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

SoildiverAgro

Soil biodiversity enhancement in European agroecosystems to promote their stability and resilience by external inputs reduction and crop performance increase

D5.1- HANDBOOK ON WP5 SETTING UP, PROTOCOLS FOR SAMPLING, SAMPLE PROCEDURE AND ANALYSIS

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

D5.1. HANDBOOK ON WP5 SETTING UP, PROTOCOLS FOR SAMPLING, SAMPLE PROCEDURE AND ANALYSIS

Summary

The WP5 aims to determine the relationship that exists between soil biodiversity and other variables such as the crop production or emission of greenhouse gases. The first step in carrying out this work package is to establish a series of standardized procedures for experinmental set up, sampling, processing of these samples, data collection and characterization, and data analysis.

This deliverable presents, in the form of an appendix, a handbook of methods to carry out all the analyzes that will allow establishing the soil and crops characteristics of the different pedoclimatic regions. Therefore, the protocols agreed by the project members are included. The aspects considered in this handbook of methods are:

- Experinmental design
- Crop growth and quality properties. This part includes the development of the crops, the yield, the incidence of diseases, and the nutritional evaluation of cereals, legumes and vegetables.Types of sampling of the soils (oriented to determine dry bulk density, biodiversity or physicochemical characteristics).
- Earthworm sampling.
- Physical characterization of the soil including determination of dry bulk density, coarse fragments, water holding capacity, humidity, soil hydraulic conductivity, particle size distribution and aggregates.
- Chemical characterization of the soil including determination of pH, salinity, and content of organic matter, organic and inorganic carbon, total and inorganic nitrogen, available phosphorus, potassium, calcium, magnesium, boron, effective exchange capacity, cations (iron, manganese, copper, zinc and molybdenum), and pesticides.
- Biological analyzes that include biodiversity measures (of worms, nematodes and microorganisms), using techniques such as DNA extraction and purification, or fatty acid analysis.
- Soil greenhouse gas emission.

Deliverable Number	Work Package
D5.1.	WP5_Impacts of soil biodiversity on crop production and other ecosystem services
Lead Beneficiary	**Deliverable Author(s)**
UCPH	Kristian Koefoed Brandt [UCPH] Lieven Waeyenberge [EV-ILVO] Diego Soto Gómez [UVIGO] David Fernández Calviño [UVIGO] Raúl Zornoza [UPTC] Silvia Martínez Martínez [UPTC]
Versions (updates)	**Date**
V1	21.10.2020
V2	19.11.2020
V3	03.11.2020

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

Deliverable Quality Check	Date
David Fernández Calviño [UVIGO]	04.12.2020
Planned Delivery Date	**Final Delivery Date**
31.05.2020	07.12.2020

Type of deliverable	R	Document, report (excluding periodic and final reports)	X
	DEC	Websites, patents filing, press & media actions, videos	
	E	Ethycs	

Dissemination Level	PU	Public	X
	CO	Confidential, only for members of the consortium	

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

Table of content

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

1 Introduction

Taking into account that, through this WP (WP5) it is intended to determine the capacity of edaphic biodiversity to solve current economic, environmental and agronomic problems, it is essential to establish a reference framework to carry out the pertinent experimental design, sampling and analyzes. Thus, through this book "HANDBOOK ON WP5 SETTING UP, PROTOCOLS FOR SAMPLING, SAMPLE PROCEDURE AND ANALYSIS" included in Annex I, the members of the SoildiverAgro project tried to standardize the methods that will be used to design field experiments, follow crop performance, and sample/characterize the soils and crops of 15 case studies in 6 different pedoclimatic regions throughout Europe. The methods included in the handbook cover techniques for experimental design, follow crop performance (growth, yield, pest incidence, and nutritional quality of vegetables, among others), soil sampling and determination of chemical properties (pH, salinity, organic matter, available cations, etc.), physical (bulk density, texture, water holding capacity, among others) and biological (nematodes, earthworms, microorganisms, etc). Moreover, method for soil greenhouse gases emission analysis is also included.

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

2 Annex 1: Handbook

HANDBOOK ON CASE STUDIES SET UP, PROTOCOLS FOR SAMPLING, SAMPLE PROCEDURE AND ANALYSIS

EDITED BY DIEGO SOTO GÓMEZ, DAVID FERNÁNDEZ CALVIÑO, KRISTIAN KOEFOED BRANDT, LIEVEN WAEYENBERGE, RAÚL ZORNOZA AND SILVIA MARTÍNEZ MARTÍNEZ.

NOVEMBER 2020

WWW.SOILDIVERAGRO.EU

This handbook is a deliverable of WP5: Impacts of soil biodiversity on crop production and other ecosystem services of the SoildiverAgro project financed by the European Union´s Horizon2020 research and innovation programme under grant agreement Nº. 817819.

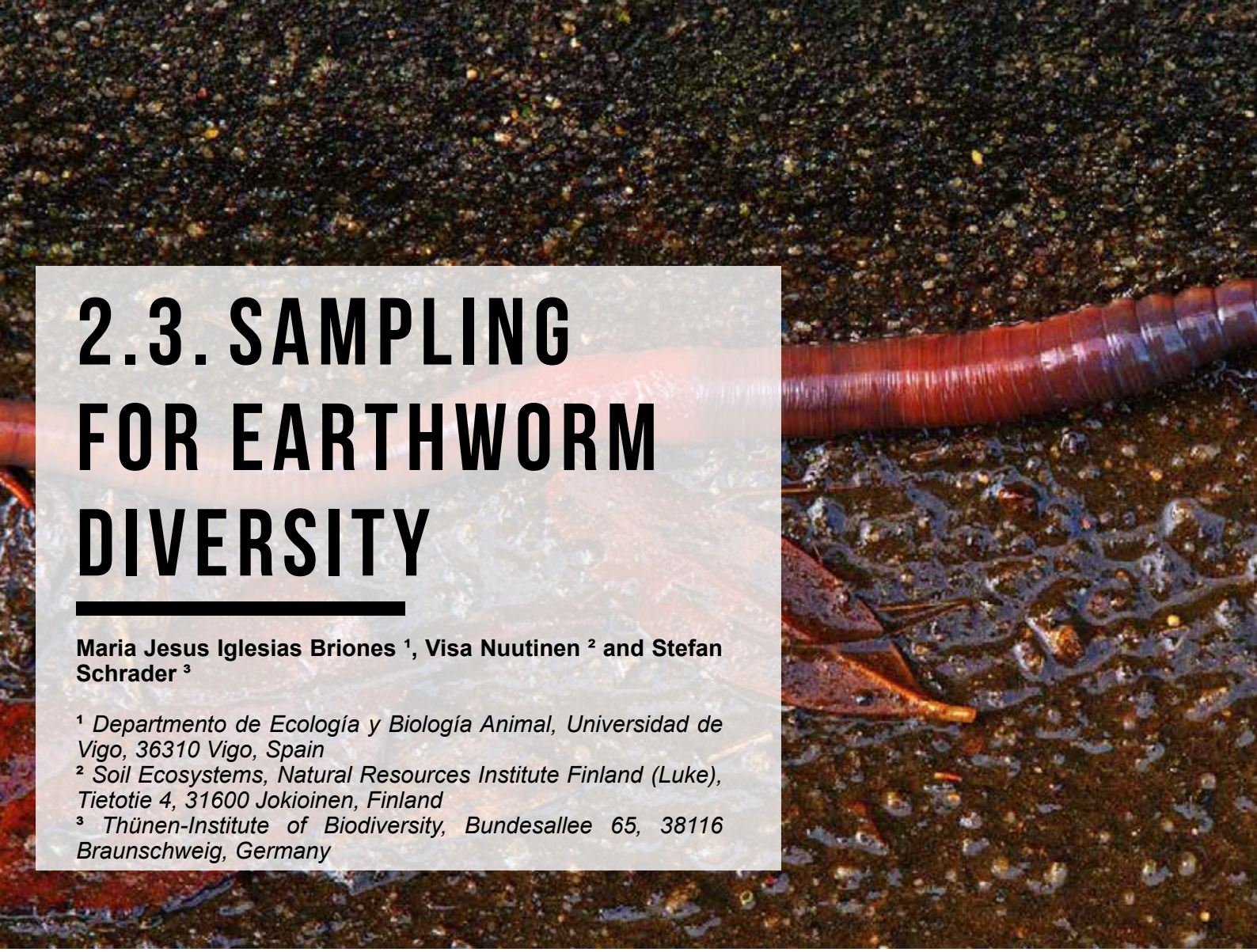
Edited by Universidade de Vigo (UVigo).

Design by Fundacion Empresa Universidad Gallega (FEUGA).

Image and icons credits: Pexels, Pixabay, Unsplash, Freepik, Flaticons.

NOVEMBER 2020
1st Edition

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



2.3. SAMPLING FOR EARTHWORM DIVERSITY

Maria Jesus Iglesias Briones [1], Visa Nuutinen [2] and Stefan Schrader [3]

[1] *Departmento de Ecología y Biología Animal, Universidad de Vigo, 36310 Vigo, Spain*
[2] *Soil Ecosystems, Natural Resources Institute Finland (Luke), Tietotie 4, 31600 Jokioinen, Finland*
[3] *Thünen-Institute of Biodiversity, Bundesallee 65, 38116 Braunschweig, Germany*

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. 2.3.1.). 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. 2.3.1. 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. 2.3.2.). 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. 2.3.2. 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. 2.3.3.).**
-

Fig.2.3.3. 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 2.3.4.) 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 5.1.

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. 2.3.4. 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 5.1.

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).

REFERENCES

- Bartlett M.D., Briones M.J.I., Neilson R., Schmidt O., Spurgeon D., and Creamer R. (2010). A critical review of current methods in earthworm ecology: From individuals to populations. European Journal of Soil Biology 46(2):67-73. doi: 10.1016/j.ejsobi.2009.11.006
- ISO 23611-1:2018 Soil quality -- Sampling of soil invertebrates -- Part 1: Hand-sorting and extraction of earthworms. International Organization of Standardization, Geneva, Switzerland.
- Pelosi C., Bertrand M., Capowiez Y., Boizard H., and Roger-Estrade J. (2009). Earthworm collection from agricultural fields: Comparisons of selected expellants in presence/absence of hand-sorting. European Journal of Soil Biology 45, 176-183. doi: 10.1016/j.ejsobi.2008.09.013
- Zaborski E.R. (2003). Allyl isothiocyanate: an alternative chemical expellant for sampling earthworms. Applied Soil Ecology 22, 87-95. doi: 10.1016/S0929-1393(02)00106-3