as the fixative because of its good preserving properties compared with e.g. alcohols. If the usage of formalin is regarded as unacceptable, fixing in alcohol is possible. Formalin cannot be used if DNA studies are planned with the material.

The sampling is done during a season when the topsoil is sufficiently moist and cool for high earthworm activity. This can be ascertained prior to sampling by observing earthworm activity in a few spadeful to a depth of 20 cm across the study site. If many earthworms are curled in balls (the aestivation position of endogeic earthworms in particular) it indicates excessively dry and warm or too cold conditions for sampling.

The location of the samples at the study sites is adjusted with the overall setting of the soil sampling program as necessary. The number of samples per plot or field needs must be determined case-by-case taking into consideration the size of the area under investigation. The field work is likely to last for several days and over this period environmental conditions may change enough to affect the activity and depth distribution of earthworms. It is therefore necessary to plan the sampling sequence so that there is no risk for bias due to temporal variation in earthworm activity.

Reagents

- Allyl-isothiocyanate (AITC, mustard oil) [synthetic grade (about 94% to 97% (volume fraction))]
- Isopropanol (2-propanol)
- Ethanol [70% (volume fraction)]
- Formalin [formaldehyde solution 4% (volume fraction)]

Warning – Appropriate precautions must be taken when working with mustard-oil and formalin. Both are highly irritating substances which should not be breathed, swallowed or come into contact with skin or eyes. Protective clothing and gloves must be used, and the use of goggles is recommendable. Preparation of the solutions should be done in the laboratory in a fume cabinet or under a local exhaustion system with an eye-wash bottle available. For further advice, please refer to operational safety bulletins of the products and your own laboratory's safety guidelines.

Materials and equipment

In the field:

- measure and pegs for determining and marking the positions of the sampling points (high-precision GPS may be used when available)
- garden scissors (for cutting the vegetation)
- frame (wire or wooden) or a board, 25 cm x 25 cm (to mark the sampling area)
- spade, preferably with a straight, flat blade (the depth of the sample, 20 cm, can be marked on the blade)
- large plastic sheets or trays, preferably white, for sorting soil blocks
- rubber gloves and forceps for the hand-sorting and collection of the worms
- containers (e.g. 2 l) and fresh water where earthworms are picked
- trowel or knife (to level the bottom of the sampling pit if necessary)
- extraction chemical in small bottles (see "Preparation of mustard oil solution" below)
- large water container, 20 litres or more (for preparing the mustard oil solution)
- sprinkling cans (for pouring the mustard oil solution)
- watch/timer (to control the length of the chemical extraction)



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- sample vials with watertight tops for each individual sample (e.g. 250 ml)
- fine-meshed sieve (to ease the transfer of earthworms from water to sample vials)
- water resistant marker/pencil and paper labels to put into sample vials (vials can be additionally marked outside)
- 4% formalin preservative (1:9 dilution of 40% formaldehyde in water)
- notebook for keeping diary

Additional notes for the field:

- If fresh water is not easily available at the site, a sufficient amount must be taken to the field.
- It is useful to measure the topsoil (e.g. 0-15 cm) temperature and moisture daily to demonstrate the suitability of general conditions for sampling.
- Hand-sorting is made more comfortable if the sample can be handled on a camp table. An all-weather work tent allows field-sorting in bad weather. In bright sunshine, too, hand-sorting in a tent is convenient (no sharp shadows disturbing the procedure).

In the laboratory:

- paper towels
- rubber gloves
- fine-meshed sieve
- Petri dishes
- forceps
- balance
- stereo microscope
- taxonomical key
- data sheets

Procedure

Field work:

- a. Preparation of mustard oil solution. (*Carefully follow safety instructions.*) In the laboratory, 2 mL of allyl- isothiocyanate is mixed into 40 mL of isopropanol in small bottles. One bottle (42 mL) of the concentrate is enough to make 20 litres of mustard oil solution used in extraction. Several bottles can be made and stored in a refrigerator. Bottles are transported to the field in a cool box.
- b. Sampling point location. (*One should take into consideration the need to adjust the points spatially with other soil sampling points.*) While the random positioning of samples could be preferable, systematic placing is practically convenient, e.g. for later sampling at the site, when sampling at the exact same points needs to be avoided. Replicate samples can be positioned along a transect at the central area of the treatment. The number of samples and their intervals depends on the area under investigation. In an experimental plot scale 4-6 samples at a few metre distances is suitable. When treatments are compared at field scale, a larger number of samples with wider distances are needed (for instance 10 samples separated so that the whole field is covered). In selecting the exact sampling spot, it is good to avoid clearly aberrant spots such as deep wheel tracks. Margin areas with high field traffic should in general be avoided. Meter readings (or coordinates) of sampling point positions are taken for later reference. When the sampling is repeated, the sampling should follow the same transect pattern, with the new samples taken at least 2-3 m away from the earlier sampling spots.



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- c. Soil block hand-sorting. Vegetation is cut and removed from the sampling point. The frame or board (25 cm x 25 cm) is placed on the ground to mark the sample area and a soil block is taken with a spade to a depth of 20 cm. Digging is done as much as possible along the sample margins, as cutting inside the block increases the proportion of injured worms. The sample is placed on the sheet or tray and earthworms are hand-sorted from the soil aiming at finding even the smallest specimens (newborn individuals can be only 10 mm in length). The bottom of the pit can be levelled with a trowel or knife to ease the subsequent chemical extraction. When the soil is not easily friable and needs to be actively broken during sorting (as in the case of plastic clay) one must decide roughly to how small pieces breaking is done ("tip of a thumb" -size, for instance). Dense roots can be hard to handle but they need to be closely sorted. Earthworms and pieces of them are placed in a deep vessel which has cool water and is kept in shadow (e.g. in a cool box). It is necessary to keep an eye on this to ensure that earthworms do not escape from the vessel.
- d. Chemical extraction. (*Done when the presence of anecic earthworm species can be expected*). Mustard oil solution is prepared in the field just before the sampling starts. The concentrate prepared in the lab (42 mL volume of AITC + isopropanol) is added in 20 litres of water and mixed thoroughly. Despite the dilution, the solution is irritating and the safety precautions apply. The amount of solution needed for the extraction depends on the infiltration rate which varies greatly depending on soil conditions. It is therefore not necessary to aim for the same application rate for all pits. A maximum of 5 litres of solution per pit should be enough and the extraction time is set to 20-30 minutes (the same for all pits) depending on the pace of earthworm emergence. One can start by pouring 1-2 litres of the solution from the sprinkling can at the bottom of the pit and follow the infiltration. When the infiltration rate is high, application is continued with fair additions over the whole extraction time. In case of extremely low infiltration, one can keep the bottom of the pit slightly covered with solution. Emerging earthworms are picked up with forceps or by hand (using rubber gloves) in a vessel with water (as above). One should pick up a worm only when it has fully emerged from its burrow, preferably touching the head end of the worm (to avoid autotomy).
- e. Preservation of the specimens. Water vessels with the collected earthworms are poured on a fine sieve and the worms are picked in sample vial with 4% formalin. Samples are coded on paper labels which are placed in the vials. It is helpful to also mark the code outside the bottle.
- f. Filling of the pit. After the sampling, the pit is filled with the hand-sorted soil.

Laboratory work:

- a. Change of preservation liquid. The samples should be kept in formalin for at least four days, but preferably for one to two weeks. After that the samples are changed to 70% ethanol where they can be stored for unlimited time waiting for identification and measurements. Rinsing the sample in fresh water before transfer into ethanol is recommendable.
- b. Determination of mass and species identification. Before weighing and identification, earthworms are rinsed in water, quickly dried on paper towel and subsequently have their mass determined using a suitable balance. The specimens are then identified as closely as possible (to species or genus) with the help of a stereo microscope and using standard keys. If no taxonomical identification is possible, a note on the pigmentation is useful ("pigmented" (epigeic and anecic species) or "non-pigmented" (endogeic species)). Developmental stage is finally recorded: adult (fully developed clitellum ("saddle")), sub-adult (tubercula pubertatis visible, no fully developed clitellum), juvenile.



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- c. Data recording. Each specimen is recorded on its own row in the raw data Excel-file. The coding of date, location, treatment, replicate etc. follow the common project guidelines. For earthworms (and pieces of earthworms) the additional data columns are (i) sample number (sample position in transect), (ii) species or genus of the specimen, (iii) ecological group (epi, endo, ane), (iv) pigmentation (when no other identification), (v) developmental stage (ad, subad, juv), (vi) mass, g (with 2-3 decimals), (vii) condition (for pieces of earthworms: tail, head, other).

Calculations

For each sample point total, species-wise and ecological-group-wise density and mass are calculated by first summing the figures in the sample appropriately and then multiplying by 16 to obtain per square metre values which are commonly used in earthworm ecology (sample area is 1/16 m²). In case of earthworm pieces, all parts are used for total mass, only head pieces for total density. For the description of community composition percentage values of species, genera or ecological groups will be calculated. In the statistical comparisons of the treatments, plot (or field) means can be used.

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12. Vegetation diversity, cover and structure

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Importance and applications

The assessment of vegetation richness and cover is an essential indicator of how cropping systems and/or management practices contribute to enhance biodiversity. Since plants act as feed source and niche for many animals, the increments in vegetation diversity and cover lead to increments in animal biodiversity (Lavelle et al., 2014). Most studies dealing with crop diversification, and mostly with intercropping, have shown that diversified cropping systems have lower populations of phytophagous pests than monocultures owing to increased vegetation richness, diversity and cover (Lopes et al., 2012). In this sense, the agroecological theory predicts that the higher the diversity of plants, the higher the diversity of herbivores. This, therefore, determines a higher diversity of predators, resulting in more complex food chains. As a consequence, the promotion of high vegetation diversity and cover can considerably reduce the use of external inputs such as pesticides (Gurr et al., 2012). Furthermore, an increase in vegetation cover is related to land protection and thus to soil conservation. The higher the vegetation cover during most of the year, the lower the periods when the soil remains bare, and so the risk for soil erosion by wind and runoff is decreased. This leads to healthier soils which can sustain higher productivity of healthy crops.

Principle

The quadrant method is one of the most common ways to sample vegetation. Quadrants make samplings more homogenous than transects. The method consists of placing a square over the vegetation to identify plant species (richness) and determine vegetation cover. To sample grasslands or cereals, the size of the square should be 1 m × 1 m. To sample annual crops or small woody species (shrubs), the size should be 2 m × 2 m, or 4 m × 4 m, depending on the dimensions of the plants. For trees, quadrants should be at least 5 m × 5 m. Evidently, the concrete size of the quadrant depends on the density of the plants.

To characterise vegetation structure, and how different cropping systems and management practices can affect it, similarity coefficients can be used. There are different similarity indices, but the oldest indices are still the most used ones, such as the Sorensen Index. This index can be easily calculated with qualitative data (presence/absence), giving important information about the effect of cropping systems or management practices on vegetation diversity and structure.



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Reagents

- None

Materials and equipment

- Guide for plant species identification.
- Quadrants of variable size depending on the density of vegetation (1 m × 1 m for grasslands and cereals, 2 m × 2 m or 4 m × 4 m for annual crops or shrubs, 5 m × 5 m for tree plantations). Quadrants can be made of rope or tape tied to four stakes.
- Hammer to place the stakes in the soil.

Procedure

- Identify, seasonally, all plant species present in your plots, including borders with native vegetation to increase biodiversity and attract beneficiary fauna. Distinguish crop species from other native or spontaneous species. Use a guide for plant species identification if needed. Guides with regional information are more effective for field identification than generalist guides. It is important that all seasons are well characterised. Record the name of all different species per season. The number of different species identified is the **Richness**.
- Place the quadrant (select the size depending on your crop type) randomly on the plot. Record the vegetation cover as the percentage of land covered by plants in relation with the entire quadrant surface, considering the entire area covered by the quadrant as 100%. Repeat this process at least four times in each plot, making sure that the selection of the quadrant location is random. Take the average of the vegetation cover for all repetitions. This procedure should be seasonally performed to assess the effect of crop cycles of the different cropping systems.

Calculations

- Richness: number of different plant species present in each plot, per season and per year.
- Vegetation cover: percentage of the total land surface covered by vegetation in each plot estimated by the quadrant method. Provide the average value of at least four different measures.
- Similarity index (SI). The similarity index is calculated as follows:

$$SI = \frac{2C}{A+B} * 100$$

A: the number of species found in plot A in the entire year

B: the number of species found in plot B in the entire year

C: the number of common species in both plots A and B in the entire year

All alternative cropping systems (plot A) should be evaluated against the current farm cropping system and management (B) to really demonstrate that diversified cropping systems change the vegetation structure of the agro-ecosystem. Different diversified cropping systems should also be compared to each other to assess how similar the vegetation is.



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