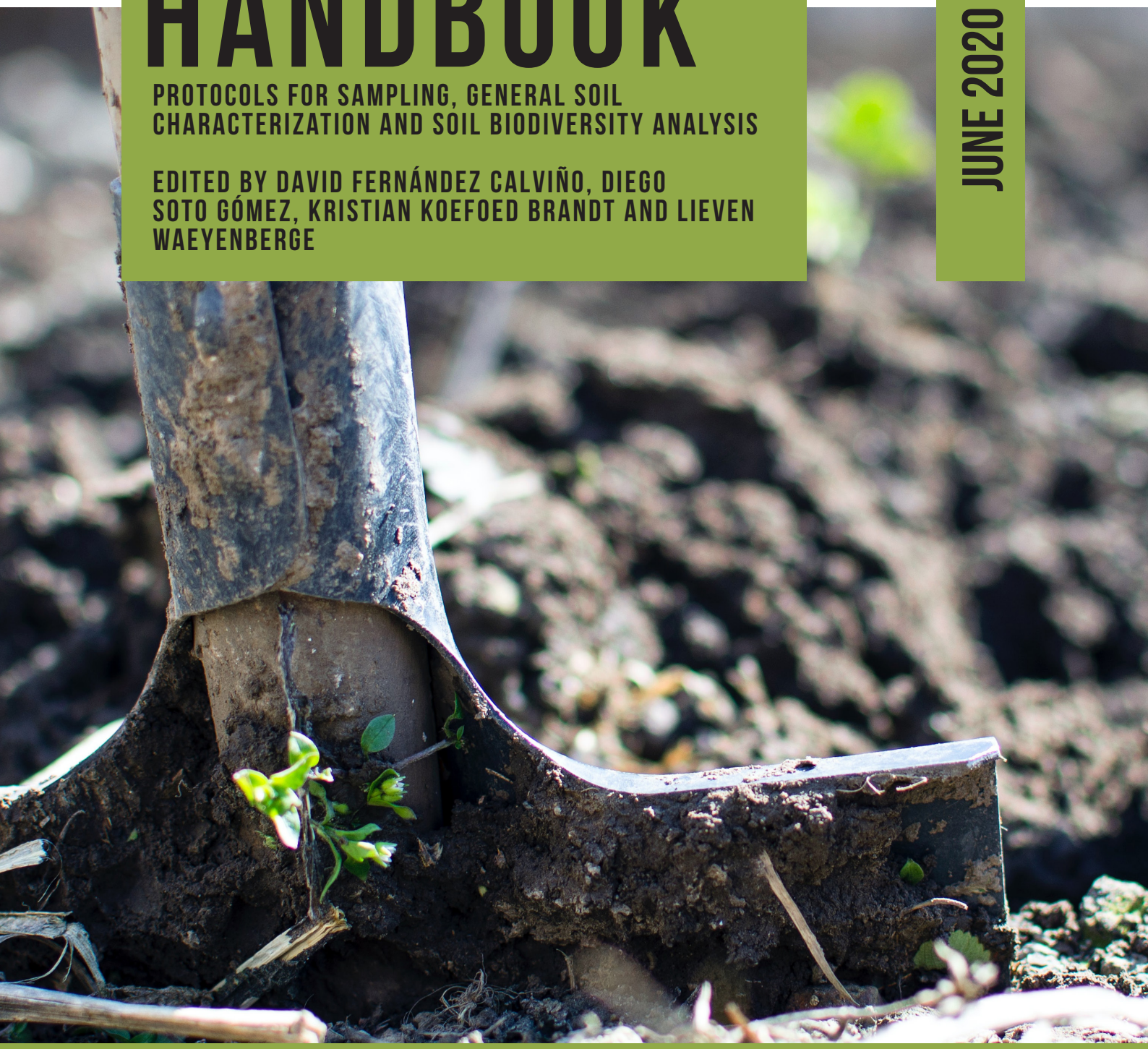
HANDBOOK

PROTOCOLS FOR SAMPLING, GENERAL SOIL CHARACTERIZATION AND SOIL BIODIVERSITY ANALYSIS

EDITED BY DAVID FERNÁNDEZ CALVIÑO, DIEGO SOTO GÓMEZ, KRISTIAN KOEFOED BRANDT AND LIEVEN WAEYENBERGE

JUNE 2020



SOIL SAMPLING

PHYSICAL ANALYSIS

CHEMICAL ANALYSIS

BIOLOGICAL ANALYSIS

WHEAT QUALITY

ANALYSIS

WWW.SOILDIVERAGRO.EU

This handbook is a deliverable of WP3: Soil biodiversity assesment in European cropping systems of the SoildiverAgro project financed by the European Union´s Horizon2020 research and innovation programme under grant agreement Nº. 817819.

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JUNE 2020
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CONTENT

Soildiver Agro

PREFACE

The idea of this handbook arises from the need to define, standardize and organize the different methodologies used in work package 3 (WP3) of the SoildiverAgro project. This work represents a compendium of methods, in which procedures for the determination of a series of soil and wheat properties are described. Each chapter of the handbook includes a first section with a slight explanation of the property to be determined, followed by a list with the necessary materials, a step-by-step explanation of the procedure in question, the calculations necessary to obtain the desired property, and a series of important tips or points to improve the procedure. Each chapter also includes a specific bibliography section.

This volume is divided into five sections. The first one is focused on the soil sampling process: a specific sampling is required for some properties such as bulk density or microbial diversity. The second section focuses on the physical characteristics of the soil and includes aspects such as particle size distribution and aggregation. The third part focuses on chemical aspects of the soil that can affect their fertility and the development of microbial communities. The fourth section focuses on the methods that allow characterizing the diversity of soil microorganisms, nematodes and earthworms. Finally, section five is dedicated to the characterization of wheat grains and focuses, above all, on quality-related properties.

Numerous people from various countries have participated in the realization of this handbook, and the editors want to express their special thanks to all of them.

THE EDITORS

1 SOIL SAMPLING

BY LIEVEN WAEYENBERGE

ILVO, FLANDERS RESEARCH INSTITUTE FOR AGRICULTURE, FISHERIES AND FOOD, PLANT SCIENCES UNIT, B-9820 MERELBEKE, BELGIUM

Soil sampling and analysis is a reliable method to determine important characteristics of agricultural fields including biological, chemical, environmental, and physical properties. Soil testing is basically a three-step process: (i) the collection of a representative sample from a field or section, (ii) proper analysis of that sample to determine a selection of soil characteristics and (iii) keeping record of the data for future data-mining. In order to standardize the methods for sampling, sample processing, data acquisition and data storage/sharing across different partners (Table 1.1.), procedures, workflows or protocols have to be selected, if needed developed or adapted, and eventually carefully noted and distributed. The following sections, each containing detailed descriptions of one or several actions belonging to the sampling event conducted for WP3 "soil biodiversity assessments in European cropping systems" of the EU-H2020 SoildiverAgro project (EU-H2020 project 817819), constitute a Handbook.

Table 1.1. List of the partners in charge for the soil sampling event par pedoclimatic zone

PEDOCLIMATIC ZONES & PARTNERS IN CHARGE FOR THE SOIL SAMPLING EVENT			
PEDOCLIMATIC ZONE	COUNTRY	PARTNER IN CHARGE	OTHER PARTNER(S) INVOLVED
Atlantic North (ATN)	Denmark	UCPH	
Atlantic Central (ATC)	Belgium	EV-ILVO	PSKW, INAGRO
Boreal (BOR)	Finland	LUKE	TT
Continental (CON)	Germany	TI	FAR
Lusitanean (LUS)	Spain	UVIGO	INORDE, RGG
Mediterranean North (MDN)	Spain	UPCT	ASJA
Mediterranean South (MDS)	Spain	UPCT	ASJA
Nemoral (NEM)	Estonia	EULS	MTÜPK
Pannonian (PAN)	Hungary, Serbia	EV-ILVO	

The main objective of WP3 is to establish a reference for biodiversity in different European pedoclimatic regions in relation to pedoclimatic zones, soil characteristics and agricultural management regimes (conventional and organic farming). The necessary data will be gathered by analysing soil samples taken from different agricultural conventional and organic management systems in different European croplands. This is accompanied by filling in a 'Soil Sample Information Sheet' with cooperation of the owner of the field sampled (Annex 1). This information sheet' assembles data about the geographical location and historical facts linked to the field in question. The historical facts include cropping rotation, tillage practices, type of fertilization, use of pesticides, weather conditions (especially anomalies like flooding's, extreme drought etc.) and notions on wheat yield and quality (obvious wheat yield and quality losses, presence of pathogens etc.) during the last 5 years.

WP3 is a one-time sampling event. It was decided to sample shortly after harvest of the main crop (winter or spring wheat 2019), with the exact timing depending on the region (environmental conditions), but preferably 1-2 weeks after harvest and before any next step in the agricultural management system. In this way, we aim to obtain comparable data by reducing temporal variation in biodiversity. However, a longer period of drought or hot weather can create non-favourable conditions for sampling for nematodes and even more so for earthworms. Hence, each partner should decide for itself whether the sampling can be done shortly after the main crop or should be postponed for a short period and if possible still before any next step in the agricultural management system. If such is unavoidable, a sample can be taken later but this should be clearly noted during data acquisition and storage on the metadata file.

Special attention is needed concerning the selection of the fields to be sampled. The aim is to select at least 10 farms, preferably 5 conventional and 5 organic, already conducting the mentioned agricultural management regime for at least 5 years. Farms following organic management for less than 5 years are allowed only if it is not possible to access suitable long-term organic farmland. Ideally, pairs of fields, 1 organic and 1 conventional, in the same area (like neighbouring farms) should be selected to ensure equal pedoclimatic conditions and similar soil characteristics. It should be avoided to sample only farms within one small region (for example 1 sub-district or town). In fact, the larger the geographic distribution of the selected pairs of farms within 1 pedoclimatic region, the better. However, to avoid excessive costs due to extreme distances, administration issues and time spent, the sampling event is allowed to remain within the borders of a country or national region.

A conventional management regime normally includes tillage practices, while an organic management regime generally tries to reduce tillage practices. Farms deviating from this should be avoided. The cropping rotation system on the selected fields should include long-term wheat production (at least 5 years). Farms setting up a field trial for WP5 “Impacts of soil biodiversity on crop production and other ecosystem services”, can be amongst the selected farms, especially when the farmer agrees to become part of the ‘Community of Practitioner’, but under the condition that wheat is part of the crop rotation system. Sampling field trials already established before the start of this project should be avoided as different treatments tested before can have a strong influence on the soil’s properties.

From each farm, 2 fields will be sampled. This will bring the total number of fields to be sampled up to 180; 9 pedoclimatic regions x 10 farms (5 conventional and 5 organic) x 2 fields. Evidently, we allow the farmer to assist upon the selection of the fields to be sampled on his farm. If the farmer has only one field on which wheat is cultivated, then the second field should be replaced by a field from an additional farm in the neighbourhood. This will increase the number of farms but maintain the number of samples collected. In some regions there are farm-cooperatives, which cover a lot of fields in a large area, which is managed centrally by the cooperative. The address is the cooperative and not the individual farm any longer. In this case it is possible to sample more than two fields in a cooperative.

The ‘WP3 Sampling Scheme’ (Annex 2) provides a practical overview of the whole procedure including the soil sampling itself, the number of samples to be taken and the pre-processing steps like transport, storage and distribution of the necessary subsamples to the different partners responsible for the analysis of a set of data (see table 1.2.1. in section 1.2.).

Importantly, each farmer should fill in a ‘document of consent’ (Information sheet/Informed consent template; Annex 3). This document gives a brief description of the aim of the SoildiverAgro-project and the collaborating project LEX4BIO (H2020 project 818309). It also provides a clarification on the purpose of the farmer’s participation including the risks, benefits and rights linked to his/her participation. Finally, it includes an explanation about the use, storage and, protection of the data. By signing the document, the farmer declares to agree upon the sampling on his/her field(s) and upon the use and distribution of the data linked to the sample(s). Of course, it remains the intention not to publish any personal information or information that can connect the data directly to the farmer as clearly mentioned in the ‘document of consent’.

ANNEX 1.1 SOIL SAMPLE INFORMATION SHEET

Soil Sample Information Sheet		
	Sampler	**Farmer**
Name		
Institute/farm		
Address		
City		
Country		
Tel.		
Cell phone		
email address		
		Community of Practitioner: ☐ Form consent signed: ☐

	Field 1 information	**Field 2 information**
Location		
Pedoclimatic region		
GPS coordinates		
Field code		
Agric. management system	Conventional ☐ Organic ☐ Other: Number of years:	Conventional ☐ Organic ☐ Other: Number of years:
Field surface (ha)		
Area sampled (ha)		
Soil color (encircle)	■ ■ ■ ■ ■	■ ■ ■ ■ ■
Topography (describe relief and surroundings)		
Weather conditions	sunny ☐ cloudy ☐ rainy ☐ windy ☐ other:	sunny ☐ cloudy ☐ rainy ☐ windy ☐ other:
temperature	<0°C ☐ 0-10 ☐ 10-20 ☐ 20-30 ☐ >30 ☐	<0°C ☐ 0-10 ☐ 10-20 ☐ 20-30 ☐ >30 ☐
vegetation	crop ☐, which: fallow ☐ herbs ☐ remnants of harvested crop ☐	crop ☐, which: fallow ☐ herbs ☐ remnants of harvested crop ☐

Important: take photographs (not location detectable), transport samples in cool box (+cool packs), desinfect augur between fields, collect 1 kg wheat grains, re-fill pit after earthworm collection

	Sampling details field 1		**Sampling details field 2**	
Sampling date		start time: end time:		start time: end time:
Augur diameter				
Core depth				
Number of cores				
Pit dimensions (*)				

(*) earthworm assessment

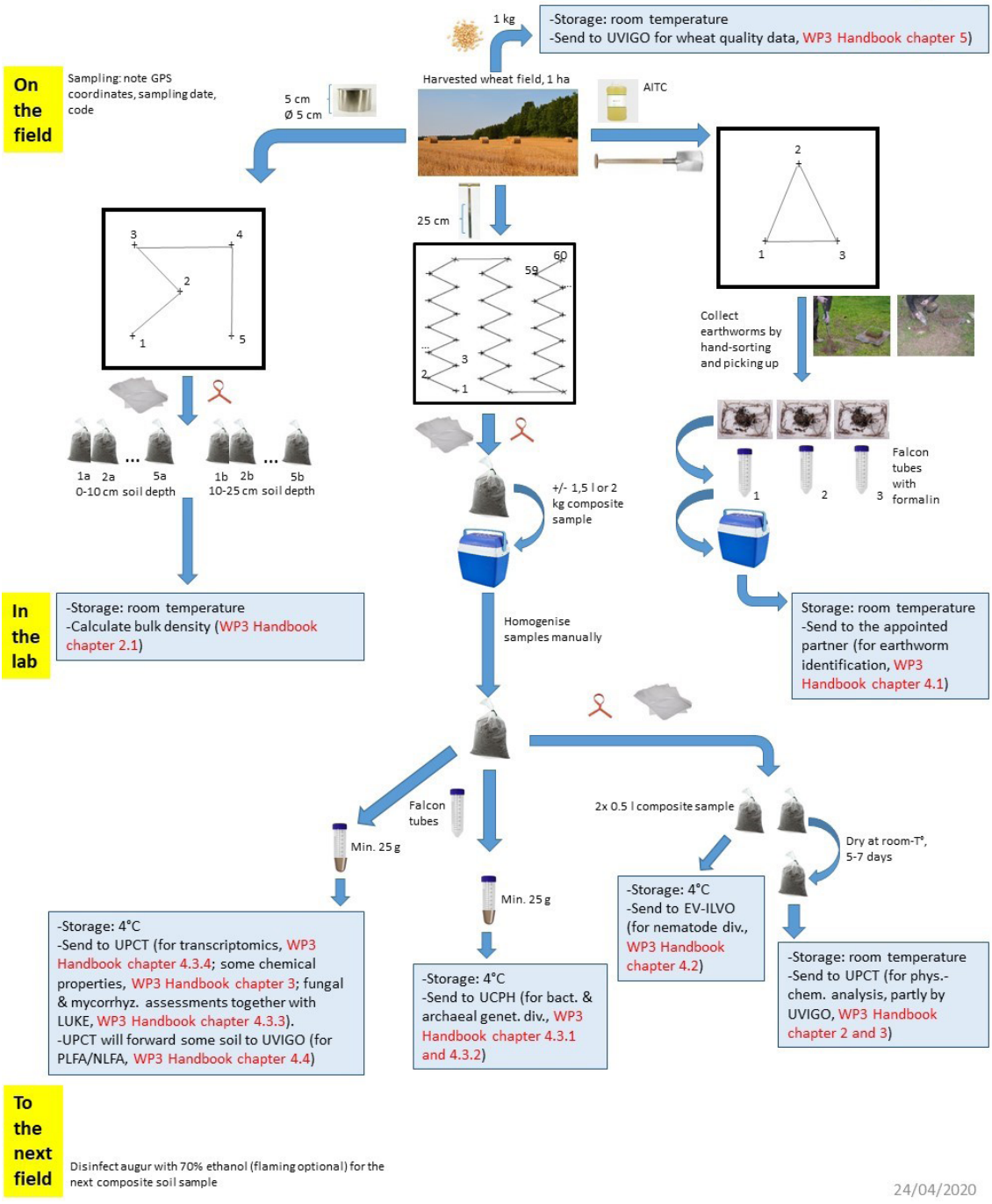
Field history (as much details as possible)

	2015	2016	2017	2018	2019	
Crop rotation system						FIELD 1
Tillage practices						
Fertilizer						
Use of Pesticides						
Climatic anomalies						
Wheat yield & quality remarks						
Pathogen detection/presence						
Crop rotation system						FIELD 2
Tillage practices						
Fertilizer						
Use of Pesticides						
Climatic anomalies						
Wheat yield & quality remarks						
Pathogen detection/presence						

ANNEX 1.2 OVERVIEW OF THE SAMPLING EVENT

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 817819

WP3 sampling scheme



ANNEX 1.3. DOCUMENT OF CONSENT

Tittle of the principal project: Soil biodiversity enhancement in European agroecosystems to promote their stability and resilience by external inputs reduction and crop performance increase – SoildiverAgro. (H2020 project 817819)

Project Coordinator: David Fernández Calviño, University of Vigo

Regional-Coordinator: [Name, Institutional affiliation]

Tittle of the collaborator project: Optimizing Bio-based Fertilisers in Agriculture – Knowledgebase for New Policies – LEX4BIO. (H2020 project 818309)

Project Coordinator: Kari Ylivainio, LUONNONVARAKESKUS,

About SoildiverAgro project: The aim of SoildiverAgro (H2020 project 817819) is to adopt new management practices and cropping systems that enhance soil genetic and functional biodiversity to reduce the use of external inputs while increasing crop production and quality, the delivery of ecosystem services and the EU agricultural stability and resilience.

About LEX4BIO project: The aim of the LEX4BIO (H2020 project 818309) is to decrease European dependency on finite and imported, apatite-based phosphorus fertilisers and energy-intensive mineral nitrogen fertilisers by optimising the use of bio-based fertilisers according to crop requirement, securing food and feed safety and human health.

Purpose of your participation: You are invited to participate in our research study on soil biodiversity (bacterial, fungal, nematodes and earthworms) and essential plant and trace elements status in wheat devoted soils in Europe under the SoildiverAgro and LEX4BIO projects. You will be requested to participate providing authorization for soil and wheat sampling in your fields, analyse them and provide us historical data on soil management practices and crop production.

Time involvement: This research study may take up to approximately five years after your participation.

Risks and benefits: The risks associated with this study are none. We cannot and do not guarantee or promise that you will receive any benefits from this study. Your decision whether or not to participate in this study will not affect your participation in other SoildiverAgro or LEX4BIO events and activities.

Payment: You will not receive any reimbursement as payment for your participation.

Rights: Your participation is voluntary and you have the right to refuse to participate and to withdraw your participation, samples or data at any time without any consequences. You have the right to refuse to answer particular questions. Your name and contact data will be always retained confidential, will never be made public, findable or accessible, and you will never be identified or linked to the samples or data. You will be always the owner of data and samples collected. The data will be made accessible to you in a personal communication and/or report.

Use of data/samples: The materials and data collected will become part of studies that may be presented at scientific or professional meetings or published in scientific or professional journals for communication and dissemination purposes of SoildiverAgro and LEX4BIO projects. Personal Data shall be processed only for those

Data storage, protection, retention and destruction: Data will be stored securely on private personal hard drives with access key and the private institutional SoildiverAgro and LEX4BIO DPVs to prevent breaches of confidentiality during the period of the project and beyond the lifetime of this project. The University of Vigo protects the DPV from viruses and data piracy and makes periodic back-ups. Natural Resources Institute (Luke) is responsible for the data used in LEX4BIO, respectively. In the event of the unlikely incidental findings, you will be communicated to decide if you aim to withdraw your data. Personal Data shall be retained until the data derived from sampling, surveys, questionnaires or interviews are scientifically processed and published, and shall be destroyed after a period of 18 months after publication. By default, Personal Data shall be destroyed after a period of 36 months after the finalisation of the SoildiverAgro and LEX4BIO projects.

Contact information: If you have any questions, concerns or complaints about this research, its procedures, risks and benefits, contact the Project Coordinator (Dr. David Fernández Calviño; davidfc@uvigo.es; +34 988 36 8888). ADD your regional contact information

Independent contact: If you are not satisfied with how this study is being conducted, or if you have any concerns, complaints, or general questions about the research or your rights as a participant, please contact Mr Anxo Moreira, Universidade de Vigo (otri9@uvigo.es, +34 986 81 2236) to speak to someone independent of the research team.

Data Protection Officer: Dr. Ana Garriga Domínguez (University of Vigo): dpd@uvigo.es; +34 988 36 8834.

Indicate Yes or No:

- I give consent for soil and wheat sampling in my fields by SoildiverAgro researchers: ……Yes ……. No
- I give consent for registration of data resulting from this study to be used at scientific or professional meetings or published in scientific or professional journals for communication and dissemination purposes: …. Yes ……No
- On default, your identity (name and contact data) will be retained anonymous and confidential, unless you give explicit consent to reveal your identity: ……Yes

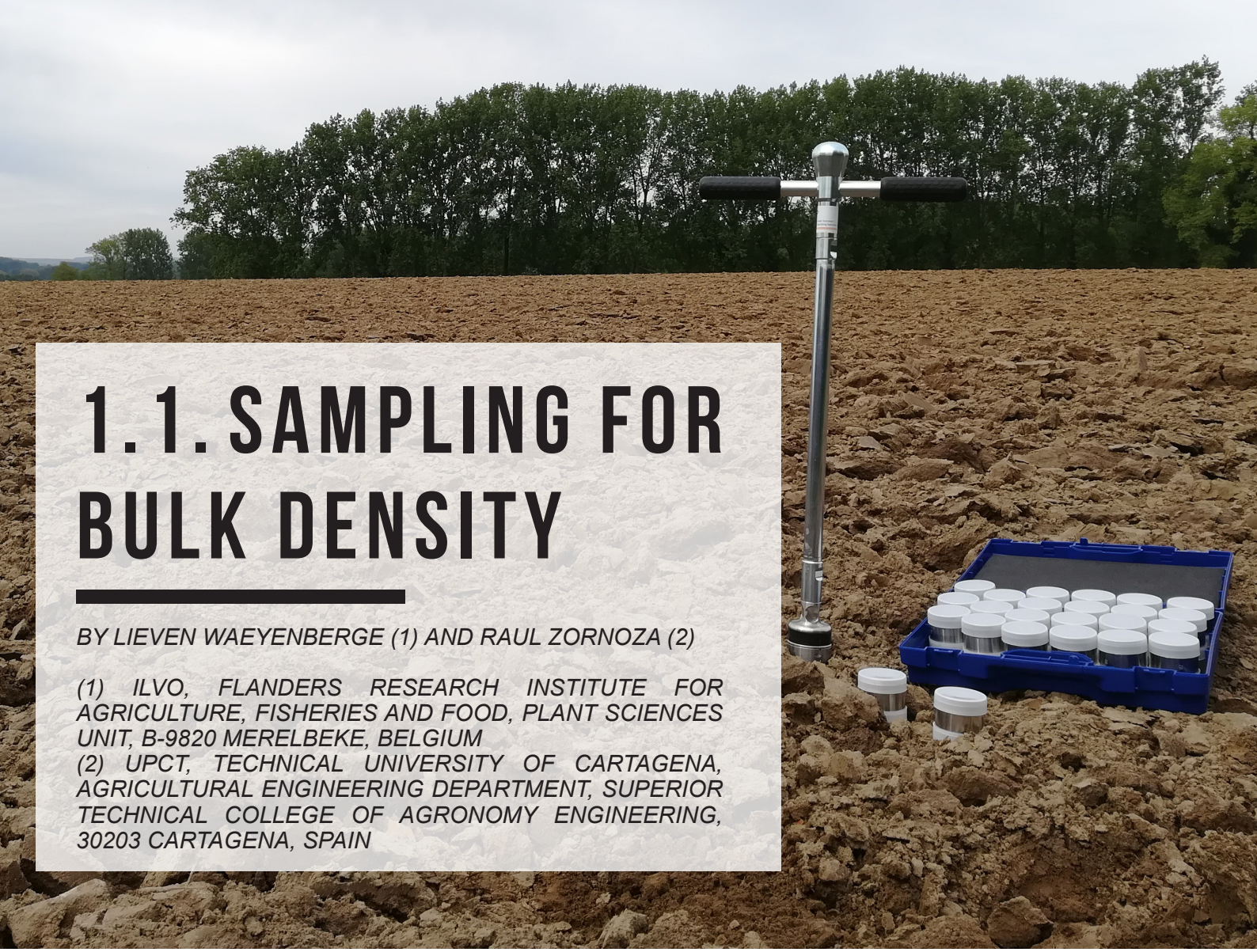
Signed, on duplicate, so that one copy of this signed and dated consent form is for you to keep.

NAME: ……

ORGANIZATION: (Use it only if necessary) ……

DATE: ……

SIGNATURE ……



1.1. SAMPLING FOR BULK DENSITY

BY LIEVEN WAEYENBERGE (1) AND RAUL ZORNOZA (2)

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(2) UPCT, TECHNICAL UNIVERSITY OF CARTAGENA, AGRICULTURAL ENGINEERING DEPARTMENT, SUPERIOR TECHNICAL COLLEGE OF AGRONOMY ENGINEERING, 30203 CARTAGENA, SPAIN

PRINCIPLE AND APPLICATION

Soil bulk density refers to the weight (mass) of soil per unit of volume and is generally expressed in g per cm^3. The weight of a soil sample can be determined easily, while collecting a certain volume of soil is less obvious. Thus, to be able to measure the soil bulk density (see section 2.1) of an agricultural field accurately, soil samples should be taken with care.

The sampling procedure selected here is the one using cylindrical cores. Basically, the cylinder is pressed or hammered into the soil. The cylinder is removed gently, thereby extracting a sample of known volume.

The use of a cylindrical core enables the sampler to take a known volume of soil. Previous studies have demonstrated that the type of cylindrical cores does not significantly influence the bulk density measurements. The key factor is that the sampler must remove the core used in such a way that the core remains completely filled with soil.

PROCEDURE

1. Prepare a flat surface at the first selected sampling spot by scraping away the upper layer with a shovel or spatula.
2. Press the ring completely into the soil, preferably till 1 cm below the prepared surface. This can be done manually or mechanically using a hammer and a piece of flat wooden plank placed on top of the ring to protect the hammer from the sharp edges of the ring.
3. Remove the ring with the help of a spatula or shovel. Be sure to avoid that soil falls down from the ring.
4. Remove excessive soil with a knife until the upper and lower surface of the soil in the cylinder is flat.
5. Close both sides of the ring if you are going to transport the soil within the ring. If you are going to use the same ring for several samples or you do not have the lids to close the rings, transfer the soil of a ring to a plastic bag and code it properly. Close the bag with a twist tie.
6. Dig out the same spot till a depth of 15 cm, press the same or another ring (depending on whether you emptied and cleaned the already used ring or not) into the prepared surface manually or mechanically using the core sampler, and repeat steps 3 till 5.
7. Repeat the whole procedure (Steps 1-6) on an additional 4 spots well spread over the selected field (approximate area of 1 ha).
8. In the lab: store the soil samples at room temperature.

The sampling procedure can also be viewed in the following video on YouTube:
https://www.youtube.com/watch?v=JQav_fx69aU

EQUIPMENT AND MATERIAL

- **Metal rings with known volume, preferably with removable lids at both ends**
- **Sampling bags and twist tie**
- **Marker**
- **Spatula and/or shovel**
- **Hammer**
- **Knife**
- **Piece of flat wooden plank**
- **Retractable tape measure**
- **Core sampler**

Photo 1.1.1 Equipment for soil bulk density sampling. Credits: Lieven Waeyenberge, ILVO

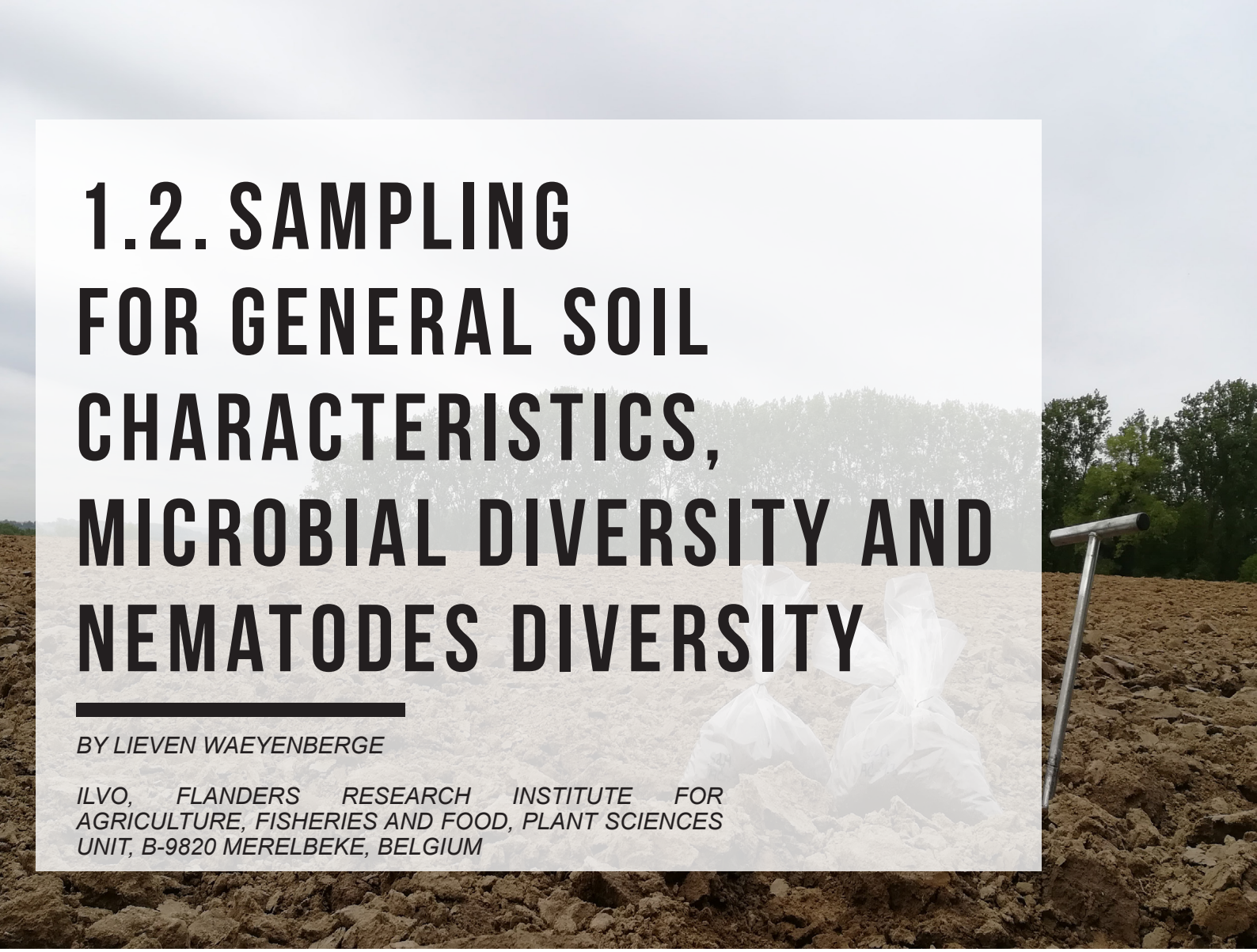
CALCULATIONS

No calculations are made during sampling and pre-processing. To measure the bulk density, section 2.1. should be consulted.

REMARKS

The method is not intended for loose, sandy soils. Additionally, dry or hard soils can shatter when the cylinder is hammered in the soil. In this case, pressing the cylinder into the soil reduces the risk of shattering the sample.
The cylindrical cores should have a thin, sharp edge to avoid compaction during sampling. If compression is excessive, the soil core may not be a valid sample for analysis. Rock fragments can damage the cores and interfere with the sampling.

1.2. SAMPLING FOR GENERAL SOIL CHARACTERISTICS, MICROBIAL DIVERSITY AND NEMATODES DIVERSITY



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PRINCIPLE AND APPLICATION

A composite soil sample is taken to assess a number of general soil characteristics and the microbial and nematode diversity. The sample is composed of several cores taken by a core sampler on a part of an agricultural field with an area of approximately 1 ha following a straight line between the remainders of the crop or following a zig-zag pattern across the field. The total number of cores taken depends on the length and the diameter of the core sampler. The length of the soil core should be 25 cm. The diameter of the core sampler is variable. However, the soil composite sample should be at least 1,5 l or 2 kg.

At arrival in the lab, after sampling, the soil samples have to be homogenized manually (not mechanically to avoid damage of nematodes) and divided into different parts (Annex 2):

- a minimum of 25 grams of each composite sample is transferred into a plastic tube (Falcon tube) using a spoon or spatula. This tube is sent to UCPH (Kristian Koefoed Brandt) for bacterial and archaeal genetic diversity analysis; a second tube again with a minimum of 25 grams is to be sent to UPCT (Raúl Zornoza) for some chemical properties and micro-organisms assessments. Before dividing the next sample, another clean spoon or spatula is used or the spoon or spatula is cleaned (70% ethanol).
- the remainder of the soil sample is split into 2 equal parts (minimum of 500 mL each). One part is sent to EV-ILVO (Waeyenberge Lieven) for the nematode functional biodiversity assessment. The other part is dried at room temperature for several nights (possibly 5 to 7 nights depending on soil moisture) and sent to UPCT (Raúl Zornoza) for soil physical and other chemical measurements.

Table 1.2.1. provides an overview of the partners receiving samples and the downstream analyses that each partner is responsible for.

Dried samples for soil physical and other chemical properties are stored at room temperature as the lack of water ensures preservation. The remaining field-moist samples are stored at 4°C until shipment to minimize changes in the analysed soil biological properties. Field-moist samples should be sent as soon as possible (express delivery) with cold packs included in the package. For nematodes, it is recommended to analyse samples within a month (better few weeks). Upon arrival in the receiving laboratory, samples for microbial diversity assessment may be stored frozen at -20ºC for several weeks or at -80ºC for several months prior to analysis. A list with sample codes and any valuable information are added in the transport box. This information is also sent by e-mail to the representative partner with the message that samples are coming. Answering with an email confirming availability for receiving samples in the coming days is desirable before sending the samples.

Table 1.2.1. Partners responsible for the acquisition of different types of data

PARTNERS IN CHARGE OF DATA ACQUISITION		
ANALYSIS TYPE	**PARTNER IN CHARGE**	**SAMPLE ORIGIN (PEDOCLIMATIC REGIONS*)**
Physical and chemical soil characteristics	UVIGO & UPCT	All
Nematode diversity	EV-ILVO	All
Whole microbial diversity	UVIGO	All
Bacterial and archaeal genetic diversity	UCPH	All
Fungal & mycorrhizal genetic diversity	LUKE	All
Microbial functional diversity	UPCT	All
Earthworm diversity	TI	ATN, CON
	EV-ILVO	ATC, PAN
	LUKE	BOR
	UVIGO	LUS, MDN, MDS
	EULS	NEM

(*) Pedoclimatic regions: ATC = Atlantic Central, ATN = Atlantic North, BOR = Boreal, CON = Continental, LUS = Lusitanian, MDN = Mediterranean North, MDS = Mediterranean South, NEM = Nemoral, PAN = Pannonian.

PROCEDURE

EQUIPMENT AND MATERIAL

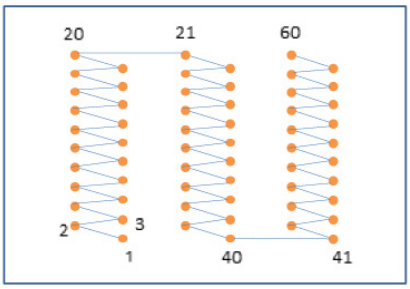
- **Sampling bags and twist tie**
- **Marker**
- **Core sampler with thumb**
- **Spray bottle with 70% ethanol**
- **Roll of paper**
- **Cool box with cool packs (when temperature is above 15°C)**

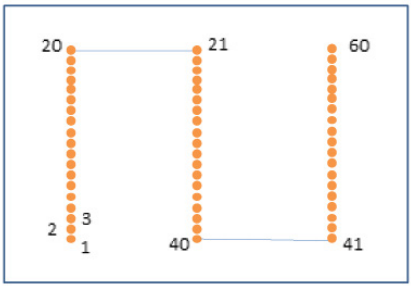
Photo 1.2.1 Equipment for soil sampling.
Credits: Lieven Waeyenberge, ILVO

1. Before going to the field, label strong plastic sampling bags appropriately: code of pedoclimatic region + 'C' or 'O' for conventional or organic farming + unique code of the field. One field = one plastic bag.

2. After arriving at the location, note the GPS coordinates if not known yet.

3. Take a soil sample spread all over an area of 1 ha. The easiest way is to follow a zig-zag pattern (A) or a pattern with straight lines (B). Go 3 times from one end to the other end of the field, trying to cover the whole area of 1 ha but avoiding coming to close to the borders. Each time you cross the field, take 20 cores using the core sampler. Thus, in total 60 cores, one at each 60 sampling points, are taken. Move approximately 6-7 steps (representing 5 m) between each core taken. When the field has an irregular form, adapt your sampling pattern accordingly.





4. At each sampling point, press the core sampler vertically in the soil. Use both hands if needed.

5. Twist the core sampler half turn and pull it out in a slight angle in an attempt to keep the soil core positioned into the core sampler.

6. Move the core sampler into the already labelled plastic bag and use the thumb to release the soil from the core sampler into the plastic bag.

7. Go to the next sampling point and repeat this procedure until all sampling points are sampled.

8. Close the sample bag with a twist tie and place it in a cool box, with cool packs when the weather is more than 15°C, to protect the sample from heat and light.

9. Before going to the next field, the sampling material (core sampler and thumb) is disinfected with 70% ethanol and wiped dry with paper. This should especially prevent carry-over of soil containing organisms from one sample to the other.

CALCULATIONS

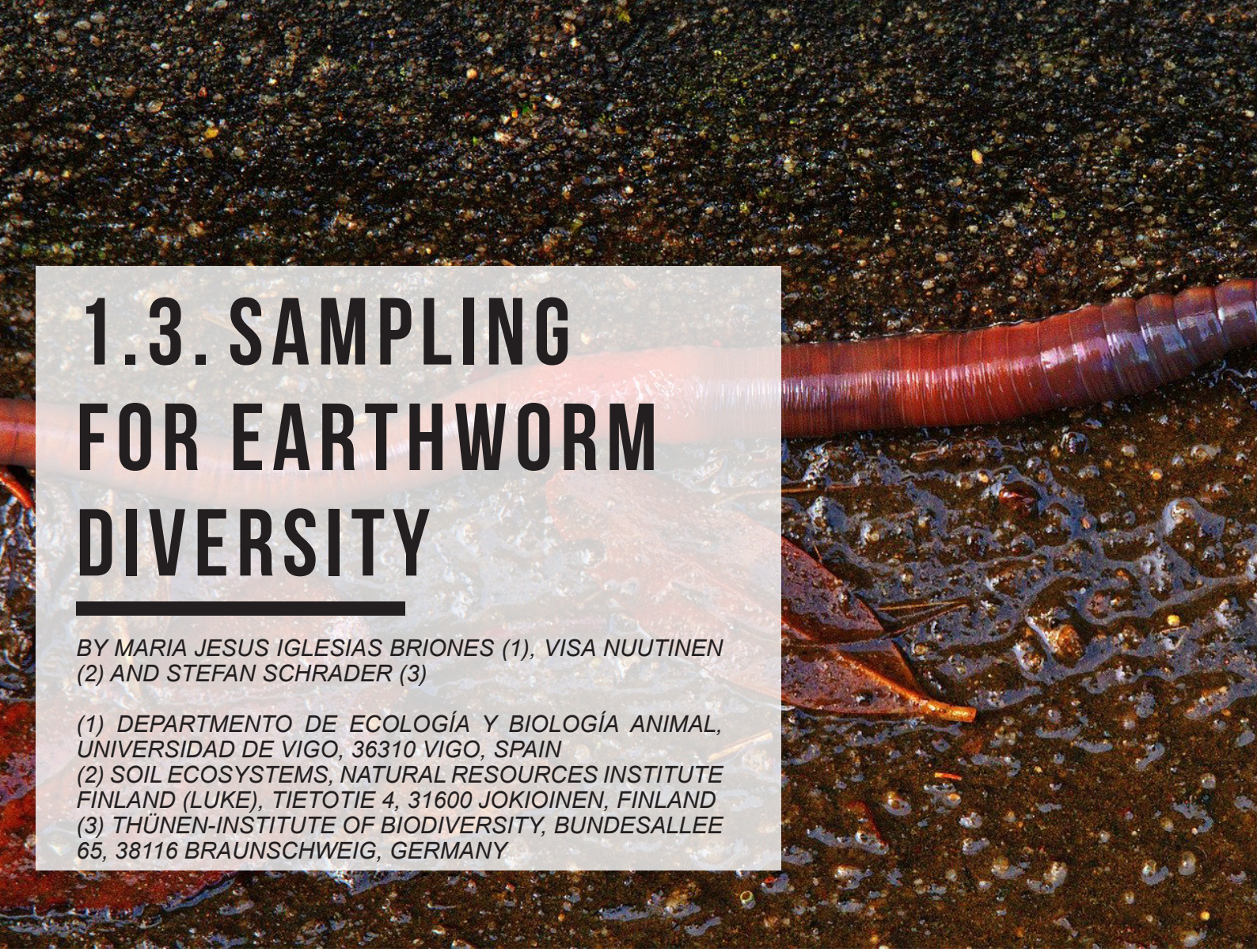
No calculations are made during sampling and pre-processing. Consult the next chapters for more information concerning the subsequent analyses.

REMARKS

It should be avoided to take soil samples that are very wet or very dry. If conditions are not favourable, it is recommended to postpone the sampling by a couple of days.

It is recommended not to use sampling bags with a zip lock. The zip lock is not strong enough to keep the bag closed at all times. It can easily cause spilling of soil into the cool box or even cause carry-over of soil from one sampling bag into the other.

Photo 1.2.2 Demonstrating how to take a soil sample (lower parts) and how to close the sampling bag properly (upper part). Credits: Lieven, Waeyenberge, ILVO.



1.3. SAMPLING FOR EARTHWORM DIVERSITY

BY MARIA JESUS IGLESIAS BRIONES (1), VISA NUUTINEN (2) AND STEFAN SCHRADER (3)

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PRINCIPLE AND APPLICATION

Digging and hand-sorting of soil is the most widely used standard method to collect earthworms. However, it is not very efficient in capturing deep burrowing anecic earthworms, and for this reason, it is usually combined with chemical expelling by a vermifuge (Bartlett et al. 2010); here the active agent in mustard, allyl isothiocyanate (AITC; mustard oil) will be used as described in ISO 23611-1:2018. From each sampling site three replicate samples has to be taken (i.e. sampling of 3 different quadrats) to account for spatial variability.

In the following, the systematic procedure is described and explained in three steps:

- **Hand-sorting of soil samples in the field**
- **Chemical expelling of deep burrowers from the same quadrats**
- **Processing of the collected live material in the field or laboratory**

PROCEDURE

Hand-sorting in the field

Hand-sorting is a physical or passive method where the earthworms are directly removed from the soil by hand.

Place the first quadrat on the soil surface at a random location or mark the area (Fig. 1.3.1.). Do not sample areas where you have walked just before. Cut and remove the aboveground vegetation (do not pull the roots out). If there is a litter layer, please check it carefully for any earthworms.

Fig. 1.3.1. Area for earthworm sampling defined by a quadrat (left) or marked (right).
Photo credits: http://www.ucd.ie/agbiota/studies/worms.htm (left); https://worms.educ.ualberta.ca/quadrat.html (right)

Excavate a 50 cm x 50 cm x 25 cm deep soil block (cut along edges of your marked area), unless the clay content of your soil is more than 50%, in which case the sampled area can be reduced to 25 x 25 x 25 cm. Place the intact soil block on the plastic sheet (e.g. bin liner) or a large tray (Fig. 1.3.2.). This is needed to prevent earthworms from escaping. Sort through soil manually; carefully check the roots. Please wear disposable protective gloves. The sampled earthworms should be soon transferred into labelled plastic containers, which should be filled with moist soil from the pit. Once the soil is sorted return it back into soil pit and leave the spot in a tidy state.

To avoid mortality keep the containers with the earthworms in cool conditions (e.g. in a cool box) and away from direct sunlight until they can be processed. Repeat this procedure at two other random locations. Keep each replicate sample separate throughout the sorting process.

EQUIPMENT AND MATERIAL

- **Wire or wooden frames (50 cm x 50 cm) or simply mark the area with sticks**
- **Garden scissors (secateurs)**
- **Spade (flat blade if possible)**
- **Large plastic sheets (e.g. a large bin bag or trays)**
- **Tweezers/forceps, labels, permanent markers**
- **Plastic containers with lids (Tupperware, lock&lock)**
- **Cool box and ice packs**
- **Disposable protective gloves**

Fig. 1.3.2. Excavated soil placed on a large tray (left) and large plastic sheet (right). Photo credit: https://soils.sectormentor.com/case-study/building-soil-health-5-key-soil-tests-to-get-you-started/

EQUIPMENT AND MATERIAL

- **Allyl-isothiocyanate (AITC), synthetic grade (about 94% to 97% (volume fraction)). [Aldrich 37,743-0]**
- **Isopropanol [2-propanol] 100 % (volume fraction).**
- **Test tubes or vials (50 mL) for stock solution**
- **F-style jugs or watering cans (Fig. 1.3.3.).**
-

Fig.1.3.3. F-style jug (left) and watering can (right). Image credits: https://www.containerandpackaging.com/products/65/pvc-f-style-bottle/B048 (left) and https://www.spottygreenfrog.co.uk/Set-of-Four-Watering-Cans/p-204-105-361-1020/

- **Tap water**
- **Plastic containers with lids (Tupperware, lock&lock)**
- **Tweezers/forceps, labels, permanent markers**
- **Cool box and ice packs**
- **Disposable protective gloves**

Chemical expelling of deep burrowers in the same quadrats

Chemical expelling is an active method where earthworms are irritated and forced to leave the soil.

The stock solution should be prepared under a laboratory fume hood for safety reasons because AITC is a toxic irritant. Please wear disposable protective gloves, strictly avoid all skin contact and inhalation and carefully protect eyes. In the lab, mix 2 mL allyl- isothiocyanate into 40 mL isopropanol (to provide a 5 g L^{-1} stock solution) in small bottles that can be easily transported to the field (in cool boxes). Because AITC is not readily soluble in water, alcohol acts as an emulsifier when AITC is added to water. Store the stock solution in a fridge and no longer than 5 days before usage.

Just before application in the field, dilute the stock solution (42 ml (2 ml AITC + 40 ml propanol) with 20 L water to give a final concentration of approximately 0.1 g L^{-1} (Zaborski 2003; Pelosi et al. 2009) in F-style jugs or watering cans and mix vigorously.

Use 10 L of the mixture per quadrat (or 20 L depending on the soil conditions; e.g. if the soil is too dry) by pouring it down into the bottom of the pit after the soil block has been excavated. Optional: pour half of the mustard solution evenly across the quadrat, and after about 15 minutes, pour the remaining solution. In case of a very low infiltration rate, less than 10 litres of mixture will suffice.

Sit next to the sampling spot and collect the expelled earthworms with forceps from inside of the sampling area as they emerge (only collect earthworms once they have left their burrows completely). Transfer the collected worms to containers containing clean tap water to rinse off the irritant. Soon after, they can be placed in labelled plastic containers filled with moist (clean) soil.

After the whole AITC solution is added, continue to monitor the plot because earthworms might crawl out; the biggest ones often take the longest time to emerge. Collect all the earthworms for 15-20 minutes before moving to the next plot.

Processing the collected live material in the field or laboratory

Rinse each subsample of earthworms with tap water and blot on paper towels. Place the live earthworms in a deep Petri dish/ plastic container containing a fixing solution (1:1; 4% formalin:96% ethanol) for 2 minutes or until they stop moving.

Put 1-3 earthworms one at a time (rather than a whole handful all at once) so they do not get tangled up into a big mess of earthworms. Please wear disposable protective gloves and avoid inhalation and skin contact.

Thereafter, carefully extend every specimen onto a flat surface (or the upturned lid of a Tupperware; Figure 1.3.4.) and after 3-5 minutes they can be placed in a leak proof vial containing 4% formalin labelled both outside and inside (pencil written label in the liquid). Store the vials in horizontal position for at least 24 hours to allow enough time for the soft tissues to be fixed.

Once the earthworms have been in formalin for at least 24 hours, change the solution (if after that time it becomes too cloudy). The worms can now be stored in the formalin 4% until further identification (long-term storage). Alternatively, 70% ethanol (volume fraction) can be used instead for long-term storage.

In addition to species identification, the total biomass of the preserved individuals collected per replicate is a useful parameter and can be determined using a balance with a precision of 0.01 g. For details see section 4.1.

EQUIPMENT AND MATERIAL

- **Tissue paper/paper towels**
- **Analytical balance (0.01 g)**
- **Petri dishes or a plastic container**
- **Formalin, formaldehyde solution 4 % (volume fraction), for storage purposes only**
- **Ethanol 70% and 96%**
- **Tweezers/forceps, labels, permanent markers, pencil**
- **Test tubes or vials**
- **Disposable protective gloves**

Fig. 1.3.4. Earthworms placed onto a flat upturned lid of a Tupperware for careful extension before storage. Photo credit: https://www.earthwormsoc.org.uk/sampling

CALCULATIONS

Present all data on abundance and biomass as individuals per square meter (ind m^{-2}) and grams per square meter (g m^{-2}), respectively. For details see section 4.1.

REMARKS

Labelling
Note the coding for site and plot (= sampling pit), sampling date, coordinates, dominant vegetation, and person in charge.

Timings
- Marking and cleaning each quadrat (10 minutes per replicate)
- Collecting earthworms i.e. hand-sorting and chemical expelling (30-60 minutes)
- Washing the worms, drying and weighing the worms (5 minutes per replicate depending on the numbers)
- Fixing the material and placing it in labelled vials (15 minutes per replicate depending on the numbers)

Recommendations
- Take samples when soil is sufficiently moist, and the earthworms are active. These conditions vary by region, climate, vegetation, land use, etc., but usually coincide with the rainy season. For temperate regions in the Northern Hemisphere, the best time would be spring and fall; in areas with only dry and wet seasons, the end of the wet season is recommended.
- In case of sunny weather, an umbrella for protecting the crawling earthworms in the pit from direct UV-radiation is recommendable.
- The fixing of earthworms can also be done in the field for logistic reasons; for instance, when processing the collected specimens needs to be delayed (e.g. long distances between the sampling site and the laboratory) or if there is a risk that temperatures during storage get too high. If this is the case, earthworms can be transferred to cool water (instead of fresh soil) and from there to the fixing solution and then carefully extended (see above).

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2. SOIL PHYSICAL ANALYSES

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Soil physics is the branch of the soil science responsible for the study of soil texture and structure, as well as the movements of energy and mass that take place in this environment (Hillel 2004). The soils, in addition to water and air, contain mineral and organic particles, elements that are not dispersed and usually intertwine to form aggregates. In turn, these aggregates are ordered to conform the porosity of the soil. The organization and size of the soil particles and aggregates will condition important properties like aeration, nutrient and contaminant storage, and water flow. In the soil, we can distinguish the following levels of organization of the structure: individual soil materials (clays and bonding agents) (≈ 0.2 µm); microbial wastes coated with inorganic particles, adhered in the surface with the help of polysaccharides produced by microbes (Ranjard and Richaume 2001) (≈ 2 µm); remains of hyphae, plants and bacteria bound with inorganic particles (≈ 20 µm); aggregates of 20 µm bound by plant roots and fungal mycelia (≈ 200 µm); aggregates are organized in the soil forming porous systems with channels that aerate and moisten the soil (≈ 2000 µm) (Tisdall and Oades 1982).

The physical properties that will be considered in this work are summarized in Table 2.1., as well as the project members in charge of each analysis.

Table 2.1. Physical analysis used to characterize the samples and partners in charge of the analysis.

Physical properties measured	
Analysis type	Partner in charge
Dry bulk density	UPCT
Coarse fragments determination	UPCT
Soil moisture	UPCT
Particle size distribution and texture	UVIGO
Aggregate stability and size distribution	UPCT

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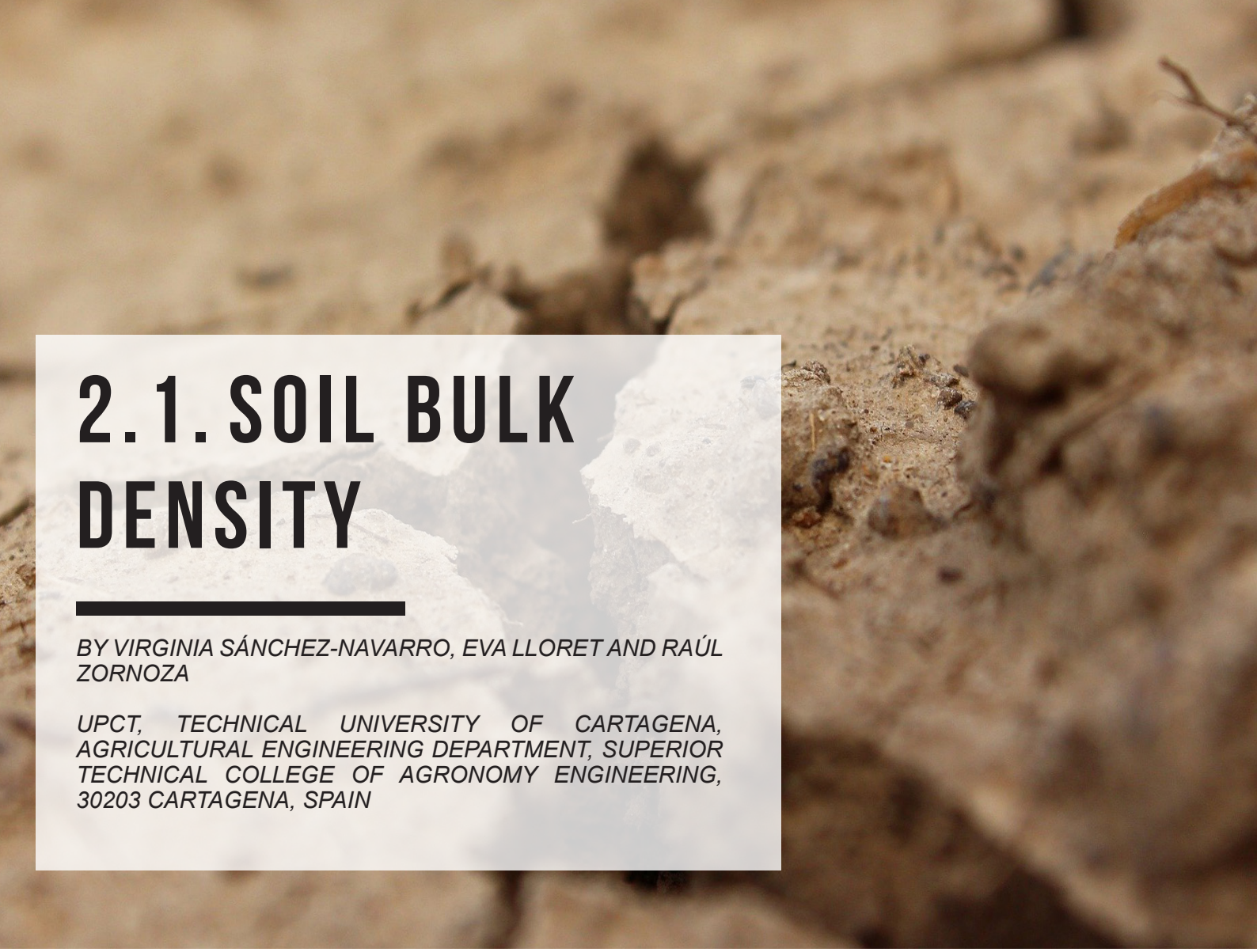

2.1. SOIL BULK DENSITY

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PRINCIPLE AND APPLICATION

Soil bulk density is defined as soil mass per unit of volume. This volume is the sample volume in the field. Soil bulk density is interesting from the point of view of soil management, since it informs about the compaction of each horizon, and it allows the identification of difficulties for the emergency, rooting and air and water circulation. Soil bulk density is directly related to the structure and therefore it depends on the same control factors.

Soil bulk density is often determined separately for the different soil horizons due to the relative ease by which it can be determined. However, it should be noted that soil bulk density has important limitations as a soil quality indicator, since it does not provide information about the size of the spaces, about the connection between them, or about the forces that have led to a specific soil structure. These aspects are of importance when aiming to predict the movement of soil water and aggregate stability. Soils with the same values of bulk densities may have different responses to external forces. To obtain information about this, specific studies on porosity must be used (Porta et al. 1999).

The method presented here is the so-called cylinder method (Campbell and Hensall 1991). A cylinder of known volume with thin and rigid walls and with a bevelled edge towards the outside, is used. The method is to take a sample by inserting the cylinder into the horizon to be studied.

PROCEDURE

1. Follow the indications for soil sampling included in section “1.1. Sampling for soil bulk density”

2. In the laboratory, the contents of the cylinder or plastic bags are transferred into a beaker of known weight.

3. Put the glass in the oven for at least 24 hours at 105 ºC until constant weight.

4. Weigh the beaker and dry soil on the analytical balance (this measure corresponds to the true bulk density), noting the exact weight.

EQUIPMENT AND MATERIAL

- **Metal cylinder of known volume (100-500 cm^3)**
- **Bulk Density Sampler Cup and Cap**
- **Hammer**
- **Spatula**
- **Analytical balance (0.01 g)**
- **Oven**

CALCULATIONS

$\rho b = (m2-m1) / V$

- **ρb is the bulk density of the soil [$g\ m^{-3}$]**
- **m1 is the weight of the metal ring [g]**
- **m2 is the weight of the metal ring + soil after drying [g]**
- **V is the volume of the metal ring [cm^{-3}]**

REMARKS

In the case of soils with gravels and boulders (Regosols, Leptosols, Technosols), coarse constituents hinder sampling and increase measurement error.

In the case of soils with swelling clay minerals, correction calculation is needed related to the field volume/dry volume ratio.

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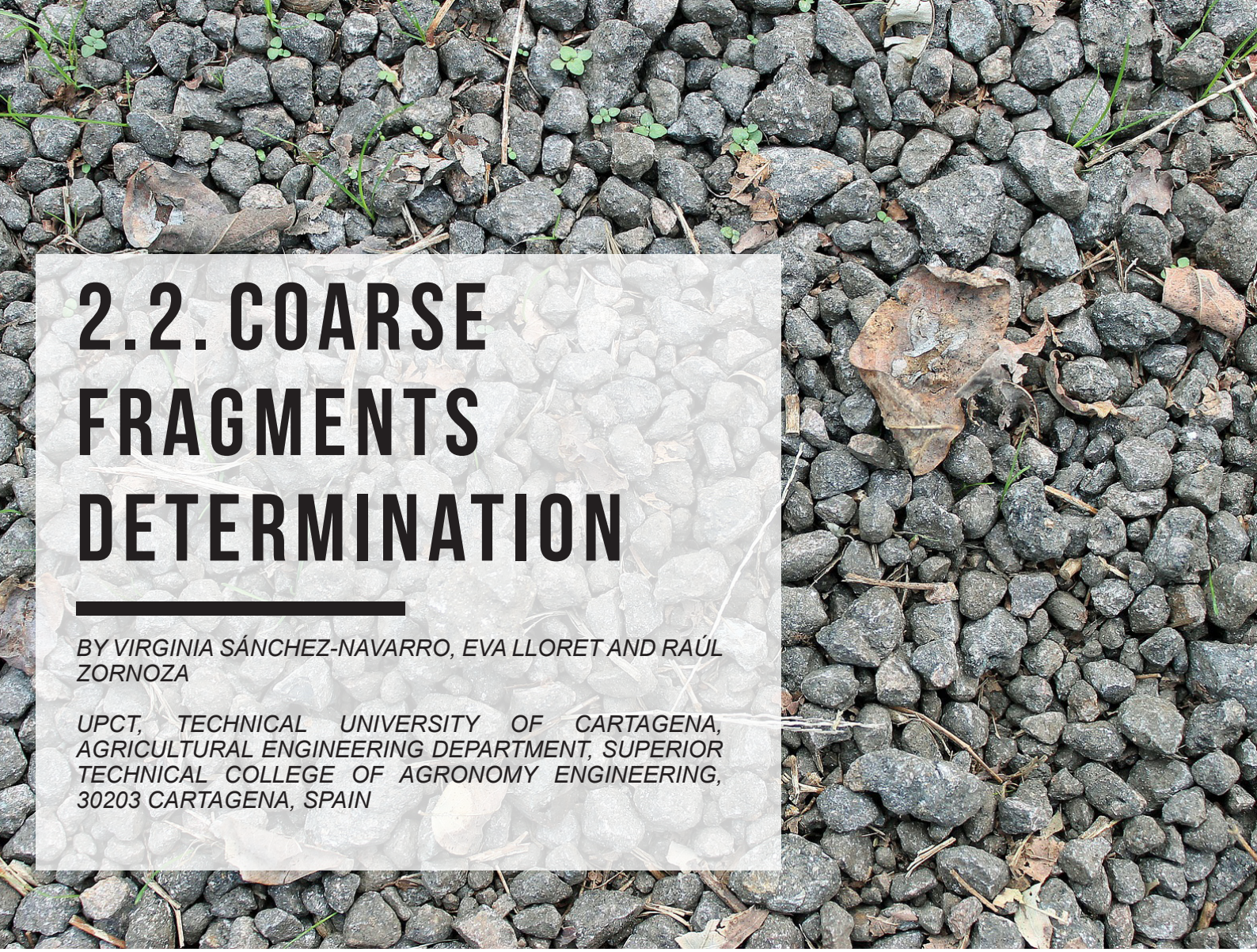

2.2. COARSE FRAGMENTS DETERMINATION

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PRINCIPLE AND APPLICATION

Particle size analysis of soils containing coarse fragments (particles > 2 mm) requires sufficient amounts of soil material. Coarse fragments from soil have the same effect as other mulching materials in protecting the soil against the impact of raindrops (Poesen et al. 1990).

This method is based on the determination of the percentage of rock fragments and gravels in a soil through sieving (Taubner et al. 2009)

PROCEDURE

1. Weigh the whole soil sample (record precise weight). Sample has to be previously air-dried.
2. Sieve the sample at < 2 mm.
3. Weigh the sieve non-passing fraction (record precise weight).

EQUIPMENT AND MATERIAL

- **Sieve (< 2 mm)**
- **Analytical balance (0.01 g)**

CALCULATIONS

Calculate the percentage of coarse fragments by dividing the weight retained on the sieve by the original sample mass.

REFERENCES

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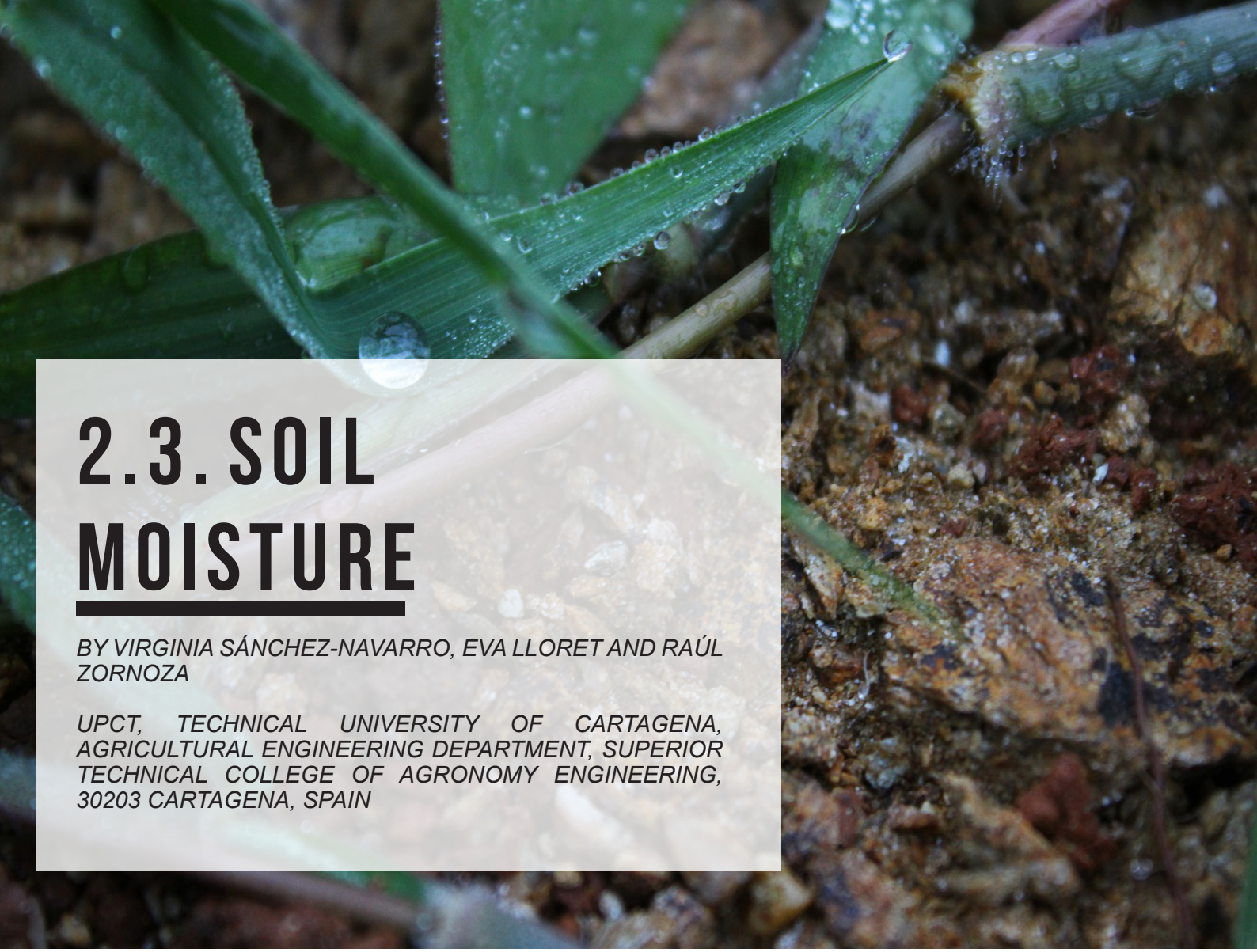

2.3. SOIL MOISTURE

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PRINCIPLE AND APPLICATION

Soil moisture is the amount of water contained in the soil at sampling time. It is usually expressed as a percentage. It can be expressed in volume (percentage of the volume of water included in 100 cm3 of soil) or gravimetrically (percentage of the mass of water included in 100 g of soil). The method here proposed is the determination of gravimetric soil moisture, weighing a soil sample before and after drying (Porta et al. 1986).

PROCEDURE

1. Weigh the crucibles using the analytical balance, noting the exact weight (Pc).
2. Weigh approximately 10 g of fresh soil sample in each crucible using the analytical balance, noting the exact weight (Ps).
3. Place the crucibles with the soil in the oven at 105 ºC for at least 10 h until constant weight.
4. Once the time has elapsed, take out the samples and put them in a desiccator until they cooled down.
5. Weigh the crucibles with the dry soil, noting the exact weight (Pf).

EQUIPMENT AND MATERIAL

- **Glass crucibles**
- **Analytical balance (0,01g)**
- **Oven**

CALCULATIONS

To determine the percentage of soil moisture, the following formula is applied:

$$\text{Soil moisture (\%)} = [Ps - (Pf-Pc))/Ps] \times 100$$

- **Ps = weight of wet soil + crucible**
- **Pf = weight of crucible + dry soil**
- **Pc = weight of crucible**

REMARKS

It is recommended to determine soil moisture on freshly sampled soil sieved < 2 mm to avoid gravels, since most soil analyses will be carried out in soil sieved < 2 mm.

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2.4. PARTICLE SIZE DISTRIBUTION AND TEXTURE

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PRINCIPLE AND APPLICATION

The particle size distribution (PSD) of a soil is a characteristic that influences the soil dry bulk density, the amount of water and nutrients that the soil is able to retain and the soil structure (Mukhopadhyay and Maiti 2018). The PSD is one of the features most used by scientists to describe the distribution in the soil of mineral particles smaller than 2 mm on the basis of relative amount of clay (<0.002 mm), silt (0.05-0.002 mm) and sand (2-0.05 mm) presented by that soil (S. Mukhopadhyay et al. 2019). From PSD, soil texture can be defined (Figure 2.4.1).

Figure 2.4.1. USDA textural classification triangle (Adapted from Groenendyk et al. 2015)

The USDA system shown in Figure 2.4.1 is one of the most used for soil texture classification. The percentage of material belonging to each group (sands, silts and clays) is represented on each side of the triangle. The texture is determined by the intersection between the three lines that cut the sides of the triangle at the point corresponding to the value of each element represented. Soil texture has a major influence on soil water flow and retention. As the amounts of clays and silts increase, the water flow through the soil is reduced, improving the soil water retention capacity.

There are several methods available for determining PSD, but the idea is always to separate the different fractions (by size) and determine their quantity in each fraction. This can be achieved by sieving, by sedimentation of a soil suspension, by differences in the density of a suspension, or by laser diffraction methods (Taubner, Roth, and Tippkötter 2009). One of the most used methods for the PSD determination is the pipette method: the largest fractions (sands) are separated by sieving, using sieve meshes of 0.2 mm (for coarse sand) and 0.05 mm (for fine sand); and the finest ones (silt and clays) are determined according to their sedimentation rate calculated by Stokes law (Carter and Gregorich 2008).

EQUIPMENT AND MATERIAL

- Sieves (0.2 and 0.05 mm)
- Bottles
- Shaker
- Sodium hexametaphosphate, $(NaPO_3)_6$
- Sodium carbonate, Na_2CO_3
- Distilled water
- Hydroxide peroxide, H_2O_2
- Test tubes (1 L)
- Crucibles
- Robinson pipette
- Hand shaker
- Chronometer
- Oven (muffle)

REMARKS

It is common to destroy the carbonates in the sample using a pre-treatment with HCl. However, this acid can affect the size and structure of some soil components.

PROCEDURE

Before starting the PSD characterization, it is necessary to prepare a solution of sodium hexametaphosphate and sodium carbonate in water that will be used as dispersant. For this aim, 35.70 g of (NaPO3)6, and 7.94 g of Na2CO3 are diluted in one litre of distilled water, to have a solution 0.06 M of (NaPO3)6 and 0.08 M of Na2CO3.

For the determination of the particle size distribution by the pipette method, 20 g of dry soil (sieved by 2 mm) are weighed. It is necessary to understand that the distribution of particle size is contributed only to the mineral material, so a previous step is necessary to eliminate the organic matter. This part can be removed from the sample by dry combustion (employing a muffle) or wet combustion (by adding H2O2, carefully until the reaction is stopped).

Once the mineral part is separated, a volume of 100 mL of distilled water and 50 mL of the solution of sodium hexametaphosphate and sodium carbonate are added. This suspension is shaken for 2 hours in order to disperse aggregates.

After that step, the suspension is wet sieved using two mesh sizes, 0.2 and 0.05 mm, to separate the coarse and fine sands respectively. It is necessary to wash these sands to be sure that all materials of a smaller size end up in the effluent. It is very important to manage the use of water properly so that less than a litre is used. The sand fractions are taken to a previously tared crucible, where they are dried in an oven at 105°C until constant weight.

The effluent, which only contains silts and clays, is transferred to a one-litre test tube. With the help of a manual shaker, the content of the test tube is agitated. Samples of 20 mL are taken after 0, 4.8 minutes and 8 hours in a previously tared crucible, and dried in an oven at 105°C until constant weight. In each case, it is necessary to adjust the position of the Robinson pipette to always take the samples at ten centimetres below the surface.

In the end, all the dried samples in the crucibles are weighed and the calculations are performed to determine the proportion of each particle size in the soil.

CALCULATIONS

In the case of sands, the calculation is as follows:

$$CSa = CSa_w - CSa_c$$

$$FSa = FSa_w - FSa_c$$

Where:

- **CSa is the weight of the coarse sand.**
- **CSaw is the weight of the coarse sand plus the crucible.**
- **CSac is the weight of the crucible used to dry the coarse sand.**
- **FSa is the weight of the fine sand.**
- **FSaw is the weight of the fine sand plus the crucible.**
- **FSac is the weight of the crucible used to dry the fine sand.**

The following formulas were used for coarse silt, fine silt and clays:

$$CSi = \frac{(CSi_w - CSi_c)}{V_s} \cdot V_1$$

$$FSi = \frac{(FSi_w - FSi_c)}{V_s} \cdot (V_1 - V_s)$$

$$CL = \frac{(CL_w - CL_c)}{V_s} \cdot (V_1 - 2\,V_s)$$

Where:

- **CSi is the weight of the coarse silt.**
- **CSiw is the weight of the coarse silt plus the crucible.**
- **CSic is the weight of the crucible used to dry the coarse silt.**
- **FSi is the weight of the fine silt.**
- **FSiw is the weight of the fine silt plus the crucible.**
- **FSic is the weight of the crucible used to dry the fine silt.**
- **CL is the weight of the clay.**
- **CLw is the weight of the clay plus the crucible.**
- **CLc is the weight of the crucible used to dry the clay.**
- **Vs is the volume of sample taken with the Robinson pipette.**
- **V1 is the volume of the test tube.**

The results of this type of analysis are usually expressed as percentages with regard to the total sample. Moreover, the two types of sand and silts are added together to determine the type of texture in the USDA triangle (Figure 2.4.1.). Thus:

$$\%Sa = \frac{(CSa + FSa)}{CSa + FSa + CSi + FSi + CL} \cdot 100$$

$$\%Si = \frac{(CSi + FSi)}{CSa + FSa + CSi + FSi + CL} \cdot 100$$

$$\%CL = \frac{(CL)}{CSa + FSa + CSi + FSi + CL} \cdot 100$$

Where:

- **%Sa is the percentage of sand in the sample.**
- **%Si is the percentage of silt in the sample.**
- **%CL is the percentage of clay in the sample.**

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2.5. AGGREGATE STABILITY AND SIZE DISTRIBUTION

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PRINCIPLE AND APPLICATION

Individual soil particles are not randomly arranged, but they form clods and lumps joined by colloidal material, which have a certain internal organization and a characteristic external shape, called aggregates. The nature and distribution of aggregate size and pore space is called soil structure and strongly affects physicochemical and biological properties of soil The soil structure is related to the mutual arrangement of the individual soil particles, the stability of the aggregate state and the pore size. Aggregates stability refers to their ability to maintain their shape when they are subjected to artificially induced forces, in particular those derived from wetting or the impact of raindrops (Díaz et al. 1994). TThis method analyses the aggregateAggregate stability and size distribution based oncan be determined using a wet sieving method of air-dried soil (Elliot 1986).

PROCEDURE

1. Take 80 g subsample from air-dried soil (weigh and record precise weight).
2. Fill up white basin with water until water level is approximately 1 cm above 2000 µm sieve-mesh.
3. Spray soil evenly out on sieve and wait for 5 minutes.
4. After 5 minutes, sieve the soil for two minutes by moving the sieve up and down (approx. 3 cm amplitude) 50 times with a slight angle to ensure that water and small particles pass through the mesh.
5. Let the sieve stand for 30s in order to let small particles settle down.
6. Aspirate off the floating litter into the flask attached to the vacuum line.

EQUIPMENT AND MATERIAL

- **8 mm sieve**
- **Two white basins with diameter of 50 cm and height of 8 cm**
- **2000 µm, 250 µm, 53 µm sieves with diameter of 30 cm**
- **Vacuum pump connected to a flask**
- **Aluminium pans for drying soil samples**
- **Air-forced drying oven (60°C)**
- **Spatula and brush**
- **Rinsing bottle**
- **Analytical balance (0.01 g)**

CALCULATIONS

The results are expressed as a percentage showing the proportions of the different aggregate size classes.

7. Take the sieve out of the water and rinse off the sides plus the bottom of the sieve with water in order to have all particles in suspension.

8. Backwash > 2000 µm aggregates (i.e. large macroaggregates) into a pre-weighed small drying pan with sufficient water.

9. Put the drying pan with the large macroaggregates into the 60°C forced air oven (overnight).

10. Pour the water and particles that went through the 2000 µm sieve remaining in the white basin onto a 250 µm sieve, which is held above the second white basin, and repeat the sieving procedure (in 2 minutes the sieve is moved up and down (approx. 3 cm amplitude) 50 times with a slight angle to ensure that water and small particles pass through the mesh).

11. Take the sieve out of the water and rinse off the sides plus the bottom of the sieve with water in order to have all particles in suspension.

12. Backwash 250-2000 µm aggregates (i.e. small macroaggregates) into a pre-weighed small drying pan.

13. Put the drying pan with the small macroaggregates into the 60°C forced air oven (overnight).

14. Pour the water and particles that went through the 250 µm sieve remaining in the white basin onto a 53 µm sieve, which is held above a white basin, and repeat the sieving procedure (in 2 minutes move the sieve up and down (approx. 3 cm amplitude) 50 times with a slight angle to ensure that water and small particles pass through the mesh).

15. Take the sieve out of the water and rinse off the sides plus the bottom of the sieve with water in order to have all particles in suspension.

16. Backwash 53–250 µm aggregates (i.e. microaggregates) into a pre-weighed small drying pan.

17. Put the small drying pan with microaggregates into the 105°C forced air oven (overnight).

18. Pour the water + < 53 µm particles (i.e. silt + clay) remaining in the white basin into a pre-weighed large drying pan.

19. Put the large drying pan with silt + clay particles into the 105°C forced air oven (overnight).

20. The following day weigh all fractions.

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3. SOIL CHEMICAL ANALYSES

BY DIEGO SOTO GÓMEZ, MANUEL ARIAS ESTÉVEZ AND DAVID FERNÁNDEZ CALVIÑO

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Soil is composed of organic and inorganic matter, air and water, and it is common for these elements to experience reactions that include adsorption, weathering, complexation, ion exchange and precipitation (Hanrahan 2012). This is crucial from the point of view of fertility and environmental safety, since the compounds and nutrients that are introduced in the soil can undergo a series of transformations that will be conditioned by the characteristics of that soil. However, that also means that if the chemical characteristics of a soil are known, it is possible to predict the behaviour and fate of these external inputs.

Soil chemistry is a scientific discipline aiming to study chemical processes in soil (Arai 2016). Knowledge on these processes is crucial for understanding soil fertility and soil quality in agroecosystems.

The chemical properties that will be considered in this work as well as the project members in charge of each analysis are summarized in Table 3.1.

Table 3.1. Chemical analysis used to characterize the samples and partners in charge of each analysis.

CHEMICAL PROPERTIES MEASURED	
ANALYSIS TYPE	PARTNER IN CHARGE
Soil pH	UVIGO
Organic matter and carbon content	UVIGO
Particulate organic carbon and mineral associated organic carbon fractions	UPCT
Total nitrogen	UVIGO
Inorganic nitrogen (nitrates and ammonium)	UPCT
Available P in the soil (Bray / Olsen)	UVIGO
Effective cation exchange capacity and Ca, Mg and K availability	UPCT
Available Fe, Mn, Cu and Zn	UVIGO
Total pesticides	UVIGO
Glyphosate and AMPA	UVIGO

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3.1. SOIL PH

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PRINCIPLE AND APPLICATION

The pH is the negative logarithm of the concentration of hydrogen ions (H+) expressed in mol L-1, assuming that concentration and activity of an ion are comparable in strongly diluted aqueous solution. The pH of a soil is conditioned by the base-forming cations (Ca2+, Mg2+, Na2+ and K+), and by the acid-forming cations (Fe2+ or Fe3+, H+ and Al3+) (Mccauley, Jones, and Olson-Rutz 2017). The pH is a fundamental property of soils since it determines solubility, concentration in solution, mobility and ionic form of nutrients (Fageria and Nascente 2014), and, therefore, their availability (Horrocks and Vallentine 1999). Some elements are more available at acidic pHs, such as nitrogen, iron or copper, and others such as calcium, magnesium and sodium are easily leached at acidic pH and are more available in basic soils (Figure 3.1.1.).

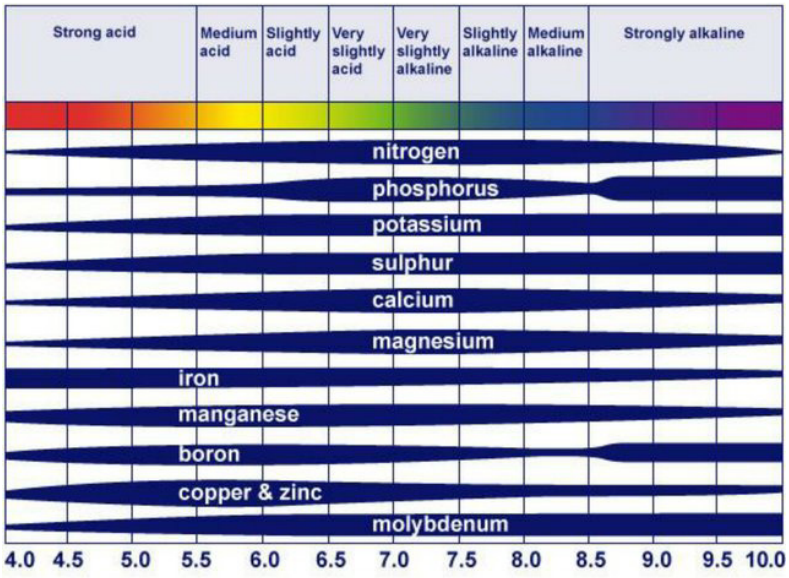

Figure 3.1.1. Effect of the soil pH in the availability of some nutrients. The wider the blue line, the more available the element (from Roques et al. 2013)

Usually, a 1:5 soil suspension in distilled water is used to measure soil pH (Ditzler, Scheffe, and Monger 2017). However, certain electrolytes can also be used for different purposes. Sometimes, instead of water, a 0.01M solution of calcium chloride (CaCl2) is applied because it dampens the suspension effect, the variations in the soil:solution ratios and the initial saline concentration. The use of potassium chloride (KCl) is also common due to its ability to displace H+ and Al3+ cations, adsorbed to exchangeable sites of soil colloids, to the solution leading to lower pH values (Kome et al. 2018). Soil pH values measured in neutral salt solutions (CaCl2 or KCl) is often referred as exchangeable acidity, being narrowly related to the degree of the cation exchange complex by aluminium. Soil pH has an strong influence on crop growth and thus, some crops develop properly in water with a pH in the range of 6-7 (Daniels 2016), while others prefer soils with more acidic conditions (Miller 2016).

EQUIPMENT AND MATERIAL

- Analytical balance (0.01 g)
  Greiner tubes of 15mL
- Spatula
- Shaker
- Distilled water
- Potassium Chloride (KCl)
- Calcium Chloride (CaCl2)
- pH-meter (preferably with an automatic temperature compensation probe)
- Buffers (pH 4.00, 7.02 and 9.21)

CALCULATIONS

The difference between the soil pH measured in KCl and measured in H2O (ΔpH = pH KCl - pH H2O) is calculated to determine if the negative charges in soil colloids predominate, conferring it as cation exchange capacity (ΔpH <0). Otherwise, if ΔpH >0, soil has an anion exchange capacity due to the predominance of positively charged colloids (Kome et al. 2018).

REMARKS

At the start of pH measurements (or when a pronounced change in pH value between two successive samples is observed), it is recommended to extent the pH measurement period for a few minutes (using the continuous mode if it is available) in order to favour the stabilization of the measurement before recording the final value. It is possible to establish a correlation between the organic matter content of a soil and its pH in CaCl2 (Fuentes et al. 2019).

PROCEDURE

The procedure to measure pH is as follows:

1. Weigh 2 g of soil (sieved, 2 mm).
2. Add 10 mL of distilled water.
3. Shake for 1 h.
4. Let the suspension rest for 1 h.
5. Calibrate the pH-meter with pH 4.00, 7.02 and 9.21 standards
6. Measure with a pH-meter before the next 2 h.

The procedure is repeated twice replacing distilled water with a 1M solution of KCl, and a 0.01 M solution of CaCl2, always maintaining the same weight:volume ratio (1: 5). Hence, three soil pH values measured in different solutions (water, 1 M KCl or 0.01 M CaCl2) are routinely obtained. Two hours of equilibration are needed before measuring the pH.

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3.2. ORGANIC MATTER AND CARBON CONTENT

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PRINCIPLE AND APPLICATION

The soil organic matter (SOM) is a complex type of natural material of crucial importance for the soil filtering function, soil aggregate stability, carbon cycling, and soil fertility (Simpson and Simpson 2012). SOM is composed of living plants, animals and microorganisms, as well as humus and residues of dead plants, animals, and microorganisms in different stages of decomposition (Hillel 2004). Humus is an important part of SOM (between 60 and 80%), it contains a large part of the nitrogen in this medium and is usually bound to inorganic materials of the matrix, so it is degraded quite slowly. SOM-content can be drastically changed by anthropogenic additions like compost, industrial organic by-products, farm yard manure, biomass of water purification systems, etc.

SOM contains a large proportion of organic carbon (around 58%), and these forms of carbon constitute the so-called soil organic carbon (SOC) (Iglesias Jiménez and Pérez García 1992). SOC is directly related to the quality of the environment, erosion resistance, water retention and plant productivity

(Han et al. 2017). SOC has a direct effect on plant growth and is the source of energy of many of the organisms that live in the soil (Anderson and Domsch 1985). Tillage can accelerate the loss of this type of carbon by favouring mineralization or oxidation as well as losses from leachate (Lal 2002).

In addition to this SOC, soil inorganic carbon (SIC) can also be found in soil, especially in the form of carbonates in high-pH soils. SIC has a lower interest from an agricultural perspective, but nevertheless constitutes an important part of the soil carbon pool (Tan et al. 2014).

SOM is usually determined by the method of loss-on-ignition (LOI) or wet oxidation (WO) (Hoogsteen et al. 2015). These methods are based on the difference in soil mass before and after oxidation (mineralization) of SOM. The difference between the two techniques (LOI and WO) rely on the way of eliminating the organic matter. SOM is more sensitive to heat than the mineral soil constituents implying that controlled SOM combustion can be carried out to eliminate organic matter without affecting the mass of remaining inorganic soil components. Another option is to use an oxidizing agent such as hydrogen peroxide to remove organic components. However, both methods have certain limitations. For example, the oxidation of organic matter by WO is usually incomplete and it is necessary to apply a correction factor that depends largely on the type of soil used. In the case of LOI, the water contained in the clays can lead to overestimation of SOM.

To determine the amount of SOC and SIC, a CHN elemental analyzer is usually employed (Duan et al. 2004). For soil carbon determination the samples are burned at high temperatures to release N2, SO2, O2, NOx, CO2 and steam. These gases are separated using a series of columns and temperature variations, and they are passed through a thermal conductivity detector that, with the help of a series of patterns, allows to calculate the amount of carbon in the samples. In order to specifically determine SOC and SIC, total soil carbon is determined twice: i.e. before and after specific removal of organic matter.

EQUIPMENT AND MATERIAL

- Elemental Analyzer
- Muffle
- Porcelain crucible
- Analytical balance (0.01 g)
- Oven
- Spatulas
- Soil mill
- Tin capsules

CALCULATIONS

In order to calculate the percentage of organic matter in the sample (%O.M.) the following formula is applied:

$$\%\text{O. M.} = \frac{m_{DS-}m_{BS}}{m_{DS-}m_{C}} x100$$

Where

- mDS is the mass of the dry soil and the porcelain crucible.
- mBS is the mass of the burnt soil and the porcelain crucible.
- mC is the mass of the porcelain crucible.

The total carbon content (TCC) and the soil inorganic carbon (SIC) are obtained in percentage by weight (g of C in 100 grams of dry soil), a unit that can be converted to g of C per kilogram of dry soil by multiplying it by 10. The SOC is calculated by:

SOC = TCC – SIC

REMARKS

Flat-bottom crucibles are better suited to the determination of organic matter, since, by extending the soil sample over a larger area, soil burning is optimized.

PROCEDURE

A) Organic matter

First, it is necessary to dry the soil to prevent errors derived from moisture loss. For this, a porcelain crucible is weighed (mC). Then, about 10 grams of 2 mm sieved soil are also weighted (two replicates are made per sample). This soil is dried in an oven at 105°C until constant weight (mDS). Once the soil moisture has been removed, the soil is weighed to obtain the initial mass, and then the dried soil is introduced into a muffle at 450°C for 4 hours (LOI). When finished, the soil is reweighed (mBS). The values of organic matter are usually given as a percentage (with regard to the dry soil mass).

B) Total soil carbon

To determine the total soil carbon, it is necessary, first, to grind a few grams of 2 mm sieved soil. About 50 mg of the grinded soil are weighed in tin capsules and introduced into the Elemental Analyzer. This process must be repeated with carbon standards of known concentrations. The proportions obtained with this method have to be converted into mg of total carbon per gram of dry soil.

This process is repeated once the organic matter has been selectively removed by LOI to determine the amount of inorganic carbon present in the sample. The amount of SOC is calculated as the difference between total soil carbon and SIC.

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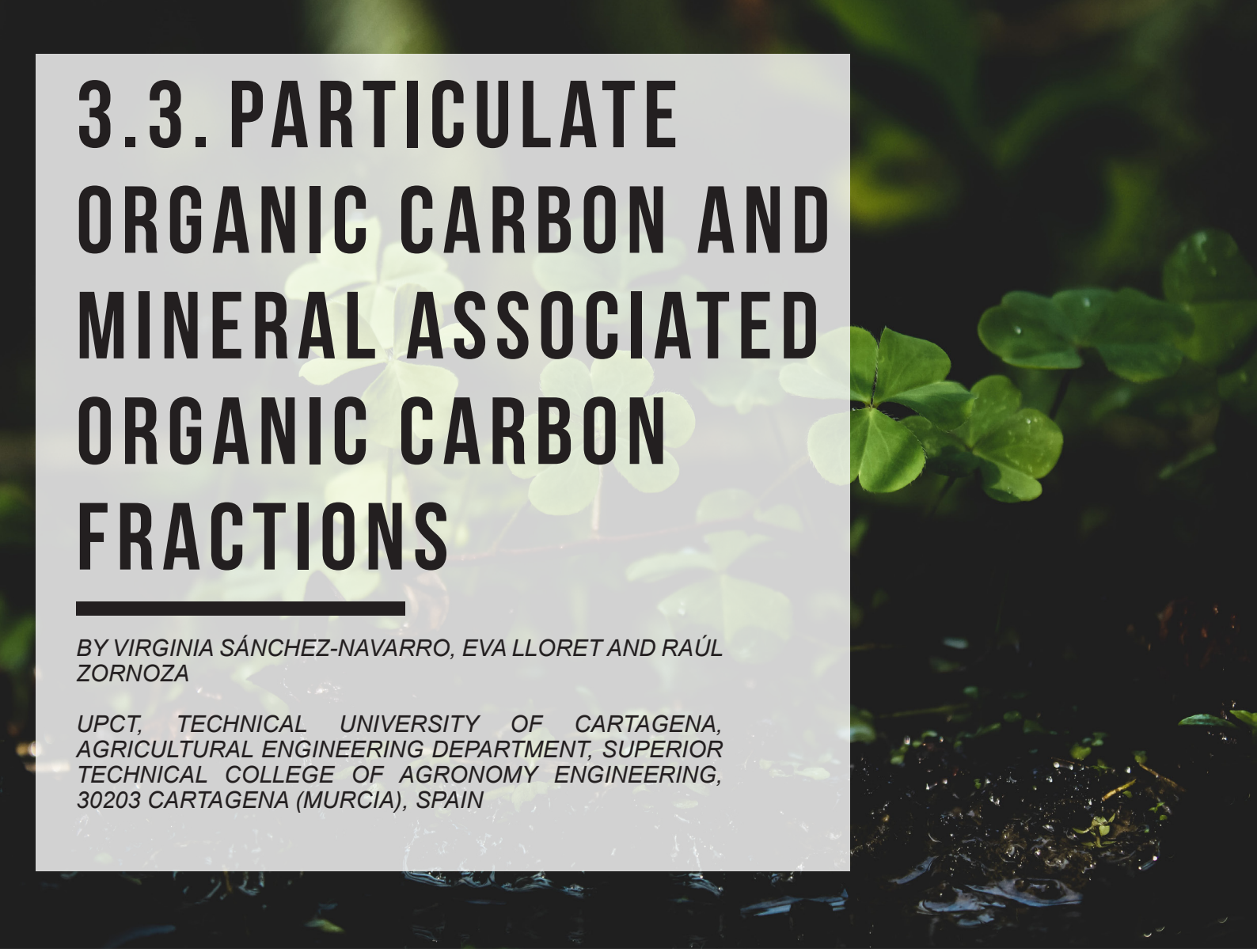

3.3. PARTICULATE ORGANIC CARBON AND MINERAL ASSOCIATED ORGANIC CARBON FRACTIONS

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PRINCIPLE AND APPLICATION

The interest for knowledge of the different carbon fractions and their resistance and ease of degradation, lies in their importance with respect to the characterization of the C cycle and the soil as a C sink (Forbes et al. 2006; Kuyakov et al. 2009). Soil organic matter (SOM) in part consists of particulate organic matter (POM) or particulate organic carbon (POC). POC represents the fresh or decomposing organic material (e.g. fine root fragments and other organic debris), and it serves as a readily decomposable substrate for soil microorganisms. The mineral associated organic carbon fraction (MOC) represents a more recalcitrant fraction, since it is highly resistant to microbial decomposition, and thus this fraction is linked to soil C storage and sequestration (Laganiere et al. 2010).

The method presented here is based on the soil physical fractionation, which is useful for distinguishing specific C pools responsive to agricultural soil management.

PROCEDURE

1. Air-dry soil samples.
2. Sieve dry soil at < 2 mm.
3. Weigh 20 g of dry mineral soil and disperse by shaking overnight in a 100 mL solution of sodium hexametaphosphate (5 g L-1).
4. Sieve the mixture through a 0.053 mm sieve and gently wash the material retained by the sievewith deionized water (use 1 L of water). The material retained by the sieve is collected for POC determination, the soil particles passing through the 0.053 mm sieve is collected for MOC determination.
5. Weigh the filters.
6. Filter the retained material (> 0.053 mm) and put it in the oven at 60ºC for 24 h for POC determination. Weight the soil + filter.
7. Put the soil particles (<0.053 mm) in the oven until the soil sample is dry for MOC determination. Weigh the dry soil.
8. Grind the soil using a mortar and store it for carbon analysis. Concentrations of C in the isolated fractions will be determined using a C and N analyzer

EQUIPMENT AND MATERIAL

- **Reciprocal shaker**
- **0.053mm sieve**
- **Porcelain crucibles 10–15 cm diameter**
- **Whatman filter paper 541. Hardened Ashless. CAT No. 1541-125**
- **Oven**
- **Agate mortar**
- **Analytical balance (0.01 g)**
- **Wash water bottle**
- **C and N analyzer**

REAGENTS

- **Deionised water**
- **Sodium hexametaphosphate (5 g L-1): dissolve 5 g of sodium hexametaphosphate in deionised water, complete to 1 L and shake well.**

CALCULATIONS

POC fraction (g C kg-1 soil) is calculated using the following expression:

$$POC\ (g\ kg\text{-}1) = ((M1 \times 100 \times C1)/Mt)/10$$

Where:

- **M1 is the mass of soil > 0.053 mm (g)**
- **Mt is total mass of soil used at the beginning of the analysis (g)**
- **C1 is the C concentration of the soil > 0.053 mm (%)**
-

MOC fraction (g kg-1) is calculated using the following expression:

$$MOC\ (g\ kg\text{-}1) = ((M2 \times 100 \times C2)/Mt)/10$$

Where:

- **M2 is the mass of soil < 0.053 mm (g)**
- **Mt is total mass of soil used at the beginning of the analysis (g)**
- **C2 is the C concentration of the soil < 0.053 mm (%)**

REMARKS

MOC fraction can also be determined by the formula MOC = TOC – POC, where TOC is total organic carbon, although this approach is less accurate.
The time in the oven will depend on the quantity of POC and MOC obtained (24 hours is the minimum).
As an alternative to removing the stones by hand, the dry soil may be sieved with a 1 mm sieve.

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3.4. TOTAL NITROGEN

BY DIEGO SOTO GÓMEZ, ANTÍA GÓMEZ ARMESTO, FLORA ALONSO VEGA, MANUEL ARIAS ESTÉVEZ AND DAVID FERNÁNDEZ CALVIÑO

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PRINCIPLE AND APPLICATION

Nitrogen (N) is one of the essential elements for crop development, together with potassium (K) and phosphorus. It (P). N is distributed across the atmosphere, the biosphere and the lithosphere, but, unlike K and P, N is not found in the rocks, so it must be introduced into the soil through fertilization or through the fixation of the atmospheric N2 (Figure 3.4.1.) (Hofman and Cleemput 2004). The total soil N content is usually between 0.05 and 0.2% by weight, but only a small proportion can be directly assimilated by plants, that (mainly assimilate the inorganic N species ammonium, (NH4++) or nitrate, (NO3-).

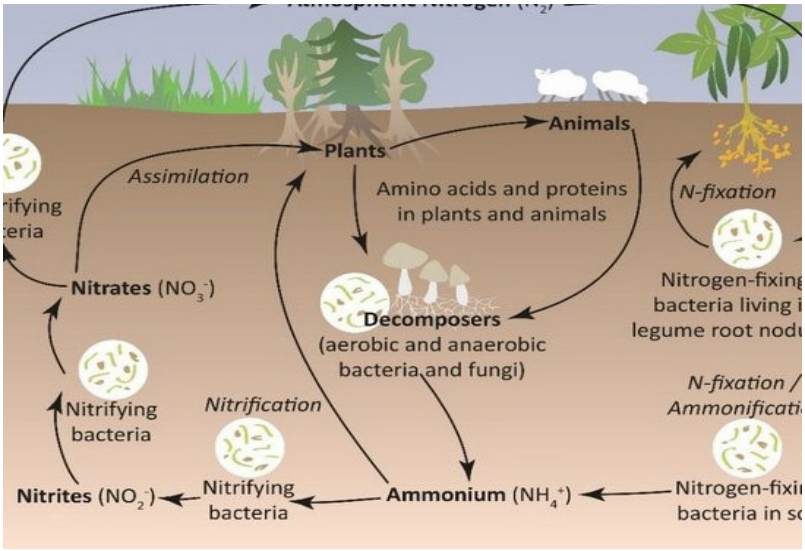

Figure 3.4.1. Simplified diagram of the terrestrial N cycle (from Lappalainen et al. 2016)

N deficiency is associated with lower crop yields and quality via reduced protein content (Dalal et al. 1991). There is a big difference in available N between different soils, as it is conditioned by many factors including weather, season, depth, vegetation, crop history, slope, soil texture ...etc (Lin 2006).

N deficiencies are common in unfertilized soils (Hofman and Cleemput 2004), and it is therefore necessary to fertilize the soil providing this element. N fertilization (in the form of synthetic fertilizers) in intensive agriculture reaches 600 kg per hectare and year in the field, and about 2000 kg in the greenhouse (Escanhoela et al. 2019). It is also possible to provide N by using organic fertilizers or rotations with N-fixing legumes (Crews and Peoples 2004). As can be seen in Figure 3.4.1.,, the bacteria symbiotically associated with the roots of legumes are a crucial part of the soil N cycle, since they are capable of fixing atmospheric nitrogen.

Total soil N can be determined by a CHN elemental analyzer (see also Section 3,2). Soil samples are burned at high temperatures in order to liberate the nitrogen, hydrogen and carbon gases. The gases are separated and the amount of nitrogen is measured trough a thermal conductivity detector, since the thermal conductivity varies from one element to another.

PROCEDURE

The method used to determine the total nitrogen is the same as the method used for soil carbon (3.2. Organic Matter and Carbon Content). About 50 mg of grinded dry soil is weighed in tin capsules and measured with the Elemental Analyzer. The nitrogen standards are also weighed and analysed. The proportions obtained with this method have to be converted into grams of total nitrogen per kilogram of dry soil.

EQUIPMENT AND MATERIAL

- **Elemental Analyzer**
- **Analytical balance (0.0001 g)**
- **Oven**
- **Spatulas**
- **Soil mill**
- **Tin capsules**

CALCULATIONS

The results of total nitrogen are obtained in percentage by weight (g of N in 100 grams of dry soil), a unit that can be converted to grams of N per kilogram of dry soil by multiplying by 10.

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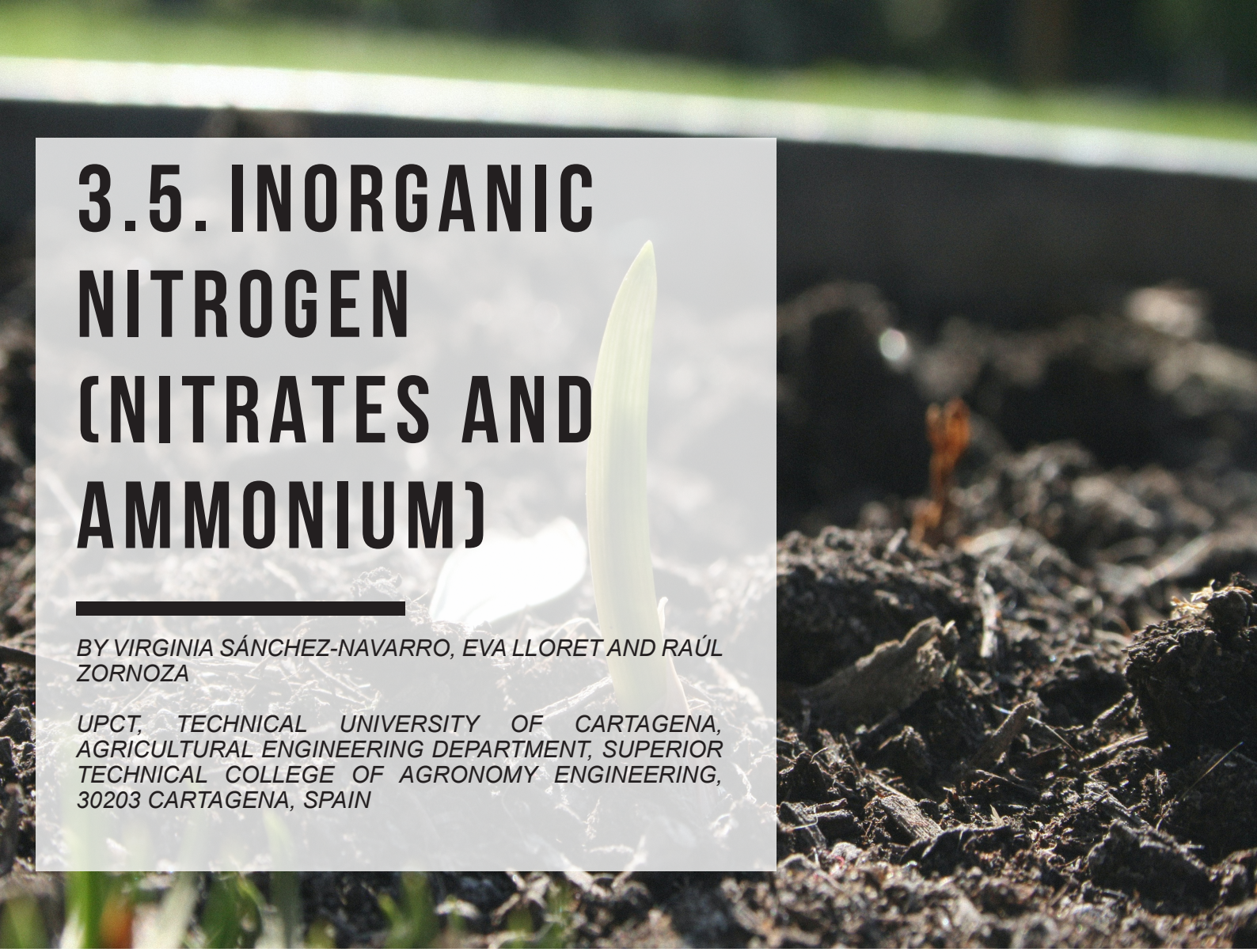

3.5. INORGANIC NITROGEN (NITRATES AND AMMONIUM)

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PRINCIPLE AND APPLICATION

(N) is an essential element for the nutrition of plants and microorganisms. Most soil nitrogen is present as organic N compounds with only a small fraction being found in the mineral forms that are directly usable by crop plants. The process of mineralization of organic nitrogen, necessary for its assimilation as a plant nutrient, occurs mainly through ammonification (organic N mineralization to ammonium) and nitrification (oxidation of ammonium into nitrite and nitrate).

Nitrate and ammonium are extracted from soil samples using a 2 M solution of potassium chloride (KCl), and subsequent analysed by ion chromatography (nitrate and nitrite) and spectrophotometry (ammonium) (Sempere et al. 1993; Kandeler and Gerber 1988; Keeny and Nelson 1982). Soil used for the determination of inorganic nitrogen may be kept at 4 ºC at field-moist conditions if it is analysed within 3 days of sampling. Alternatively, soil may be stored at -20°C to avoid the loss of mineral N.

PROCEDURE

Nitrate determination

1. Homogenize the soil sample (manually or mechanically). The soil sample must be transferred into a refrigerated container.
2. Weigh 2.5 g of sieved field-moist soil (< 2 mm) in a plastic vial, recording the exact weight. Add 25 mL of the deionized water (soil / extractant ratio 1:10).
3. Shake for 60 min on the rotating plate.
4. Centrifuge the samples for 2 min at 3000 g. Filter supernatant using syringe filters.
5. The extract obtained is measured in the ion chromatography equipment.
6. Prepare a calibration curve from the NO3- stock solution.

EQUIPMENT AND MATERIAL

- Analytical balance (0.01 g)
- Plastic vials (50 mL)
- Volumetric flask (100 mL)
- Rotating plate
- Plastic test tubes with cup (10 mL)
- Centrifuge
- Syringe filters (0.2 µm)
- Syringe (10 mL)
- Ion chromatography equipment

REAGENTS

- Stock or commercial nitrate solution (1000 mg L-1 NO3-)

CALCULATIONS

NO_3^- (mg kg-1) = (C × V × df) / m

- C is the concentration of nitrate obtained from ion chromatography (mg L-1)
- V is the total volume of the added deionised water (25 mL)
- df is the dilution factor. If it is not applied, it is 1
- m is the weight of the dry soil sample (g) (moisture correction is needed by determination of soil moisture)

EQUIPMENT AND MATERIAL

- Analytical balance (0.01 g)
- Plastic vials (50 mL)
- Plastic test tubes with cup (10 mL)
- Volumetric flasks (100, 500 and 1000 mL)
- Plastic buckets (2.5 mL)
- Vortex shaker
- Centrifuge
- Automatic pipette (1-5 mL)
- Rotating plate
- Visible / UV spectrophotometer

CALCULATIONS

$NO3\text{-}\ (mg\ kg\text{-}1) = (C \times V \times df) / m$

- C is the concentration of nitrate obtained from ion chromatography (mg L-1)
- V is the total volume of the added deionised water (25 mL)
- df is the dilution factor. If it is not applied, it is 1
- m is the weight of the dry soil sample (g) (moisture correction is needed by determination of soil moisture)

Ammonium determination

1. Homogenize the soil sample (manually or mechanically). The soil sample must be transferred in a refrigerator container.

2. Weigh 2.5 g of sieved soil (< 2 mm) in a plastic vial, recording the exact weight. Add 25 mL of the 2M KCl solution (soil / extractant ratio 1:10).

3. Shake for 60 min on the rotating plate.

4. Centrifuge the samples 2 min at 3000 g.

5. Take a 5 mL aliquot of the supernatant from each sample. For samples with a high ammonium content, pipette 2.5 mL (in this case the dilution factor is 2). Pour the aliquot in a 10 mL test tube. Add 2.5 mL of deionized water to the test tube if 2.5 mL has been pipetted to have a final volume of 5 mL in all tubes.

6. Prepare tubes to determine the calibration curve. The standards will have concentrations of 0, 0.5, 1, 1.5, 2.5 and 5 mg L-1 of NH4 +, and are prepared from the stock solution of 100 mg NH4 + L-1 (Table 3.5.1.). These concentrations will be the dependent variables and the corresponding sabsorbance values will be the independent variable.

Table 3.5.1. Preparation of standards from the 100 mg stock solution NH4 + L-1 (Final volume of 10 mL).

NH4 + standard concentration (mg L-1)	
0	0
0.5	0.05
1	0.10
1.5	0.15
2.5	0.25
5	0.5

7. Add 2.5 mL of Salicylate-Na/NaOH solution and 1 mL of 0.1% sodium dichloroisocyanide to all tubes.

8. Cover, shake (using the vortex shaker), and let stand for 30 min in darkness.

9. Then, turn on the spectrophotometer and adjust the wavelength (λ) to 690 nm. Perform the auto-zero with the 0 mg NH4 + L-1 standard.

10. Measure all standards, including 0 mg NH4 + mL-1, to perform the calibration curve in which the NH4 + concentration of the samples and blanks will be calculated.

11. Measure the absorbance of the samples in the spectrophotometer. In the event that any sample gives an absorbance greater than the maximum value of the calibration curve, it is necessary to take less volume of supernatant to make a dilution (step 5).

REAGENTS

- **Solution of potassium chloride (2 M KCl): dissolve 149 g of potassium chloride (KCl) in a 1000 mL graduated flask containing approximately 800 mL of H2O. Fill up to the final volume with deionised water.**
- **Sodium hydroxide (0.3 M NaOH): dissolve 12 g of NaOH in a 1000 mL graduated flask containing approximately 800 mL of H2O. Fill up to the final volume with deionized water.**
- **Sodium salicylate solution: dissolve 85 g of sodium salicylate (C7H5NaO3) and 600 mg of sodium nitroprusside (Na2 [Fe (CN)5 NO]). Fill up to 500 mL with deionized water. Store at 4°C.**
- **Salicylate-Na / NaOH solution: mix equal volumes of 0.3 M NaOH, sodium salicylate and deionized water (1: 1: 1). Prepare daily.**
- **Solution of sodium dichloroisocyanide (0.1% C3Cl2N3NaO3) (w:v): dissolve 0.1 g of sodium dichloroisocyanide in 100 mL of deionized water. Prepare daily.**
- **Ammonium stock solution (100 mg L-1 NH4+): dissolve 0.297 g of NH4Cl in 1000 mL of 2M KCl**

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3.6. AVAILABLE P IN THE SOIL (BRAY / OLSEN)

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PRINCIPLE AND APPLICATION

After nitrogen, (N), phosphorus (P) is the second-most important element for plant nutrition (Kauffman 2002). P is a component of nucleic acids (DNA and RNA) and of adenosine triphosphate (ATP); an energy storage molecule essential for life. In plants, P is a crucial element in development (a significant amount of phosphorus is needed for cell division), flowering, fruit ripening and photosynthesis (Weil and Brady 2017).

Intensive agriculture (without the addition of phosphorus) usually results in low P-containing soils on which plant growth is strongly constrained. This type of soils remains unprotected from erosion (Weil and Brady 2017). However, it is also common to find the opposite scenario such as agricultural systems where more phosphorus is added than removed through harvest. In many cases, this phosphorus surplus is mobilized towards groundwater by seepage and finally ends up in freshwaters (rivers and lakes), contributing to generate eutrophic conditions and putting at risk the quantity and quality of drinking water.

In soils, P normally occur in low quantities, and it appears in forms which are not usually available for plant uptake. In addition, highly soluble P forms are used for soil fertilization (as slurries, for example), it is insolubilized quite easily. after contact with the soil. This is one of the reasons why agricultural soils are usually fertilized with P in excess.

As only a small proportion of total P in soils is available for plant uptake, more attention should be paid in determining this P fraction. The concentration of available phosphorus can be assessed using different extractant solutions (depending on soil pH). Two main methods can be distinguished depending on soil pH: the Olsen method is recommended to be applied for alkaline soils, whereas for neutral and acidic soils the Bray II method is preferred (Dari et al. 2019).

EQUIPMENT AND MATERIAL

- Magnetic stirrer
- Shaker
- Greiner tubes (50 mL)
- Dispenser
- Beakers
- Analytical balance (0.0001 g)
- Centrifuge
- 0.45 µm Filters
- Sulphuric acid, H_2SO_4
- Sodium hydroxide, NaOH
- Sodium bicarbonate, $NaHCO_3$
- p-Nitrophenol ($NO_2C_6H_4OH$)
- Ammonium molybdate $[(NH_4)_6Mo_7O_{24} \times 4H_2O]$
- Potassium antimony tartrate $[(K(SbO) \times C_4H_4O_6 \times ½ H_2O]$
- Ascorbic acid ($C_6H_4O_6$)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Spectrophotometer

CALCULATIONS

To present the results, the phosphorus content is usually referenced to grams of dry soil. For this, we can use the following formula:

$$C\_P=(A-B)\ V\_E/V\_S\ \ 50/m$$

Where:

- **CP is the extractable phosphorus content in the soil (in mg of P g-1 of dry soil)**
- **A is the P concentration in the sample (mg mL-1)**
- **B is the P concentration in the blank (mg mL-1)**
- **VE is the volume of extractant used (mL).**
- **VS is the volume of sample measured by spectrophotometry (mL).**
- **m is the soil mass (g)**

PROCEDURE

A) Olsen

Before starting it is necessary to prepare several solutions:

1. Sulphuric acid solution 2.5 M. Carefully add 140 mL of H_2SO_4 (with 96% purity) to a one-litre volumetric flask containing about 400 mL of water, and dilute to the mark with distilled water.

2. Sodium hydroxide solution 1M. Dissolve 40 mL of NaOH in distilled water. It is an exothermic solution, so it is necessary to stir and let cool before completing the volume with water.

3. Sodium bicarbonate solution. In a volume close to one litre (≈ 0.8-0.9 L), dissolve 42 g of sodium bicarbonate ($NaHCO_3$). The pH of the solution is adjusted to 8.5 (adding drops of the O2 solution) before completing the volume (1 L).

4. 0.25% solution of p-Nitrophenol. 0.25 g of p-Nitrophenol ($NO_2C_6H_4OH$) are dissolved with distilled water in a 100 mL volumetric flask, completing the dilution to mark with water.

5. Ammonium molybdate solution 40g L-1. 40 g of ammonium molybdate [$(NH_4)_6Mo_7O_{24}$] are weighed, dissolved in distilled water and made up to 1000 mL.

6. Potassium antimony tartrate solution. This solution should contain 1 mg of antimony per millilitre, so 0.2728 g of potassium antimony tartrate [$(K(SbO) \times C_4H_4O_6 \times ½ H_2O$] are dissolved in distilled water and made up to 100 mL.

7. Ascorbic acid solution 0.1 M. This solution must be prepared at the time of use. 1.76 g of ascorbic acid ($C_6H_4O_6$) are dissolved in water and made up to 100 mL.

8. Sulphomolybidic solution. This solution must be prepared at the time of use. Mix: 50 mL of O1 solution, 15 mL of O5, 30 mL of O7 and 5 mL of O6.

9. It is also necessary to prepare a phosphorus stock solution to make the standards. To prepare this solution weight 4.3938 g of KH_2PO_4 (dried at 40 °C), dissolve it in distilled water and make it up to 1000 mL. The phosphorus concentration in this solution is 1000 mg L-1.

The phosphorus determination can be divided into two parts: extraction and measurement by spectrophotometry.

To carry out the phosphorus extraction, two grams of soil are weighed (m) in a 50 mL Greiner tube and 40 mL of the O3 solution are added (VE). This is stirred for 30 minutes and filtered by a 0.45 µm filter. It is important to make a blank without soil, but following the same steps.

For the determination by spectrophotometry an aliquot of the extract is taken (the volume of this aliquot depends on the amount of P the soil has, VS), 5 drops of O4 are added and then O1 is added until the colour changes to yellow. This is diluted with water and 8 mL of O8 are added. In the end, the volume of 50 mL is completed with distilled water. Let the solution stand for 10 min and measure with a spectrophotometer, using a wavelength of 882 nm.

When measuring, it is necessary to employ the same procedure with the standards that are going to be used in the calibration, replacing the soil or blank with a known concentration of phosphorus. Standards of 0, 1, 2, 3, 4 and 5 mg L-1 are usually employed.

B) Bray II

Before starting it is necessary to prepare several solutions:

1. xtraction solution of hydrochloric acid (0.1 N) and ammonium fluoride (0.03 N). Add 1.11 g of NH4F and 8.28mL of HCl (37%) to a 1 L volumetric flask and dilute to the mark with distilled water.

2. This solution is obtained by mixing two solutions. The first one is made dissolving 0.116 g of potassium antimony tartrate in 500 mL of distilled water, and the second results from the dissolution of 4.8 g of ammonium molybdate in 250 mL of distilled water. After mixing of both solutions in a 1 L volumetric flask, 55 mL of concentrated H2SO4 are carefully added, and the resultant solution is diluted with distilled water to the mark.

3. Solution of p-Nitrophenol (0.25 %). Weigh 0.25 g of p-Nitrophenol in a 100 mL volumetric flask and dilute to the mark with ethanol.

4. Solution of NH3 (5%). Add 20 mL of NH3 in a 100 mL volumetric flask and dilute to the mark with distilled water.

5. Solution of H2SO4 (5%). Add 5.1 mL of H2SO4 in a 100 mL volumetric flask and dilute to the mark with distilled water.

6. This solution must be prepared just before the measurement process. Dissolve1 g of ascorbic acid in 100 mL of solution B2.

7. It is also necessary to prepare a phosphorus stock solution to make the standards. Weight 4.3938 g of KH2PO4 (dried at 40 °C), dissolve it in distilled water and make it up to 1000 mL. The phosphorus concentration in this solution is 1000 mg L-1.

The procedure is the following:
First, 1 g of sieved dry soil (m) is weighted in a Greiner tube and 10 mL of solution B1 is added (VE).
This suspension is shaken for 30 min and filtered by a 0.45 µm filter. In a 50 mL volumetric flask, an aliquot of the filtered suspension is added (0.1-0.5 mL). The volume used depends on the expected amount of phosphorus (VS).

The following solutions are added to the flask
0.5 mL of boric acid.
2-3 drops of B3 solution.
2-3 drops of B4 solution (until the solution turns yellow).
2-3 drops of B5 solution (until the solution becomes colourless).
5 mL of B6 solution.
Dilute to the mark with water.

It is necessary to employ the same procedure with the blank and the standards that are going to be used in the calibration. Standards, replacing the soil with a known concentration of phosphorus of 0.04, 0.08, 0.12, 0.16 and 0.24 mg L-1 are usually employed.

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3.7. EFFECTIVE CATION EXCHANGE CAPACITY AND CA, MG AND K AVAILABILITY

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PRINCIPLE AND APPLICATION

Cation exchange capacity (CEC) represents the total number of negative charges of the colloidal soil complex, expressed in cmol per kg of soil.

CEC is an index of soil fertility, since it provides information about the content of nutrients that a soil can retain by ionic exchange, and so, the nutrients that are available for the plants. CEC values of 8-10 cmol kg-1 are usually considered the minimum acceptable for Ap horizons in cultivated soils in order to obtain a satisfactory crop production (Porta et al. 1999).

The method to determine the CEC shown here is the determination of the effective cation exchange capacity and base saturation level using barium chloride solution as exchangeable salt (ISO 2018).

EQUIPMENT AND MATERIAL

- Shaker, rotary shaker (end-over-end) or horizontal
- Tightly locking polyethylene centrifuge tubes (ca. 50 mL)
- 50 or 100 mL polyethylene (PE) flasks
- Funnels
- Filter paper (Whatman No. 42, Schleicher & Schuell 595 1/2, Macherey-Nagel 261 G 1/4, or similar)
- Glass vacuum line (e.g. electric pump)
- Flame atomic absorption spectrometer (FAAS) or ICP-OES

REAGENTS

- Deionised water (electric conductivity < 0.2 mS m-1 at 25 °C)
- Barium chloride (BaCl2) solution; c(BaCl2) = 0.1 mol L-1. Preparation: Dissolve 24.43 g of BaCl2 × 2 H2O in 1000 mL of water (use volumetric flask)
- BaCl2 solution; c(BaCl2) = 0.0025 mol L-1. Preparation: Dilute 25 mL of solution BaCl2 solution (0.1 mol L-1) in 1000 mL of water
- Magnesium sulphate solution; c(MgSO4) = 0.020 mol L-1. Preparation: Dissolve 4.930 g magnesium sulphate heptahydrate (MgSO4 × 7 H2O) in 1000 mL of water (use volumetric flask). Prepare fresh solution. Magnesium sulphate can lose crystal water during storage. Therefore, wrap the flask in an additional PE bag and store the chemical in a refrigeratorHydrochloric acid, c(HCl) = 12 mol L-1 (ρ = 1.19 g cm^{-3})
- Magnesium standard solution; c(Mg) = 0.0010 mol L-1. Preparation: Add 50 mL of MgSO4 solution (0.020 mol L-1) to a 1000 mL volumetric flask and fill up with water to the mark (see Remarks).
- Acidified lanthanum solution: c(La) = 10 mg L-1. Preparation: Add 15.6 mg lanthanum nitrate hexahydrate [La(NO3)3 × 6 H2O] to a 500 mL volumetric flask, add 42 mL hydrochloric acid (12 mol L-1), and fill up with water to 500 mL
- Acidified caesium chloride solution: Dissolve 10 g caesium chloride in some water. Add 83 mL of hydrochloric acid (12 mol L-1), and make up to 1000 mL with water
- Sodium and potassium stock solution: c(Na) = 400 mg/L, c(K) = 1000 mg/L. Dissolve 1.0168 g sodium chloride and 1.9068 g potassium chloride in water. Transfer to 1000 mL volumetric flask and fill up to the mark with water (see Remarks)
- Diluted stock solution: c(Na) = 40 mg L-1, c(K) = 100 mg L-1. Pipette 25 mL of solution (c(Na) 400 mg L-1, c(K) = 1000 mg/L) in 250 mL volumetric flask and fill up with water to the mark (see Remarks).
- Hydrochloric acid, c(HCl) = 4 mol L-1. Add 333 mL of commercial HCl 37% (ρ = 1.19 g/cm^3) to water, to receive a total volume of 1000 mL.Calcium stock solution: c(Ca) = 1000 mg L-1. Dissolve 2.497 g calcium carbonate in water. Transfer to 1000 mL volumetric flask and fill up to the mark with water (see Remarks).
- Magnesium stock solution: c(Mg)

PROCEDURE

Leaching and measurement of soil bases

1. Weigh 2.50 g of air-dried soil into a polyethylene centrifuge tube of about 50 mL capacity. Close cap tightly. Note the combined mass of tube and soil (m1).

2. Add 30 mL of BaCl2 solution (0.1 mol L-1) and shake for 1 h. Subsequently centrifuge the tubes at 3,000 g for 10 min. Note: Balance tubes before centrifugation. Transfer the supernatant to a 100 mL volumetric flask. Repeat this procedure, i.e. the addition of 30 mL of BaCl2 solution, shaking and centrifugation twice more. Collect all three supernatants in the same volumetric flask. Fill up to the mark with BaCl2 (0.1 mol L-1). Mix, filter and store the extract I for the determination of the exchangeable concentration of Na, K, Ca and Mg (sum of bases).

3. Calibration for Na, K, Ca and Mg determination: Prepare solutions with 0 mL, 5 mL, 10 mL, 15 mL, 20 mL and 25 mL of the diluted stock solution (c(Na) = 40 mg L-1, c(K) = 100 mg L-1) in 50 mL volumetric flasks. Add 10.0 mL of extraction solution BaCl2 (0.1 mol L-1) and 5.0 mL of acidified caesium chloride solution. Fill up to the mark with water. The resulting concentrations of Na are 0, 4, 8, 12, 16, 20 mg L-1. The resulting concentrations of K are 0, 10, 20, 30, 40, 50 mg L-1. Prepare solutions with 0, 2, 4, 6, 8 and 10 mL of the stock solution (c(Ca) 1000 mg L-1, c(Mg) = 100 mg L-1) in 100 mL volumetric flasks. The resulting concentrations of Ca are 0, 1, 2, 3, 4, 5 mg L-1. The resulting concentrations of Mg are 0, 0.1, 0.2, 0.3, 0.4, 0.5 mg L-1.

4. Analysis: Fill 2.0 mL of the extract I and of the blank sample, respectively, into reaction tubes. Add 1.0 mL of acidified caesium chloride solution and 7.0 mL of water and mix (see Remarks). Determine bases with FAAS or ICP-EOS, with the instrument set according to the manufacturer's instructions for optimum performance.

Cleansing

1. Add 30 mL of $BaCl_2$ solution (0.0025 mol L^{-1}) to the soil pellet and shake overnight. Resulting Ba concentration in the equilibrium solution will be about 0.01 mol L^{-1}. Centrifuge tubes at 3,000 g for 10 min. Decant the supernatant liquid.

Re-exchange

2. Weigh the tube with its contents and cap (m2). Add 30 mL of $MgSO_4$ solution (0.020 mol L^{-1}) to the soil pellet and shake overnight. Centrifuge tubes at 3,000 g for 10 min. Decant the supernatant through a filter paper into a new flask and store the extract II for the determination of the concentration of excess Mg.

3. Prepare blank samples without the addition of soil in parallel and follow the above described procedure completely.

Determination of CEC

1. Pipette 0.20 mL from the extracts II of the samples and blank samples into 100 mL volumetric flasks.
2. Add 0.3 mL of the BaCl2 solution (0.1 mol L-1) and additional 10 mL of acidified lanthanum solution (10 mg L-1).
3. Fill up with water to the mark and mix.
4. For calibration, use dilutions of the Mg standard solution (10 mg L-1). Pipette 0, 1, 2, 3, 4, and 5 mL into a series of 100 mL volumetric flasks. Add 10 mL of acidified lanthanum solution (10 mg L-1) to each flask and fill up to the mark with water. Final concentration of the calibration solutions: 0, 0.01, 0.02, 0.03, 0.04, and 0.05 mmol L-1, respectively.
5. Analyse Mg using FAAS at a wavelength of 285.2 nm or using ICP-OES with instrumentation settings following the manufacturer's instructions for optimum performance of the instrument.

REMARKS

If the barium chloride extract has a yellowish-brown colour, this indicates that some organic matter has been dissolved. If this occurs, record it in the test report.

As an alternative to the preparation of standards and calibration series, respectively, certified standard solutions are commercially available; aliquots are diluted as required.

For a complete analysis of exchangeable cations, it might be reasonable to additionally determine NH4+ in fertilised agricultural soil and exchangeable Al and Fe in very acidic soil respectively.

Any other volumes can be used as well, as long as the same concentrations are obtained and the final sample volume is sufficient for analysis with FAAS or ICP-OES.

Dilutions can be prepared much faster by pipetting or using a diluter system.

CALCULATIONS

Correct the concentrations of Mg in the sample solutions for th
e volume of the liquid retained by the centrifuged soil after being treated with 0,0025 mol L-1 BaCl2 solution:

$$c2 = [c1 (30 + m2 – m1)] / 30$$

where

- **c2 is the corrected Mg concentration in the sample [mmol L-1]**
- **c1 is the Mg concentration in the sample [mmol L-1]**
- **m1 is the mass of the centrifuge tube with air-dried soil [g]**
- **m2 is the mass of the centrifuge tube with wet soil [g]**
-

Calculate the cation exchange capacity (CEC) of the soil using the following equation:

$$CEC = (cb1 - c2) 3,000 / m$$

where

- **CEC is the cation exchange capacity of the soil [cmolc kg-1]**
- **c2 is the corrected Mg concentration in the sample [mmol L-1]**
- **cb1 is the Mg concentration in the blank [mmol L-1]**
- **m is the mass of the air-dried sample [g]**

If the CEC exceeds 40 cmolc kg-1, the determination should be repeated using less soil, adjusting the calculation accordingly

Calculate the soil bases as follows:

$$c(Na, exchangeable) = 2.1749 \times (csample – cblank) / m [cmolc kg-1]$$
$$c(K, exchangeable) = 1.2788 \times (csample – cblank) / m [cmolc kg-1]$$
$$c(Ca, exchangeable) = 8.2288 \times (csample – cblank) / m [cmolc kg-1]$$
$$c(Mg, exchangeable) = 4.9903 \times (csample – cblank) / m [cmolc kg-1]$$

with the measured concentrations in the diluted sample (csample) and the diluted blank sample (cblank), respectively, and m as the soil mass in g.

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3.8. AVAILABLE FE, MN, CU AND ZN

BY DIEGO SOTO GÓMEZ, CRISTINA ÁLVAREZ ESMORÍS, FLORA ALONSO VEGA, MANUEL ARIAS ESTÉVEZ AND DAVID FERNÁNDEZ CALVIÑO

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PRINCIPLE AND APPLICATION

When assessing a soil and its fertility it is crucial to take into account the concentration of bioavailable essential metals required for plant nutrition (Zhang et al. 2018). Metal bioavailability depends on the characteristics of the organisms present, the properties of the soil and the chemical metal speciation (Ehlken and Kirchner 2002).

Frequently, to obtain the phytoavailable metals from a soil, one-step extraction is done using either ethylenediaminetetraacetic acid (EDTA) or diethylenetriaminepentaacetic acid (DTPA) as extractants (Haq, Bates, and Soon 1980). These two acids are widely used because they form very stable and water-soluble metal complexes. Considering the work of Feng et al. (2005), the extraction with DTPA is more suitable for neutral or alkaline soils. If DTPA is used for extraction of metals from an acidic soil, the buffering capacity of the soil suspension can be exceeded and metals that are not phytoavailable are likely to be released into the solution. EDTA is used for acidic soils since it forms, at low pH, stable chelates with the metals adsorbed to the soil surface (Hammer and Keller 2002). This trend to form stable soluble chelates with EDTA decreases in alkaline soils, in the presence of calcium and magnesium ions (Manouchehri, Besancon, and Bermond 2006). For this reason, when analysing the phytoavailability of metals, both extractants are usually used depending on the soil pH in water.

PROCEDURE

When extracting Fe, Mn, Cu and Zn, it is important to consider the pH of the soil in water: for soils with a pH higher than 6, DTPA is used, and for those with a lower pH EDTA is applied.

A) Soil with pH > 6

First of all, it is necessary to prepare a DTPA solution by weighing the following reagents in a 250 mL beaker:

- DTPA, 1.9667 g
- CaCl2 x 2H2O, 0,0735 g
- Triethanolamine: 14 mL (TEA 98%) or 15.6 mL (TEA 85%).

This solution is shaken by a magnetic stirrer, and distilled water is added until it reaches a volume of 900 mL. Once this is done, the pH is measured and adjusted to 7.3 by adding 37% HCl. Then distilled water is added up to 1 L.

Once the solution has been prepared, 2 grams of soil (sieved by 2 mm) are weighed in a Greiner tube and 4 mL of the DTPA solution are added (the soil:extractant ratio is 1:2). The tubes are shaken for 2 hours, centrifuged during 5 minutes at 1500 g and the supernatant filtered using a 0.45 µm filter. The filtrate is analysed by an atomic absorption spectrophotometer.

B) Soil with pH < 6

In this case, the process is similar but the extractant, and the ratio are different. First, it is necessary to prepare the EDTA solution, for which the following reagents must be weighed in a 250 mL beaker:

- EDTA, 1,8612 g.
- Ammonium acetate, 77 g.

This solution is shaken by a magnetic stirrer and distilled water is added until it has a volume of 900 mL. Once this is done, the pH is measured and adjusted to 4.65 by adding 37% HCl. Then distilled water is added up to 1 L.

Once the solution has been prepared, 2 grams of soil (sieved with a 2 mm mesh sieve) are weighed in a Greiner tube and 10 mL of the EDTA solution are added (the soil:extractant ratio is 1:5). The tubes are shaken for 1 hour, centrifuged for 5 minutes at 1500 g and the supernatant filtered using a 0.45 µm filter. The filtrate is analysed by an atomic absorption spectrophotometer or using an ICP-MS.

EQUIPMENT AND MATERIAL

- Diethylenetriaminepentaacetic acid, DTPA, ($C_{14}H_{23}N_3O_{10}$)
- Calcium chloride, $CaCl_2 \times 2H_2O$
- Triethanolamine, TEA, ($C_6H_{15}NO_3$)
- Hydrochloric Acid, HCl (37%)
- Ethylenediaminetetraacetic Acid, EDTA ($C_{10}H_{16}N_2O_8$)
- Ammonium acetate ($C_{10}H_{16}N_2O_8$)
- Magnetic stirrer
- Shaker
- Greiner tubes (15 mL)
- Dispenser
- Beakers
- Analytical balance (0.0001 g)
- 2 mm mesh sieve
- Centrifuge
- 0.45 µm Filters
- Atomic absorption spectrophotometer
- Inductively coupled plasma mass spectrometer (ICP-MS)

CALCULATIONS

The results obtained from the atomic absorption spectrophotometer are in mg mL-1 and should be converted into mg g-1 of dry soil.

To do this, if we start from the concentration value that we obtain from the measurement of each metal, CM (mg mL-1), and assume that the concentration in the filtrate is homogeneous, for DTPA:

$$C\_DS=(4\ C\_M)/w\_DS$$

Where:

- CDS is the metal concentration by gram of dry soil (mg of metal / g of dry soil).
- CM is the measured concentration in the spectrometer (in mg mL-1)
- WDS is the exact amount of soil weighted (in g).

And for EDTA:

$$C\_DS=(10\ C\_M)/w\_DS$$

These formulas will be applied to each measured metal (Fe, Mn, Cu and Zn).

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3.9. TOTAL PESTICIDES

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PRINCIPLE AND APPLICATION

Currently, at European level, there are 479 active substances that can be used as pesticides, and other 43 are pending approval (European Commission 2009). These pesticides are used to protect crops against possible pests and have contributed to increase agricultural productivity during the last decades (Silva et al. 2019). However, pesticides can reach the ground during the application or by rain (Pérez-Rodríguez et al. 2017), and, given their effects on living organisms, they have become a major threat to the soil, to biodiversity, and to food safety (Stolte, Tesfai, and Keizer 2016). Therefore, when examining the biodiversity of a soil, it is also essential to determine the pesticides present.

To measure pesticides we use the QUECHERS method (Quick, Easy, Cheap, Effective, Rugged, and Safe) adapted to measure soil samples (Mol et al. 2008; Silva et al. 2019). This method is based on the pesticide extraction with one or several solvents, the cleaning of the sample to avoid interferences and the determination of pesticides by LC-MS/MS and GC-HRMS.

PROCEDURE

EXTRACTION

1. Weigh 5 g of the soil sample in a 50 mL Greiner tube.
2. Add 50 µL of a solution of 13C3-Caffeine with a concentration of 10 µg mL−1.
3. Add 5 mL of Millipore Water and 10 mL ACN with 1% HAc.
4. Shake mechanically for 1 hour.

CLEANING THE SOLUTION

1. Add 1 g of Sodium Acetate (NaHAc) and 4 g of Magnesium Sulphate (MgSO4)
2. Stir by vortex and centrifuge for 5 min at 2500 g.
3. Collect supernatant.

The supernatant is analysed by two methods: LC-MS / MS and GC-HRMS.

LC-MS / MS

1. Add 125 µL of the supernatant obtained in the previous steps to an LC vial.
2. Add 250 µL of Millipore Water and 125 µL ACN with 1% HAc.
3. Filter using a PTFE filter.
4. Measure using LC-MS / MS.

GC-HRMS there is an extra cleaning step

1. Add 1500 µL of supernatant to an Eppendorf vial.
2. Add 38 mg of PSA, 38 mg of C18 and 250 mg MgSO4.
3. Add 38 µL of PCB-198 of a solution with a concentration of 1 µg mL−1.
4. Shake the sample by vortex and centrifuge for 15 min at 13000 g.

5. Take a 250 µL aliquot in a GC vial.

6. Measure using GC-HRMS.

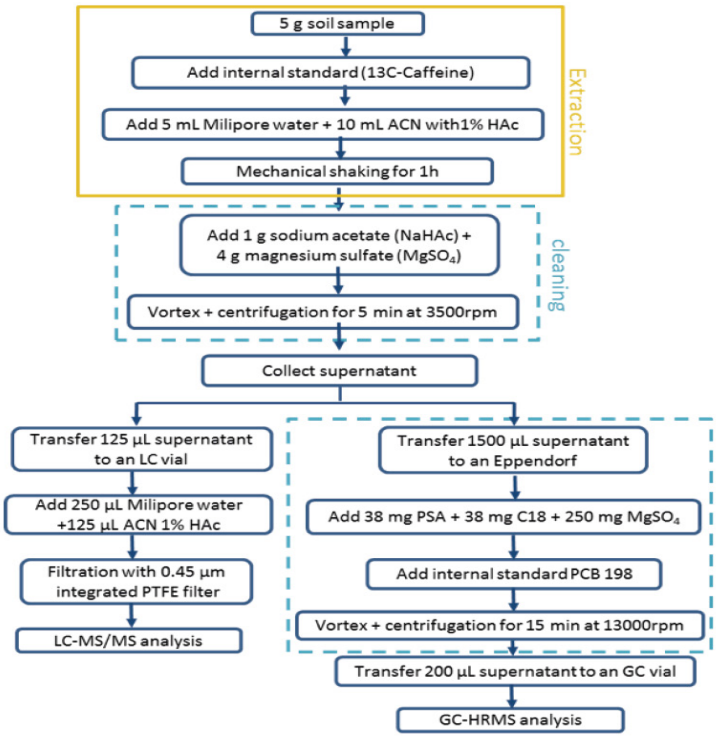

Figure 3.9.1. Scheme of the procedure followed to determine the concentration of pesticides in soils. This procedure is used to extract, clean and measure the 76 pesticides included in Table 3.9.1. (Adapted from Fuentes et al. 2019)

Analytical determinations by LC-MS / MS and GC-HRMS are made in duplicate. As for the calibrations, they are carried out with multi-pesticide standards that also included the internal standard. The standards are measured before and after measuring the samples (Reynolds et al. 2013). The pesticides measured are summarized in Table 3.9.1., taken from the work of Silva et al. (2019).

Table 3.9.1. Summary of the compounds to be measured in the soil samples with the procedure explained in Figure 3.9.1. The table also includes the type of pesticide and the chemical class.

Compound	Type	Chemical class
Abamectin	Insecticide	Botanical
Aldrin	Insecticide	Organochlorine
Atrazine	Herbicide	Triazine
Atrazine-deisopropyl	Metabolite of simazine and atrazine	Unclassified
Atrazine-desethyl	Metabolite of atrazine	Triazine
Azoxystrobin	Fungicide	Strobilurin
Boscalid	Fungicide	Carboxamide
Carbaryl	Insecticide	Carbamate
Carbofuran	Insecticide; metabolite of furathiocarb, carbosulfan and benfuracarb	Carbamate
Carbofuran, 3-hydroxy	Metabolite of carbofuran, carbosulfan and benfuracarb	Unclassified
Carbofuran, -keto	Metabolite of carbofuran and benfuracarb	Unclassified
Chlordane alpha	Insecticide	Organochlorine
Chlordane gamma	Insecticide	Organochlorine
Chlordecone	Insecticide; metabolite of mirex and kelevan	Organochlorine
Chlorfenvinphos	Insecticide	Organophosphate
Chlorpyrifos	Insecticide	Organophosphate
Chlorpyrifos-methyl	Insecticide	Organophosphate
Cymoxanil	Fungicide	Cyanoacetamide oxime
Cyproconazole	Fungicide	Triazole
Cyprodinil	Fungicide	Anilinopyrimidine
DDD op	Insecticide; metabolite of DDT	Organochlorine
DDD pp	Insecticide;	Organochlorine
	metabolite of DDT	Organochlorine
DDE op	Metabolite of DDT	
DDE pp	Metabolite of DDT	Organochlorine
DDT op	Insecticide	Organochlorine
DDT pp	Insecticide	Organochlorine
Diazinon	Insecticide	Organophosphate
Dieldrin	Insecticide, metabolite of aldrin	Organochlorine
Difenoconazole	Fungicide	Triazole
Dimethomorph	Fungicide	Morpholine
Diuron	Herbicide	Phenylamide
Endosulfan alpha	Insecticide	Organochlorine
Endosulfan beta	Insecticide	Organochlorine
Endosulfan sulphate	Metabolite endosulfan, endosulfan alpha and endosulfan beta	Unclassified
Endrin	Insecticide	Organochlorine
Epoxiconazole	Fungicide	Triazole
Ethion	Insecticide, metabolite of chlormephos	Organophosphate
Fenpropimorph	Fungicide	Morpholine
Fluometuron	Herbicide	Phenylurea
Fluroxypyr	Herbicide	Pyridine compound

Folpet	Fungicide	Phthalimide
Heptachlor	Insecticide	Organochlorine
Heptachlor epoxide	Metabolite of heptachlor	Unclassified
Hexachlorobenzene	Fungicide, metabolite of quintozene	Organochlorine
Hexachlorocyclohexane, alpha	Insecticide,	Organochlorine
	metabolite of HCH gamma	
Hexachlorocyclohexane, beta	Metabolite of HCH gamma	Unclassified
Hexachlorocyclohexane, gamma	Insecticide	Organochlorine
Imazalil	Fungicide	Imidazole
Imidacloprid	Insecticide	Neonicotinoid
Isoproturon	Herbicide	Urea
Linuron	Herbicide	Urea
Malathion	Insecticide	Organophosphate
Metalaxyl	Fungicide	Phenylamide
Metamitron	Herbicide	Triazinone
Myclobutanil	Fungicide	Triazole
Parathion	Insecticide	Organophosphate
Parathion-methyl	Insecticide	Organophosphate
Penconazole	Fungicide	Triazole
Pentachlorobenzene	Metabolite of quintozene	Unclassified
Phthalimide	Metabolite of folpet	Unclassified
Pinoxaden	Herbicide	Unclassified
Pirimiphos-methyl	Insecticide	Organophosphate
Prochloraz	Fungicide	Imidazole
Procymidone	Fungicide	Dicarboximide
Propiconazole	Fungicide	Triazole
Prothioconazole	Fungicide	Triazolinthione
Pyraclostrobin	Fungicide	Strobilurin
Quinoxyfen	Fungicide	Quinoline
Simazine	Herbicide	Triazine
Tebuconazole	Fungicide	Triazole
Terbuthylazine	Herbicide	Triazine
Terbuthylazine-desethyl	Metabolite of terbuthylazine	Unclassified
Triadimenol	Fungicide	Triazole
Trifloxystrobin	Fungicide	Strobilurin
Simazine	Herbicide	Triazine
Tebuconazole	Fungicide	Triazole
Terbuthylazine	Herbicide	Triazine
Terbuthylazine-desethyl	Metabolite of terbuthylazine	Unclassified
Triadimenol	Fungicide	Triazole
Trifloxystrobin	Fungicide	Strobilurin

EQUIPMENT AND MATERIAL

- Analytical balance (0.0001 g)
- Micropipettes
- Greiner tubes of 50 mL
- Shaker
- Distilled water
- Vortex
- Centrifuge
- LC vials
- PTFE Filters
- Liquid Chromatograph-Mass Spectrometer (LC-MS/MS)
- Eppendorf vials
- GC vials
- High-Resolution Gas Chromatograph-Mass Spectrometer (GC-HRMS)

REAGENTS

- 13C3-labeled caffeine
- PCB-198 ($C_{12}H_2Cl_8$)
- C18 (40 µm of Particle Size, Prep LC)
- Magnesium sulphate ($MgSO_4$; ≥99.8%)
- Acetic acid (CH_3COOH; ≥99.8%) (HAc)
- Sodium acetate (CH_3COONa; 99%)
- Acetonitrile (C_2H_3N; 99.95% LC grade) (ACN).
- Primary secondary amine sorbent (PSA)

CALCULATIONS

With the internal standards, a response factor can be calculated in order to determine the efficiency of the extraction procedure. The response factor is the concentration of the internal standard in the sample divided by the initial concentration of the standard. By using this response factor, it is possible to correct the concentration of pesticides measured.

The results obtained from the LC-MS / MS and GC-HRMS are in ng mL-1 and should be converted into ng g-1 of dry soil.

REMARKS

Given the importance of glyphosate and AMPA, these two substances are usually extracted by using more specific methods (Bento et al. 2016), that will be explained in detail in the following section.

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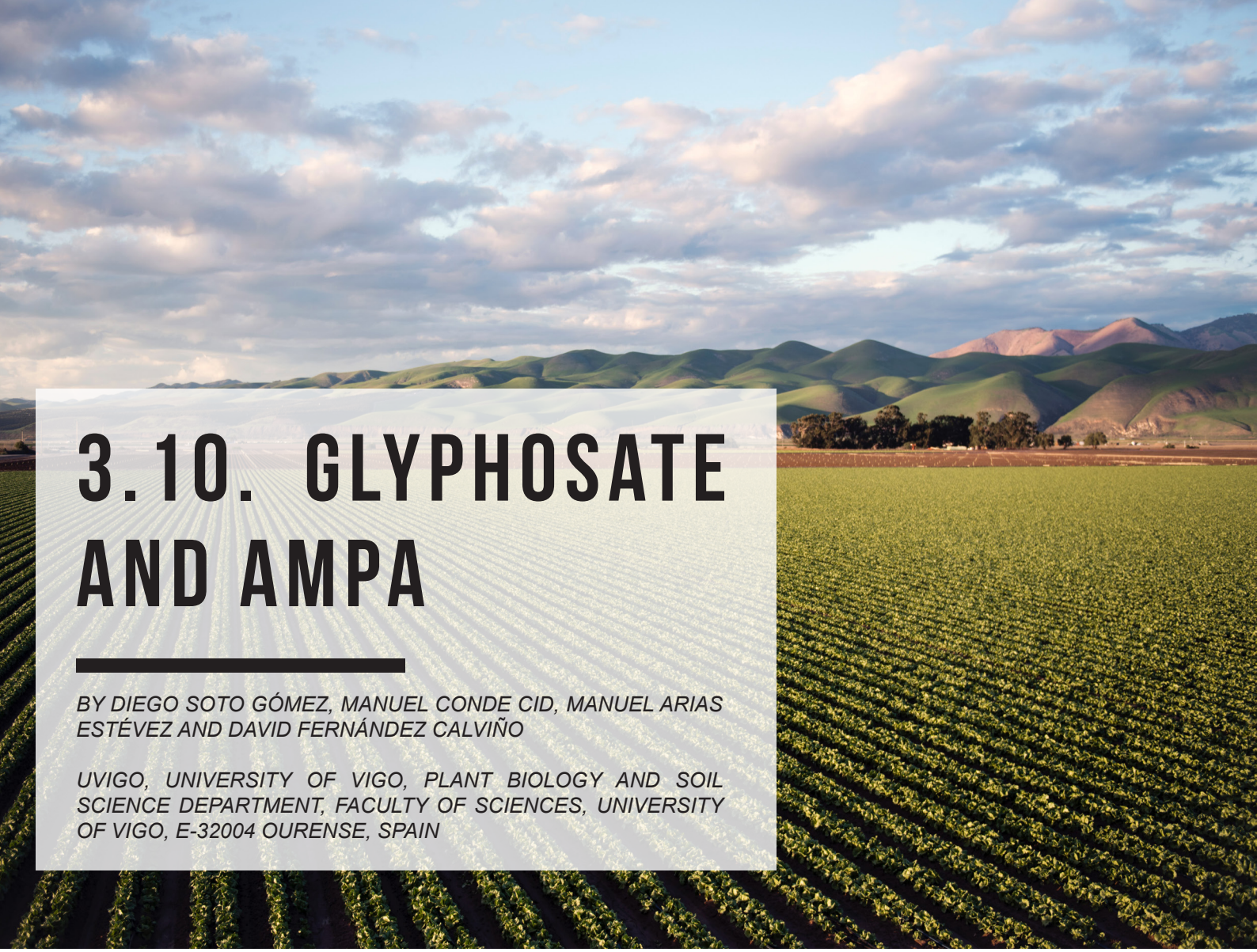

3.10. GLYPHOSATE AND AMPA

BY DIEGO SOTO GÓMEZ, MANUEL CONDE CID, MANUEL ARIAS ESTÉVEZ AND DAVID FERNÁNDEZ CALVIÑO

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PRINCIPLE AND APPLICATION

Glyphosate (N-phosphonomethylglycine, C3H8NO5) is a broad-spectrum herbicide with a higher solubility in water than in organic solvents. It is one of the most widely used herbicides since it was commercialized in the 1970s (Ghanem et al. 2007). It does not bio-accumulate, is poorly absorbed orally or through the skin, but can cause eye and skin irritation in humans (Farmer 2010). It is a systemic herbicidal substance that is used before planting the crop (before or after harvest), and is used to control the growth of annual or perennial weeds. This substance is also used to remove plants from parks and railway lines or roads (Silva et al. 2018). In Europe, it is mainly used in cereals, vineyards and oilseeds, crops that have not been genetically modified to tolerate glyphosate. In the USA and elsewhere, it is also applied to glyphosate resistant genetically engineered crops such as corn and cotton.

Glyphosate can be degraded by soil microbes into aminomethylphosphonic acid (AMPA), its main metabolite. The massive use of glyphosate can lead to high levels of AMPA in both soil and water (Grandcoin, Piel, and Baurès 2017). Its presence is frequent in the superficial part of agricultural soils, where it degrades slowly, but it can reach surface water currents adsorbed to the surface of some particles, and, rarely, it reaches groundwater. In vitro experiments performed with this element have confirmed its toxicity for

human cells (Kwiatkowska, Huras, and Bukowska 2014).
Glyphosate and AMPA have a great presence in soils (Figure 3.10.1.). In studies carried out in European soils, these two substances were found at levels higher than those predicted by EFSA (Silva et al. 2019), so they are not usually analysed with the rest of pesticides and a specific extraction is made for these compounds. For this reason, we have not included them in the previous section (3.9. Total Pesticides).
Glyphosate is a highly polar and amphoteric substance, which presents some analytical challenges. Normally, both glyphosate and its metabolite AMPA are measured through some type of derivatization. Pre-column derivatization using 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) is one of the most commonly used methods (Ghanem et al. 2007) as it facilitates chromatographic separation and subsequent analysis by mass spectroscopy or fluorimetry. FMOC-Cl is able to react with the amino groups of both AMPA and glyphosate (Garba et al. 2018). The derivatization occurs under alkaline conditions, in which the amino group hydrogen (from glyphosate and AMPA) is replaced by an aromatic FMOC ring, and FMOC-Glyphosate, FMOC-AMPA and hydrochloric acid are produced (Figure 3.10.2.).

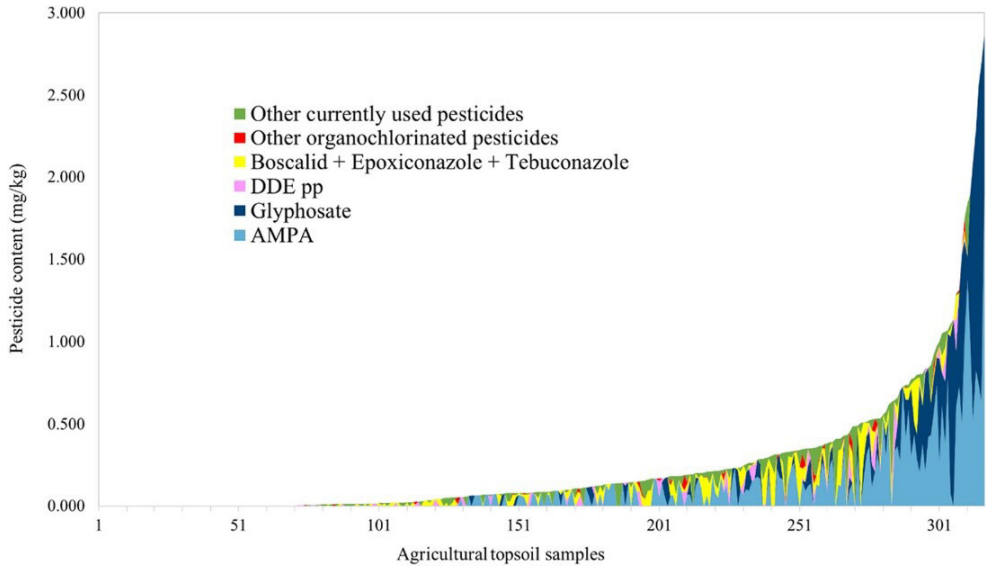

Figure 3.10.1. Amount of pesticides (in mg per kg of soil) found in 317 samples analysed throughout Europe (from Silva et al. 2019).

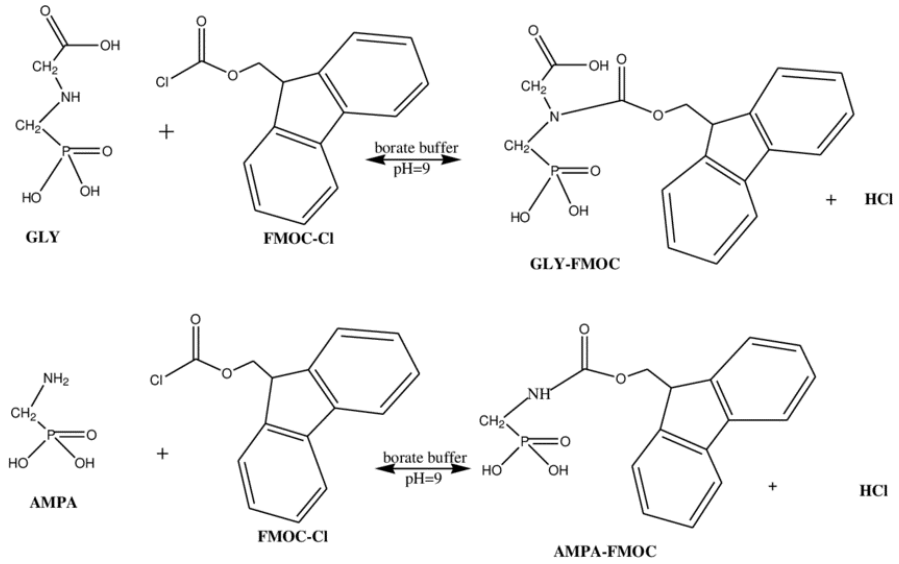

Figure 3.10.2. Derivatization reaction that occurs between FMOC-Cl and Glyphosate (GLY) and AMPA under alkaline conditions (from Garba et al. 2018).

PROCEDURE

The process includes the extraction of glyphosate and AMPA, their derivatization to make and determination by LC-MS/MS.

5. Weigh 2 g of the air-dried soil sample in a 50 mL Greiner tube.

6. Add 10 mL of a solution of potassium hydroxide 0.6 M (Extractant agent).

7. Shake for 60 min.

8. Centrifuge 30 min at 2500 g

9. Take an aliquot of 1 mL in Greiner tube of 15 mL.

10. Add 80 µL of HCl 6 M. In this step a pH of 9 (approximately) is reached.

11. Add 40 µL of a solution that includes the labelled internal standards of glyphosate and AMPA (5 µg mL−1).

12. Add 0.5 mL of borate buffer (5 %).

13. Add 0.5 mL of 9 fluorenylmethoxycarbonyl chloride 6.5 mM (Derivatization agent).

14. Vortex for 10-15 seconds.

15. Let the reaction happens for 30 min.

16. Add 50 µL of formic acid (98-100%) to stop the reaction.

17. Filtrate by 0.45 µm (using PTFE filters).

18. Transfer 0.5 mL to an LC vial and measure by LC-MS/MS.

It is also necessary to prepare standards of glyphosate and AMPA: 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 µg mL−1. Those have to be injected in the beginning and at the end of the sample measurement. It is also important to inject one or two standards in the middle of the samples.

EQUIPMENT AND MATERIAL

- Analytical balance (0.0001 g)
- Micropipettes
- 50 mL Greiner tubes of 50 mL
- 15 mL Greiner tubes of 15 mL
- Shaker
- Distilled water
- Vortex
- Centrifuge
- LC vials
- PTFE Filters
- Liquid Chromatograph-Mass Spectrometer (LC-MS/MS)

REAGENTS

- Glyphosate Standard (98%)
- AMPA Standard (98%)
- Isotope labelled internal standard of glyphosate (1, 2–13C 15N; 100 µg mL−1)
- Isotope labelled internal standard of AMPA (13C, 15N; 100 µg mL−1).
- Potassium hydroxide (KOH; 85%)
- Hydrochloric acid (HCl; 37%)
- Sodium tetraborate decahydrate (Na2B4O7·10H2O; 99.5%)
- 9 fluorenylmethoxycarbonyl chloride (FMOC-Cl; ≥99.0%)
- Formic acid (CH2O2; 98–100%)

CALCULATIONS

With the internal standards, a response factor can be calculated in order to determine the efficiency of the extraction procedure. The response factor is the concentration of the internal standard in the sample divided by the initial concentration of the standard. By using this response factor, it is possible to correct the concentration of pesticides measured.
The results obtained from the LC-MS / MS and GC-HRMS are in ng mL-1 and should be converted in ng g-1 of dry soil.

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4. BIOLOGICAL ANALYSES

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There are a lot of different biological analyses for soil biota. Each reveal the enormous richness of species and their diverse functional potential. Assessment of soil health through complete analysis of the soil food web consisting of bacteria, fungi, protozoa, nematodes, earthworms, enchytraeids, mites, insects, ants, tardigrades, collembola, moles etc. is practically daunting. Alternatively, a well-considered selection of indicator organisms is typically applied. Together they should reflect the structure and functions of ecological processes and they should exhibit sensitive responses to different soil conditions as affected by either natural or anthropogenic change.

The soil organisms selected to be investigated during the SoildiverAgro-project are important complementary bio-indicators concerning the linkage between soil biodiversity and soil functioning as described in the Technical Report-2010-049 “soil biodiversity: functions, threats and tools for policy makers” (European Commission DG ENV, DOI 10.2779/14571). One representative of each functional group in a soil ecosystem is present: bacteria and fungi as chemical engineers, responsible for the decomposition of organic material into plant nutrients; nematodes as biological regulators, responsible for the general management of the food web structure; earthworms as ecosystem engineers, responsible for the formation of biogenic soil aggregates and macropores as habitats and for controlling the soil water balance and air ventilation.

The (functional) diversity of the selected soil organisms will be determined as explained in the following sections. Table 4.1. shows an overview of the methods used by the partners in charge of a certain type of biodiversity analysis.

Table 4.1. Partners responsible for the acquisition of different types of biological data and the methods used

Methods for biodiversity assessments		
Analysis type	Partner in charge	METHOD
Nematode diversity	EV-ILVO	SSU rRNA gene amplicon sequencing
Whole microbial diversity	UVIGO	PLFA/NLFA
Bacterial and archaeal genetic diversity	UCPH	SSU rRNA gene amplicon sequencing
Fungal & mycorrhizal genetic diversity	LUKE	Fungal ITS sequencing
Microbial functional diversity	UCPT	Transcriptomic dynamics of key genes (C and N cycling)
Earthworm diversity	TI, EV-ILVO, LUKE, UVIGO, EULS	Morphological analysis

4.1. EARTHWORM DIVERSITY

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PRINCIPLE AND APPLICATION

Earthworm populations are usually characterized according to different criteria: i) developmental stage to quantify the proportion of adults and juveniles; ii) ecological groups to describe the feeding and burrowing behaviour; iii) species identification to determine species richness.

This protocol describes how:

- to identify earthworm species
- to assign each specimen to one of the established ecological groups (Bouché, 1977): epigeic, anecic and endogeic,
- to distinguish developmental stages: adult, subadult and juvenile
- to separate incomplete specimens and fragments from all others
- to determine total abundance (individuals m^{-2}) and biomass (g m^{-2})

PROCEDURE

Wear disposable protective gloves and use plastic forceps when handling earthworms. The specimens from each replicate sample should be placed into a plastic container filled with cold tap water. Before the species identification under the stereo microscope and weighing, each specimen should be cautiously dried on tissue paper.

Developmental stage

Earthworms are first separated according to their developmental stage by looking at their sexual maturity:

- Adults have a fully developed clitellum (glandular thickened section of the body wall in the middle or towards the anterior end of the body)
- Subadults do not have a developed clitellum but tubercula pubertatis (glandular thickenings on the ventral surface) are present
- Juvenile worms do not show any visible signs of clitellum nor tubercula pubertatis.

Figure 4.1.1. shows some of the morphological characters for identifying earthworms to species level, and includes the position of the clitellum and the turbercula pubertatis, two distinct characteristics of fully adult specimens.

Ecological groups

Most earthworm individuals (adults and sub-adults but often also juveniles) can be assigned to their ecological groups according to their dorsal pigmentation:

- Epigeic species are heavily pigmented small earthworms.
- Anecic species are large earthworms and moderate to heavily pigmented on their front end (until some segments behind the clitellum);
- Endogeic species are unpigmented or nearly unpigmented; in some species, the epidermis is translucence showing through inner organs

EQUIPMENT AND MATERIAL

- **Disposable protective gloves**
- **Tissue paper or paper towels**
- **Plastic forceps**
- **Flat plastic containers (vol.: ca. 1 L) with cold fresh tap water**
- **Plastic cups with cold fresh tap water**
- **Stereo microscope (magnitude: ca. 10x to 50x)**
- **Analytical balance (precision: 0.01 g)**
- **Identification keys**
- **Data sheets**

REMARKS

All fragments of earthworms in the sample must be included for determining total earthworm biomass. However, only those fragments that include the head and as best also the clitellum can be identified to the species level and if this is the case, they can be used for total abundance and diversity estimates. Any other fragments are listed as "unidentified fragments".

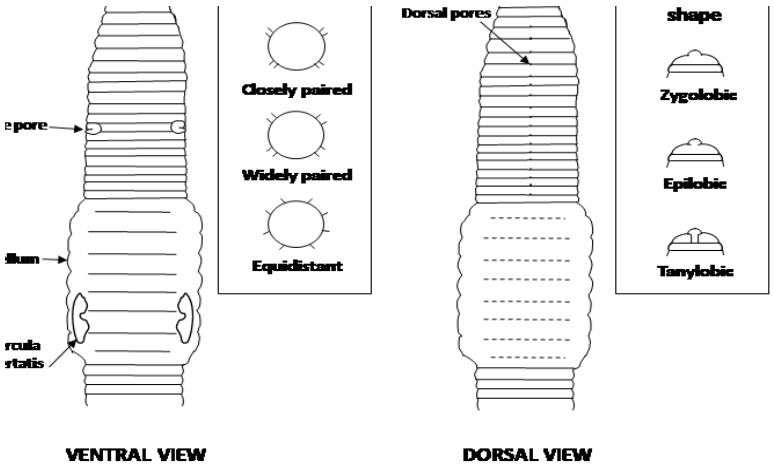

Figure 4.1.1. Some of the main morphological characters that are used in identifying developmental stage and species of earthworms

Figure 4.1.2. shows one example species, its typical pigmentation and type of burrow system for each ecological group.

Taxonomical identification

Adult earthworms can be identified to species level by using a stereo microscope (magnitude: 10x to 50x) and a taxonomic identification key. For example, the identification guide by Sims and Gerard (1999) and key to the earthworms of the UK and Ireland by Sherlock (2018) are useful for identifying the majority of the common European species. Because they do not cover completely European earthworm fauna, national or regional keys should also be used when necessary.

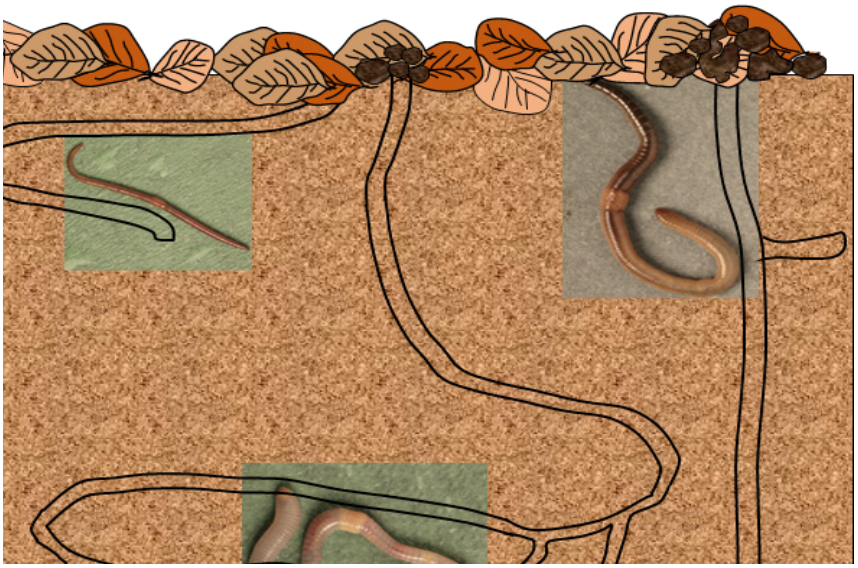

Figure 4.1.2. Earthworm ecological groups (Bouché, 1977): epigeic (*Lumbricus castaneus*; top left), anecic (*Lumbricus terrestris*; right), and endogeic (*Octolasion cyaneum*; bottom) (the size of the species not to scale). Photo credit: Guide to British earthworms (https://www.opalexplorenature.org/earthwormguide).

The most important diagnostic characters are the position and shape of the clitellum and location of the tubercula pubertatis (see Figure 4.1.1.). Other important diagnostic characters are the shape of prostomium (in front of the first segment) and the clitellum (annular, saddle), setae pattern (distance between setae on each segment) (Fig. 4.1.1.). Subadult earthworms can be identified to species in many cases, however, juvenile earthworms can only be assigned to ecological groups (see above). If the specimen cannot be accurately identified, then annotate all available information and classify it as ‘unknown’.

CALCULATIONS

Use a balance with precision of 0.01g for determination of the preserved earthworm biomass (g). Determine the biomass per species, per ecological group and in total for each sample.
Express all abundance (of individual species and ecological groups) and biomass data as individuals per square meter (ind. m^{-2}) and grams per square meter (g m^{-2}), respectively. In case of a 50 cm x 50 cm sampling quadrat, multiply the density and mass in the sample by 4. In case of a 25 cm x 25 cm sampling quadrat, multiply by 16.

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4.2. NEMATODES DIVERSITY

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Nematodes (also called roundworms) constitute a diverse species-rich group of organisms. They inhabit nearly all ecosystems including marine, freshwater, and terrestrial environments. In the soil, they occur across multiple trophic levels making them vitally important in the soil environment. Nematodes are frequently the most abundant and diverse invertebrates present in the soil. Consequently, the nematode composition contains high intrinsic information value for each soil sample (Yeates et al., 1993; Van den Hoogen et al., 2019).

While there are many general indices of biological diversity, specific indices have been developed for nematodes. For example, the Maturity Index (MI), is a weighted mean frequency of c-p scaling across the entire nematode community, a scaling based on the nematode's differential life-style, and provides the information of the likely condition of the soil environment (Bongers, 1990). Ferris et al. (2001) assigned weights to indicator nematode guilds representing basal, enriched and structured conditions of the food web. This concept led to the development of food web indices including Enrichment (EI) and Structure Index (SI). The EI describes whether the soil environment is nutrient enriched (high EI) or depleted (low EI). SI describes whether the soil ecosystem is structured with greater trophic links (high SI) or degraded (low SI) with fewer trophic links. Plotting of EI versus SI provides a model framework of nematode faunal analysis as an indicator of the likely conditions of the soil food web in a given habitat.

The characterization of a nematode community through microscopy is time-consuming and requires trained taxonomists. As a consequence, molecular techniques are increasingly preferred to unravel changes in nematode communities. Such a technique combined with a practical understanding from the research generated is believed to help in choosing farming strategies for improved ecosystem services directly or indirectly influenced by the nematode community.

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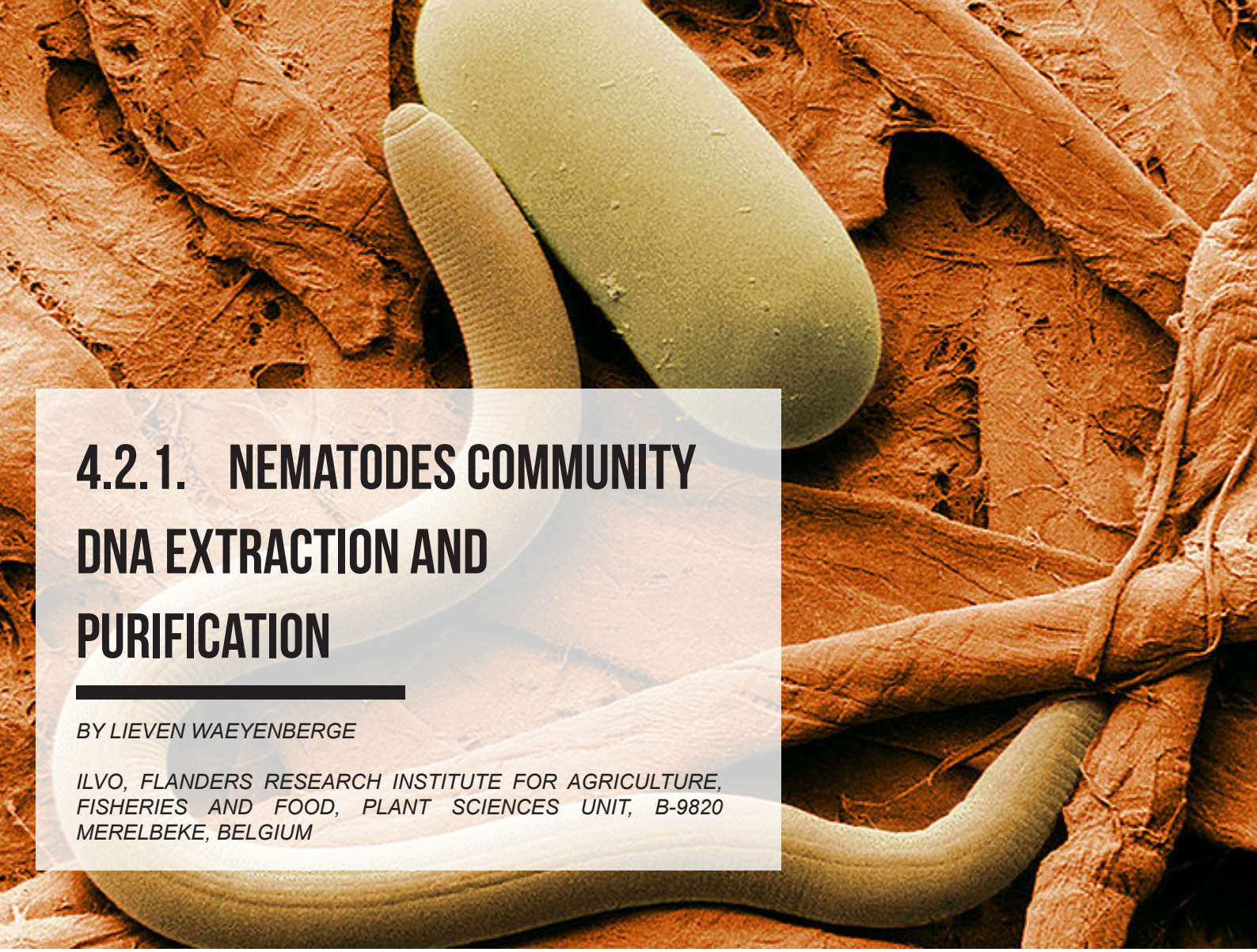

4.2.1. NEMATODES COMMUNITY DNA EXTRACTION AND PURIFICATION

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PRINCIPLE AND APPLICATION

Nematode community DNA is extracted from a nematode suspension isolated from a soil sample (enrichment procedure). The suspension is obtained by extracting the nematode community from a 100 ml soil sample using the technique of automated zonal centrifuging (AZC, Hendrickx, 1995). The suspension is then concentrated and stored at -20°C until DNA-extraction.

The nematode suspension is concentrated by gravity force. Centrifugation is not recommended because strong forces can cause nematodes to burst open and be lost while pipetting away the upper layer (supernatant) during multiple steps.

To extract DNA, the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) is used following the protocol “Purification of total DNA from animal tissues “according to the manufacturer’s instructions with minor adaptations. Samples are first lysed using proteinase K. The buffer added next, provides optimal DNA-binding conditions before the lysate is loaded onto the DNeasy Mini spin column. During centrifugation, DNA is selectively bound to the DNeasy (silica-based) membrane while contaminants pass through.

Remaining contaminants and enzyme inhibitors are removed in two wash steps and DNA is then eluted in water or buffer, ready for use.

EQUIPMENT AND MATERIAL

- Large volume pipet or suction machine
- 50 mL conical tubes
- 15 mL conical tubes
- 1.5 and 2 mL Eppendorf tubes
- Pasteur pipets (glass pipets)
- A set of Micropipettes
- DNeasy Blood & Tissue Kit from Qiagen, hereafter called 'Qiagen-kit'
- Vortex
- Centrifuge (min. 20,000 rcf)
- Warm water bath (56°C)
- Refrigerator (4°C)
- Freezer (-20°C)

REAGENTS

- Ethanol (96-100%)

CALCULATIONS

No calculations are made during DNA-extraction.

REMARKS

The quality and quantity of the DNA do not have to be measured. The subsequent PCR-reaction and electrophoresis demonstrate whether the extracted DNA is amplifiable and thus provides an impression of the DNA quality and/or yield.
The use of an optimal DNA extraction protocol is crucial. The Qiagen-kit scored the highest yield compared with several other commercial DNA-extraction methods. However, there is still room to further optimize the DNA-extraction procedure (Waeyenberge et al., 2019).

PROCEDURE

1. Place the beakers (150 mL) holding the nematode suspensions obtained from the AZC endrickx, 1995), on a bench at room temperature.

2. After 4 hours, reduce the volume till 40 mL without disturbing the nematodes at the bottom of the beaker. Several devices can be used: from large volume pipets till a suction machine.

3. Swirl the beaker and transfer the nematode suspension to a 50 mL conical tube.

4. Store the tubes at 4°C for one night.

5. Remove the supernatant till a volume of 5 mL. Again several devices can be used.

6. Swirl the tube and transfer the nematode suspension to a 15 mL conical tube.

7. Store the tubes at 4°C for one night.

8. Remove gently (!) the supernatant till a volume of 2 mL using a micropipette. Be careful not to suck up nematodes.

9. Homogenize the 2 mL nematode suspension with a Pasteur pipet and divide it equally (250 µL each) into 2 mL Eppendorf tubes.

10. Store the 8 Eppendorf tubes with nematode suspensions at -20°C until use.

11. In the morning, take 2 Eppendorf tubes out of the freezer and add in each 180 µL buffer ATL and 20 µL proteinase K (Qiagen-kit).

12. Vortex and incubate at 56°C for 3 hours. Preferably, mix the suspensions by turning the tubes now and then during incubation.

13. Vortex 15 s and add in each tube 200 µL buffer AL (Qiagen-kit).

14. Mix thoroughly by vortex.

15. Add 200 µL ethanol (96-100%) in each tube and mix thoroughly by vortex.

16. Pipet maximally 700 µL mixture into one DNeasy Mini spin column placed in a collection tube (Qiagen-kit).

17. Centrifuge at 6000 g for 1 min and discard the flow-through.

18. Repeat steps 16 and 17 until the mixtures from both Eppendorf tubes have passed the same DNeasy Mini spin column.

19. Place the spin column in a new collection tube and add 500 µL buffer AW1 (Qiagen-kit).

20. Centrifuge at 6000 g for 1 min and discard the flow-through.

21. Place the spin column in a new collection tube and add 500 µL buffer AW2 (Qiagen-kit).

22. Centrifuge at 20,000 g for 3 min and discard the flow-through.

23. Place the spin column in 1.5 mL Eppendorf tube, labelled properly.

24. Elute the DNA by adding 75 µL buffer AE (Qiagen-kit) to the centre of the spin column membrane and by incubating for 1 min at room temperature.

25. Centrifuge at 6000 g for 1 min and discard the DNeasy Mini spin column.

26. Close the 1.5 mL Eppendorf tube and store the purified DNA at -20°C until further use.

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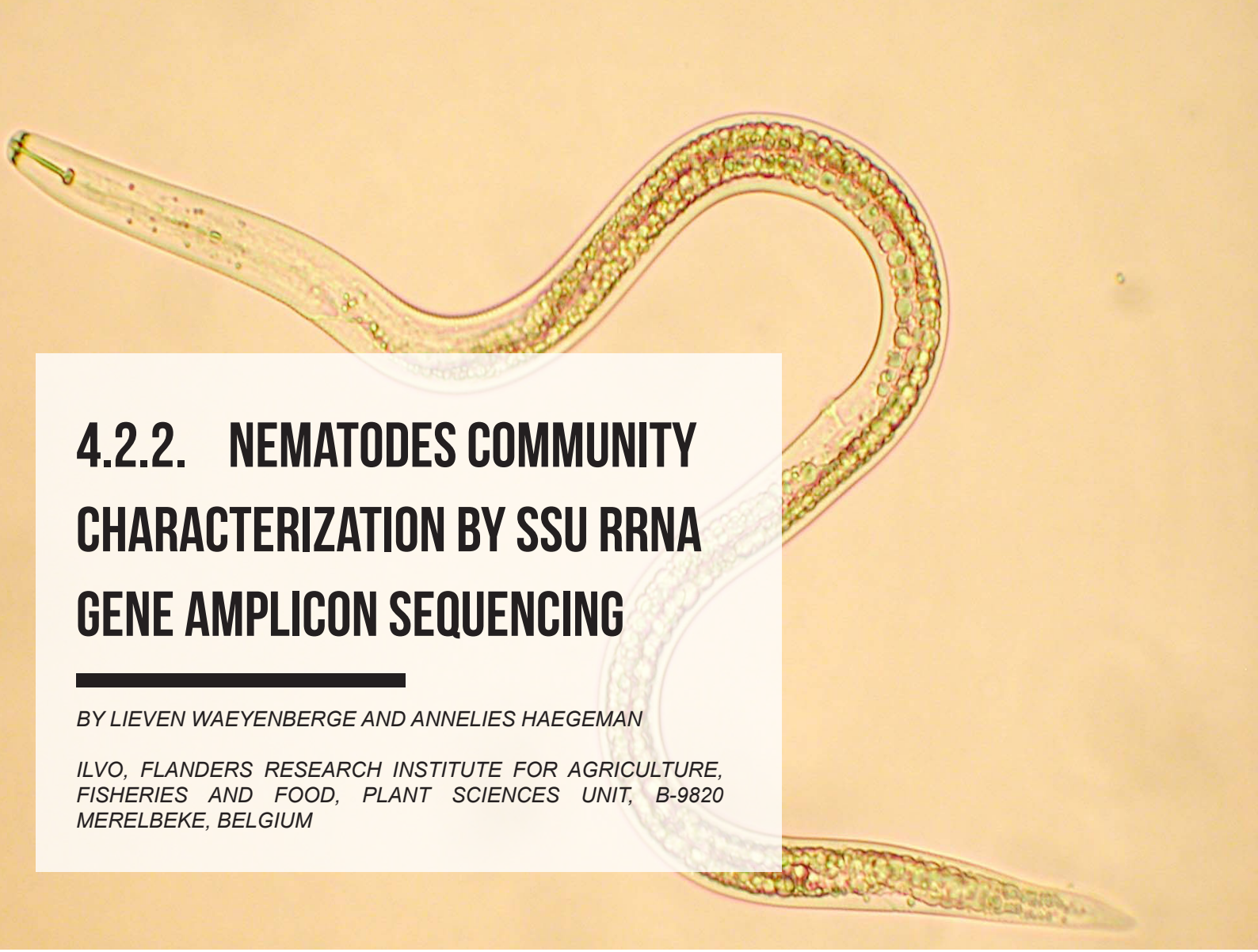

4.2.2. NEMATODES COMMUNITY CHARACTERIZATION BY SSU RRNA GENE AMPLICON SEQUENCING

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PRINCIPLE AND APPLICATION

Within the relatively conserved 18S rDNA gene, variable sequence regions covering the required taxonomic resolution for nematodes, are alternated by short conserved sequences useful for universal primer design (Hadziavdic et al, 2014). Further, an extensive number of 18S rDNA sequences of different nematode species are available for diagnostic assignment (Van Megen et al., 2009). As a consequence, ssu rRNA gene amplicon sequencing is currently the mostly used high throughput method to identify nematode communities from various habitats (Waeyenberge et al., 2019).

The method for nematode community characterization is based on the amplification and sequencing of a variable region within the ssu or 18S rRNA gene. The amplicon libraries of approximately 300 different nematode communities are pooled and sequenced using a Next Generation Sequencing (NGS) technology on an Illumina MiSeq platform with PE300 reads (70% ≥ Q30). The sequences are subsequently analysed using a bio-informatics pipeline and characterised by comparison with a nematode 18S sequence database. This results in the determination of the nematode genera present in all the nematode suspensions processed and their relative abundances. Index primers incorporated in the amplicons, containing a unique 8 bp multiplex identifier per nematode suspension, make it possible to group all sequences from one nematode community together.

An open-source R package, DADA2 (Callahan et al., 2016), is used for sequence data analysis. This cluster-free pipeline not only infers amplicon sequence variants (ASV) directly, the reads are 'corrected' by a quality-aware model of Illumina-amplicon errors. Whether this approach yields a better evaluation of soil nematode communities compared to the use of OTUs (Operational Taxonomic Units) is yet unknown. However, the authors stated that in mock communities DADA identifies more real variants and outputs fewer spurious sequences than other methods. Additionally, it has been recently reported for marine nematodes that the DADA2 pipeline produced the most realistic estimates of species richness yet taxonomic assignment of ASVs were reduced (Macheriotou et al., 2018).

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EQUIPMENT AND MATERIAL

- LThermocycler
- 1.5 or 2 mL Eppendorf tubes
- 0.2 mL PCR tubes, strips or multiwell plates
- A set of Micropipettes
- Vortex
- Centrifuge (min. 20,000 rcf)
- Refrigerator (4°C)
- Freezer (-20°C)
- Microwave
- Gel electrophoresis apparatus including a casting gel tray, an electrophoresis tank, few combs and a power supplier
- Gel Imaging System (for example, Azure Biosystems c150, Dublin, CA, USA)
- Fluorometer (for example, Quantus, Promega, WI, USA)

REAGENTS

- 2X KAPA HiFi HotStart ReadyMix (Roche Diagnostics)
- 18S rRNA forward primer with Illumina adapter (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3')
- 18S rRNA reverse primer with Illumina adapter (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3')
- MilliQ water
- Agarose
- TAE buffer (ready-to-use)
- Midori Green Advanced DNA stain (Nippon Genetics Europe GmbH, Düren, Germany)
- Loading dye (for example, 6X Orange DNA Loading Dye from Life Technologies Europe B.V.)
- DNA marker (for example, GeneRuler 100 bp DNA Ladder, ready-to-use, from Life Technologies Europe B.V.)
- PCR-purification method with magnetic beads (CleanNGS, GC Biotech BV., Alphen aan den Rijn, the Netherlands)
- Index primers, Illumina Nextera XT Index Kit v2, set A-D (Illumina Inc., San Diego, CA, USA)
- QuantiFluor ds DNA System (Promega, WI, USA)

CALCULATIONS

DNA quantities are calculated by the software provided with the Quantus (Promega, WI, USA).

PROCEDURE

1. Execute a PCR (= amplicon-PCR) using the next mixture:
12.5 µL of 2X KAPA HiFi HotStart ReadyMix
0.75 µM of both the forward and reverse 18S rRNA gene primer
MilliQ water up to a volume of 23 µL.
To reduce pipetting steps, make a master mix in a 1.5 or 2 mL Eppendorf tube(s) with a volume depending on the number of reactions. Include at least 3 negative controls. Then distribute 23 µL of the master mix in a number of 0.2 mL PCR tubes, strips or multiwell plates.

2. Add 2 µL DNA-extract obtained from a nematode community (see section 4.2.1). The final volume is now 25 µL.

3. Put the tubes, strips or multiwell plate in a thermocycler programmed as follows:
95 °C for 3 min
35 cycles of 98 °C for 20 s, 64 °C for 15 s, 72 °C for 30 s
72 °C for 2 min
Hold temperature at 10°C

4. During PCR, a 1.5% agarose gel is prepared by melting agarose powder in 1X TAE-buffer in a microwave. After cooling partially, Midori Green Advanced DNA stain is added to the gel at a ratio of 8 µL per 100 mL of agarose gel.

5. Pour the prepared gel on a casting tray and place one or more combs.

6. Transfer the gel tray with the completely solidified gel into an electrophoresis tank filled with 1X TAE buffer; The gel should be completely submersed.

7. Remove the combs gently.

8. Take the PCR tubes, strips or multiwell(s) with amplicons and transfer per sample 5 µL amplicon to another tube, strips or multiwell(s) and mix each with 1 µL loading dye.

9. Load this mixture in the different gel wells (one sample mixture per well).

10. Also load a (100 bp) DNA marker in a separate well.

11. Turn on the power supplier (100V) and let the electrophoresis run until the amplicons have migrated sufficiently.

12. Place the gel in a gel imaging system, visualise the amplicons with UV-light and take a photo. Evaluate the amplicons by investigating the photo.

13. Take again the PCR tubes with amplicons and clean the amplicons by separating them from unincorporated primers, dNTPs, and primer dimers using a PCR-purification method with magnetic beads. Follow the manufacturer's instructions.

14. Execute a second PCR (= index-PCR) using the next mixture:
12.5 µL of 2X KAPA HiFi HotStart ReadyMix
0.75 µM of both a forward and reverse index primer
MilliQ water up to a volume of 23 µL.
Making a master mix is not possible since for each sample another combination of index primers has to be used.

15. Add 2 µL purified amplicons as DNA-source. The final volume is now 25 µL.

16. Put the tubes, strips or multiwell plate in a thermocycler and run the same temperature profile as used for the amplicon-PCR (see step 3) but reduce the number of cycles from 35 till 15 only.

17. Repeat step 4 till 10 for a selection of samples to visualize the amplification after index-PCR.

18. Repeat step 11 to purify the amplicons from the index-PCR

19. Quantify the amplicons using a fluorometer and a double-stranded DNA quantification system (QuantiFluor ds DNA System, Promega, WI, USA) following the manufacturer's instructions.

20. Pool the amplicons of each sample in equimolar concentrations (10 nM) in a 2 mL Eppendorf tube and send (part of) the pooled sample to a sequence service provider. Follow the instructions of the provider for storage and transportation.

21. After receiving the raw sequence data from the sequence service provider, the data is analysed and quality checked as follows:
Remove primer sequences from the obtained reads using Trimmomatic v0.32 (Bolger et al., 2014). Perform all subsequent analyses in R 3.4.3 (R Core Team) using the package DADA2 (Callahan et al., 2016) containing a full amplicon workflow starting from filtering, dereplication, chimera identification, until merging paired-end reads. In the filtering step, forward and reverse reads are trimmed. Finally, the sequences are compared with our sequence database (own produced 18S rRNA gene sequences added to the most recent SILVA release from which only sequences belonging to the phylum Nematoda are retained) to link them to a certain nematode taxon using the naive Bayesian classifier method (Wang et al., 2007) assigning taxonomy across multiple ranks (e.g. Kingdom to Genus) with minimum bootstrapping support of 80. In addition, all sequences are blasted against the non-redundant database of Genbank to confirm their taxonomic assignment. Relative abundances for each sequence variant per sample are calculated and exploratory barplots and other graphs are produced.

4.3. MICROORGANISMS DIVERSITY

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Microbial diversity is immense in soils and refers to the number of different bacterial, archaeal, and fungal species and their relative abundance (Maron et al., 2018). Microorganisms are crucial for the stability and productivity of agroecosystems as they are involved in key processes such as the formation of soil structure, organic matter decomposition, biodegradation of organic pollutants and cycling of essential elements such as carbon, nitrogen, phosphorus, and sulphur (Garbeva et al., 2004). Soil microorganisms can furthermore be highly beneficial for plants as they can suppress soil-borne diseases and enhance plant growth. Microbial diversity is, therefore, often linked to plant and soil quality, and constitute an important indicator for evaluating soil health (Abawi and Widmer, 2000; Schloter et al., 2003). Physicochemical properties of soil are considered key determinants for microbial diversity and can be affected by soil management and treatment, such as tillage, crop rotation (Lupwayi et al., 1998), pesticide application (Jacobsen and Hjelmsø, 2014) and fertilization (Zhong et al., 2010).

Traditionally, cultivation based methods have been used to investigate microbial diversity, but these methods are time-consuming, and only a small fraction of microorganisms are cultivatable (Kirk et al., 2004). Advancements in molecular technologies such as direct DNA extraction from soil, PCR amplification of conserved gene targets and next-generation DNA sequencing now allow for comprehensive, high-throughput cultivation-independent analyses of soil microbial diversity (Schöler et al., 2017). Gene targets include the small sub-unit ribosomal RNA (ssu rRNA) gene (taxonomic gene marker) or a range of different functional gene markers indicative of the functional diversity of the soil microbiome. Collectively, these techniques have proven highly useful for assessing soil microbial diversity.

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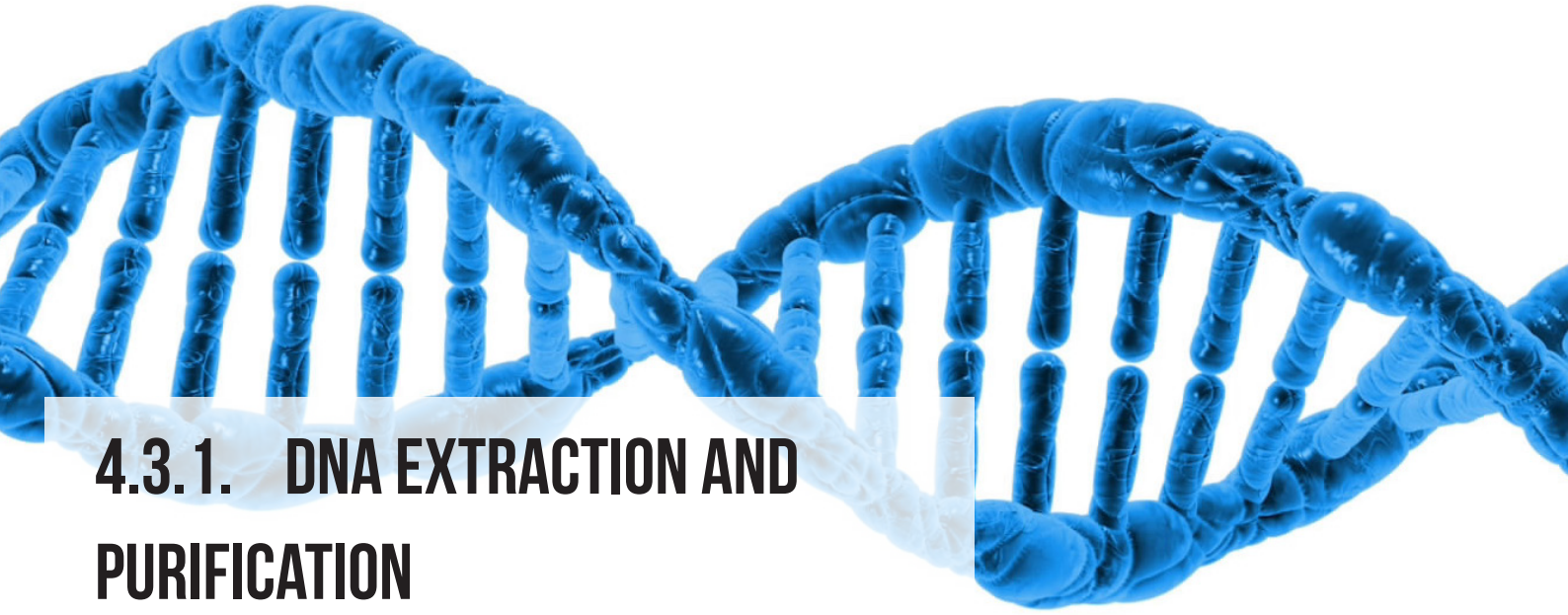

4.3.1. DNA EXTRACTION AND PURIFICATION

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PRINCIPLE AND APPLICATION

To extract metagenomic DNA from soil for subsequent ssu rRNA gene amplicon sequencing, we used the DNeasy PowerSoil Kit according to the general protocol with minor deviations (Vishnivetskaya et al. 2014; Ramírez, Graham, and D'Hondt 2018). Nanodrop (ThermoFisher Scientific) and D Qubit® 2.0 Fluorometer (Invitrogen) were used for subsequent analysis of DNA yield and quantity.

PROCEDURE

DNA Extraction

1. To the PowerBead Tubes provided, add 0.25 grams of soil sample.
2. Gently mix the tubes using a Ribolyzer (1 min, 5.5 m/s).
3. Check Solution C1. If Solution C1 is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of Solution C1 and invert several times or vortex briefly.
5. Secure PowerBead Tubes in the MP Fastprep-24 instrument, and run the recommended program for soil samples (30s, 5.5 m/s).
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature.
7. Transfer the supernatant to a clean 2 ml Collection Tube
8. Add 250 µl of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean 2 ml Collection Tube.

11. Add 200 µl of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean 2 ml Collection Tube.

14. Shake to mix Solution C4 before use. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 seconds.

15. Load approximately 675 µl onto a Spin Filter and centrifuge at 10,000x g for 1 minute at room temperature. Discard the flow through add an additional 675 µl of supernatant to the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature.

16. Add 500 µl of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x g.

17. Discard the flow through.

18. Centrifuge again at room temperature for 1 minute at 10,000 x g.

19. Carefully place the spin filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.

DNA Extraction

20. Add 100 µl of Solution C6 to the centre of the white filter membrane.

21. Centrifuge at room temperature for 30 seconds at 10,000 x g.

22. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

23. Long-term storage of DNA at -80°C.

Determination of DNA quantity and quality using Nanodrop

1. Make sure the measuring arm on the Nanodrop apparatus is in a down position.

2. Open the ND-1000 program by double-clicking on the NanoDrop ND1000 icon.

3. Choose “Nucleic Acid measurements” and “DNA”
Lift up the measuring arm on the apparatus and remove the tissue paper.
Check that both measuring surfaces are clean (if not then clean with a soft piece of paper (Kleenex))
Apply 1.5 µl of MQ water
Close the measuring arm

4. Click “OK”
To calibrate
Lift up the measuring arm
Clean of the measuring surface with a soft piece of paper (Kleenex)
Apply 1.5 µl of solution C6 as a blank, close the measuring arm.Click on “blank”
To measure the DNA concentration:
Lift up the measuring arm
Clean of the measuring surface with a soft piece of paper (Kleenex)
Apply 1.5 µl of the extracted DNA sample, close the measuring arm.
Write the sample name in the field on the right-hand side of the screen
Click on “measure”
Write down the measured concentration (or print a report after the last sample)
Repeat from step a) to f) for all samples

Determination of DNA quantity using Qubit® 2.0 Fluorometer

1. Prepare High Sensitive (HS) standards and DNA samples according to the manual supplied by the Invitrogen™ Qubit™ dsDNA HS Assay Kit.
2. Calibrate the Qubit® 2.0 Fluorometer according to the manual using the prepared HS standards.
3. Measure the DNA concentration in all samples according to the Qubit® 2.0 Fluorometer manual.

EQUIPMENT AND MATERIAL

Equipment necessary (* provided with the DNeasy PowerSoil kit)

- **Analytical balance (0.01 g)**
- **Micropipettes**
- **Collection tubes of 2 mL ***
- **PowerBead tubes ***
- **Spin Filter tubes ***
- **Distilled water**
- **Vortex**
- **Centrifuge**
- **Refrigerator**
- **Ribolyzer**
- **MP Fastprep-24TM instrument**
- **Nanodrop 1000 spectrophotometer**
- **Qubit® 2.0 Fluorometer**
- **Invitrogen™ Qubit™ dsDNA HS Assay Kit**
- **500 µL thin-walled PCR tubes**

REAGENTS

- **70% ethanol**
- **Solutions C1, C2, C3, C4, C5, and C6 ***

CALCULATIONS

The DNA concentration of all samples is calculated by the Nanodrop and Qubit 2.0 software. The quality of the DNA is determined by the A260/280 and A260/230 ratios quantified by the nanodrop software.

REMARKS

The use of an optimal DNA extraction protocol is crucial. The PowerSoil Kit method is the most universally applicable DNA extraction kit, resulting in high-quality data, with lower bias than other methods (Hermans, Buckley, and Lear 2018). Nevertheless, all DNA extraction protocols will be biased to some extent. It is thus crucial to always standardize DNA extraction protocols for samples to be directly compared with each other.

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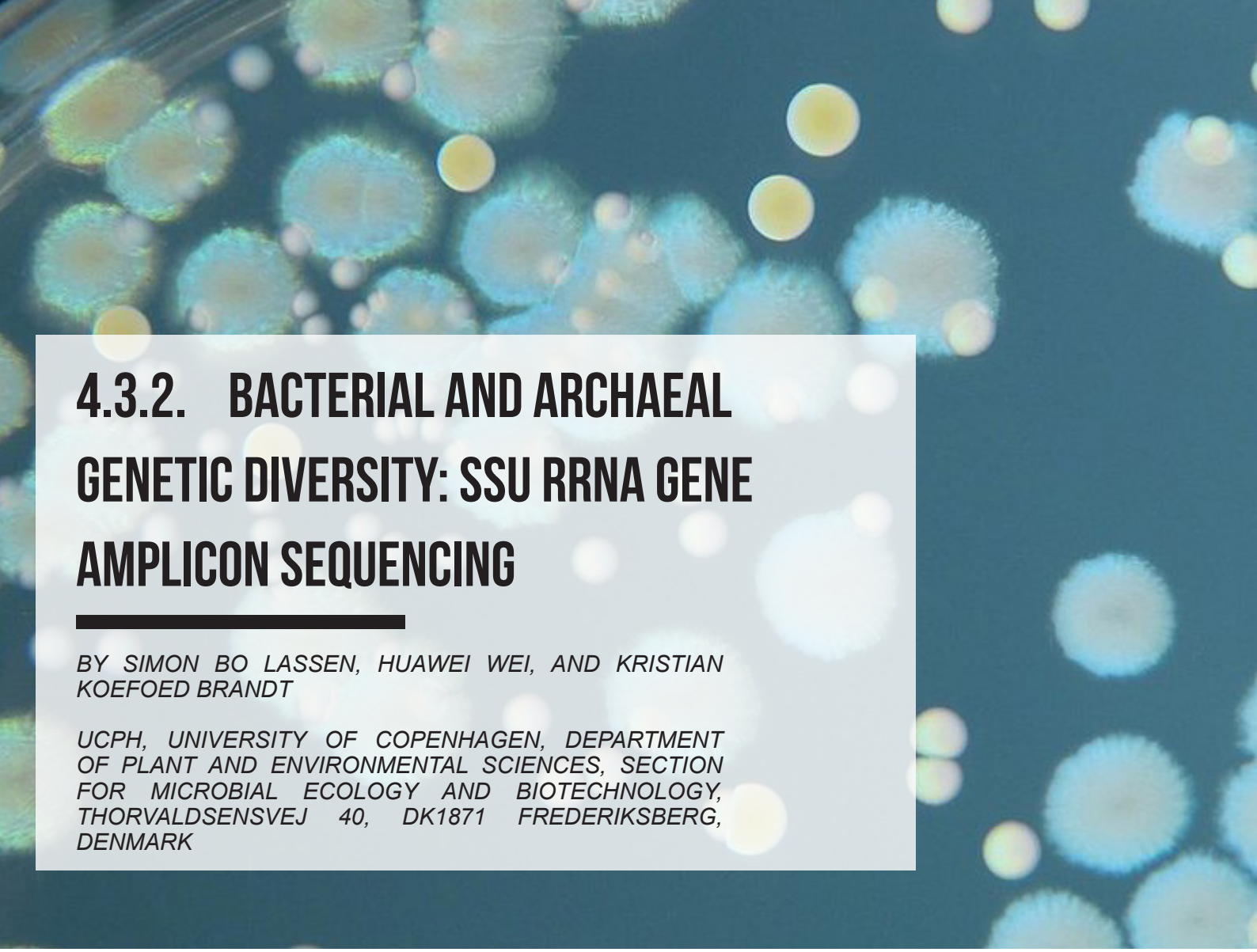

4.3.2. BACTERIAL AND ARCHAEAL GENETIC DIVERSITY: SSU RRNA GENE AMPLICON SEQUENCING

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PRINCIPLE AND APPLICATION

Small subunit (ssu) rRNA amplicon sequencing is a widely used high throughput method to identify prokaryotic taxa in different environments allowing for cultivation-independent determination of bacterial and archaeal community composition (Caporaso et al., 2012). The technique is based on PCR amplification of hypervariable regions of the ssu rRNA gene (also termed 16S rRNA gene in bacteria), which are then analysed using Next Generation Sequencing (NGS) technology and compared against DNA sequence databases to determine the identity and abundance of prokaryotic taxa. Within the SoildiverAgro Project, UCPH has opted to use a commercial next-generation sequencing provider for our sequencing needs in order to enhance our ability to meet tight deadlines for our project deliverables.

PROCEDURE

1. For amplicon sequencing, send the high-quality DNA samples obtained by DNA extraction (minimum concentration of 10 ng/μL) to NOVOGENE (HK) COMPANY LIMITED.

2. At Novogene, PCR amplification will be conducted using the modified bacterial/archaeal primer pair 515F -GTGYCAGCMGCCGCGGTAA and 806R-GGACTACNVGGGTWTCTAAT targeting the V4 region of the bacterial 16s rRNA gene (Parada et al., 2016; Walters et al., 2016).

3. The PCR products will subsequently be purified and used for library preparation.

4. The prepared library will be sequenced on an Illumina HiSeq2500 platform, and 250-bp paired-end reads will be generated with at least 50.000 raw reads per sample (Q30≥ 75%).

5. The obtained reads will subsequently be quality checked and analysed using the following methods and software:

Standard Analysis	Software
Data split and reads merging	FLASH
Data quality control: data filtration and chimera removal	QIIME
OTUs(Operational Taxonomic Units) Clustering and Species annotation, including OTUs Heatmap, GraPhlAn display, Taxonomy Tree, KRONA results	Uparse, PyNast, Mothur
Species distribution, including Ternary plot, Species Abundance Heatmap, the evolutionary tree in genus.	RDP
Alpha-diversity Analysis, including Alpha Indices table (Observed species, Good's coverage, Chao1, ACE, Shannon, Simpson, PD whole tree), Species diversity curves, Species accumulation boxplot, Venn and Flower diagram(Up to 5 figures for free)	QIIME, R
Beta-diversity Analysis, including Beta diversity heatmap, (un)weighted unifrac distance analysis, PCA (Principal Component Analysis), PCoA (Principal Co-ordinates Analysis), UPGMA (Unweighted Pair-group Method with Arithmetic Means), MetaStat, LEfSe analysis	QIIME, R

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4.3.3. FUNGAL/MYCORRHIZA GENETIC DIVERSITY: FUNGAL ITS ILLUMINA AMPLICON SEQUENCING

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PRINCIPLE AND APPLICATION

Amplicon sequencing is a highly targeted approach that enables the analysis of genetic variation in specific genomic regions. This method, that employs DNA metabarcoding, allows the paralleled amplification and sequencing of potentially thousands of different barcoded regions belonging to different species. The barcoded region corresponds to a short fragment within the genome of an organism, whose sequence is conserved within a species, but varies among different species. By comparing the sequences obtained to those of a reference database, it is possible to gather information about the community composition of a complex sample. The ultra-deep sequencing of PCR products (amplicons) allows efficient variant identification and characterization. Illumina amplicon sequencing uses oligonucleotide probes designed to target and capture regions of interest, followed by next-generation sequencing (NGS). NGS overcomes the limits of the cultivation-based methods and allows profiling the entire microbiome by directly sequencing the DNA taken from a wide range of different and complex samples.

In this protocol, we chose the fungal Internal Transcribed Spacer (ITS) region for fungal amplicon sequencing. ITS is part of the non-transcriptional region of the fungal rRNA gene. The ITS sequences used for fungal identification

usually include ITS1 and ITS2 because in fungi, 5.8S, 18S, and 28S rRNA genes are highly conserved, whereas ITS can tolerate more mutations in the evolutionary process and exhibits extremely wide sequence polymorphism in most eukaryotes. At the same time, the conservative type of ITS is relatively consistent within species, the differences between species are easily detectable and they have been widely used in phylogenetic analysis of different fungi (Kim et al. 2013).

In this protocol, the Illumina MiSeq system with a read length of 2 x 300 bp and an output of 22-25 M reads, was chosen to characterize the fungal diversity. Illumina sequencing by synthesis (SBS) chemistry is the most widely adopted NGS technology, generating approximately 90% of global sequencing data (Illumina 2015).

PROCEDURE

1.

Amplicon library preparation

This protocol is based on the protocol described in the Earth Microbiome Project (http://www.earthmicrobiome.org/protocols-and-standards/its/). The libraries are prepared following a 2-step PCR protocol. For the first PCR, the fungal ITS1- ITS2 region with an amplicon of ~450 bp (Bokulich and Mills 2013; Hoggard et al. 2018) is amplifed using the primers ITS1f (5' - CTTGGTCATTTAGAGGAAGTAA - 3') (Gardes and Bruns 1993) and ITS2 primer (5' - GCTGCGTTCTTCATCGATGC - 3') (White et al. 1990). These primers include an oligonucleotide extension at their respective 5' ends in order to make the amplicons suitable for sequencing in an Illumina MiSeq sequencer. The complete primer sequences are shown in table 4.3.3.1. The reaction mixture and the cycling conditions of the first PCR are described in table 4.3.3.2. and 4.3.3.3, respectively. The indices, which are required for multiplexing different samples in the same sequencing run will be added in a second PCR with identical conditions but with only 5 cycles and 60 ºC as the annealing temperature.

A negative control containing no DNA needs to be included in every PCR run to check for contamination during the library preparation.

The libraries are verified by electrophoresis on 2 % agarose gel stained with GreenSafe (NZYTech), and imaged under UV light to verify the library size.

The libraries are purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), following the instructions provided by the manufacturer. Then, they will be pooled in equimolar amounts according to the quantification data provided by the Qubit dsDNA HS Assay (Thermo Fisher Scientific).

Table 4.3.3.1. PCR primers

PCR #	Primer name	Primer sequence (5' – 3')*	Reference
PCR1	ITS1f	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns 1993
	ITS2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTGCGTTCTTCATCGATGC	White et al. 1990
PCR2	Forward primer	AATGATACGGCGACCACCGAGATCTACACNNNNNNNNACACTCTTTCCCTACACGA	
	Reverse primer	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTG	

* N represent the index sequences, which will differ among samples.

Table 4.3.3.2. PCR reaction mixture

Reagent	InitialConc.	Vol/rxn (µl)	Final Conc.
Supreme NZYTaq 2x Green Master Mix (NZYTech)	2x	12.5	1x
Forward Primer	10 µM	1.25	500 nM
Reverse Primer	10 µM	1.25	500 nM
Sterile H2O	***	7.5	***
Total volume		25	

Table 4.3.3.3. PCR cycling conditions

Step	# of cycles	Temperature	Time
Denaturation	1	95 ºC	5 min
Denaturation	35	95 ºC	30 sec
Annealing		52 ºC	30 sec
Extension		68 ºC	30 sec
Extension		68 ºC	10 min

2.

High-throughput sequencing
The pooled DNA will be sequenced in a MiSeq PE300 run (Illumina). The MiSeq run includes a 5-10 % control PhiX library (Illumina) to increase the sequence diversity among amplicons.

EQUIPMENT AND MATERIAL

- Digestion tubes
- Digester unit (Bloc-Digest)
- Fume collector/extractor
- Erlenmeyer flasks
- Distiller
- Burette
- Laboratory mill
- Balance

REAGENTS

- Sulfuric Acid (95-98%)
- NaOH solution (35%)
- Mixed indicator
- Kjeldahl Catalyst
- Boric Acid
- Phenolphthalein
- HCl solution (0.31 N)

CALCULATIONS

The % of nitrogen is calculated with the following equation:

$$\% \text{ Nitrogen} = (1.4 \times (V_1 - V_0) \times N)/P$$

Where:

- P is the weight of the sample (g).
- V1 is the volume of HCl used in the titration of the sample (mL).
- V0 is the volume of HCl used in the titration of the blank (mL).
- N is the normality of the HCL.

And with the %Nitrogen is possible to calculate the % of protein:

$$\%\text{Protein} = \%\text{Nitrogen} \times F$$

Where F is 5.7, the conversión factor for the wheat (Kowalczewski et al., 2019).

REMARKS

The quantity and quality of the extracted metagenomic DNA is crucial. Low DNA yield or poor DNA quality will likely lead to an inaccurate estimation of microbial diversity (Claassen et al. 2013; Ínceošlu et al. 2010). To avoid this, DNA 260/280 and 260/230 ratios should be higher than >1.8. At the same time, the quantified DNA should have a minimum concentration of 10 ng µl-1 with at least 200 ng, and should be re-suspended in water, 10 mM Tris HCl pH 8.5 or TE buffer (although water should be avoided to prevent acid hydrolysis).

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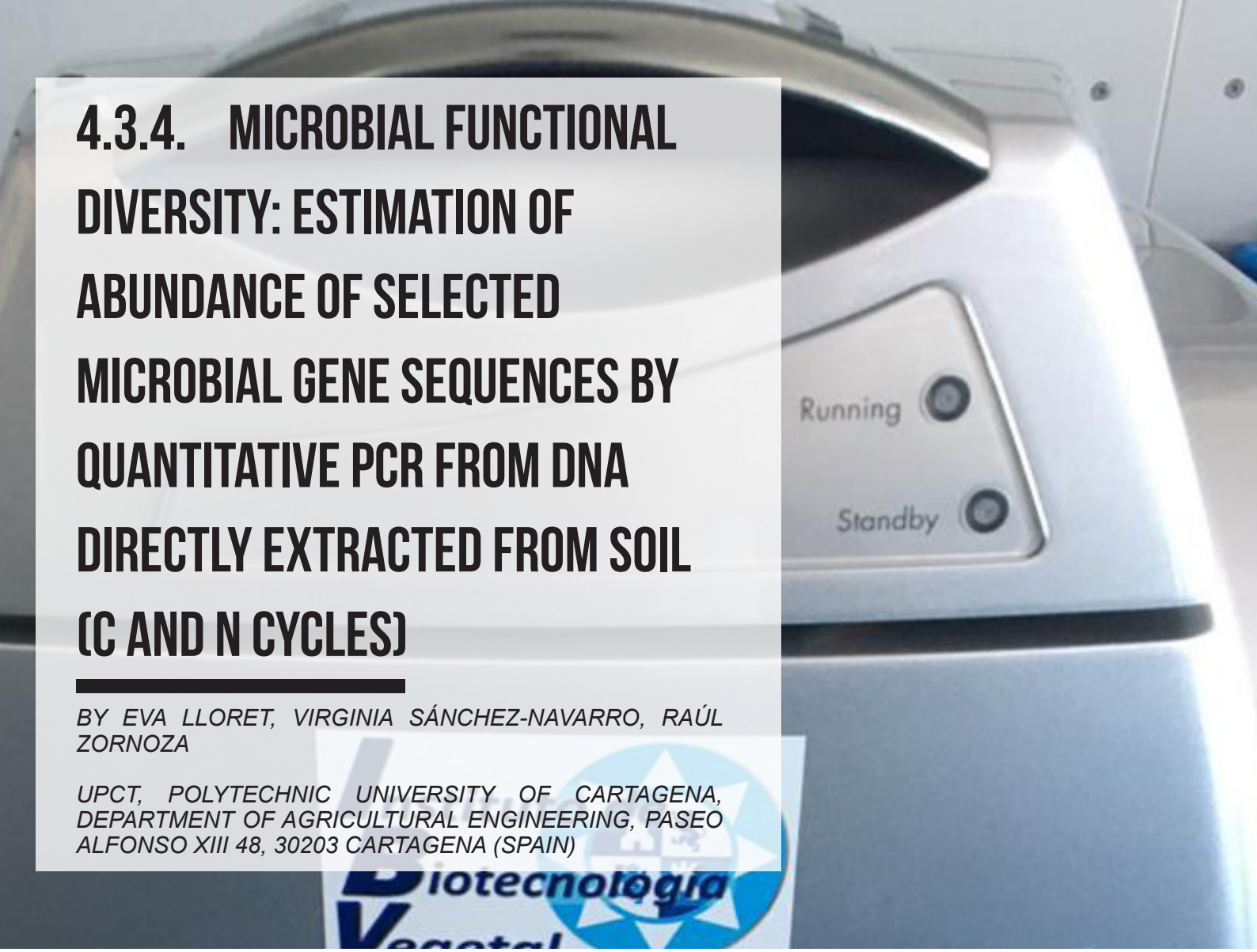

4.3.4. MICROBIAL FUNCTIONAL DIVERSITY: ESTIMATION OF ABUNDANCE OF SELECTED MICROBIAL GENE SEQUENCES BY QUANTITATIVE PCR FROM DNA DIRECTLY EXTRACTED FROM SOIL (C AND N CYCLES)

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PRINCIPLE AND APPLICATION

Carbon and nitrogen biogeochemical cycles are mostly driven by microbial activities (Burgin et al. 2011). In this protocol, microbial C and N cycling gene abundance is determined by qPCR targeting the genes ureC, amoA, narG, nirK, and nifH involved in the N cycle and the genes cbbL and GH7 involved in the C cycle.

Ammonification is the process whereby organic N is mineralized to ammonium (NH_4^+). It is the first step in organic N decomposition and is often referred to as N mineralization (Hopkinson and Giblin 2008). Ammonium is then available to be assimilated and incorporated into amino acids or used for other metabolic purposes (Strock 2008). Urease is a nickel-containing enzyme that catalyses the conversion of urea to ammonia and carbonic acid (Reed 2001). The ureC gene is the largest of the genes encoding urease functional subunits and contains several highly conserved regions (Su et al. 2013).

Nitrification is the biological oxidation of ammonia (NH3) or ammonium (NH4+) to oxidized N in the form of nitrite (NO2−) and further to nitrate (NO3−). Nitrification is of pivotal importance in agricultural systems since it increases the mobility of N through the soil matrix, strongly influencing N retention in the system (Norton and Ouyang 2019). Ammonia oxidation is considered to be the rate-limiting step of nitrification and is catalysed by the ammonia monooxygenase enzyme (AMO), which is encoded by the amoA gene (Li et al. 2015).

Denitrification is the reduction of nitrate (NO3−) to nitrous oxide or dinitrogen and is the major biological mechanism by which fixed nitrogen returns to the atmosphere from soil and water. This removal of soluble nitrogen oxide (N2O) from the biosphere is of great importance in agriculture, where it can account for significant losses of nitrogen fertilizer from soil (Henry et al. 2004). A phylogenetically broad group of bacteria can reduce nitrate (NO3−) into nitrite (NO2−), which can be then reduced into gaseous nitrogen compounds by denitrification or into NH4+ by dissimilatory nitrate reduction in ammonium (DNRA). Denitrification is the dominant process in soils and lake sediment. The ability of facultative anaerobic bacteria to respire nitrate when oxygen is limiting has been ascribed mainly to the activity of a membrane bound nitrate reductase encoded by the narGHJI operon (Philippot and Højberg 1999). For nitrite reduction, two different types of nitrite reductase have been characterized: copper nitrite reductase encoded by the nirK gene and a cytochrome cd1-nitrite reductase encoded by the nirS gene (Zumft 1997).

Nitrogen-fixing microorganisms are globally significant in that they provide the only natural biological source of fixed nitrogen in the biosphere. These organisms enzymatically transform dinitrogen gas from the atmosphere (N2) into ammonium (NH4+) equivalents needed for biosynthesis of essential cellular macromolecules. The nifH gene, encoding the nitrogenase reductase subunit, is the most widely sequenced marker gene used to identify nitrogen-fixing bacteria and archaea (Gaby and Buckley 2012).

Carbon fixation is the assimilation of inorganic C (CO2) to organic compounds by living organisms. The Calvin-Benson-Bassham cycle is the major pathway for CO2 fixation (Shively et al. 1986), and the most abundant protein on Earth, ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO), catalyses the first, rate-limiting step in the Calvin cycle (Ellis 1979). The large subunit of form I RubisCO is encoded by the cbbL gene in bacteria (Kusian and Bowien 2006). I RubisCO proteins can be subdivided into two major groups, the green-like and red-like groups (Watson and Tabita 2006). In this protocol, we choose the cbbL red-like genes to quantify carbon-fixing microorganisms.

Carbon degradation, as plant litter decomposition, is a major source of organic carbon in soils and a key step in nutrient recycling (Berg et al. 2001). Fungi are considered key players in organic matter decomposition in soil because of their ability to produce a wide range of extracellular enzymes, which allows them to efficiently attack the recalcitrant lignocellulose matrix that other organisms are unable to decompose (Kjøller, Struwe, and Kjoller 1982; De Boer et al. 2005). Cellulose is the main structural component of higher plant cell walls, representing 35–50 % of plant dry weight, and through decomposition and transformation makes up much of soil organic matter (Lynd, Wyman, and Gerngross 1999). The Glycoside hydrolase encoding GH7 gene is widely used to evaluate cellulose degradation (Merlin et al. 2014; Hu et al. 2019; Tian et al. 2017).

REAGENTS

1. Soil DNA extracted according to the method described in chapter 4.3.1 of this Handbook.
2. Ice
3. 70% Ethanol
4. PCR Buffer
5. BSA
6. Primers
7. 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.
8. TE buffer × 1: 100 mL of TE buffer × 10 in 900 mL of H2O.
9. Molecular-biology-grade water
10. Agarose
11. SYBR Safe DNA stain
12. DNA ladder with known lengths and concentrations of fragments.
13. NucleoMag® NGS Bead Suspension
14. pGEM®-T Easy Vector Systems with competent cells (Promega)
15. LB plates with ampicillin/IPTG/X-Gal
16. LB Broth
17. Glycerol
18. Ampicilline sodium, C16H18N3NaO4S (CAS No. 69-52-3)
19. Qubit™ dsDNA HS Assay Kit (Invitrogen™)
20. ISOLATE II Plasmid Mini Kit (Bioline)
21. SYBR Green® qPCR kit

EQUIPMENT AND MATERIAL

- PCR hood
- PCR thermocycler
- Qubit® 2.0 Fluorometer (Invitrogen™)
- Horizontal electrophoresis system
- Gel imaging system
- Water bath
- Shaking incubator
- Autoclave
- Oven
- Real-time PCR thermocycler
- Filtered tips
- Pipettes
- Low DNA binding eppendorfs
- PCR tubes
- Syringe filters
- qPCR tubes
- Cold well-loading block

PROCEDURE

1.

qPCR standard preparation
qPCR standards targeting a sequence of the microbial gene of interest (GOI) are obtained from DNA extracted from soil samples according to chapter 4.3.1 of this Handbook. To select the appropriate amplicon to settle down each qPCR assay, primer pairs listed in Table 4.3.4.1 will be used for each GOI. First, using the DNA mentioned above as the template, PCR reactions will be conducted with the primer sets from Table 4.3.4.1, the reaction mixture described in Table 4.3.4.2. and the corresponding PCR cycling conditions described in Tables 4.3.4.3 to 4.3.4.9.

Table 4.3.4.1. PCR primers set for quantitative analysis

Function	Target gene	Gene definition	Primer name	Primer sequence (5' – 3')	Annealing temp. (°C)	Amplicon size (bp)	Reference
Ammonification	ureC	urease	ureC-F ureC-R	TGGGCCTTAAAATHCAYGARGAYTGGG GGTGGTGGCACACCATNANCATRTC	55	340	Reed et al. (2001)
Nitrification	amoA	ammonia monooxygenase	amoA1F amoA1R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	57	491	Rotthauwe et al. (1997)
Denitrification	narG	nitrate reductase	narG1960m2F narG250m2R	TAYGTSGGGCAGGARAAACTG CGTAGAAGAAGCTGGTGCTGTT	56	110	López-Gutiérrez et al. (2004)
Denitrification	nirK	nitrite reductase	nirK876F nirK1040R	ATYGGCGGVCAYGGCGA GCCTCGATCAGRTTRTGGTT	58	165	Henry et al. (2004)
N2 fixation	nifH	nitrogenase reductase	PolF PolR	TGCGAYCCSAARGCBGACTC ATSGCCATCATYTCRCCGGA	55	342	Poly et al. (2001)
Carbon fixation	cbbL	cbbL red-like gene	cbbL-RedF cbbL-RedR	AAGGAYGACGAGAACATC TCGGTCGGSGTGTAGTTGAA	57		Selesi et al. (2005)
Carbon degradation	GH7 (fungal)	cellulose degradation	GH7-F GH7-R	GAGATCAAGCGCYTCTAYGTBCA GTCRAGCCASAGCATGTTGG	56	242	Tian et al. (2017)
Cloning site			SP6 T7	ATTTAGGTGACACTATAG TAATACGACTCACTATAGGG			

Table 4.3.4.2. qPCR reaction mixture.

Reagent	InitialConc.	Vol/rxn (uL)	Final Conc.
Sybr Green Bioline	2x	10	1x
BSA	20 mg/mL	0.6	0.6 mg/mL
Forward Primer	10 uM	0.8	400 nM
Reverse Primer	10 uM	0.8	400 nM
DNA template	2 ng/ul	5	10 ng
Sterile H2O	***	2.8	***
Total volume		20	

Table 4.3.4.3. ureC cycling conditions.

Step	# of cycles	Temperature	Time
Denaturation	1	95 ºC	10 min
Denaturation	40	95 ºC	30 sec
Annealing		55 ºC	30 sec
Extension		72 ºC	30 sec

Table 4.3.4.4. amoA PCR cycling conditions.

Step	# of cycles	Temperature	Time
Denaturation	1	95ºC	10 min
Denaturation	35	95ºC	25 sec
Annealing		57ºC	25 sec
Extension		72ºC	40 sec
Extension		72ºC	10 min

Table 4.3.4.5. narG cycling conditions.

Step	# of cycles	Temperature	Time	
Denaturation	1	95 ºC	10 min	
Denaturation	6	95 ºC	15 sec	
Annealing		65 to 60 ºC	30 sec	Touchdown (annealing temperature progressively decreased by 1C by cycle)
Extension		72 ºC	30 sec	
Reading		80 ºC	15 sec	
Denaturation	40	95 ºC	15 sec	
Annealing		60 ºC	30 sec	
Extension		72 ºC	30 sec	

Table 4.3.4.6. nirK cycling conditions.

Step	# of cycles	Temperature	Time	
Denaturation	1	95 ºC	10 min	
Denaturation	6	95 ºC	15 sec	
Annealing		63 to 58 ºC	30 sec	Touchdown (annealing temperature progressively decreased by 1C by cycle)
Extension		72 ºC	30 sec	
Reading		80 ºC	15 sec	
Denaturation	40	95 ºC	15 sec	
Annealing		58 ºC	30 sec	
Extension		72 ºC	30 sec	

Table 4.3.4.7. nifH cycling conditions.

Step	# of cycles	Temperature	Time
Denaturation	1	95 ºC	10 min
Denaturation	40	95 ºC	60 sec
Annealing		55 ºC	60 sec
Extension		72 ºC	60 sec

Table 4.3.4.8. cbbL cycling conditions.

Step	# of cycles	Temperature	Time
Denaturation	1	95 ºC	10 min
Denaturation	40	95 ºC	60 sec
Annealing		57 ºC	2 min
Extension		72 ºC	2 min

Table 4.3.4.9. GH7 cycling conditions.

Step	# of cycles	Temperature	Time
Denaturation	1	95 ºC	10 min
Denaturation	40	95 ºC	10 sec
Annealing		56 ºC	30 sec
Extension		72 ºC	30 sec

The expected size of the qPCR standard amplicons (PCR products from the above reactions) are verified by electrophoresis on 2% agarose gel in TBE buffer stained with SYBR Safe® DNA gel stain. Amplicons are purified with NucleoMag® NGS Bead Suspension according to the manufacturer instructions and quantified with Qubit™ dsDNA HS Assay Kit following the manual. Make sure you get at least 10 ng/µl of PCR product.

2.

Cloning of qPCR standard

- The same day after purifying the PCR product obtained in the PCR reactions mentioned above (Tables 4.3.4.3 to 4.3.4.9.), the ligation should be started. If the PCR product is stored, the A-Overhangs left by the polymerase may detach from the product and ligation efficiency will go down.
- The ligation is made using the pGEM®-T Easy Vector Systems kit from Promega following the manufacturer instructions but incubating the ligation reaction overnight instead of a few hours.
- Transform your competent cells (108 cfu/µg DNA) that are provided with the kit, with the ligation mixture following the kit's instructions and plate 100 µL aliquots of the cells suspension onto LB/Amp/IPTG/X-Gal solid medium. Incubate the Petri dishes at 37°C overnight in the dark for no more than 13 h. This process will facilitate white or blue colour development of the colonies.
- For screening of the recombinant clone, pick three white colonies per GOI (colonies containing the PCR product) and a blue one. Transfer them to liquid LB media with Ampicillin and incubate overnight at 37°C.
- Extract the plasmid using the Isolate II Plasmid Mini Kit from Bioline.
- To make sure you get the desired product size, run a test PCR for the white and blue colonies for each GOI using the primers listed in Table 4.3.4.1. with the reaction mixture of Table 4.3.4.2 and the cycling conditions described in Tables 4.3.4.3 to 4.3.4.9.
- Quantify the plasmids with Qubit™ dsDNA HS Assay Kit.

3.

qPCR standard curve

The qPCR standard curve is performed on serial dilution of the standard plasmid ranging from 108 to 101 copies per reaction. The GOI is amplified by using the specific primers pair listed in Table 4.3.4.1., the reaction mixture described in Table 4.3.4.10, and the cycling program described in Tables 4.3.4.3. to 4.3.4.9. replacing the final extension by a dissociation stage (melt analysis) by gradually increasing the temperature from 60°C to 95°C. Standard dilutions and non-template controls (NCT) made of molecular grade water should be amplified in triplicate.

Table 4.3.4.10. qPCR reaction mixture.

Reagent	Initial Conc.	Vol/rxn (µL)	Final Conc.
SYBR Green®	2x	10	1x
BSA	20 mg/mL	0.6	0.6 mg/mL
Forward Primer	10 µM	0.8	400 nM
Reverse Primer	10 µM	0.8	400 nM
Plasmid standard	***	2	***
Sterile H2O	***	5.8	***
Total volume		20	

At the end of the assay, the results are analysed using the automatic option of the thermocycler. The qPCR is validated with the following observations:

(1) no amplification in NTC reactions,
(2) a single dissociation peak for each dilution of qPCR standard,
(3) a linear calibration curve (standard curve) with R2 ≥ 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. The efficiency of the qPCR assay, which is calculated by the thermocycler software, is estimated from the calibration curve formula:

$Ct = \alpha \times q + c$

where,

Ct is measured cycle threshold;
α is the slope of the calibration curve;
q is the log copy number of qPCR standard;
c is the ordinate at the origin (Ct for 1 copy of qPCR standard).

$E = 10^{(-1/\alpha)} - 1$

where E is the efficiency of the calibration assay and α is the slope of the calibration curve.

It shall be stated that a calibration curve having a slope equal to -3.32 is 100 % efficient. Therefore, a 10-fold-dilution of a given DNA template gives a Ct difference of 3.32 for a qPCR assay 100 % efficient (i.e. if 108 Ct = 10, so 107 Ct = 13.32).

4.

DNA inhibition test

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. Inhibition is tested the following way:

- Spike 104 copies of plasmid DNA to soil DNA and perform a qPCR using SP6 and T7 primers.

- The amplification reaction is performed with the reaction mixture described in Table 4.3.4.10 including the soil DNA dilutions to be tested, three positive controls containing only plasmid DNA, and three NTC. The qPCR cycling program is shown in the following table and it will include a final dissociation stage (melt analysis).

Table 4.3.4.12. Inhibition test qPCR cycling conditions.

Step	# of cycles	Temperature	Time
Denaturation	1	95ºC	15 min
Denaturation	30	95ºC	15 sec
Annealing		55ºC	30 sec
Extension		72ºC	30 sec
Melt curve	1	65-95ºC	2-5 sec/step*
*temperature progressively increased by 0.25ºC increments			

The inhibition test is validated by observing the following:

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.

5.

qPCR assays

Once we have come out with a proper standard curve and obtained a DNA template free of inhibitors, the qPCR assay can be run for our DNA samples extracted from soil according to chapter 4.3.1 of this Handbook. 108 to 101 dilutions of the standard plasmid are run in every qPCR assay to create the standard curve and obtain an absolute quantification. The qPCR assay targeting the GOI will be performed on each soil DNA template at the dilution showing no inhibition exactly as described in "3. qPCR standard curve" with the only difference that both standards and DNA templates will be performed in duplicate.

CALCULATIONS

In this section the calculation of the copy number of the GOI in the soil DNA extract is described. 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:

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

I (GOI/ng DNA) = (GOI in assay)/(volume of template in assay (µL) x concentration of template in assay (ng/µL))

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

II (GOI/g soil) = (I x extracted DNA from soil (ng))/(soil sample from which DNA is extracted (in g of dry mass equivalent))

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4.4. WHOLE MICROBIAL DIVERSITY: PLFA AND NLFA

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PRINCIPLE AND APPLICATION

The study of the structure and diversity of soil microorganisms is a tool widely used to know the changes produced on microbial communities by different environmental disturbances (Kirk et al. 2004). One widely used technique to determine the changes in the microbial community structure is the analysis of the fatty acids (chain length between 14 and 20 C atoms) of different lipid groups: phospholipid fatty acids (PLFA) and neutral lipid fatty acids (NLFA) (Malosso et al. 2004). These lipids play important roles in living cells as either structural components of the cellular membranes (PLFA) or as energy (storage compounds (NLFA) (Ruess and Chamberlain 2010).

Within the neutral lipids, the triglycerides, formed by three fatty acids linked by ester bonds to a single glycerol molecule, are the most abundant. Since the polar parts are linked (the carboxyl groups of fatty acids and the alcohol group from the glycerol) these neutral lipids are nonpolar and not soluble in water (Nelson et al. 2005).

The phospholipids are formed by fatty acids, glycerol or sphingosine (depending on the type of phospholipids) and a phosphate group. Since 1980, phospholipid fatty acids (PLFA) have been frequently used as biomarkers and to determine both the microbial biomass (total biomass and

biomass of specific groups of microorganisms) and the diversity of microbial communities due to the fact that while some fatty acids are unspecific, others are characteristic of determined microorganisms (White and Findlay 1988). Some fatty acids are more common in prokaryotes and others in eukaryotes, while the triglycerides (and the fatty acids that compose them, NLFAs) are exclusive to eukaryotic organisms (fungi) as storage compounds. The analysis of both types of fatty acids (PLFA and NLFA) helps to improve the microbial community characterization (Bååth 2003).

Table 4.1. Fatty acid biomarkers (adapted from Frostergård et al. 1996 and Kaur et al. 2005)

Group	Biomarker
Bacteria	i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ω7t, i17:0, a17:0, 17:0, cy17:0, 18:1ω7, cy19:0
Gram-positive Bacteria	br17:0, br18:0, i17:0, a17:0, i16:0, i16:1, 10Me16:0, 10Me17:0, i15:0, a15:0, 16:1ω9, 16:1ω7c, 16:1ω5*, 18:1ω7 and C19:1
Gram-negative Bacteria	cy17:0, cy19:0, r-hydroxy fatty acids, 16:1ω9, 16:1ω7, 16:1ω5, 18:1ω7 and 19:1
Fungi	18:2ω6 (linoleic acid)
Actinobacteria	10Me16:0, 10Me17:0 and 10Me18:0
Anaerobic Bacteria	Non-ester linked phospholipid fatty acids (NEL-PLFA)
Sulphate Reducing Bacteria (Desulfobacter sp.)	cy17:0 and 10Me16:0 without high level of 10Me18:0
Methanogens: Type I	16:1ω8
Methanogens: Type II	18:1ω8

*characteristic of arbuscular mycorrhiza within roots (Frostegård et at, 2011)

The carbon chains of PLFA can be saturated (carbons appear linked by simple bonds) or unsaturated (when double bonds appear between carbons) (Benatar et al. 2011). Carbon α is the one that belongs to the carboxyl group, while the furthest is carbon ω. However, when numbering them the 1 is the furthest from the carboxyl group (the ω carbon). When naming them, the nomenclature usually used is “total carbon number of the fatty acid:number of double bondsωposition of double bonds from the methyl end of the molecule separated by commas”. To indicate a cis or trans configuration, the letter “c” or “t” is usually added. The prefixes “i” and “a” indicated iso- or anteiso-branching; “br” indicates unknown methyl branching position (Ran-Ressler et al. 2014). If there is a methyl group in carbon 10, “10Me” is added, “cy” if there is a cyclopropane, and “di” if is a dicarboxylic fatty acid. The prefixes “p” and “r” point that the OH groups of the OH fatty acid are in the positions 2 and 3 respectively. If there is a strange or difficult-to-describe configuration, “br” is added (Bååth 2003; Kaur et al. 2005).

PLFA and NLFA soil microbial community analysis can be divided into four parts: extraction of lipids from the soil, fractionation of the different types of lipids (neutral lipids, glycolipids and polar lipids), mild alkaline methanolysis of the different lipids to convert their components, the fatty acids, into volatile methyl esters, and analysis of these by gas chromatography (Frostegård et al. 1993; Díaz-Raviña et al. 2006).

PROCEDURE

Preparation of reagents

- 5 N NaOH solution. Weight 200 g of NaOH in a volumetric flask and bring up to 1000 ml with deionized water.
- 0.15 M citrate buffer, pH 4: dilute 28.82 g of citrate acid in 850 ml of water and add the 5 N NaOH solution to adjust the pH to 4 before dilute to 1000 ml with deionized water. This solution must be prepared at the time of use.
- Bligh and Dyer (B&D) reagent: mixt CHCl3:MeOH:citrate buffer in a proportion 1:2:0.8 (v/v/v).
- Methanol:toluene: Mix both solutions in a proportion 1:1 (v/v)
- Hexane:CHCl3 Mix both solutions in a proportion 4:1 (v/v)
- Acetic acid 1 M. Add 57.2 ml of acetic acid in a volumetric flask and bring up to 1000 ml with deionized water.
- KOH 0.2 M in methanol. Weight 11.22 g of KOH in a volumetric flask and bring up to 1000 ml with methanol. This solution must be prepared at the time of use.
- Standard methyl nonadecanoate (Std19:0, molecular weight 312.54 g). Dissolve 115 µg of the standard in 5 ml of hexane (concentration 23 µg ml-1). This solution must be prepared daily.
- Detergent free of phosphates (2% in deionized water type II).

1. Lipid extraction

- Weight between 0.5 and 6 grams of dry soil (0.5-1.5 g if high OM (Organic Matter) content, 3-6 g if low OM content) and place in 50 ml Teflon tubes. Moist, air-dried, frozen or freeze-dried soil samples are used. Blanks without soil, treated under the same conditions, are prepared in each batch of samples.
- Add 10 ml of B&D reagent, shake with vortex for 1 min and keep for 2 hours at room temperature to allow the compounds to be extracted.
- Centrifuge for 11 minutes at 1677 x g and transfer the supernatant to a 50 ml Teflon or glass tube.
- To complete the extraction, repeat the process, adding 5 ml of B&D to the residue. Add the supernatants to the 50 ml tube mixing both supernatants.
- Add 4 ml of chloroform and 4 ml of citrate buffer to the extract obtained and to shake in the vortex for 1 min.
- The suspension is left overnight at room temperature to extract the lipids in the organic phase. Another option is to centrifuge for 11 min at 1677 x g.
- The lower phase (organic) is extracted with a Pasteur pipette. An aliquot of 3 ml (high OM content soil) or 5 ml (low OM content soil) of this phase is dried at 40 ºC under a flow of nitrogen in a dry block heater. The rest of the extract is stored at 4 ºC until the end of the procedure.
- Dry samples are frozen a -20 ºC. In case of continuing with the fractionation on the same day, it is not necessary to freeze.

2. Fractionation

The second step consists in separating the different lipid fractions.

- Dried samples (and thawed) are re-suspended using 100 µl of chloroform and agitation in the Vortex. This suspension is added to a silica column (SPE-SI), previously placed on glass tubes of 25 ml. Repeat this process twice by adding 100 µL of CHCl3.
- Once the lipids have been retained in the silica column, reagents of increasing polarity are applied successively to eluate the different lipid fractions collected in different glass tubes:

  - 1.5 ml of chloroform to elute the neutral lipids
  - 6 ml of acetone in two 3 ml steps to separate the glycolipids
  - 1.5 ml of methanol to eluate the polar lipids
- At the end of the fractionation, the contents of the tubes are dried at 40 ºC under a flow of nitrogen in a dry block heater

In this method, the first (neutral lipids) and last (polar lipids) fractions will be analysed and the procedures detailed below are the same for both and must be performed with each of the samples.

3. Mild alkaline methanolysis (Transesterification)

The third step involves a transesterification reaction to convert the fatty acids of the phospholipids (PLFA) and neutral lipids (NLFA) into volatile derivatives that can be measured by gas chromatography.

- Add 100 µl of the Std19:0 (23 µg ml-1 concentration) to all samples
- Evaporate the hexane under a flow of nitrogen.
- Add 1 ml of the MeOH:toluene (1:1) mixture and shake in a vortex for 5 s.
- Add 1 ml of 0.2 M KOH in methanol, shake in a vortex 5 seconds and incubate in a bath (at 37°C) for 15 min. Let the samples cool some minutes at room temperature.
- Add 2 ml of hexane:chloroform (4:1), 0.3 ml of 1M acetic acid and 2 ml of water.
- Stir for 1 min in a vortex, check that the pH of the lower phase is around 6, centrifuge 11 min at 1677 x g and remove with a Pasteur pipette the upper phase to a glass container. This step must be repeated by adding 2 ml of hexane:chloroform, stirring and centrifuging again.
- Evaporate the solvents in the samples under a flow of nitrogen without heating. It is important to remove the samples when the last drop of the solvent evaporates to avoid losses of the more volatile fatty acids. Freeze the dry samples at -20 ºC until its further analysis.

4. GC Analysis

The last step is the sample analysis on a gas chromatograph with a flame ionization detector (GC-FID). A HP5 (phenilmethyl silicone) capillary column (50 m longitude, 0.2 mm internal diameter, 0.33 µm film thickness) is used.

- Chromatographic conditions (Frostergård et al. 1993):
  - Carrier gas was He (flow 0.8 ml/min)
  - Detector temperature: 230 ºC
  - Injector temperature: 230 ºC, injection was made in splitless mode
  - Oven temperature program: initial temperature 80 ºC for 1 min, increasing first with 20ºC min-1 to 160 ºC and after with 5ºC min-1 to reach 270ºC, remain at this temperature 5 min.
- The frozen sample is thawed to room temperature.
- Add 100 µl of hexane to dissolve the sample and transfer to GC vials.
- 1 µl of this solution is analysed by GC-FID by duplicated.
- The PLFAs and NLFAs were separated, identified by the relative retention times compared with those obtained for the standards and the peak areas are quantified by adding, before the transesterification step, methyl nonadecanoate (Std19:0) as internal standard.

EQUIPMENT AND MATERIAL

- 50 ml Teflon centrifuge tubes with screw cap
- Glass tubes with screw cap
- Erlenmeyer flasks
- Beakers
- Volumetric flasks
- Graduated cylinder
- Pasteur pipettes
- Micro-centrifugation tubes of 1.5 or 2 ml
- Petri dishes
- GC vials
- Spatulas and tweezers
- Racks
- LCR Cartridges Bond Elut SI (SPE) normal phase (polar)

REAGENTS

- Citric acid ($C_6H_8O_7$)
- Sodium hydroxide (NaOH)
- Potassium hydroxide (KOH)
- Acetone (C_3H_6O)
- Acetic acid (CH_3COOH)
- Chloroform ($CHCl_3$)
- Hexane (C_6H_{14})
- Methanol (CH_3OH)
- Toluene (C_7H_8)
- Methyl nonadecanoate (Std19:0, $C_{20}H_{40}O_2$)
- Water deionized type I (Resistivity 18 Ω cm-1, C <5 ppb) used to prepare the reagents and water deionized type II (Resistivity > 5 Ω cm-1, TOC < 30 ppb) to wash the material
- Phosphate-free detergent

REMARKS

Due to the high sensitivity of the method, impurities from the reagents and ineffective cleaning of the material used can cause serious contamination problems that can be detected in the blanks (without soil).
It is important to wash the material with a phosphate-free detergent. All clean and dry glass material (non-volumetric) must also undergo a heat treatment at 400°C for at least 6 hours to eliminate any other organic matter including lipid contaminants.
All reagents used are analytical grade, except the n-hexane, which is solvent to GC chromatography.
During the process, the following security measures must be taken into account: a) work in the gas extraction cabinet and, if it is not possible, use a mask with a filter to organic solvents, b) wear dust mask to weigh the powder reagents and c) use gloves and safety glasses whenever there is any possibility of splashes.

CALCULATIONS

Each identified PLFA is quantified (nmol g-1 of dry soil) in each sample using the following equation:

$$PLFA\ (nmol\ g^{-1}) = \frac{Area\ PLFA sample}{Area\ 19{:}0 sample} \times CIStd19{:}0\ added\ \times\ R\ \times V\ \times \frac{1}{dry\ soil\ weight}$$

Where:

- **Area PLFAsample is the peak area of each PLFA identified in the sample**
- **Area 19:0sample is the peak area of PLFA19:0 in the same sample**
- **CIStd19:0 is the amount of internal standard, methyl nonadecanoate (Std19:0) added in step 3 to each sample, expressed in nmol.**

$$CIStd19{:}0 added\ (nmol) = \frac{23000\ ng\ ml^{-1} \times 10^{-1}\ ml}{312.54\ ng\ nmol^{-1}} = 7.36\ nmol$$

- **R is the correction factor determined by the recovery of the internal standard to each sample, calculated as:**

$$R = \frac{Area 19{:}0 blank}{Area 19{:}0 sample}$$

Where

- **Area19:0blank is the peak area of PLFA 19:0 in the blank**
- **Area19:0sample is the peak area of PLFA 19:0 in the sample**
- **V is the factor obtained from the quotient between the total volume of CHCl3 used in the lipids extraction, 7.95 ml, and the aliquot volume used to fractionation (3 or 5 ml) (step 1).**

The procedure for the quantification of neutral lipids fatty acids (NLFAs) is the same as described for the PLFAs.

Total PLFA microbial biomass estimations

The sum of the content of all the PLFAs (around a total of 30-35 unspecific and specific to different microbial groups) identified and quantified of each sample were used to obtain the total PLFA biomass (nmol g-1 of dry soil)

Microbial PLFA biomass of specific microbial groups

The content of each PLFAs biomarkers characteristic of the specific microbial groups are summed to obtain the PLFA biomass of these groups.

For example, the bacterial PLFA biomass is determined following the equation (Frostegard et al. 1996)

$$TB = \Sigma\ i15{:}0 + a15{:}0 + 15{:}0 + i16{:}0 + 16{:}1\omega9 + 16{:}1\omega7t + i17{:}0 + a17{:}0 + 17{:}0 + cy17{:}0 + 18{:}1\omega7 + cy19{:}0$$

TB, is the bacterial PLFA biomass (nmol g-1 of dry soil)

In the same way, the biomass of other microbial groups was obtained by the sum of their specific PLFAs biomarkers (fungi, Gram-positive bacteria, Gram-negative bacteria, actinobacteria, anaerobic bacteria, etc.) (see Table 4.1.). It should be noticed that the criteria of the PLFAs characteristics of specific microbial groups could differ among authors (Frostegård et al. 1993; 1996; Zelles 1999; Kaur et al. 2005; Díaz-Raviña et al. 2006); thus, in these biomass estimations the reference used should be included.

Ratios between different biomarker fatty acids

The relationships between some specific fatty acids are used to obtain information on the composition or the microbial status of soil microbial communities. The mostly used ratios are the following:

- ***Fungal PLFA/Bacterial PLFA ratio***: provides information about the predominance of these main microbial groups, which play a different role in soil (Frostegård et al. 1996; 2011).
- Gram-negative to Gram-positive bacterial PLFA ratio: provides information concerning the microbial status of microorganisms (Zelles 1999; Kirk et al. 2004; Frostegård et al. 2011)
- Ratios of PLFAs trans/PLFAs cis of monounsaturated fatty acids (16:1ω7t/16:1ω7c, 18:1ω7t/18:1ω7c) or cyclo/mono-unsaturated precursor (cy17:0/16:1ω7c and cy19:0/18:1ω7c): provides information on stress or starvation (Zelles 1999; Kirk et al. 2004).
- NLFAs/PLFAs ratios for specific fatty acids: provides information on nutrient status or physiological conditions of soil fungi (Bååth 2003).
- PLFA or NLFA diversity indexes
- The richness (R) of the fatty acids is expressed as the total number of PLFAs or NLFAs present in each sample. The diversity of the FAs is calculated with the Shannon index H.

$$H = \sum_{i=1}^{R} pi \ ln \ pi$$

Where pi is the relative abundance of each fatty acid in the total sum and R is the number of detected fatty acids.

The equitability of the fatty acids is calculated with the Shannon's evenness E.

$$E = \frac{H}{Ln(R)}$$

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5. WHEAT QUALITY ANALYSES

BY DIEGO SOTO GÓMEZ(1), KENIA ANTA RODRÍGUEZ(2), MARÍA MUINELO MARTÍNEZ(2), SERVANDO ÁLVAREZ POUSA(2), MANUEL ARIAS ESTÉVEZ(1) AND DAVID FERNÁNDEZ CALVIÑO(1)

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Wheat (Triticum aestivum) is a basic cereal that is consumed by humans in all parts of the world as it is used as the main ingredient in basic food products such as bread and pasta (Mahesh et al., 2008). In 2018, more than 700 million tons of wheat was produced worldwide, and more than 214 million hectares were used for the cultivation of this cereal (FAO, 1997).

When determining the ripening of the wheat and its quality, a series of characteristics are used. They include, among others, the weight of the grain, the humidity, the amount of gluten, and the milling performance (Mutlu et al., 2011). These wheat characteristics depend on the variety of wheat, the type of soil, the climate, the type of fertilization used, and other factors (Carew et al., 2009)

Throughout this section the following characteristics of wheat grain (summarized in Table 5.1.) will be analysed:

- Wheat grain moisture, i.e., the percentage of water that each grain has, which is a characteristic that determines the period that the grain can remain stored.
- Wheat grain specific weight, i.e., the volume of a certain amount of wheat.
- Wheat seed index, or the weight of one thousand grains.
- Wheat nitrogen and protein content, characteristics related with the fate of the grain and its market value.

Table 5.1. Wheat analysis used to characterize the quality of the wheat samples and partners in charge of the analysis.

WHEAT QUALITY PROPERTIES MEASURED	
ANALYSIS TYPE	PARTNER IN CHARGE
Wheat grain moisture	UVIGO
Wheat grain specific weight	UVIGO
Weight of thousand grains (or seed index)	UVIGO
Nitrogen and protein content of wheat grain	UVIGO

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5.1. WHEAT GRAIN MOISTURE

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PRINCIPLE AND APPLICATION

The moisture of the wheat grain is a fundamental aspect that indicates the maturation of the kernel and can be used to point the moment of the harvest. The amount of water is the characteristic that determines the period that the grain can remain stored (May and Van Sanford, 1992). During the maturation of the grains the amount of dry matter typically increases steadily while water filling occurs mainly in the first days (Schnyder and Baum, 1992). When the grain reaches 33% of dry matter (in weight), it stops accumulating water and continues storing dry material until it reaches a proportion of 55% in weight. The loss of ability to absorb water from the grain coincides with the end of the nuclear division of the endosperm.

PROCEDURE

The moisture determination is executed following the UNE-EN ISO 712 standard “Cereals and derived products. Determination of moisture content. Reference method”. The procedure is the following:

- First, an aluminium tray and about 25 g of wheat are weighed. It is important to write down the exact weight.
- The tray with the wheat is introduced in an oven at 130 °C until constant weight.
- The tray is reweighed when constant weight is reached.

EQUIPMENT AND MATERIAL

- **Aluminium tray**
- **Analytical balance (0.01 g)**
- **Oven**
- **Spatula**

CALCULATIONS

The percentage of moisture in the wheat is calculated with the equation:

$$\%H = \frac{M_T - M_{dry}}{M_T} \; x \; 100$$

Where,

- **MT is the weight of wheat at room temperature.**
- **Mdry is the weight of dry wheat.**

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5.2. WHEAT SPECIFIC WEIGHT

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PRINCIPLE AND APPLICATION

Apart from moisture, one of the best indicators of the quality of wheat is the specific weight (Hook, 1984), i.e., the volume occupied by a given quantity (mass) of grain. It is a characteristic that some authors associate with the potential flour yield (Baker et al., 1965). Depending on the variety of wheat, the density of the endosperm and the amount of water, the specific weight can vary greatly, between 60 and 90 Kg hL-1. For example, the amount of nitrogen in the soil increases the specific weight of some varieties of wheat (Clarke et al., 2004). The specific weight is a very useful feature when storing grain.

PROCEDURE

For the determination of the specific weight, we can follow the UNE-EN ISO 7971-2: 2019 “Cereals. Determination of the volumetric density, called weight per hectolitre”.

The procedure is the following:

- A 0.5 L test tube is weighted.
- The test tube is filled to the mark with wheat.
- The weight in grams of the sample is determined on a balance.

EQUIPMENT AND MATERIAL

- **0.5 L test tube**
- **Analytical balance (0.01 g)**
- **Spatula**

CALCULATIONS

In order to calculate the specific weight (in Kg hL-1), the following equation is employed:

$$Specific\ Weight = \frac{Wheat\ Weight\ (g)}{0.5\ (L)} x\ 0.1$$

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5.3. WEIGHT OF THOUSAND GRAINS (OR SEED INDEX)

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PRINCIPLE AND APPLICATION

The seed index, i.e., the weight of a thousand grains, is a characteristic that allows the quality of the cereal to be evaluated (Shahwani, 2014). The value of this property is conditioned by the cereal variety and fertilizer application, and it has been found to be highly correlated with the yield (Hadjichristodoulou, 1990).

PROCEDURE

To determine the seed index, the procedure is the following:

- Weight a beaker.
- With the help of tweezers, 100 grains of the sample (taken randomly) are collected in the previously weighed beaker.
- The beaker with the grains is weighted again.
- The weight of the 100 grains is determined by difference.

EQUIPMENT AND MATERIAL

- **Analytical balance (0.01 g)**
- **Tweezers**
- **Beaker**

CALCULATIONS

For the calculations, it is necessary to multiply the weight of one hundred grains by 10, and so the seed index is obtained.

REMARKS

It is crucial to select the seeds randomly in order to obtain representative results.

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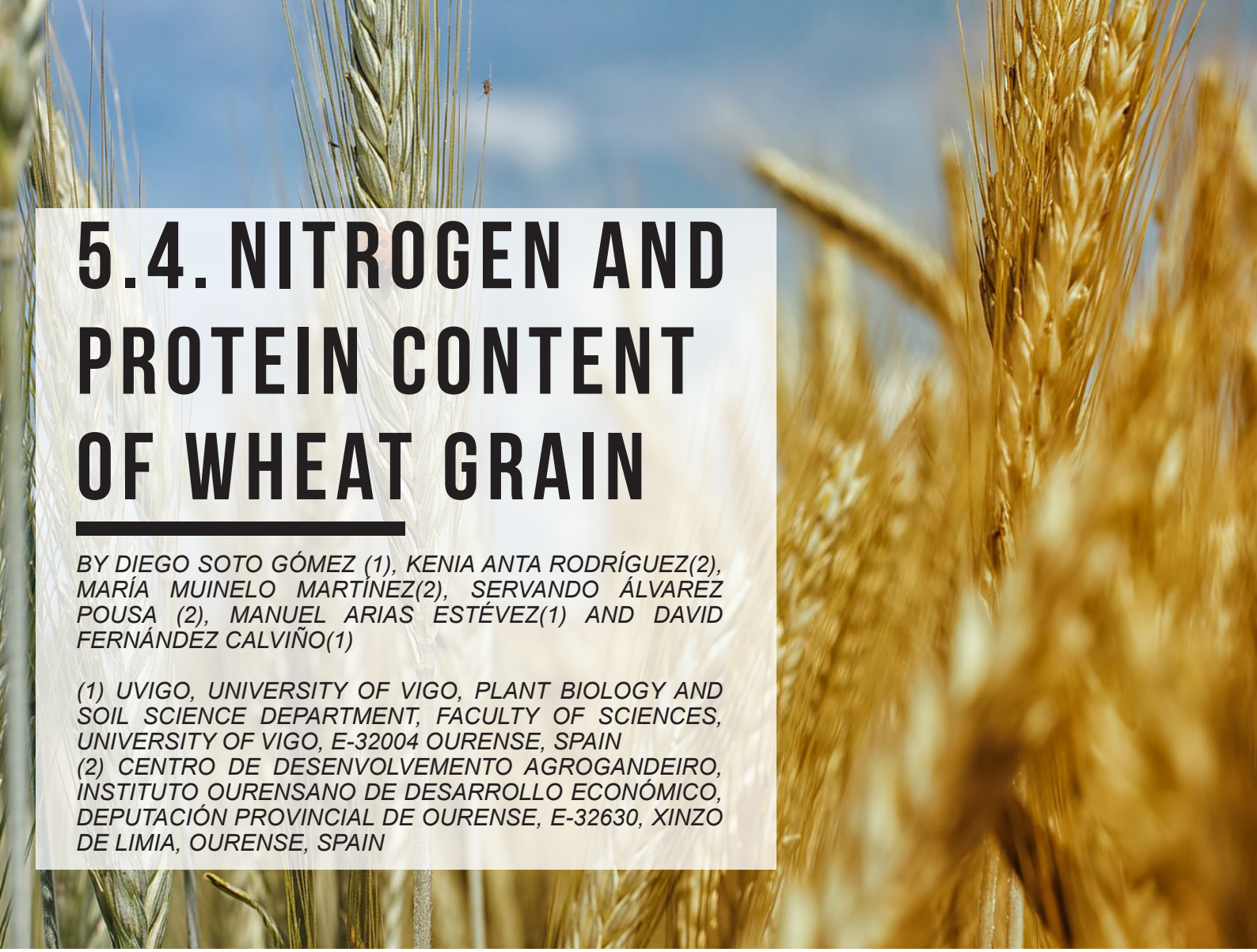

5.4. NITROGEN AND PROTEIN CONTENT OF WHEAT GRAIN

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PRINCIPLE AND APPLICATION

The protein content of a grain is a characteristic that greatly conditions its quality, the destination that will be given to that grain and its market value (Hare, 2017). For example, a grain that has a protein content lower than 11.5% (by weight) is not suitable for pasta production.

There is a very close relationship between the amount of protein in a grain and its richness in nitrogen: the availability of nitrogen causes crops to produce protein-rich grains (Jones and Olson-Rutz, 2012).

This is because nitrogen is an essential component of amino acids. It has been proven that, in addition to nitrogen fertilization, some environmental factors such as drought or high temperatures during the ripening of the grain produce cereals with higher protein content, but in these last cases, the yield is usually affected.

The amount of protein in the grain can be determined through the nitrogen content of the sample. The Kjeldahl method (ISO 20483:2013, 2013) can be used to determine the amount of nitrogen. Then, by multiplying the nitrogen content by 5.7, which is the factor of conversion for wheat (Kowalczewski et al., 2019), the amount of protein is obtained.

EQUIPMENT AND MATERIAL

- Digestion tubes
- Digester unit (Bloc-Digest)
- Fume collector/extractor
- Erlenmeyer flasks
- Distiller
- Burette
- Laboratory mill
- Analytical balance (0.01 g)

REAGENTS

- Sulphuric Acid (95-98%)
- NaOH solution (35%)
- Mixed indicator
- Kjeldahl Catalyst
- Boric Acid
- Phenolphthalein
- HCl solution (0.31 N)

PROCEDURE

The procedure to determine the amount of nitrogen in wheat begins with the digestion, which consists of the following steps:

- Weigh 1 g of sample (grinded and homogenized) and place it in a digestion tube.
- Add 5 g of Kjeldahl catalyst to the tube with the sample, and 10 mL of 95-98 % sulphuric acid.
- Place the digestion tubes with the samples in a digester unit (Bloc-digest) with the smoke collector running, and perform the digestion at a temperature of 400ºC for 30 minutes.
- Allow the sample to cool to room temperature.
- Slowly add 50 ml of distilled water. This should be done carefully, slowly dropping water through the walls of the tube.

The next part of the procedure includes neutralization and distillation:

- Add 25 mL of boric acid in a 250 mL Erlenmeyer flask and 2 or 3 drops of mixed indicator.
- Submerge the refrigerant extension in the Erlenmeyer with the boric acid solution.
- Place the tube with the sample on the left side of the distiller, and add 2 or 3 drops of phenolphthalein.
- Once the sample tube and the Erlenmeyer are placed with boric acid, add the solution of NaOH (35%) until the colour change and start the distillation. Distillation should be prolonged long enough for a minimum of 150 mL to be distilled, approximately 5 to 10 minutes.

The last step is the titration:

- The distillate obtained is titrated with 0.31N hydrochloric acid, until the solution turns from green to violet.
- The same is done with a blank.

CALCULATIONS

The % of nitrogen is calculated with the following equation:

$$\%\ Nitrogen = \frac{1.4\ x\ (V_1 - V_0)\ x\ N}{P}$$

Where:

- P is the weight of the sample (g).
- V1 is the volume of HCl used in the titration of the sample (mL).
- V0 is the volume of HCl used in the titration of the blank (mL).
- N is the normality of the HCl.

With the % of Nitrogen known, it is possible to calculate the % of protein:

$$\%Protein = \%Nitrogen\ x\ F$$

Where:

- F is 5.7, the conversion factor for wheat (Kowalczewski et al., 2019).

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