Universidad Politécnica de Cartagena

European Commission

DIVERFARMING

HANDBOOK OF PLANT AND SOIL ANALYSIS FOR AGRICULTURAL SYSTEMS

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

Jorge Álvaro-Fuentes, Dénes Lóczy, Sören Thiele-Bruhn, Raúl Zornoza.
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Enero 2019
I.S.B.N.: 978-84-16325-86-3

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No 728003

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PART 1. PLANT AND CROP ANALYSES

PART 1. PLANT AND CROP ANALYSES

1.1. CROP GROWTH, INCIDENCE OF PESTS AND DISEASES AND FARM YIELD EVALUATION

1.1.1. Tree height

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Importance and applications

Tree height is associated with growth form, position of the species in the vertical light gradient of the vegetation, competitive vigour, reproductive size, whole-plant fecundity, potential lifespan, and whether a species is able to establish and attain reproductive size between two disturbance events (such as e.g. fire, storm, ploughing, grazing).

Principle

The growth of trees is a key agronomical parameter; it is very important as an indicator of agronomical conditions. Tree height is defined as the vertical distance between two horizontal planes: one plane passing through the highest twig and the other through the base of the tree at mid-slope. Tree height is not synonymous with tree length (Figure 1.1).

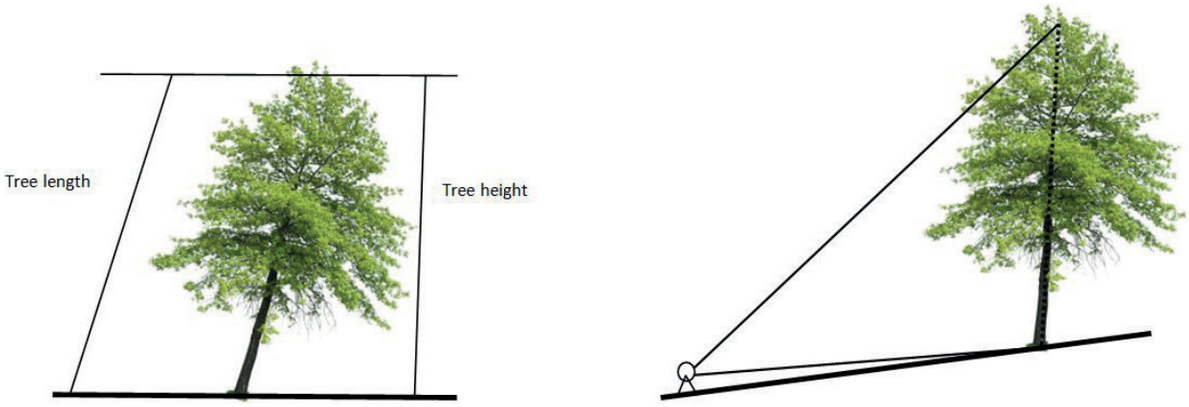

Figure 1.1. To be observed when measuring tree height on broadleaved and leaning trees.

Materials and equipment

Digital photography and/or metre.

Procedure

Tree height measurement may be carried out by means of several instruments such as: metre, dendrometric table, Blume-Leiss, Suunto, Haga, Blitterlich Relascope. Height measurement is made during several stages:

a. Tree distance (at 15, 20, 30 or 40 m). To avoid measurement errors, the distance from the tree must be at least equivalent to the tree height.
b. Observation of the tree crown.
c. Addition or subtraction of the two observation results depending on the case: addition if the

operator is standing uphill, subtraction if the operator is standing downhill in relation to the tree.

d. Slope correction (if needed). Hint: for leaning trees, it is advisable to take height measurements perpendicular to the direction of leaning (see fig. 1.2). When a tree stands on a slope, it is advisable to take height measurements from the same contour line as the tree base or from above. Starting the measurement from the mark at diameter at breast height (DBH) and adding 1.3 m to the result eliminates errors originating from different perceptions of ground.

e. The tree height will be determined in three trees per repetition.

Calculations

The tree height can be calculated (12 m for a, b, and c, and 11.7 m for d):

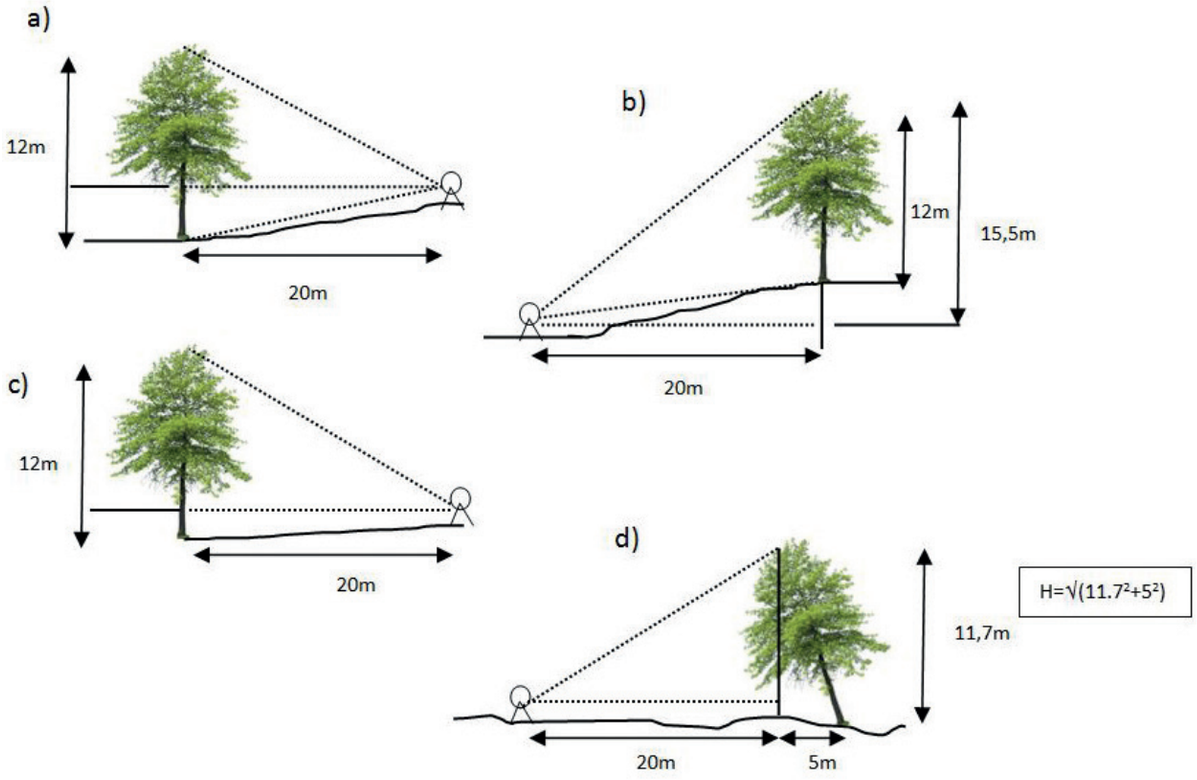

Figure 1.2. Different situations to measure the height of a tree.

- By adding the readings of the tree top and the tree base, if they are on both sides of the horizontal line: cases a) and c) in Figure 1.2.
- By subtracting the reading of the tree base from the reading of the tree top, if they are both above the horizontal line: case b) in Figure 1.2.
- For an inclined tree (case d in Figure 1.2), once the height (h) has been calculated between the tree top and the ground, just below the vertical projection of the tree top, then measure the distance (D) from the tree base to the point located just at the vertical of the tree top, and

calculate the tree height (H) by applying the formula:
H= ¬√(h2+D2).

References

FAO., 2009. Monitoring and Evaluation of National Forest Resources - Handbook for the integrated field data collection. Version 2.2. Monitoring and Evaluation Working Paper National Forest Resources Assessment, NFMA 37/S. Rome.

FAO., 2012. National Forest Monitoring and Assessment – Manual for integrated field data collection. Version 3.0. National Forest Monitoring and Assessment Working Paper NFMA 37/E. Rome.

1.1.2. Trunk cross-sectional area

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Importance and applications

This method is used to describe the tree's size, calculate its fertiliser requirements and determine the tree's potential value as a source of wood.

Principle

This parameter can be linked to external as well as internal factors serving as a proxy parameter for the reaction of trees and stands to changes in site and environmental conditions. Tree diameter is measured over bark, at 1.3 m breast height above the ground (DBH - diameter at breast height) except in the cases mentioned below. Measurement may be carried out with the help of a diameter tape (tape whose diameter unit is in centimetres), or with the use of a calliper. To avoid overestimation of the volume and to compensate measurement errors, diameter is measured in cm, and adjusted in a decreasing sense (e.g.: 16.8 cm become 16 cm).

Materials and equipment

- Tape measure or calliper.

Procedure

a. Measure 1.3 m (Figure 2.1) up the trunk of the tree to locate the point at which to measure the tree's diameter. If the tree trunk splits below this height, the two separate trunks must be most purposes, and must be measured independently. If a branch occurs at this height, take either 30 cm below the branch or above the branch where the swelling around the branch junction no longer exists.

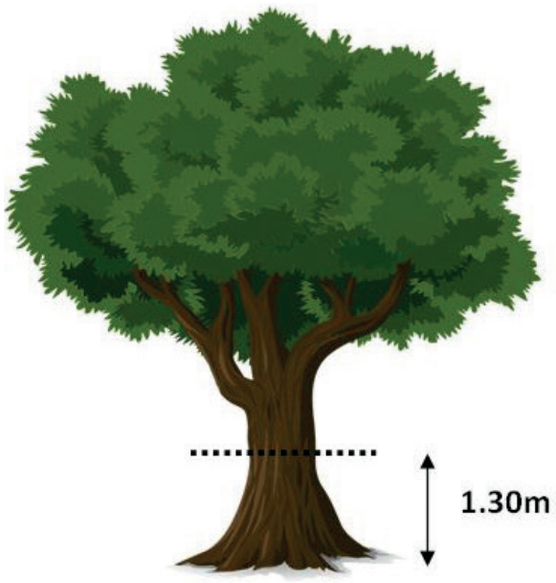
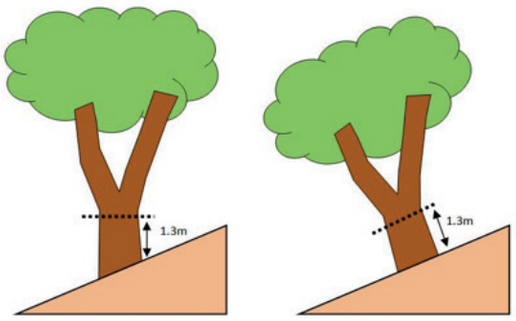
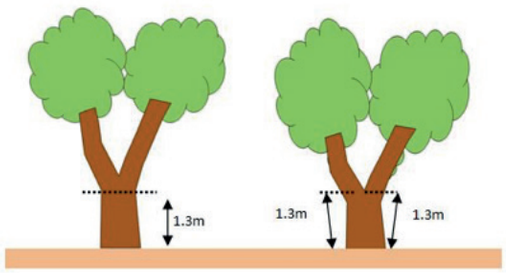
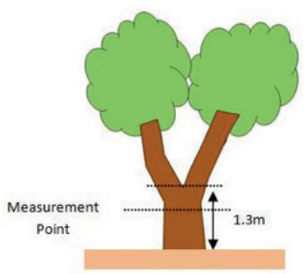


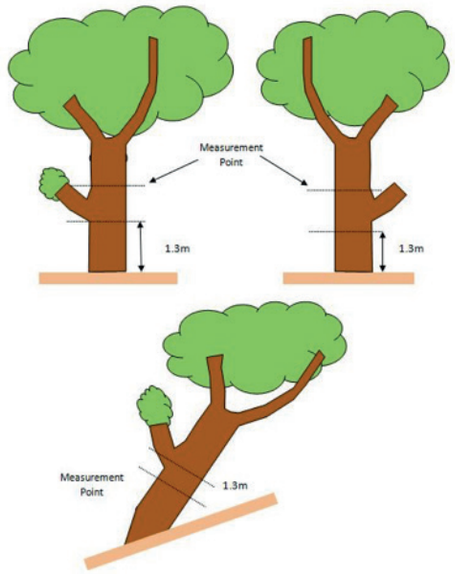
Figure 2.1. Position for diameter measurement at breast height in flat terrain

b. Wrap the cloth measuring tape around the tree trunk. Avoid accidentally wrapping it at an angle or catching it on any twigs. A "hugging" method with both arms reaching around the tree and feeling for any obstacles often proves most efficient and provides the most accurate, level measurement.

c. Read the number, in centimetres, where the measuring tape reaches the starting point and end of the tape. This is the tree's circumference at breast height.

Position for diameter measurements. Particular cases:

Case	Description of diameter measurement	Figure
On inclined terrain	DBH tree measurement at 1.3 m is taken from an uphill position.	
Fork tree	Several cases exist, depending on the point where the fork divides the stem. • If the fork begins (the point where the core is divided) below 1.30 m height, each stem having the diameter required (20 cm in the whole plot, 10 cm for rectangular subplots) will be considered as a tree and will be measured. Diameter measurement of each stem will be taken at 1.3 m height. • If the fork begins above 1.3m height, the tree will be counted as a single tree and diameter measurement is carried out at 1.3m. • If a fork occurs at or immediately above 1.3 m, the tree will be counted as a single tree and diameter is measured below the fork just beneath any swelling that could inflate the DBH.	 

Tree with irregular stem at 1.3m	Trees with bulges, wounds, hollows and branches, etc. at breast height, are to be measured just above the irregular point, where the irregular shape does not affect the stem	

The vegetative growth will be evaluated at the end of the growing season in 3 trees per repetition (12 trees per treatment), from the trunk diameter and the weight of the wood from pruning. The trunk cross-section area (TCSA) will be obtained from the measurements of the diameter of the trunk and branches. Pruning work will be carried out in the moments decided upon by the grower.

Calculations

$$TCSA\ (cm^2) = \pi \cdot R^2$$

Where R:

$$R = \frac{(Perimeter\ (cm)}{2 \cdot \pi}$$

References

FAO., 2009. Monitoring and Evaluation of National Forest Resources - Handbook for the integrated field data collection. Version 2.2. Monitoring and Evaluation Working Paper National Forest Resources Assessment, NFMA 37/S. Rome.

FAO., 2012. National Forest Monitoring and Assessment – Manual for integrated field data collection. Version 3.0. National Forest Monitoring and Assessment Working Paper NFMA 3

1.1.3. Trunk diameter fluctuations

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Importance and applications

The measurement of trunk diameter fluctuations (TDF) has attracted great interest, both for evaluating the water behaviour of the plant as well as for managing irrigation water more accurately (Katerji et al., 1990; Simonneau et al., 1993; Tardieu & Simonneau, 1998; Fereres & Goldhamer, 2003; Goldhamer & Fereres, 2004; Intrigliolo & Castel, 2004; 2006b; Ortuño et al., 2004; Moreno et al., 2006; Garcia-Orellana et al., 2007).

Principle

Seasonal variations in the trunk diameter depend principally on growth processes (Kozlowsky & Winget, 1964). However, cycles of contraction and expansion of the trunk also occur at daily scale (Kozlowski, 1967), partly due to the thermal effect (McCracken & Kozlowski, 1965) but mainly caused by changes in the moisture content of the plant tissues (Simonneau et al., 1993). According to Irvine and Grace (1997), more than 90% of the daily fluctuations in trunk diameter take place in tissues of the phloem.

During the day, due to the continual transpiration of the plant leaves, there is a horizontal diffusion of water in the tissues of the bark towards the xylem (Parlange et al., 1975), generating a progressive reduction in the diameter. During the evening the absorption of water by the plant exceeds the losses by transpiration, so there is a recovery in the xylem water potential and a gradual increase in the diameter. Therefore, short-term trunk diameter variations reflect changes in the xylem water potential (Klepper et al., 1971). The magnitude of the daily trunk contraction also provides valuable information on the intensity of the stress.

Several indicators of plant water stress are obtained from the TDF. The maximum daily trunk contraction (MCD) is calculated as the difference from the maximum trunk diameter (MXDT) which occurs at the first hour of the morning minus the minimum trunk diameter (MNDT) which generally occurs after the maximum transpiration (generally in the evening). This has been the most commonly used indicator (Fereres & Goldhamer, 2003; Goldhamer & Fereres, 2004; Nortes et al., 2005, in almond trees; Ortuño et al., 2004; 2006; García-Orellana et al., 2007, in lemon trees; Ferreira et al., 1996; Remorini & Massai, 2003; Conejero et al., 2007, in peach trees).

The difference between both values, maximum amplitude, is denominated maximum daily contraction of the trunk diameter (MCD) and represents the radial diffusion of water in the tissues of the bark towards the xylem (Parlange et al., 1975), which generates a progressive reduction in the diameter. The magnitude of MDC depends on several factors such as: i) the modulus of elasticity and the water diffusion properties of the phloem tissues (Parlange et al., 1975; Gènard et al., 2001), ii) thickness of the phloem and tree

size (Naor & Cohen, 2003; Intrigliolo & Castel, 2005) and iii) productive load (Moriana & Fereres, 2004; Intrigliolo & Castel, 2007), probably as a consequence of water losses by transpiration by the fruits (Berger & Selles, 1993; Link et al., 1998).

Another parameter derived from the daily variations in trunk diameter is the trunk diameter daily growth rate (TCD), (Goldhamer & Fereres, 2001), which is given by the difference between two maximum trunk diameters of two consecutive days. As observed by Nortes et al., (2005) and Moriana and Fereres (2002) in young almond and olive trees, respectively, this indicator seems to offer greater sensitivity to detect water stress in young trees. In adult trees, the TCD seems to be less sensitive to detecting the water stress than the rest of the indicators evaluated (Intrigliolo & Castel, 2006). According to these authors, the absence of sensitivity of the TCD in adult trees is due to a lower trunk growth rate than in young trees, independently of the plant water status.

De la Rosa et al., 2016, proposed a new indicator obtained from the trunk fluctuations that showed, together with the stem potential, to be more sensitive than the other traditional indicators of FTD in nectarine. This indicator, denominated EDS (early trunk contraction) was measured between 09:00 and 12:00 solar hour.

The following figure schematically represents the parameters derived from trunk contractions:

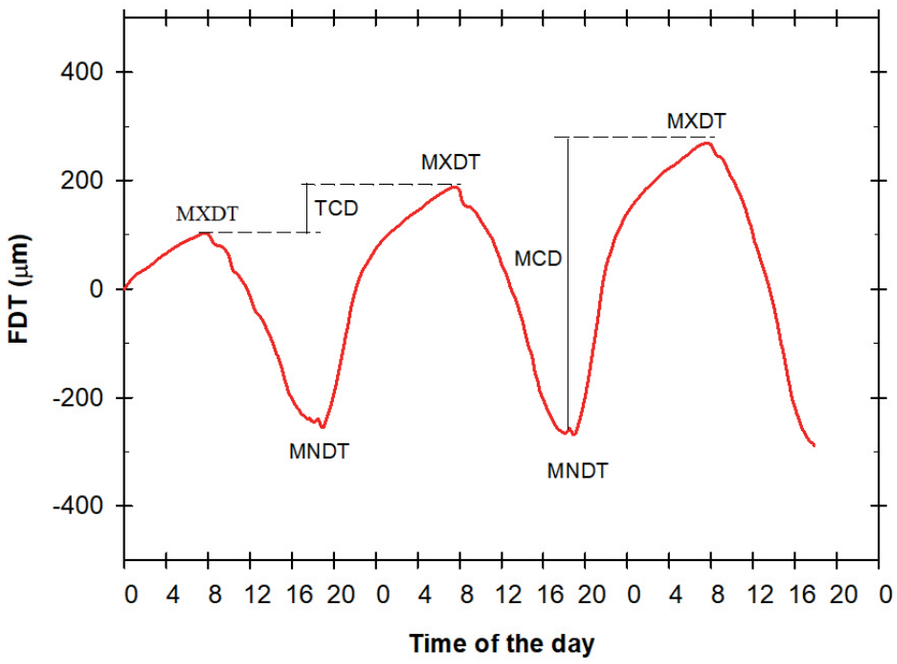

Figure 3.1. Parameters derived from trunk diameter fluctuations (FDT): maximum daily contraction (MCD), trunk growth rate (TCD), maximum (MXDT) and minimum (MNDT) daily trunk diameter.

These measurements, using LVDT-type transformers (linear variable differential transformer), can be easily automated and used for irrigation scheduling (González-Altozano, 1998).

The automation capacity of the indicators derived from the FDT is one of the factors that convert them into

indicators to consider for use as a tool in irrigation scheduling. However, the high cost of the sensors, sensor holders, dataloggers, communication system… together with the complexity in processing the information explains why they are still not used in commercial plots.

Materials and equipment

- Linear variable differential transformer (LVDT; Solartron Metrology, Bognor Regis, UK, model DF ±2.5 mm, accurate to ± 10 µm.
- Datalogger (CR1000, Campbell Scientific Inc., Logan, USA).
- Invar sensor holders (iron and nickel alloy with a minimal thermal dilation coefficient -1.7·10-6 ºC-1).
- 4-wire cables.

Procedure

These sensors consist of a magnetic core that is moved driven by a rod in contact with the plant organ to be measured. Said core runs through the interior of a cylinder, between a primary coil and a secondary coil. A carrier signal (alternating current) is applied to the primary coil which produces a magnetic field around the core, and this magnetic field induces an alternating voltage in the secondary coil. As in any transformer, the voltage of the signal induced in the secondary coil is linearly related with the number of turns exposed.

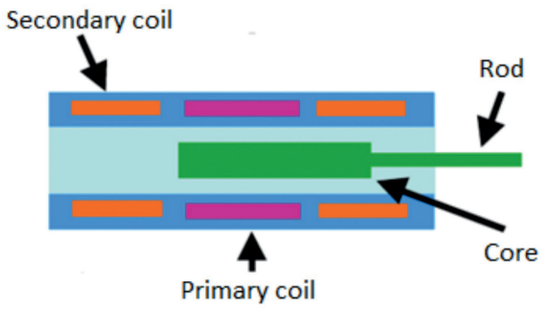

Figure 3.2. Cross-section of an LVDT sensor (www.researchgate.net)

In this way, depending on the displacement of said rod in this core a certain voltage or another is generated. This voltage, with the corresponding equation, is translated into a displacement of the trunk. The readings are taken every 30 seconds and the mean is stored each 10 minutes in a datalogger (CR1000), obtaining MXDT, MNDT and, thus, MCD.

References

Berger, A. y Selles, G., 1993. Diurnal fruit shrinkage: a model. En: Borghetti, M., Grace, J. y Raschi, A. (eds). Water transport in plants under climatic stress. Cambridge University Press. pp. 261–269.

Conejero, W., Alarcón, J.J., García-Orellana, Y., Abrisqueta, J.M., Torrecillas, A., 2007. Daily sap flow and maximum daily trunk shrinkage measurements for diagnosing water stress in early maturing peach trees during the post-harvest period. Tree Physiology 27, 81-88.

De la Rosa, J.M., Conesa, M.R., Domingo, R., Aguayo, E., Falagán, N., Pérez-Pastor A., 2016. Combined effects of deficit irrigation and crop level on early nectarine trees. Agricultural Water Management 170, 120-132

Fereres, E. y Goldhamer, D.A., 2003. Suitability of stem diameter variations and water potential as indicators for irrigation scheduling of almond trees. Journal of Horticultural Science & Biotechnology 78, 139- 144.

Ferreira, M.J., Valancogne, C., Daudet, F., Ameglio, T., Pacheco, C.A., Michaelsen, J., 1996. Evapotranspiration and crop-water relations in a peach orchard. En: Camp, C.R., Sadler, E.J., Yoder, R.E. (eds). Proc. International Conference on evapotranspiration and irrigation scheduling. November 3-6. San Antonio, Texas, 61-68.

Garcia-Orellana, Y., Ruiz-Sánchez, M.C., Alarcón, J., Conejero, W., Ortuño, M.F., Nicolás, E., Torrecillas, A., 2007. Preliminary assessment of the feasibility of using maximum daily trunk shrinkage for irrigation scheduling in lemon trees. Agricultural Water Management 89, 167-171.

Génard, M., Fishman, S., Vercambre, G., Huguet, J.G., Bussi, C., Besset, J., Habib, R., 2001. A biophysical analysis of stem and root diameter variations in woody plants. Plant Physiology, 126, 188–202.

Goldhamer, D.A., Fereres, E., 2001. Irrigation scheduling protocols using continuously recorded trunk diameter measurements. Irrigation Science, 20, 115-125.

Goldhamer, D.A., Fereres, E., 2004. Irrigation scheduling of almond trees with trunk diameter sensors. Irrigation Science, 23, 11-19.

González-Altozano, P., 1998. Riego deficitario controlado en Clementina de Nules: relaciones hídricas y respuesta agronómica. Tesis doctoral. Universidad Politécnica de Valencia. 173 pp.

Intrigliolo, D.S., Castel, J.R., 2004. Continuous measurement of plant and soil water status for irrigation scheduling in plum. Irrigation Science 23, 93-102.

Intrigliolo, D.S., Castel, J.R., 2005. Effects of regulated deficit irrigation on growth and yield of young Japanese plum trees. Journal of Horticultural Science & Biotechnology 80, 177–182.

Intrigliolo, D.S., Castel, J.R., 2006a. Performance of various water stress indicators for prediction of fruit size response to deficit irrigation in plum. Agricultural Water Management 83, 173-180.

Intrigliolo, D.S., Castel, J.R., 2006b. Usefulness of diurnal trunk shrinkage as a water stress indicator in plum trees. Tree Physiol 26, 303-311.

Intrigliolo, D.S., Castel, J.R., 2007. Crop load affects maximum daily trunk shrinkage of plum trees. Tree Physiology 27, 89–96.

Irvine, J., Grace, J., 1997. Continuous measurement of water tensions in the xylem of tres based on the elastic properties of wood. Planta 202, 455-461.

Katerji, N., Schoch, P.G., Rimgoto, P., Lhotel, J.C., 1990. Diagnosis via stem microvariations of water-stress periods in greenhouse-grown eggplants. Agronomie 10, 541-549.

Klepper, B., Browing, U.D., Taylor, H.M., 1971. Stem diameter in relation to plant water status. Plant Physiology, 48, 683-685.

Kozlowski, T.T., 1967. Diurnal variations in stem diameters of small trees. Botanical Gazette, 128 (1): 60–65.

Kozlowski, T.T., Winget, C.H., 1964. Diurnal and seasonal variation in radii of tree stems. Ecology 45, 149–155.

Link, S.O., Thied, M.E., Van Bavel, M.G., 1998. An improved strain-gauge device for continuous field measurement of stem and fruit diameter. Journal of Experimental Botany 49, 1583–1587.

McCracken, I., Kozlowski, T.T., 1965. Thermal contraction in twigs. Nature 208, 910.
Moreno, F., Conejero, W., Martin-Palomo, M.J., Giron, I.F., Torrecillas, A., 2006. Maximum daily trunk shrinkage reference values for irrigation scheduling in olive trees. Agricultural Water Management 84, 290-294.
Moriana, A., Fereres, E., 2002. Plant indicators for scheduling irrigation of young olive trees. Irrigation Science, 21, 83-90.
Moriana, A., Fereres, E., 2004. Establishing reference values of trunk diameter fluctuations and stem water potential for irrigation scheduling of olive trees. Acta Horticulturae 664, 407–412.
Naor, A., Cohen, S., 2003. Sensitivity and Variability of Maximum Trunk Shrinkage, Midday Stem Water Potential, and Transpiration Rate in Response to Withholding Irrigation from Field-grown Apple Trees. HortScience 38, 547–551.
Nortes, P.A., Pérez-Pastor, A., Egea, G., Conejero, W., Domingo, R., 2005. Comparison of changes in stem diameter and water potential values for detecting water stress in young almond trees. Agricultural Water Management 77, 296-307.
Ortuño, M.F., Alarcón, J.J., Nicolás, E., Torrecillas, A., 2004. Interpreting trunk diameter changes in young lemon trees under deficit irrigation. Plant Science 167, 275-280.
Ortuño, M.F., García-Orellana, Y., Conejero, W., Ruiz-Sánchez, M.C., Alarcón, J.J., Torrecillas, A., 2006. Stem and leaf water potentials, gas exchange, sap flow, and trunk diameter fluctuations for detecting water stress in lemon trees. Trees-Structure and Function 20, 1-8.
Parlange, J.Y., Turner, N.C., Waggoner, P.E., 1975. Water uptake, diameter change and non-linear diffusion in tree stems. Plant Physiology 55, 247–250.
Remorini, D., Massai, R., 2003. Comparison of water status indicators for young peach trees. Irrigation Science 22, 39-46.
Simonneau, T., Habib, R., Goutouly, J., Huguet, J., 1993. Diurnal changes in stem diameter depend upon variations in water content: direct evidence in peach trees. Journal of Experimental Botany 44, 615–621.
Tardieu, F., Simonneau, T., 1998. Variability among species of stomatal control under fluctuating soil water status and evaporative demand: modelling isohydric and anisohydric behaviours. Journal of Experimental Botany 49, 419-432.

1.1.4. Leaf area index

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Importance and applications

The leaf area index is defined as the ratio between the total leaf area of a tree and the area of soil occupied by the same tree. It is a parameter that allows measuring the productive efficiency of the soil occupied by the crop.

Principle

Leaf area index (LAI) measurements are a fundamental part of research in plant physiology, agriculture and dendrology (Broadhead et al., 2003). The leaf area is associated with most agronomic, biological, environmental and physiological processes, including growth analysis, photosynthesis, transpiration, light interception, biomass allocation and energy balance (Kucharik et al., 1998).

Plant physiologists, biologists and agronomists demonstrated the importance of leaf area in the estimation of plant growth, in the determination of phenological stages, in the estimation of biological and agronomic yield potential, in the calculation of the efficient use of solar radiation, as well as in the calculation of the efficient use of water and mineral nutrition (Sonnentag et al., 2008).

This method is a useful tool for developing predictive harvest models and an accurate way to estimate the light-capture capacity of the canopy; the distribution of the leaves can affect the efficiency of light use. The leaf area index also serves to evaluate the development and growth of crops as well as bioenergy efficiency or to determine the damage caused by pests and diseases on the foliage. The estimation of the yield in different crops can be based on the leaf area index determined at some phenological stage and previously correlated by some determination method.

The LAI of vegetation depends on species composition, development stage, and seasonality. Furthermore, the LAI is strongly dependent on the prevailing site conditions and the management practices. LAI can be assessed directly using harvesting methods such as destructive sampling. As the leaf area is determined through repeated area measurements on single leaves and area accumulation, these methods are hence considered the most accurate (Chen et al., 1992).

After collection of ten leaves, leaf area can be calculated by means of either planimetric or gravimetric techniques (Daughtry, 1990). The planimetric approach is based on the principle of the correlation between the individual leaf area and the number of area units covered by that leaf in a horizontal plane. To do so, a leaf can be horizontally fixed to a flat surface, its perimeter can be measured with a planimeter, and its area can be computed from this perimeter assessment.

Procedure

- Select different branches from the middle of five trees. In general, sampling must be carried out in such way that all the orientations are represented in the set of sample trees. Four measurements are taken for each tree (one per quadrant), which will be repeated a total of three times each.
- The recommended minimum quantities are 10-20 g of fresh leaves (resulting in 5-10 g of dry material) for each sample.
- Calculate the area of the leaves.
- Dry the leaves in an oven at 80ºC to determinate the dry weight of samples of known area, until constant weight is achieved.
- Calculate the specific area of the leaves.
- Take all the leaves present on a planimeter or known area.
- Calculate the leaf area index.

Calculations

The specific leaf area of a tree species (SLA) is its leaf area (Ad) divided by the corresponding dry mass (Wd):

$$SLA = \frac{Ad}{Wd}$$

Therefore, the leaf area index is the specific leaf area multiplied by the dry mass of a known surface area.

$$LAI = SLA * Wd$$

SLA: Specific leaf area
Ad: leaf area
Wd: dry weight of leaves
LAI: leaf area index

References

Fleck, S., Raspe, S., Cater, M., Schleppi, P., Ukonmaanaho, L., Greve, M., Hertel, C., Weis, W., Rumpf S.. 2013. Leaf area measurements. Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests. Part XVII. UNECE ICP Forests Programme Co-ordinating Centre, Hamburg, DE. 37 pp.

Kucharik, C.J., Norman, J.M. and Gower, S.T., 1998. Measurements of branch area and adjusting leaf area index to indirect measurements. Agricultural and Forest Meteorology 91, 69-88.

Chen, J.M., Black, T.A., 1992. Defining leaf-area index for non-flat leaves. Plant Cell. Environ. 15, 421–429.

1.1.5. NDVI

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Importance and applications

Different spectral indices have been proposed in recent decades, which, depending on the characteristic that one seeks to evaluate, has generated a large number of expressions applied to different studies in the vegetation. Among the most used is the Normalised Difference Vegetation Index (NDVI), applied for the first time by Rouse (1973), developed to highlight the spectral signatures of vegetation between Red and NIR (Jensen, 2000). Its application is extensive, with numerous prior studies that show the viability in the estimation of a large number of properties of the vegetation. Typical examples include the estimation of the leaf area index, the biomass, the concentration of chlorophyll in leaves, the productivity of the plants, the fractioned vegetation cover, the accumulated precipitation, etc. (Ye et al., 2012).

Principle

The vegetation indices or green indices are transformations that imply carrying out a mathematical combination between digital levels stored in two or more spectral bands of the same image. (Esperanza & Zerda, 2002).

The development of these indices responds to the observation of the consistency in the response of the reflectance to red and infrared light of green vegetation: the higher the amount of chlorophyll, the greater the absorption of red incident light; the greater the leaf volume, the greater the reflectance in near infrared, and others with high absorption in red, the use of only one band may lead to errors. However, only live vegetation invariably produces both responses since, if the quotient of the infrared reflectance with the red is calculated, or its difference, as the former always increases as the second decreases, the quotient (or difference) will be greater the more vegetation there is, by the additive effect that a greater abundance of vegetation produces (Towers, 2002)

The NDVI (Rouse et al., 1974) is the most used vegetation index for all manner of applications. The fundamental reason for this is that it is easy to calculate, and has a fixed range of variation (between –1 and +1), which enables thresholds to be established and to compare images, etc. This index gives rise to isolines of vegetation of increasing slope and convergent in the origin (Sánchez et al., 2000).

With respect to more complex vegetation indices, the NDVI has the advantages of being simple to calculate and to facilitate the direct interpretation of the biophysical parameters of the vegetation. Additionally, it enables the comparison among data obtained by different researchers. On the other hand, it has the drawback of having little capacity to minimise the influence of the soil and the atmosphere. The NDVI

allows the presence of green vegetation on the surface to be identified and its special distribution to be characterised, as well as the evolution of its status over time. This is determined fundamentally by the climate conditions. The interpretation of the index must likewise consider the phenological cycles and the annual development, to distinguish natural oscillations in the vegetation with changes in the time and spatial distribution caused by other factors. Therefore, the interpretation of the NDVI values obtained can be summarised as follows:

- The water has greater reflectance in infrared than in red, therefore, negative values of NDVI.
- Uncovered land and with scraggly vegetation presents positive values, although not very high.
- Dense, healthy and well-developed vegetation presents the greatest values of NDVI.
- Clouds present values similar in the R and IRC, so their NDVI is close to 010–30 150 mL beakers.

Materials and equipment

A multispectral camera (Sentera Quad) installed on a DJI Phantom 3 Advanced drone will be employed for the determination of the NDVI and with which images will be taken in the different spectral bands and subsequently the NDVI will be determined by means of the following equation:

$$NDVI = \frac{(IR-R)}{(IR+R)}$$

IR = pixel values of the infrared band
R = pixel values of the red band

Procedure

The procedure to determine the NDVI starts with the taking of aerial images of the zone of interest with the multispectral camera installed on a drone. Once the images have been obtained, they are processed with an appropriate software (PIX4D) to obtain the values of NDVI corresponding to the study plot. A flight will be carried out every 15 days coinciding with measurements of the rest of the variables of the plant water status.

References

Esperanza, F., Zerda, H., 2002. Potencialidad de los índices de vegetación para la discriminación de coberturas forestales. Universidad Nacional de Santiago del Estero, Facultad de Ciencias Forestales, Santiago del Estero.

Jensen, J., 2000. Remote Sensing of the Environment: An Earth Re- Source Perspective. UK: Prentice Hall in Geographic Info. Science, 2nd ed. Chapter 10-13.

Rouse, J.W., Haas, R.W., Schell, J.A., Deering, D.H., Harían, J.C., 1974. Monitoring the vernal advancement and retrogradation (Greenwave effect) of natural vegetation. Greenbelt, MD. USA, NASA/GSFCT.

Rouse, J. 1973. Monitoring vegetation systems in the great plains with ERTS. Third ERTS Symposium.

NASA SP-351.
Sánchez, E., Torres, M., Palacios, A., Aguilar, M., Pino, S., Granado, L., 2000. Comparación del NDVI con el PVI y el SAVI como Indicadores para la Asignación de Modelos de Combustible para la Estimación del Riesgo de Incendios en Andalucía. Tecnologías Geográficas para el Desarrollo Sostenible Departamento de Geografía. Universidad de Alcalá.
Towers, P., 2002. Conceptos Iniciales sobre Teledetección y su Aplicación al Agro. Buenos Aires, 138p.
Ye, X., Sakai, K. 2012. Fruit yield estimation through multispectral imaging. Advance in citrus nutrition 2012, 453–473.

1.1.6. Stem water potential

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Importance and applications

The leaf water potential (Ψ) is, perhaps, the most frequently used parameter to define the water status of plants (Goode & Higgs, 1973; Klepper, 1968) and to determine the moment to irrigate (Peretz et al., 1984). It is commonly used as an indicator of the water status of fruit trees, and is affected by other factors, both of an environmental origin as well as of endogenous character (Jones, 1990), which causes variations in its levels based on the moment of the day (Elfving et al., 1972) and throughout the growing season (Winkel & Rambal, 1993), on the leaf age (Knipling, 1967) and the orientation and position they occupy on the tree (Sánchez-Blanco, 1989). The measurement of Ψ is universally accepted as being the fastest, most reliable and most economical means of assessing the water status of plants (Ruiz-Sánchez & Girona, 1995).

Principle

The instrument used to measure the Ψ in the field is the pressure chamber (Scholander et al., 1965). Hsiao (1990) states that the designation of the Ψ measured with the pressure chamber as Ψ of the xylem is only valid in those cases where the water balance existed before scission; in this case the Ψ of the leaf is equal to the Ψ of the xylem and the pressure measured in the chamber (P) represents the pressure of the sap in the xylem prior to the cut. As the osmotic potential (Ψo) of the sap is very small it is usually rejected so that:

$$- P = \Psi xylem\ (\Psi t\ ) = \Psi leaf\ (without\ transpiration)$$

Fundamentally, the leaf water potential at midday (Ψmd) and the leaf water potential before dawn (Ψa) have been used as indicators of the water status of the plant. The values of Ψmd vary greatly since they depend on the climate conditions, whilst Ψa is more stable, but with the limitation that it is not indicative of the state the plant is in at the moment of greatest demand.

Shackel et al. (1997) propose the use of the stem xylem potential, Ψt (leaves bagged for at least 2 hours, without transpiration). Its advantages include its lower variability, better correlation with the vapour pressure deficit (VPD and that it better reflects the lack of water in the soil than the leaf water potential (Ψ).

The threshold value Ψt, corresponding to an adequate water supply, is around -0.8 MPa for fruit species during the sensitive physiological processes and of -1.0 MPa for the most tolerant species (Villalobos et al., 2002). More concretely and for well-irrigated trees, Ψt reaches midday values of between -0.4 and -1.0 MPa in nectarine trees (Pérez Pastor et al., 2016) and -1.0 and -1.5 MPa in apricot trees (Pérez-Sarmiento et al., 2010).

Materials and equipment

The equipment used in the field to measure Ψ is the pressure chamber (Scholander et al., 1965).

Procedure

The stem potential (Ψt) will be determined in healthy, adult, shaded leaves, close to the principal branches, with a pressure chamber (Soil Moisture Equip. Corp., model 3000). The leaves will previously be wrapped with a polyethylene film and covered with aluminium foil at least two hours prior to the measurement (Illustration 1). The measurements will be taken around 10 solar hours (when the stomata of the leaves are open). The stem water potential (Ψt) will be measured every 7-10 days at the solar midday with a pressure chamber in two leaves per repetition (8 per treatment), close to the trunk and in the shaded part.

Figure 6.1. Detail of leaf covered with aluminium foil

Calculations

The result is directly expressed in MPa in the vacuum gauge of the pressure chamber.

References

Elfving, D.C., Kauffmann, M.R., Hall, A.E., 1972. Interpreting leaf water potential measurements with a model of the soil-plant-atmosphere continuum. Physiologia Plantarum 27, 161–168.

Goode, J.E., Higgs, K.H., 1973. Water, osmotic and pressure potential relationships in apple leaves. Journal of Horticultural Science 48, 203–215

Hsiao, TC., 1990. Measurements of plant water status. In Irrigation of Agricultural Crops. Eds. B A Steward y D R Nielsen. American Society of Agronomy, Madison, USA. pp. 243–279.

Jones, H.G., 1990. Physiological aspects of the control of water status in horticultural crops. HortScience 25, 19–26.

Klepper, B., 1968. Diurnal pattern of water potential in woody plants. Plant Physiology 43, 1931–1934

Knipling, E.B., 1967. Measurement of leaf water potential by the dye method. Ecology 48, 1038–1041.

Peretz, J., Evans, R.G., Proebsting, E.L. 1984. Leaf water potentials for management of high frequency irrigation on apples. Trans. ASAE 437-442.

Pérez-Pastor, A., Ruiz-Sánchez, M.C., Conesa, M.R., 2016. Drought stress effect on woody tree yield.

Water Stress and Crop Plants: A Sustainable Approach, Volume 2, First Edition. Edited by Parvaiz Ahmad. John Wiley & Sons, Ltd. pp. 356-374.

Perez-Sarmiento, F., Alcobendas, R., Mounzer, O., Alarcón, J.J., Nicolás, E., 2010. Effects of regulated deficit irrigation on physiology and fruit quality in apricot trees. Spanish Journal of Agricultural Research 8, 86–94.

Ruiz-Sánchez, M.C., Girona, J., 1995. Investigaciones sobre riego deficitario controlado en melocotonero. En: Riego deficitario controlado, Fundamentos y aplicaciones. Eds Mundi-Prensa, Madrid, España, pp. 67–95.

Scholander, P.F., Hammel, H.T., Bradstreet, E.D., Hemingsen, E.A., 1965. Sap pressure in vascular plants. Science 148, 339–346

Sánchez-Blanco, M.J., Torrecillas, A., León, A., Del Amor, F., 1989. The effect of different irrigation treatments on yield and quality of Verna lemon. Plant and Soil 120, 299–302.

Shackel, K.A., Ahmadi, H., Biasi, W., Buchner, R., Goldhamer, D., Gurusinghe, S., Hasey, J., Kester, D., Krueger, B., Lampinen, B., McGourty, G,. Micke, W., Mitcham, E., Olson, B., Pelletrau, K., Philips, H., Ramos, D., Schwankl, L., Sibbett, S., Southwick S., Stevenson, M., Thorpe, M., Weinbaum, S., Yeager, J., 1997. Plant water status as an index of irrigation need in deciduous fruit trees. HortTechnology 7, 23–29.

Villalobos, F.J., Mateos, L., Orgaz, F., Fereres, E., 2002. Fitotecnia. Bases y tecnologías de la producción agrícola. Mundiprensa eds. Madrid, España. 496 pp.

Winkel, T., Rambal S., 1993. Influence of Water Stress on Grapevines Growing in the Field: from Leaf to Whole-plant Response. Australian Journal of Plant Physiology 20, 143–57.

1.1.7. Net CO_2 fixation rate, transpiration rate and stomatal conductance

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Importance and applications

The stomatal conductance (g_s) is the variable that measures the degree of opening of the stomata situated on the leaves, by regulating the gaseous exchange with the atmosphere that surrounds it. This indicator of stress is affected by a large number of factors. Thus, its values depend on the light intensity, the temperature, the difference in absolute humidity between the leaf and the air, the age of the leaf, the concentration of CO_2 and the water potential itself (Jones, 1983; Ruiz-Sánchez et al., 2000).

Photosynthesis is one of the most important processes in the plant response to water deficit conditions (Azcón-Bieto et al., 1983). It implies the coordination of different subprocesses such as the absorption of CO_2, the capturing of light by the chlorophyll-protein complexes, the synthesis of NADPH and the synthesis of ATP, among others.

Principle

Water stress affects the stomatal opening, so therefore it can be an index of plant water stress; it is moreover well correlated with the rate of photosynthesis (Farquhar & Sharkey, 1982; Harrison et al., 1989; Yoon & Richter, 1990), which also depends on the water status (Wong et al., 1979). The measurements of gs are very useful to detect the recovery of the plants after water stress (Gebre & Kuhns, 1992) and after flooding (Savé & Serrano, 1986; Ruiz-Sánchez et al., 1996; Domingo et al., 2002). Additionally, more or less complex models have been developed to estimate the transpiration based on the measurements of a few leaves (Williams et al., 1996). gs shows a circadian evolution throughout the day, which is more pronounced the greater the climatic demand is (Torrecillas et al., 1988; Ruiz-Sánchez et al., 2007). In this way, the stomata open with the sunrise (increase in the photosynthetically active radiation, PAR) increasing gs and reaching its maximum between 10 and 12 solar hour, after which it progressively decreases. The maximum value of gs is reached sooner in water stress conditions; the time of maximum stomatal opening is lower (Henson et al., 1982).

With respect to photosynthesis, when the water deficit is slight it causes a partial closing of the stomata, increasing the photorespiration and decreasing the relation of CO_2/O_2, which makes for a faster recovery of photosynthesis after the disappearance of the stress. (Ruiz-Sánchez et al., 2000; Medrano & Flexas, 2004).

The net CO_2 assimilation rate or the photosynthesis rate (P_n) is measured with infrared gas analysers, some of which are portable, which enables direct measuring in field conditions.

Materials and equipment

The measurement of both photosynthesis as well as for the stomatal conductance require a portable system to measure the gaseous exchange CIRAS 2® (PP Systems, Hitchin, Hertfordshire, UK), into which an infrared gas analyser (IRGA) will be incorporated. A leaf cuvette, model PLC6 (U) (PP Systems, Hitchin, Hertfordshire, UK), will be used with a measuring area of 1.7 cm^2. The CO_2 concentration of the air will be controlled using the injection system of the CIRAS 2® and compressed CO_2 cylinders. The levels of PAR sought were obtained acting on a source of red/blue light (LED) incorporated into the leaf cuvette.

Procedure

The instructions provided in the manual of the CIRAS2 will be followed in order to carry out the measurements of both the net photosynthesis as well as the net conductance. Two leaves will be selected for each repetition (8 per treatment) for that purpose. Measurements will be taken every 7-15 days, mid-morning, prior to the stomata closing as is habitual at the solar midday.

References

Azcón-Bieto, J. Lambers, H., Day, D.A., 1983. The effect of photosynthesis and carbohydrate status on respiration rates and the involvement of the alternative path in leaf respiration. Plant Physiology 72, 598-603.

Domingo, R., Pérez-Pastor, A., Ruiz-Sánchez, C., 2002. Physiological responses of apricot plants grafted on two different rootstocks to flooding conditions. Journal of Plant Physiology 159, 725-732.

Farquhar, G.D., Sharkey, T.D., 1982. Stomatal conductance and photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology 33, 317-345.

Gebre, G.M., Kuhns, M.R., 1992. Effects of water stress preconditioning on gas exchange and water relations of Populus deltoides clones. Can. J. For. Res. 23, 1291-1297.

Harrison, R.D., Daniell, J.W., Chesire, J.M., 1989. Net photosynthesis and stomatal conductance of peach seedlings and cuttings in response to changes in soil water potential. J. Amer. Soc. Hort. Sci. 114, 986-990.

Henson, I.E., Alagarswamy, G., Bidinger, F.R., Mahalakshmi, V., 1982. Stomatal responses of pearl millet (Pennisetum americacum L. Leeke) to leaf water status and environmental factors in the field. Plant Cell and Environment 5, 64–74.

Jones, H.G., 1983. Estimation of an effective soil water potential at the root surface of transpiring plants. Plant, Cell and Environment 6, 671–674.

Medrano, H., Flexas, J., 2004. Relaciones hídricas de las plantas. En La ecofisiología vegetal, una ciencia de síntesis. Madrid: Thompson Editores. Paraninfo S.A, pp. 1141–1174.

Ruiz-sanchez M.C., Domingo, R., Morales, D., Torrecillas, A., 1996. Water relations of Fino lemon plants on two rootstocks under flooded conditions. Plant Sci. 120, 119-125.

Ruiz-Sánchez, M.C., Domingo, R., Pérez-Pastor, A., 2007. Daily variations in water relations of apricot

trees under different irrigation regimes. Biologia Plantarum, 51, 735–740.
Ruiz-Sánchez, M.C., Torrecillas, A., Pérez-Pastor, A., Domingo, R., 2000. Regulated deficit irrigation in apricot trees. Acta Horticulturae 537, 759–766.
Savé, R., Serrano, L., 1986. Some physiological and growth responses of Kiwi fruit (Actinidia chinensis) to flooding. Physiol. Plant. 66, 75-78.
Torrecillas, A., Ruiz-Sánchez, M.C., León, A., García, A.L., 1988b. Stomatal response to leaf water potential in almond trees under drip irrigated and non-irrigated conditions. Plant Soil 112, 151-153.
Williams, M., Rastetter, E.B., Fernandes, D.N., Goulden, M.L., Wofsy, S.C., Shaver, G.R., Melillo, J.M., Munger, J.W., Fan, S.M., Nadelhoffer, K.J., 1996. Modelling the soil-plant-atmosphere continuum in a Quercus-Hacer stand at Harvard forest: the regulation of stomatal conductance by light, nitrogen and soil/plant hydraulic properties. Plant Cell Environ. 19, 911-927.
Wong, S.C., Cowan, I.R., Farquhar, G.D., 1979. Stomatal conductance correlates with photosynthetic capacity. Nature 282, 424-426.
Yoon, T.M. y Richter, H., 1990. Seasonal changes in stomatal responses of sweet cherry and plum to water status in detached leaves. Physiol. Plant. 80, 520-526.

1.1.8. Fruit growth

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Importance and applications

Monitoring the fruit growth is a measure that many authors consider an indicator to take into account for irrigation scheduling, given that it allows us to differentiate the different crop phenological phases, even more so in those crops where the period that elapses from the fruit set until its harvest is extensive, as in the case of citrus or almond. It is likewise a measure that enables us to know the fruit status and predict much earlier when to harvest the crop.

Principle

Furr (1955) and Oppenheimer and Elze (1941) proposed the evolution of the fruit growth as an index for irrigation scheduling in citrus. Ebel et al. (1995) used the evolution of the fruit growth as an indicator for changes in irrigation, once a threshold value has been reached, in strategies of controlled deficit irrigation in apple. It is difficult to accurately signal the critical periods of each crop. Some authors indicate the fruit growth phases as periods of maximum sensitivity to water deficit. In citrus, two critical periods are highlighted, the first which goes from the flowering to the set, in which the water deficit conditions the number of fruits, and a second period of greater transcendence which corresponds to the phase of rapid fruit growth and determines their final size (Shalhevet et al., 1979; Domingo et al., 1996).

Materials and equipment

- Digital calliper

Procedure

The fruit growth will be monitored from the fruit set (April-May) until harvest (January). The fruits will be randomLy chosen among those at eye-level and measured at their equator. The measurements will be taken every two weeks with a digital calliper and in 10 fruits per repetition (30 per treatment).

References

Furr, J.R., 1955. Responses of citrus and dates to variations in soil moisture conditions at different seasons. 14th Int. Hort. Congress 1: 400-412.

Oppenheimer, H.R. Elze, D.L., 1941. Irrigation of citrus trees according to physiological indicators. Rehovoth Agr. Res. Sta., Bull. 31.

Ebel, R.C., Proebsting, E.L., Evans, R.G., 1995. Deficit irrigation to control vegetative growth in apple and monitoring fruit growth to schedule irrigation. HortScience 30, 1229-1232.

Shalhevet, J., Mantell, A., Bielorai, H., Shimshi, D., 1979. Irrigation of Field and Orchard Crops under Semi-arid Conditions. IIIC. nº 1 (revised version). Israel. Canada. 124 pp.

Domingo, R., Ruiz-Sánchez, M.C., Sánchez-Blanco, M.J., Torrecillas, A., 1996. Water relations, growth and yield of Fino lemon trees under regulated deficit irrigation. Irrig. Sci. 16, 115-123.

1.1.9. Grape development

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Importance and applications

These observations provide information about the physiological development of the grapevine and grapes, indicating the maturity stage. This may show possible competition or benefit through the intercrop.

Principle

To describe the maturity stage and yield potential of the vine, the grape size, number of grapes per vine plant and the colour of berries are the most visible. Having this information, ripening processes can be described identifying influences of intercrops.

Materials and equipment

- The grape size is measured by tape
- Counting grapes per vine plant
- Colour is assessed using a scheme.

Procedure

Three different data are collected on selected vine plants (6 per repetition) starting when grapes begin fruit development (BBCH 70) (Lorenz et al., 1994).

a. Grape size is measured by two measurements: cross sectional (d = 2*r) on top of the grape and total height (h)
b. Counts of grapes are collected on defined vine plants (6 per repetition).
c. Colour scheme for maturity stages (BBCH 70-89) (Lorenz et al., 1994). If berries of a grape show different colours, the dominant colour should be counted.

	COLOUR OF BERRIES
1	green
2	light green
3	light yellow
4	yellow
5	purple (Botrytis cinerea)
6	brown / rotten

Calculations

The measurement of grape size can be calculated as the volume of a cone.

Therefore, this formula can be used: $V = 1/3 * \pi r^2 * h$, resulting in a single data set for this parameter.

References

Lorenz, D.H., Eichhorn, K.W., Blei-Holder, H., Klose, R., Meier, U., Weber, E., 1994. Phänologische Entwicklungsstadien der Weinrebe (Vitis vinifera L. ssp. vinifera). Vitic. Enol. Sci. 49, 66-70. cited on: https://ojs.openagrar.de/index.php/BBCH/article/view/483, 16.4.2018

1.1.10. Sprout / stem diameter

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Importance and applications

This parameter is easy to measure and provides information about the physiological development of the vine plant during early development stages. The sprout / stem diameter indicates growth-conditions in terms of availability of nutrients and water also as weather conditions (temperature, wind…) and may show some impact of the intercrop.

Principle

Sprouts, young stems of the vine grape, grow fast if growing conditions are favourable. This parameter is correlated to the fruit setting (Currle, 1983). Measuring sprout diameters provides information about stress factors such as water stress. If water stress is present, the vine is able to reduce water consumption resulting in lower gains in sprout / stem diameters.

Materials and equipment

- Calliper

Procedure

a. Measuring the diameter of the sprout / stem basis.
b. Take 6 measures per vine plant on 6 selected plants per repetition

References

Currle, O., Bauer, O., Hofäcker, W., Schumann, F., Frisch, W., 1983. Biologie der Rebe, Meininger Verlag, Neustadt a.W. ISBN 3-87524-031-6, 90.

1.1.11. Flowering height

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Importance and applications

This parameter is the main observation for the maturity stage of herbal crops, defining the harvest time. For Origanum vulgaris L. and Thymus vulgaris L. the best relation between yield mass and etheric oil concentration is present at beginning to full flowering (Pahlow, 2006). The height of the inflorescences – in addition to the plant width - is a good indicator for plant growth and establishment success.

Principle

Herbal intercrops are measured in height and widths from May to summer until the flower is overblown. The plant maturity stage (flowering) is visually checked to define harvest dates.

Materials and equipment

- Measure tape

Procedure

a. Measuring height and cross-sectional width of herbal intercrops
b. Visual assessment of flowering

Calculations

Plant volume is calculated with this formula: ($V = \pi r^2 * h$); height (h); width (r*2).

References

Pahlow, M., 2006. Das Grosse Buch der Heipflanzen. Weltbild Verlag 317, 220-221.

1.1.12. Crop establishment

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Importance and applications

The field establishment of grain crops is the percentage of sown seed that goes on to produce established plants (Peltzer, 2018). Successful crop establishment is crucial to achieve maximum potential yield. Factors affecting the establishment percentage include management factors such as sowing depth, row spacing, seed size and herbicide application, as well as environmental factors such as soil moisture and temperature (Peltzer, 2018). Timeliness of sowing is the most important factor followed by an evenly established and uniform plant stand. The procedure to measure the establishment rate is very easy, inexpensive and requires little equipment.

Principle

The number of plants established in the field relative to the number of plants sown is the final assessment of success of a planting operation.

Reagents

No reagents needed

Materials and equipment

- Measure tape
- Balance
- Plastic or wooden pegs
- Data recording sheet/Note book
- Recording material (pen, pencil, …)

Procedure

a. Obtain the weight of 1,000 seeds (grains).
b. On each experimental plot, randomLy select three representative 0.5-m long rows. If the row spacing is, for instance, 0.2 m, the total sampling area at each sampling point will be 0.1 m^2.
c. Count and record the number of seedlings at each sampling point. Be careful not to count tillers or other grass species.

Calculations

The establishment rate is the average number of seeds that are established in the field after planting.
Establishment rate = Number plants/Area (m^2)

To calculate crop establishment, expressed as percentage of emergence, proceed as follows.

Example:
If the establishment rate is 240 plants m^{-2} (average of the three sampling points), the seed rate is 170 kg ha^{-1}, and the weight of 1000 seeds is 40 g, calculate the emergence percentage as follows:

- Number of plants emerged:
  - 240 plants m^{-2} x 10,000 m^2 ha^{-1}= 2,400,000 plants ha^{-1}

- Number of seeds sown:
  - Seed rate: 170 kg ha^{-1} x 1,000 = 170,000 g ha^{-1}
  - 1,000 seeds…….. 40 g
    X seeds ……. 170,000 g, then X= 4,250,000 seeds

- Percentage of emergence:
  - 4,250,000 seeds sown…….…. 100%
  - 2,400,000 plants emerged …… X%, then X= 56.47%

References

Peltzer, S., 2018. Factors affecting grain crop field establishment. Available at: https://www.agric.wa.gov.au/barley/factors-affecting-grain-crop-field-establishment?nopaging=1

1.1.13. Above-ground biomass

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Importance and applications

The crop growth of arable crops can be controlled with measurement of the above-ground biomass. Although above-ground biomass can be measured in different moments of the crop, measuring this parameter at flowering provides an excellent indicator of the crop growth at a critical crop development stage.

Principle

The above-ground biomass measurement consists in the collection of the total above-ground crop biomass within a known surface. Once in the laboratory, this fresh biomass is dried out in an oven and weighed.

Reagents

- No reagents

Materials and equipment

- 0.5 m ruler
- Scissors
- Labelled paper or plastic bags
- Aluminium trays
- Oven
- Balance

Procedure

a. Place the ruler in the crop interrow area. Three replicates per plot are recommended to account for spatial variability in the field.
b. Clip all crop plants from one side of the 0.5 m ruler and place them in the paper or plastic bag.
c. Dry at 65°C for 48 h.
d. Weigh the biomass.

Calculations

$$\textit{Above-ground biomass (in g DM } m^{-2}) = \frac{\textit{dry biomass weight (g)}}{\textit{(0.5 * distance between rows } (m^2)}$$

1.1.14. Carbon and nitrogen in leaves

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Importance and applications

A C and N elemental analysis provides a means for the rapid determination of C and N in organic and inorganic matrices. It is capable of handling a wide variety of sample types (including solids, liquids and viscous samples) in the fields of agriculture, food, chemicals, environment, pharmaceuticals and energy. It is a regulation mechanism of many metabolic and development processes in plants.

Principle

This simultaneous C and N analysis requires high temperature combustion in an oxygen-rich environment and is based on the classical Dumas method. In this combustion process (furnace Tª around 1000ºC), carbon is converted to carbon dioxide and nitrogen to nitrogen gas/ oxides of nitrogen. The combustion products are swept out of the combustion chamber by inert carrier gas (helium) and passed over heated high-purity copper. The function of this copper is to remove any oxygen not consumed in the initial combustion and to convert any oxides of nitrogen to nitrogen gas. The gases are then passed through the absorbent traps in order to leave only carbon dioxide and nitrogen.

Detection of the gases can be carried out by gas chromatography (GC) separation followed by quantification using thermal conductivity detection (TCD) of individual compounds. Quantification of the elements requires calibration for each element using high-purity analytical standard compounds.

Materials and equipment

- A EuroVector Elemental Analyser EA3000 (EuroVector SpA, Milan, Italy) for CN microanalysis of samples (between 0.5 and 10 mg per sample).
- A EuroVector Elemental Analyser EA3000 (EuroVector SpA, Milan, Italy) for CN macroanalysis of samples (above 20 mg per sample).

Both of them are equipped with Callidus software (EuroVector SpA, Milan, Italy) for running the instrument, storing the data, and for processing the results obtained.

Procedure

Before the C and N analysis, samples must be dried and fine crushed. Every sample is weighed in a tin capsule using a microbalance. After that, the tin capsule with the sample is sealed to avoid air inside the capsule.

Each sample is combusted in a reactor at about 1000°C in a temporarily enriched oxygen atmosphere. The combustion products are carried by a carrier gas (helium) that passes through a glass column packed with an oxidation catalyst and a copper reducer. At this temperature, the nitrogen oxides are reduced to N^2. The N^2, and CO^2, are then transported by the helium to a packed column, separated by GC and quantified with a TCD detector (set at 90°C).

Calculations

Previously, the chromatographic responses for each element are calibrated against standards, weighed and analysed. The C and N chromatography areas for each sample are applied to the calculated calibration curve to obtain the C and N composition. These elemental contents are reported in weight percent and the detection limit for this analysis is around 0.2%.

Remarks

C and N elemental analysis is used extensively across a wide range of applications such as

- Determination of nitrogen (as a surrogate for protein) in agricultural samples.
- Determination of C/N ratio, total carbon (TC) and total organic carbon (TOC).
- Quantitative analysis of total C and N in samples.

References

Serrano, S., Rincón, F. and García-Olmo, J., 2013. Cereal protein analysis via Dumas method: standardization of a micro-method using the EuroVector Elemental Analyser. J. Cereal Sci., 58, 1, 31–36. doi: 10.1016/J.JCS.2013.04.006.

AMC Technical Brief No 29, CHNS Elemental Analysers, The Royal Society of Chemistry, Cambridge, UK, 2008.

ASTM D5291-10, Standard Test Methods for Instrumental Determination of Carbon, Hydrogen, and Nitrogen in Petroleum Products and Lubricants, ASTM International, West Conshohocken, PA. 2010

1.1.15. Monitoring Pests and Diseases

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Importance and applications

The objectives of sampling or monitoring are to detect the presence or absence of pests, quantify their abundance and their natural enemies and follow the progress of an arthropod population through time by regular, periodic sampling. The goal of monitoring is to reach a decision as to whether, or when, a pest population requires control action.

Monitoring means checking the field to identify which pests are present, how many there are, or what damage they have caused. Correctly identifying the pest is key to knowing whether a pest is likely to become a problem and to determining the best management strategy.

After monitoring and considering information about the pest, its biology, and environmental factors, a decision can be taken as to whether the pest can be tolerated or whether it is a problem that warrants control. If control is needed, this information also helps in selecting the most effective management methods and the best time to use them.

Pest populations vary from field to field, crop to crop and year to year. Managing pests requires flexibility and an absolute commitment to pest monitoring. Pest monitoring is site, crop and pest-specific. Each situation will require specialised knowledge and tools.

Principle

Knowing the exact number of pests in a field is rarely possible, and so the pest levels will have to be estimated. To reach this estimate, the field population is sampled. How well the actual population is estimated will greatly depend on how well the samples are taken.

Scouting for pests in the area can start before establishing the crop. By inspecting weeds and other surrounding vegetation, potential pests and natural enemies can be identified and possibly treated to prevent them from becoming a problem early in the crop.

For proper diagnosis of unfamiliar pests and natural enemies, identification guides are available with photographs of common pests. The local Ministries of Agriculture and extension services can also provide help in identification.

The PARAMETERS most frequently used are:

PEST POPULATION. Insect populations can be estimated using a number of different sampling devices. The method selected depends on the insect or mite and the habitat. Procedures are shown below.

PLANTS AFFECTED. Incidence (of a pest) is the proportion or number of units in a sample, consignment,

field or other defined population that is affected by a pest.
DISEASE INCIDENCE. Diseases present can be assessed by estimating the proportion of plant area that is affected (i.e. disease severity), or the proportion of the total number of plants that are affected (disease incidence) in the field.

DAMAGE PROPORTION. Pests are responsible for several kinds of damage to growing crops, causing significant losses. Loss data are the prerequisite for economic management of pests and for evaluating the efficacy of the present crop protection practices. This parameter is mandatory in the project, so the general procedure is explained below.

On the other hand, measuring the effect of pest damage and pest products such as skin casts, frass, nests, etc. is an indirect way of sampling pests. It is necessary to associate the visible damage with the insects or mites present.

Materials and equipment

For identification: Hand lens, Digital camera, Binocular lent, Identification guides (Books, Internet)
For sampling: Aerial net, Vacuum blowing, Electric vacuum, Pitfall, Bails and Pheromone traps (Delta, Mc Pall or, Funnel traps), Emergence traps, Yellow sticky trap, Pheromones. Berlese funnel
For removing samples: Plastic bags, Envelopes, Jars, Tubes, Bottles, Cups. Alcohol, Propylene glycol.

Procedure

There are several ways to **SELECT WHERE TO SAMPLE** in the field.

The most recommended method is systematic sampling. Divide the area to be sampled into smaller areas or sub-plots. Take the first sample from a randomLy selected sub-plot. Take the remaining samples from sub-plots at regularly spaced, fixed intervals. The samples may be taken along set lines called transects (e.g. an imaginary “X” or “M” or “Z” can be drawn on the field, and samples taken along those imaginary lines). Subsequent samples can be taken by changing the starting position or the orientation of the transect line.

The method of unrestricted random sampling (URS) selects sample units with equal chance. A table of random numbers is typically used.

Sampling areas or populations where there are obvious factors that can affect distribution can be effective for stratified sampling. Divide the population or sample area into smaller groups or areas known as strata. The strata are formed based on shared attributes or characteristics (such as slope, degree of shading, and border versus middle plants). Take a number of samples proportional to the stratum’s size from each stratum.

SAMPLING PROCEDURES FOR PEST POPULATIONS

Measures for sampling pests could be direct and indirect.

Direct counting of pests, collection by netting or trapping, extraction from soil, etc. Indirect measuring of the effect of pest damage and pest products, such as skin, casts, frass, nests, etc. The entire plant, or parts of it, may be used to estimate the pest population.
Whole plant – counting the number of plants damaged by the pest or number of missing plants that are due to the pest's (feeding) activity, in order to obtain an indication of the pest density.
Stem – pests may damage stems by gnawing, eating, tunnelling, etc. Assess the level of damage to obtain an indication of the pest population.
Leaf and flower – similarly, the area of a leaf/flower or the number/proportion of leaves/flowers damaged (consumed, mined, covered by lesions) or missing due to the pest's presence can give an estimate of pest incidence on the crop or the severity of the attack.
For fruit and seed pests, estimate the feeding and tunnelling damage.
Depending on where the population of insects to be sampled is, the procedure followed is different.

1. Air sampling

The most used are traps. There are many types of traps, especially for insects, that are available or easily made. Traps can attract (light, pheromone, baited), or be passive (pitfall, water).

a. Pheromone and Baited Traps

Food attractants or pheromones are usually used to capture insects. Pheromone traps are an effective pest monitoring tool that is used to help control insect infestations. Especially known are lepidoptera, tortricid and noctuide pheromones, and also some coleoptera such as some coccidus diaspinos. These sex pheromones are usually very specific and are marketed together with traps for the capture of insects. Food attractants are specially used for monitoring dipterous pests.

A pheromone trap usually consists of a small glue trap or a mild killing agent that is impregnated with sex pheromone or it comes with a small vial of sex pheromone that will be placed on the trap. Sex pheromones are hormone scents that are usually emitted by the female insect and picked up by the male as a cue for mating. Male pests are drawn to the trap for the purpose of mating and are then caught.

Pheromone traps are usually simple to use. Some traps only require you to peel the protective paper from the glue area. Other traps also require you to place the pheromone vial on the trap to attract the insects. Traps should be placed in the area where the target pest is a problem.

b. Yellow Sticky Traps

They can be an effective monitoring tool. Yellow sticky traps can be used for monitoring most pests, including whiteflies, thrips, winger aphids, leafminer, scales and many others. They may also capture parasitic wasps.

c. Pitfall and water traps (used for crawling insects)

They are simply containers or trays with smooth sides sunk into the ground with the edges at the same level as the ground.

Water traps are filled with water and detergent to break the surface tension of the water so that the insects cannot walk on the water surface, and thus they sink to the bottom of the container and drown. In some of them, like Moericke traps, the colour of the container is what attracts the insects and can be used for aphids and their parasitoids located at the height of the crop.

d. Light traps that attract moths

2. Plant sampling

Knowing the biology or behaviour of the pest can be very helpful when choosing the strata or parts of the plant in which the population is more abundant, presents a more homogeneous distribution, and/or shows a closer relation with the total population. It is also useful to know the state or stage of development of the most representative arthropod of the total population.

It must be noted that the common dispersion patterns of pests in the field are random, uniform, and clumped, even many pest populations tend to be clumped, as well as deciding the sampling accuracy. All this facilitates the choice of the sampling unit.

Several devices can be used for monitoring pests, however one of the most used is direct counting.

a. Direct counting. Visual checking

In most cases, no specialised or expensive equipment is required for the direct counting of pests. The number of pests observed in a sample unit can be counted and recorded. The sample unit may be part of a plant (such as the leaves, branches, roots, flowers, fruits, seeds), the entire plant, or part (where the pest is known to occur). Counting may be conducted in the field, or the plant part may be removed and taken back to the laboratory for counting.

Some dissection of certain samples, such as fruits, seeds, and roots, may be necessary in order to expose the pest. In other instances, the plant may be shaken (manually or with a beating stick) a given number of times over a container or sheet of cloth (white or black). This is a common tool for leaf feeding insects. A cloth is placed beneath the foliage beaten to dislodge insects so that they fall onto the sheet. Fallen insects are counted immediately or collected.

If the pest numbers are too numerous or the pest is small, other methods, such as the use of an aspirator (or potter) or washing off with soapy water, may be employed to remove and collect the pest from the plant and take it to the laboratory for counting.

An alternative to the method of counting the insects of a sample is the binomial method. Binomial or presence-absence sampling consists in counting the sampling units occupied by arthropods of the total observed sampling units. The method is based on the generally existing relationship between the proportion of occupied sampling units and the density of individuals of a species in the sampling unit.

b. Sweep nets

They are usually made of cotton and have a long handle. Sweeping through the crop canopy a fixed number of times in a specific period of time, insects fly or fall into the net.

c. Tullgren or Berlese funnel

Tullgren or Berlese funnels can be used to extract live soil arthropods from leaf litter and soil samples. A heat or light source is placed over the funnel containing the soil sample. Organisms move downward in the funnel, away from the heat and drying soil, and into a collecting vial with a preserving liquid, such as 70% ethanol.

3. Soil monitoring

The Berlese-Tullgren funnel is one of the best ways of monitoring microarthropods that live on the soil, especially in ecological or biodiversity studies.

Emergence traps, which are transparent tubes or containers sealed on the surface of the ground, trap emerging insects which pass through a developmental stage underground.

PROCEDURES FOR DISEASE INCIDENCE

A major problem of assessing disease is the complex nature of disease development. The nature of the disease determines whether the disease incidence or disease severity (or both) is measured.
Samples of crop units (plants, leaves, fruit, etc.) can be taken randomLy from a plot.
To determine disease incidence, take samples and count the number of plants, leaves, flowers, etc., that are infected or dead.
For diseases that cause varying degrees of damage to plants throughout the crop, assess disease severity by estimating the proportion of total area of the plant that is diseased.

DAMAGE PROPORTION

Crop losses may be quantitative and/or qualitative. Quantitative losses result from reduced productivity, leading to a smaller yield per unit area. Qualitative losses from pests may result from the reduced content of valuable ingredients, reduced market quality, e.g. due to aesthetic features (pigmentation), reduced storage characteristics, or due to the contamination of the harvested product with pests, parts of pests or toxic products of the pests (e.g. mycotoxins). Crop losses may be expressed in absolute terms (kg/ha, financial loss/ha) or in relative terms (loss in %). The loss rate may be expressed as the proportion of attainable yield, but sometimes the proportion of the actual yield is calculated (Oerke, 2006).

The procedure will vary depending on the crop, but in general terms, once a month, the number of damaged plants (whose production would be null) at each field will be counted, trying to identify the species responsible for the depredation based on tracks and distinctive tooth marks. Losses will be presented as the percentage

of damaged plants out of the total planted, for each crop field, and plot type. Harvest extended over several weeks and total biomass harvested will be recorded by farmers, differentiating yields from exclosures and controls.

References

Dent, D., 2000. Insect Pest management- 2nd ed. CABI Publishing International, New York.
Pedigo, L.R., 1999. Entomology and Pest management- 3nd ed. Prentice Hall Upper Saddle River, NJ 07458
Southwood, T.R.E., 1996. Ecological Methods- 2nd ed. Chapman & Hall, London
Oerke, E.C., 2006. Crop losses to pests. Journal of Agricultural Science144, 31-43
https://www.agriskmanagementforum.org/sites/agriskmanagementforum.org/files/Caribbean%20Risk%20Model%202.3%20Monitoring%20for%20pest%20management.pdf
http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/GUIAUVADETRANSFORMACION_tcm30-57934.pdf
http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/GUIAOLIVAR%20(2)_tcm30-57939.pdf
http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/GUIACITRICOS_tcm30-57942.pdf
http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/guiadealmendroweb_tcm30-57951.pdf
http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/GUIA%20de%20CEREALES%20WEB_tcm30-57956.pdf
http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/guias-gestion-plagas/horticolas/default.aspx

1.1.16. Fruit production

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Importance and applications

It is a parameter that enables us to quantify the harvest to know the influence of the treatments in the trial. In addition, it serves to know yields of the harvest and commercial yields. The productive yield is an indicator that can be used to assess the crop response to the climate and/or other factors related with abiotic stresses, such as water deficit or saline stress. The different sizes of the fruit will be determined among the different treatments in order to determine the commercial value of the harvest.

Principle

The bibliography shows different productive responses depending on the intensity and moment that deficit is applied in citrus. González-Altozano and Castel (1999) with water savings of between 6 and 22%, obtained in clementine similar production and quality to the control, applying the deficit in summer. Romero et al. (2006) observed a decrease in the production of Clemenules mandarin trees grafted on Cleopatra after three years applying water deficit, in which irrigation was suppressed in the phases I and III of the fruit growth, reaching minimum Ψt values of -2 MPa, with the number of fruits per tree being the component that was most affected.

Treeby et al. (2007) did not observe differences in the harvest load (kg fruit per m^2 of canopy) nor in the number of fruits per m^2 but they did obtain fruits of a lower fresh weight and diameter after two years of continued water deficit, in which the deficit treatment received half the amount of water that the control received, in Navel orange grafted on different rootstocks.

Pérez-Pérez et al. (2008b) did not observe differences in production until the third year of applying deficit, although in the second year the number of fruits increased significantly with the water deficit, reducing the mean fruit weight as compared to the control.

García-Tejero et al. (2010) showed a clear influence of the irrigation treatment on the production, observing that severe water stress applied during the flowering phase reduced the number of fruits per tree, whilst a water deficit applied during the fruit growth phase produced a reduction in the fruit size.

Materials and equipment

Hand scales SANDA

Procedure

To determine the production obtained, the total of all the fruits harvested from 4 trees per repetition will be weighed (16 per treatment) at the moment of harvesting. To do so, all the fruits from one tree will be deposited in the boxes needed and they will be weighed; the same operation will be repeated for all 12 trees

per treatment monitored.

Given the particulars of the market for this crop and the way that the fruits of this crop are harvested, it is not possible to directly distinguish and determine the different calibres at the moment of harvesting. However, given that the fruit growth will be measured with the calliper until just before the harvest time, the different sizes of the fruit can be determined among the different treatments.

References

García-Tejero, I. Romero-Vicente, R., Jiménez-Bocanegra, J.A. Martínez-García, G., Durán-Zuazo, V.H., Muriel-Fernández, J.L., 2010. Response of citrus trees to deficit irrigation during different phenological periods in relation to yield, fruit quality, and water productivity. Agricultural Water Management 97, 689–699.

González-Altozano, P., Castel, J.R., 1999. Regulated deficit irrigation in 'Clementina de Nules' citrus trees. I. Yield and fruit quality effects. Journal of Horticultural Science & Biotechnology 74, 706–713.

Pérez-Pérez, J.G., Romero, P., Navarro, J.M. y Botía, P., 2008b. Response of sweet orange cv Lane late to deficit-irrigation strategy in two rootstocks. II: Bibliografía 177 Flowering, fruit growth, yield and fruit quality. Irrigation Science 26, 519– 529.

Romero, P. y Botía, P., 2006. Daily and seasonal patterns of leaf water relations and gas exchange of regulated deficit-irrigated almond trees under semiarid conditions. Environmental and Experimental Botany 56, 158–173.

Treeby, M.T., Henriod, R.E., Bevington, K.B., Milne, D.J., Storey, R., 2007. Irrigation management and rootstock effects on navel orange (Citrus sinensis (L.) Osbeck) fruit quality. Agricultural Water Management 91, 24–32.

1.1.17. Marketable yield

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Importance and applications

It is parameter that enables us to quantify the harvest once those fruit which are not suitable for sale have been eliminated, to know the influence of the treatments in the trial.

Principle

The bibliography shows different productive responses depending on the intensity and moment that deficit is applied in citrus. González-Altozano and Castel (1999) with water savings of between 6 and 22%, obtained in clementine similar production and quality to the control, applying the deficit in summer. Romero et al. (2006) observed a decrease in the production of Clemenules mandarin trees grafted on Cleopatra after three years applying water deficit, in which irrigation was suppressed in the phases I and III of the fruit growth, reaching minimum Ψt values of -2 MPa, with the number of fruits per tree being the component that was most affected.

Treeby et al. (2007) did not observe differences in the harvest load (kg fruit per m^2 of canopy) nor in the number of fruits per m^2 but they did obtain fruits of a lower fresh weight and diameter after two years of continued water deficit, in which the deficit treatment received half the amount of water that the control received, in Navel orange grafted on different rootstocks.

Pérez-Pérez et al. (2008b) did not observe differences in production until the third year of applying deficit, although in the second year the number of fruits increased significantly with the water deficit, reducing the mean fruit weight as compared to the control.

García-Tejero et al. (2010) showed a clear influence of the irrigation treatment on the production, observing that severe water stress applied during the flowering phase reduced the number of fruits per tree, whilst a water deficit applied during the fruit growth phase produced a reduction in the fruit size.

Materials and equipment

Hand scales SANDA accurate to 10 grams

Procedure

The production was evaluated from the weight and number of fruits per tree. During the harvest the fruits which have remained on the trees will be accounted for and weighed; these fruits are considered by the workers to be unsuitable for sale. To determine the value of the commercial yield we shall obtain a final value of commercial kilograms per hectare.

References

García-Tejero, I. Romero-Vicente, R., Jiménez-Bocanegra, J.A. Martínez-García, G., Durán-Zuazo, V.H., Muriel-Fernández, J.L., 2010. Response of citrus trees to deficit irrigation during different phenological periods in relation to yield, fruit quality, and water productivity. Agricultural Water Management 97, 689–699.

González-Altozano, P., Castel, J.R., 1999. Regulated deficit irrigation in 'Clementina de Nules' citrus trees. I. Yield and fruit quality effects. Journal of Horticultural Science & Biotechnology 74, 706–713.

Pérez-Pérez, J.G., Romero, P., Navarro, J.M., Botía, P., 2008b. Response of sweet orange cv Lane late to deficit-irrigation strategy in two rootstocks. II: Bibliografía 177 Flowering, fruit growth, yield and fruit quality. Irrigation Science 26, 519– 529.

Romero, P., Botía, P., 2006. Daily and seasonal patterns of leaf water relations and gas exchange of regulated deficit-irrigated almond trees under semiarid conditions. Environmental and Experimental Botany 56, 158–173.

Treeby, M.T., Henriod, R.E., Bevington, K.B., Milne, D.J., Storey, R., 2007. Irrigation management and rootstock effects on navel orange (Citrus sinensis (L.) Osbeck) fruit quality. Agricultural Water Management 91, 24–32.

1.1.18. Cover crop yield

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Importance and applications

Cover crops are one of the most important agricultural practices that farmers can use to improve soil quality and increase the sustainability of their production system. Cover crops provide many benefits, including reducing erosion, fixing nitrogen (if legumes are included), and providing a habitat for pollinators and beneficial insects. Their use also increases soil organic matter, infiltration rates, and nutrient availability. Knowing how much biomass (biomass dry weight per m^2) there is in a field is a critical piece of information for cover crop management.

Principle

The dry weight of the cover crop will be assumed as the cover crop yield, expressed as weight of dry matter per unit area (g DM m^{-2}). The method consists in collecting total plant biomass production (green manure or spontaneous ground covers) at peak growing season from several replicated quadrants of a known surface placed randomLy at each management treatment. Both quadrat size and number of replicates depend on the observed heterogeneity in plant composition and the field size. The more homogeneous the plant composition is, the smaller the quadrats can be. We use clippers to cut the cover crop biomass at ground level, excluding soil, cash crop residues, or weeds from the sample. We must try to capture only cover crop biomass and then place it in a labelled paper/plastic bag. Later, in the laboratory, the collected cover crop biomass from each quadrat is placed in separate labelled trays and oven dried at 60ºC for 72 h or until constant weight is reached.

Reagents

- No reagents

Materials and equipment

- Frame: 0.5 m x 0.5 m or 1 m x 1 m, depending on the crop size
- A pair of scissors, clippers, sharp knife, and/or machete
- Labelled paper or plastic bags
- Plastic bucket
- Clean tarpaulin
- Data sheet
- Aluminium trays
- Oven
- Balance

Procedure

a. Toss the frames randomLy into the different treatment plots. Between four and six replicates are recommended to account for spatial variability in the field.
b. Clip all plant material inside the quadrat and place it in the paper or plastic bag. If you have a great amount of biomass you can use a clean tarpaulin to collect it. Try not to include soil on the base of the cover crop.
c. Dry at 60°C for 72 h.
d. Weigh the biomass and keep it for C and N analyses.

Calculations

$$Biomass\ Dry\ weight\ (in\ g\ DM\ m^{-2}) = \frac{dry\ biomass\ weight\ (g)}{surface\ (m^2)}$$

Table 1. Range of values in different agricultural soils.

Aboveground biomass (g m^{-2})	Cover crops	Soil type	Reference
138-183	Common vetch and barely	Calcisol	Almagro et al., (2016)
12.87-290.03	Rice and Trios	Cumulic Haploxeroll	Steenwerth & Belina, 2008
185-663	Barely and Clover	Oxyaquic xerorthent	Peregrina et al., 2014
110-366	Barely and Hairy vetch	Fluventic haplustept	Tosti et al., 2014

Remarks

- If you want to determine plant residue C and N contents subsamples should be ground and analysed using an elemental C/N analyser (procedure explained in section 2.2.7). The annual plant carbon and nitrogen inputs will be calculated as a product of the C and N concentration and total biomass production of each treatment.
- If you want to obtain belowground biomass there are two options: 1) you can sample the belowground component of the cover crops at the same time that the aboveground component is collected; or 2) you can apply a known specific belowground-aboveground ratio (also called root:shoot ratio) to estimate root biomass from aboveground biomass.

References

Almagro, M., Vente de, J., Boix-Fayos, C., García-Franco, N., Aguilar de, J. M., González, D., Solé-Benet, A., Martínez-Mena, M., 2016. Sustainable land management practices as providers of several ecosystem services under rainfed Mediterranean agroecosystems. Mitig Adapt Strateg Glob Change 21,1029–1043.
Peregrina, F., Pérez-Álvarez, E. P., García-Escudero, E., 2014. Soil microbiological properties and its stratification ratios for soil quality assessment under different cover crop management systems in a semiarid vineyard. SJAR 12, 1000-1007
Steenwerth, K., Belina K.M., 2008. Cover crops enhance soil organic matter, carbon dynamics and microbiological function in a vineyard agroecosystem. Applied Soil Ecology 40, 359–369
Tosti, G., Benincasa, P., Farneselli, M., Tei, F., Guiducci, M., 2014. Barley–hairy vetch mixture as cover crop for green manuring and the mitigation of N leaching risk. Eur J Agron 54, 34-39.

1.1.19. Land equivalent ratio

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Importance and applications

The land equivalent ratio (LER) is an essential indicator to assess the efficiency of intercropped agricultural systems. Intercropping is the cultivation of two or more crop species simultaneously in the same field for the entire or a part of their growing period. It is expected that intercrops use land and other resources more efficiently than monocrops. In fact, intercropping is a practical application of the principle of productivity increase by biodiversity (Cardinale et al., 2007). To really assess if intercropping is using resources more efficiently and delivers higher production per unit of land, the LER appears. LER is defined as the area of monocrops that would be required to obtain the same yield of the component crops as a unit area of intercrop (Mead & Willey, 1980). Thus, LER is useful to evaluate the benefit of intercropping compared to monocultures.

Principle

The land equivalent ratio compares the yields from growing two or more crops together (intercropping) with yields from growing the same crops in monocultures (Mead & Willey, 1980). This ratio indicates the quantity of land needed to grow two or more crops together compared to the quantity of land needed to grow pure stands of each. A LER > 1 indicates that intercropping is favourable and efficient, while a LER < 1 normally indicates a disadvantage. For example, a LER of 1.10 suggest that a field grown as a monoculture would require 10% more land to produce the same yield as the same area grown as intercropping. A LER of 3.0 would indicate that intercropping would produce three times the yield of the monoculture (Kantor, 1999).

Reagents

No reagents needed

Materials and equipment

- Equipment for weighing crop yields in each crop (balance or scale).

Procedure

a. Yield data should be collected when the crop is ready for market. For crops harvested multiple times (such as fava beans, tomatoes, etc.), yield data should be collected when a sizeable portion has reached the market size and at regular intervals over the harvest period. For crops harvested multiple times, the same area must be harvested at each harvest.

b. Measure and record the total weight of each crop harvested.

Calculations

To calculate the LER, follow the following equation:

$$LER = \frac{I_1}{M_1} + \frac{I_2}{M_2} + \frac{I_n}{M_n}$$

I_1, I_2 and I_3 are the yields (per unit of total area of the intercrop) of species 1, 2 and n of the intercrop.
M_1, M_2 and Mn are the yields of the same species grown in monocultures (per unit of area of the respective sole crop).

References

Cardinale, B.J., Wright, J.P., Cadotte, M.W., Carroll, I.T., Hector, A., Srivastava, D.S., Loreau, M., Weis, J.J., 2007. Impacts of plant diversity on biomass production increase through time because of species complementarity. Proc. Natl. Acad. Sci. 104, 18123–18128.

Kantor, S., 1999. Comparing yields with land equivalent ratio (LER). Cooperative Extension Washington State University. King County. Agriculture and Natural Resources Fact Sheet #532.

Mead, R., Willey, R.W., 1980. The concept of a land equivalent ratio and advantages in yields from intercropping. Exp. Agric. 16, 217–228.

1.1.20. Crop yield

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Importance and applications

Crop yield is a measurement of the amount of agricultural production harvested per unit of land area. Crop yield is the measurement most often used for cereal crops and is normally expressed in metric tons or kilograms per hectare (bushels or pounds per acre in the US). Alternatively, crop yield, which is sometimes referred to as "agricultural output", can be defined as the amount of useful parts of a crop harvested at an appropriate development stage on a unit area. According to Fischer (2015), crop yield is the weight of grain or other economic product, at some agreed standard moisture content, per unit of land area harvested per crop. Standard moisture content varies between crops but is 8–16% in grains. In all cases, grain moisture content is calculated on a fresh weight basis (Fisher, 2015).

Principle

To estimate the crop yield, the amount of harvested product (grain, tuber…) for a given crop is measured in a sample area. The harvested product is then weighed, and the crop yield of the entire field is extrapolated from the sample.

Reagents

None

Materials and equipment

- Measuring tape
- Plastic or wooden pegs
- Scissors
- Basket, container, bags, …
- Combine harvester
- Oven
- Balance
- Data recording sheet/Notebook
- Recording material (pen, pencil, …)

Procedure

Hand-harvest

a. On each experimental plot, and immediately prior to harvest, randomLy select three representative 0.5-m long rows. If the row spacing is, for instance, 0.2 m, the total sampling area at each sampling point will be 0.1 m^2.

b. Hand-harvest total plants from each row and separate the ears.
c. After oven-drying them at 65ºC for 48 h, thresh the ears to collect the grain.
d. Weigh the dry grain collected from each row.

Combine harvester

Alternatively, grain yield can be measured by harvesting partially or completely each experimental plot using either a commercial combine harvester or an experimental combine harvester. If you use a combine harvester, measure the width and length of the harvested area on each plot and pay attention that the machine does not lose too much grain. Harvest all the grain in the harvesting area and place it in a bag, and, finally, weigh the grain collected (CIMMYT, 2013).

Calculations

Firstly, determine the harvest area:

$$Area\ (m^2) = width \times length$$

To determine the crop yield, divide the total grain weight (kg) by the harvest area (ha):

$$Crop\ yield\ (kg/ha) = Total\ grain\ weight\ /\ Area$$

Remarks

- Since harvest grains present different moisture contents, yield values must be expressed according to a fixed moisture content (e.g., small grain crops 10%; maize 14%).
- In multiple cropping or intercropping systems, the yield of each crop obtained within a given year and piece of land must be summed to obtain an annual land production value.

References

Fischer R.A., 2015. Definitions and determination of crop yield, yield gaps, and of rates of change. Field Crops Research 182, 9-18.

CIMMYT., 2013. Yield and yield components. A Practical Guide for Comparing Crop Management Practices. International Maize and Wheat Improvement Center, 23.p. Available at: https://repository.cimmyt.org/xmLui/bitstream/handle/10883/3387/98391.pdf?sequence=1

1.2. CROP QUALITY AND NUTRITIONAL EVALUATION

1.2.1. Fruit weight

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Importance and applications

It is an indicator of fruit quality and additionally enables us to know parameters such as the yield in kg ha^{-1} or kg $tree^{-1}$.

Principle

The fruit quality is of vital importance when it is directed at fresh consumption. The bibliography points out that the soil moisture has a determinant effect on the quality of citrus fruits, so Levy et al. (1979) used parameters of grapefruit fruit quality to diagnose the degree of water stress. On the other hand, it has been demonstrated that moderate water stress can improve the fruit quality in certain fruit trees (Goldhamer, 1989).

In general, the parameter of fruit size is associated to the variables of weight, equatorial diameter and volume (Bain, 1985).

Materials and equipment

- Balance, accurate to 1 mg.

Procedure

To determine the fruit weight, when weighing the production, the number of fruits for each tree will also be counted, so that using the ratio “weight of tree production/ number of fruits per tree” the mean weight of the fruits for each tree can be obtained and thus that of the different treatments.

The fruits are weighed in a weighing balance, accurate to ± 0.01g. The mean weight per fruit is obtained by dividing the weight of the sample by the number of fruits.

References

Bain, J., 1985. Morphological, anatomical and physiological changes in the developing fruit of the Valencia orange, Citrus sinensis L. Osbeck. Australian Journal of Botanic 6, 1-24.

Levy, Y., Shalhevet, J., Bielorai, H., 1979. Effect of irrigation regime and water salinity on grapefruit quality. Journal of the American Society for Horticultural Science 104, 356–359.

Goldhamer, D.A., 1989. Drought Irrigation Strategies for Deciduous Orchards. Cooperative Extension. University of California, Div. Agric. and Natural Resources. Publication nº 21453, 15 pp.

1.2.2. Total soluble solids

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Importance and applications

The Brix scale is used in the agri-food sector to measure the approximate amount of sugars in fruit juice, wine or processed liquids within the agri-food industry, since in reality what is in fact determined is the total soluble solids (TSS) content. This indicator is used to monitor in situ the evolution of fruit ripening and the optimum moment for the harvest. In this way, the fruit TSS content is an indicator which allows us to know the juice's organoleptic properties.

Principle

To estimate the sugars content in citrus the value of the total soluble solids has habitually been used, since between 75 and 85% of the TSS of orange, mandarin, grapefruit, and lemon juices are sugars (Agustí, 2003).

With regard to this, Hagenmainer and Baker (2004) state that the flavour of the fruits is related with the TSS. The TSS and titratable acidity (TA) are important elements in the estimation of the flavour and nutritional quality of citrus (Li et al., 2012).

The increase in TSS and TA in citrus is particularly noteworthy when deficit irrigation strategies (such as controlled deficit irrigation) are applied (Ginestar & Castel, 1996; Gonzalez-Altozano & Castel, 2003; Pérez-Pérez et al., 2009). However, in some cases of water deficit, there may be a greater increase in the acidity than in the sugars, thus diminishing the sugars/acidity ratio and therefore, reducing the fruit quality (Maotani & Machida, 1977; Mougheith et al., 1977; Levy et al., 1978 and 1979).

Hardy and Sanderson (2010) mentioned that the soluble solids content increases principally due to the accumulation of sucrose, in the ripening phase. The same behaviour was reported by Agustí et al. (2003), who pointed out that in early ripening varieties, the content in sugars increases rapidly and the fruits continue ripening when the temperature falls (in sub-tropical regions); but in late varieties the ripening occurs when the temperature tends to rise, and the sucrose content increases relatively little in the fruit (Agustí et al., 2003).

Materials and equipment

- Manual refractometer ATAGO N-1E.

Illustration 2. Manual refractometer manual

Procedure

For this case, the determination of the TSS will be carried out in a small portion of juice using a manual refractometer. The refractometer measures the refraction index, which indicates the proportion of a bright light that is delayed upon passing through a liquid (in this case juice), and has a scale where this refraction index is directly observed, expressed generally as ºBrix or % TSS. In this case, the TSS concentration of the juice will be expressed in ºBrix. The relationship between TSS and sugars corresponds to a solution of sucrose at 1% and at 20ºC has one degree Brix.

References

Agustí, M., 2003. Citricultura. Eds. Mundi-Prensa. 422 pp.

Ginestar, C. y Castel, J.R., 1996. Response of young 'Clementine' citrus trees to water stress during different phenological periods. Journal of Horticultural Science 71, 551–559.

González-Altozano, P., Castel, J.R., 2003a. Riego deficitario controlado en 'Clementina de Nules'. I. Efectos sobre la producción y la calidad de la fruta. Spanish Journal of Agricultural Research 1, 81–92.

Hagenmainer RD, Baker RA., 2004. Quality of fresh citrus fruit. Quality of fresh and processed foods. Kluwer academic.

Li, Q., Wu, F., Li, T., Su, X., Jiang, G., Qu, H., Jiang, Y., Duan, X. 2012. Methylcyclopropene extends the shelf-life of 'Shatangju' mandarin (Citrus reticulate Blanco) fruit with attached leaves. Postharvest Biol Tec 67, 92–95.

Maotani, T., Machida, Y. 1977. Studies on leaf water stress in fruit trees. VII. Effects of summer water potential on Satsuma mandarin trees on fruit characteristics at harvest time. Journal of the Japanese Society for Horticultural Science 46, 145–152.

Pérez-Pérez, J.G., Robles, J.M., Botía, P. 2009. Influence of deficit irrigation in phase III of fruit growth on fruit quality in 'lane late' sweet orange. Agricultural Water Management 96, 969–974.

1.2.3. Juice pH

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Importance and applications

The pH is an indicator of the juice quality that allows us to know the organoleptic properties of the fruit. This indicator, together with the acidity, is one of the most important to assess the fruit quality given that it is closely related with the content of acids present, the capacity for microbial proliferation in conservation (low values will enable a longer useful life) since it will act on the fruit at physiological level as a natural physiological barrier against microbial action.

Principle

The pH value is used as an indicator of the acid content that exists in a specific food; the value varies between 0 and 14. In this way when a food or drink presents a pH value lower than 7 it is considered acid. The fruit quality is of vital importance when it is directed at fresh consumption. The bibliography points out that the soil moisture has a determinant effect on the quality of citrus fruits, so Levy et al. (1979) used parameters of grapefruit fruit quality to diagnose the degree of water stress. On the other hand, it has been demonstrated that moderate water stress can improve the fruit quality in certain fruit trees (Goldhamer, 1989).

Materials and equipment

- pH meter (multiparameter) PC 80+ DHS STIRRER Bench With Cell VPT 80-1 and Standard electrode

Procedure

To determine the pH of juice, after the harvest, between 10 and 20 fruits per repetition will be randomLy selected and taken to the laboratory. In the laboratory, the juice will be obtained by means of a squeezer. The juice from these fruits will then in part be placed in a beaker and the multiparameter pH-meter will be used to obtain a direct measurement of the juice obtained, for each repetition.

References

Levy, Y., Shalhevet, J., Bielorai, H., 1979. Effect of irrigation regime and water salinity on grapefruit quality. Journal of the American Society for Horticultural Science 104, 356–359.

Goldhamer, D.A. 1989. Drought Irrigation Strategies for Deciduous Orchards. Cooperative Extensión. University of California, Div. Agric. and Natural Resources. Publication nº 21453, 15 pp.

Parámetros de calidad interna de hortalizas y frutas en la industria agroalimentaria. Grupo cooperativo cajamar. Septiembre 2014. Número 5.

1.2.4. Titratable acidity

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Importance and applications

The pH is an indicator of the juice quality that allows us to know the organoleptic properties of the fruit.

Principle

The total acidity of the juice is expressed as the number of grams of acids contained in one litre of the juice, evaluating the predominant acid. In citrus juice the predominant acid is citric acid (Agustí, 2003).

The acidity can be affected by multiple variables, amongst which is the amount of irrigation applied to the crop.

According to Davies and Albrigo (1994), the organic acids significantly contribute to the total acidity of the juice, with citric acid being the predominant organic acid (70-80% of the total). The organic acids are considered to be an important source of acid flavour in the fruit and a source of energy in the plant cell (Landanilla, 2008).

The acids generally decrease during ripening, given that they can be used as respiratory substrates or converted into sugars, although they are also used for the formation of aromatic and flavour compounds (Cañizares et al., 2003; Landanilla, 2008). In the fruit ripening phase, the free acids progressively decrease as a consequence, fundamentally of a dilution process (Agustí et al., 2003), which happens as the fruit grows in size and in juice content (Landanilla, 2008). It is important to point out that the titratable acidity is commonly used as a component to calculate the ripeness index, more than as an independent parameter (Acevedo, 2008).

Materials and equipment

- Burette
- NaOH
- Beaker
- Phenolphthalein

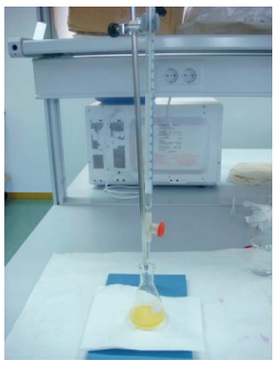

Figure 27.1. Detail of a burette

Procedure

To determine the titratable acidity of the juice, after the harvest, between 10 and 20 fruits will be taken per repetition, selected randomLy and these will be taken to the laboratory for chemical analysis.

The titratable acid (TA) will be determined by acid-base evaluation, neutralising the juice's acidity filtered with sodium hydroxide (NaOH) 0.1 N until a final pH of 8.2 is reached. Phenolphthalein is used as indicator (2- 3 drops). The titratable acidity as a function of the dominant acid will be calculated from the mL of NaOH consumed in the assessment.

Calculations

With V being the volume of NaOH 0.1N in mL consumed in the assessment and V1 the volume of juice employed in the dissolution.

$$Acidity\ (g \cdot L^{-1}) = \frac{V \cdot 0.1 \cdot \frac{192}{3}}{V_1}$$

References

Agustí, M., 2003. Citricultura. Eds. Mundi-Prensa. 422 pp.

Davies, F.S., Albrigo, L.G., 1994. Citrus. CAB International, Wallingford, U.K. 254p.

Landanilla, M.S. 2008. Fruit morphology, anatomy and phisiology. Capitulo 2-7. Citrus fruit. Biology, Tecnhonolgy, and Evaluation. First edition. Academic Press. India.

Cañizares, A., Puesme, R., Laverde, D., 2003. Determinación de la curva de crecimiento y desarrollo del fruto de la lima persa (Citrus latifolia Tanaka) en el estado de Monagas. XV Congreso Venezolano de Botánica. pp:54-55.

Agustí, M., Martínez-Fuentes, A., Mesejo, C., Juan, M., Almela, V., 2003. Cuajado y Desarrollo de los Frutos Cítricos. GENERALITAT VALENCIANA. Serie Divulgación Técnica No. 55:1-82.

Acevedo, Y.P., 2008. Eventos fisiológicos asociados a la madurez y calidad de frutos cítricos en Cuba y su relación con los productos transformados de la industria. Instituto de Investigaciones en Fruticultura Tropical. FAO: 1-21

1.2.5. Percentage of juice

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Importance and applications

This method provides information by means of the ratio of juice weight/fruit weight. It is an indicator of the fruit quality and additionally allows us to know other parameters such as the total titratable acidity, total soluble solids among others.

Principle

The fruit quality is of vital importance when the production is for fresh consumption. The bibliography points out that the soil moisture has a determinant effect on the quality of citrus fruits, so Levy et al. (1979) used parameters of grapefruit fruit quality to diagnose the degree of water stress. On the other hand, it has been demonstrated that moderate water stress can improve the fruit quality in certain fruit trees (Goldhamer, 1989).

With regard to the fruit composition, it is known that water stress has an influence on reducing the juice content and increases the skin thickness in grapefruit (Levy et al., 1979), lemon Verna (Sánchez-Blanco et al., 1989), Clemenules mandarin (González-Altozano & Castel, 2003a) and Lane late orange (Pérez-Pérez et al., 2009). According to Orduz-Rodríguez et al. (2006), the juice contents are considered high for mandarin when they are above 27.4% (value recorded for mandarin Ponkan). Likewise, for oranges and tangelos the minimum percentage accepted in fruit destined for juice making must be over 40% (Orduz Rodríguez et al., 2011).

Materials and equipment

- Squeezer (Mod. 4, 220V, Lomi)
- Balance, accurate to 1 mg
- Nylon muslin of 1 µm

Procedure

After the harvest, between 10 and 20 fruits will be randomLy selected per repetition and taken to the laboratory for chemical analysis. In this case, said fruits will be weighed using a “sartorius AX623” weighing balance, accurate to 1 mg. Once the fruits have been weighed then the juice will be extracted. Subsequently, the juice obtained from these fruits will be weighed and the percentage weight of juice for each repetition will be obtained using the ratio “juice weight/fruits weight *100”.

The juice extraction is performed manually, using a squeezer (Mod. 4, 220V, Lomi). The juice is separated

from the pulp by sieving through a nylon muslin of 1 µm. In this way, two fractions of juice are obtained, that which is called filtered, which is what has passed through the muslin by gravity, and the syphoned, which is what is retained by the pulp, then separated by natural pressure. The total juice volume is obtained by summing the filtered and syphoned volumes.

Figure 28.1. Detail of the juice extraction

Calculations

The total volume of the juice is obtained by means of the sum of the volume filtered and syphoned. The ratio “juice weight/ fruits weight *100” will give the percentage in weight of juice obtained for each repetition.

References

Levy, Y., Shalhevet, J., Bielorai, H., 1979. Effect of irrigation regime and water salinity on grapefruit quality. Journal of the American Society for Horticultural Science, 104: 356–359.

Pérez-Pérez, J.G., Robles, J.M., Botía, P., 2009. Influence of deficit irrigation in phase III of fruit growth on fruit quality in ‘lane late’ sweet orange. Agricultural Water Management, 96: 969–974.

González-Altozano, P., Castel, J.R., 2003a. Riego deficitario controlado en ‘Clementina de Nules’. I. Efectos sobre la producción y la calidad de la fruta. Spanish Journal of Agricultural Research 1, 81–92.

Goldhamer, D.A., 1989. Drought Irrigation Strategies for Deciduous Orchards. Cooperative Extensión. University of California, Div. Agric. and Natural Resources. Publication nº 21453, 15 pp.

1.2.6. Degree of acidity

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Importance and applications

The determination of free fatty acids in olive oils is an important quality factor and has been widely used as a criterion for the classification of olive oil into various commercial categories.

Principle

The degree of acidity of oil is the percentage of free fatty acids in oil. Any fat from the chemical point of view is composed of triglycerides, i.e. esters of fatty acids and glycerine. The hydrolysis reaction causes the breakdown of these, losing fatty acids and giving diglycerides and monoglycerides. In vegetable oils, this percentage is expressed as if all free acids were oleic acid (CHO).
The method consists of dissolving a sample in a mixture of solvents and the free fatty acids present titrated using an ethanolic solution of potassium hydroxide.

Reagents

- Diethylether
- Ethanol 96%
- Potassium hydroxide
- Phenolphthalein

Materials and equipment

- 250 mL conical flask
- Analytical balance
- 10 mL burette, graduated in 0.05 mL

Procedure

a. Dissolve the sample in 50 to 150 mL of the previously neutralised mixture of diethyl and ethanol.
b. Titrate while stirring with the 0.1mol L^{-1} solution of potassium hydroxide (see Note 2) until the indicator changes (the pink colour of the phenolphthalein persists for at least 10 seconds).

Calculations

Acidity as a percentage by weight is equal to:

$$V * c * \frac{M}{1000} * \frac{100}{m} = \frac{V * c * M}{10 * m}$$

where:

V = the volume of titrated potassium hydroxide solution used, in millilitres;

c = the exact concentration in moles per litre of the titrated solution of potassium hydroxide used;

M = the molar weight in grams per mole of the acid used to express the result (= 282);

m = the weight in grams of the sample.

Remarks

- The titrated ethanolic solution of potassium hydroxide may be replaced by an aqueous solution of potassium or sodium hydroxide provided that the volume of water introduced does not induce phase separation.
- If the quantity of 0.1mol/Lpotassium hydroxide solution required exceeds 10 mL, use the 0.5 mol/L solution.
- If the solution becomes cloudy during titration, add enough of the solvents to give a clear solution.

References

Boskou, D., 2006. Olive oil: Chemistry and Technology (2º ed.). Edit. AOCS PRESS. Illinois.

Commission of the European Community., 1991. Regulation 2568/91, On the Characteristics of Olive Oil and Olive-residue Oil and on the Relevant Methods of Analysis.

Commission Implementing Regulation., 2015. (EU) 2015/1833 of 12 October 2015 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis.

1.2.7. Quality of fatty matter

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Importance and applications

Spectrophotometric examination in the ultraviolet radiation can provide information on the quality of a fat, its state of preservation and changes brought about by technological processes.

Principle

The absorption at the wavelengths specified in the method is due to the presence of conjugated diene and triene systems resulting from oxidation processes and/or refining practices. These absorptions are expressed as specific extinctions E1 % 1 cm (the extinction of 1 % w/v solution of the fat in the specified solvent, in a 10 mm cell) conventionally indicated by K (also referred to as “extinction coefficient”).
A sample is dissolved in the required solvent and the absorbance of the solution is measured at the specified wavelengths with reference to pure solvent. The specific extinctions at 232 nm and 268 nm in iso-octane or 232 nm and 270 nm in cyclohexane are calculated for a concentration of 1 % w/v in a 10 mm cell.

Reagents

- During the analysis, unless otherwise stated, use only reagents of recognised analytical grade and distilled or demineralised water or water of equivalent purity.
- Solvent: Iso-octane (2,2,4 trimethylpentane) for the measurements at 232 nm and 268 nm and cyclohexane for the measurements at 232 nm and 270 nm, having an absorbance less than 0.12 at 232 nm and less than 0.05 at 270 nm against distilled water, measured in a 10 mm cell.

Materials and equipment

- A spectrophotometer suitable for measurements at ultraviolet wavelengths (220 nm to 360 nm), with the capability of reading individual nanometric units. A regular check is recommended for the accuracy and reproducibility of the absorbance and wavelength scales as well as for stray light.
- Wavelength scale: This may be checked using a reference material consisting of an optical glass filter containing holmium oxide or a holmium oxide solution (sealed or not) that has distinct absorption bands.

  The reference materials are designed for the verification and calibration of the wavelength scales of visible and ultraviolet spectrophotometers having nominal spectral bandwidths of 5 nm or less. The measurements are carried out against an air blank over the wavelength range of 640 to 240 nm, according to the instructions enclosed with the reference materials. A baseline correction is performed with an empty beam path at every slit width alteration. The wavelengths of the standard are listed in the certificate of the reference material.

- Absorbance scale: This may be checked using commercially available sealed reference materials consisting of acidic potassium dichromate solutions, in certain concentrations and certified values of absorbance at its λmax (of 4 solutions of potassium dichromate in perchloric acid sealed in four UV quartz cells to measure the linearity and photometric accuracy reference in the UV). The potassium dichromate solutions are measured against a blank of the acid used, after baseline correction, according to the instructions enclosed with the reference material. The absorbance values are listed in the certificate of the reference material.
- Another possibility to check the response of the photocell and the photomultiplier is to proceed as follows: weigh 0.2 g of pure potassium chromate for spectrophotometry and dissolve in 0.05 N potassium hydroxide solution in a 1000 mL graduated flask and make up to the mark. Take precisely 25 mL of the solution obtained, transfer it to a 500 mL graduated flask and dilute up to the mark using the same potassium hydroxide solution.
  Measure the extinction of the solution thus obtained at 275 nm, using the potassium hydroxide solution as a reference. The extinction measured using a 1 cm cuvette should be 0.200 ± 0.005.
- Rectangular quartz cuvettes, with covers, suitable for measurements at ultraviolet wavelengths (220 to 360 nm) having an optical path-length of 10 mm. When filled with water or other suitable solvent the cuvettes should not show differences between them of more than 0.01 extinction units.
- One-mark volumetric flasks, capacity 25 mL, class A.
- Analytical balance, capable of readings to the nearest 0.0001 g.

Procedure

a. The sample must be perfectly homogeneous and without suspended impurities. If not, it must be filtered through paper at a temperature of approximately 30°C.
b. Weigh accurately approximately 0.25 g (to the nearest 1 mg) of the sample thus prepared into a 25 mL graduated flask, make up to the mark with the specified solvent and homogenise. The resulting solution must be perfectly clear. If opalescence or turbidity is present, filter quickly through paper.
c. If necessary, correct the baseline (220-290 nm) with solvent in both quartz cells (sample and reference), then fill the sample quartz cell with the test solution and measure the extinctions at 232, 268 or 270 nm against the solvent used as a reference.
d. After measuring the absorbance at 268 or 270 nm, measure the absorbance at λmax, λmax + 4 and λmax– 4. These absorbance values are used to determine the variation in the specific extinction (ΔK).

Calculations

Record the specific extinctions (extinction coefficients) at the various wavelengths calculated as follows:

$$K\lambda = \frac{E\lambda}{c * s}$$

where:
Kλ = the specific extinction at wavelength λ;
Eλ = the extinction measured at wavelength λ;

c = the concentration of the solution in g/100 mL;
s = the path length of the quartz cell in cm;
Variation of the specific extinction (ΔK)
The variation of the absolute value of the extinction (ΔK) is given by:

$$\Delta K = KmV - \left(\frac{K\lambda m - 4 + K\lambda + 4}{2} \right)$$

where Km is the specific extinction at the wavelength for maximum absorption at 270 nm and 268 nm depending on the solvent used.

Remarks

- Generally, a mass of 0.25 to 0.30 g is sufficient for absorbance measurements of virgin and extra virgin olive oils at 268 nm and 270 nm. For measurements at 232 nm, 0.05 g of sample are usually required, so two distinct solutions are usually prepared. For absorbance measurements of olive pomace oils, refined olive oils and adulterated olive oils, a smaller portion of sample e.g. 0.1 g is usually needed due to their higher absorbance.
- The extinction values recorded must lie within the range 0.1 to 0.8 or within the range of linearity of the spectrophotometer which should be verified. If not, the measurements must be repeated using more concentrated or more dilute solutions, as appropriate.
- λmax is considered to be 268 nm for isooctane used as solvent and 270 nm for cyclohexane.

References

Boskou, D., 2006. Olive oil: Chemistry and Technology (2º ed.). Edit. AOCS PRESS. Illinois.
Commission of the European Communities, Regulation 2568/91., 1991. On the Characteristics of Olive Oil and Olive-residue Oil and on the Relevant Methods of Analysis.
Commission Implementing Regulation (EU) 2015/1833 of 12 October 2015 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis (2015).

1.2.8. Essential oils in aromatic species

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Importance and applications

Essential oils (EOs) are complex mixtures of volatile compounds extracted from plants, with a great interest in areas such as medicine due to their biocidal activities and medicinal properties, the pharmaceutical field where they are part of pharmaceutical base formulations, the food industry for food preservation and the textile industry where microspheres of EOs are used to improve the properties on textiles (Burt, 2004; Martins et al., 2014). EOs are highly volatile compounds, which are protected from external factors through encapsulation processes, and thus increasing their action duration (Hong & Park, 1999).

Principle

Supercritical fluid extraction (SFE) is the most widely used technique to isolate EOs from plants (Munshi & Bhaduri, 2009). The extraction is generally carried out using CO_2 as a solvent at temperatures and pressures above the critical point of CO_2 (7.4 MPa and 31.1°C or near to this region). CO_2 is a good solvent for non-polar compounds, but it has a lower polar compounds affinity. In this case, a co-solvent (ethanol or other low molecular weight alcohols) can be added to CO_2, and thus improve its power over polar molecules. SFE is a semi-continuous process, where the solvent flows through the particles of vegetable material in the extractor and dissolves soluble substances. The supercritical solvent with the solutes extracted flows through a depressurisation valve to a separator (S1) where the pressure is lower, and thus the extracts are separated from the CO_2 and collected. In order to quantify the essential oil composition of the supercritical extract, the different fractions are analysed with a GC-MS (Fornari et al., 2012; Sovová, 2012). The method described here is based and modified from Fornari et al. (2012).

The chemical compounds of EOs are classified as terpenes and terpenoids (carbures, alcohols, esters, phenols, ethers, ketone and aldehydes), aromatic compounds (phenols, aldehydes, alcohol, metoxiderivates) and sulphur or nitrogen-containing compounds (thiosulfinates, allyl sulphides, pyracines, isothiocyanates) (Dima & Dima, 2015).

Reagents

- Liquid nitrogen
- Standards (for example thymol, linalool, terpineol…)
- Ethanol (HPLC grade)
- CO_2 (N38 quality)
- Helium (99.99%)

Materials and equipment

- Mill
- Sieve (1000-500 µm)
- Balance
- Supercritical fluid extractor
- GC-MS equipped with a split/splitless injector, electronic pressure control, auto injector, mass spectrometer detector, GC-MS solution software, and a column of 30 m x 0.32 mm ID and 0.25 µm phase thickness.

Procedure

a. Fresh plants are air dried in the shade or at room temperature (20°C).
b. The plant is ground into powder using mill under cryogenic conditions.
c. The ground plant material is passed through a sieve (1000-500 µm).
d. The samples are stored at – 20°C until use.
e. Extraction in carried out using supercritical extraction equipment, with a 2 l cylinder extraction cell and 2 different separators each with a capacity of 0.5 l, with independent control of temperature and pressure.
f. Cryogenically milled and sieved plant material is placed into the extraction vessel (0.6 kg).
g. The extraction takes place at 30 MPa of pressure and 40°C of temperature, with a flow rate of 2.4 kg h^{-1}.
h. In the first separator (S1), the pressure is maintained at 10 MPa and S2 at ambient pressure (0.1 MPa).
i. The solid fractions collected within S1 and S2 are recovered and placed in vials.
j. Both separators are washed with ethanol in order to ensure a precise determination of extraction yield. The residual material recovered in each separator is added to its corresponding solid fraction.
k. Ethanol is eliminated by evaporation (35°C).
l. The obtained solid samples are kept under N_2 at – 20°C in the dark until analysis.
m. Supercritical extract is analysed by GC-MS. Helium is used as a carrier gas at a flow of 1 mL min^{-1}. Oven temperature is first programmed at 60°C (4 min), then increased to 106°C (2.5°C min^{-1}), from 106°C to 130°C (1°C min^{-1}) and finally from 130°C to 250°C (20°C min^{-1}), this temperature is kept constant for 10 min.
n. Sample injection (1 µL) is performed in split mode (1:20).
o. The inlet pressure of helium is 57.5 KPa, while the interface temperatures are 230 and 280°C, respectively.
p. The mass spectrometer is used in TIC mode and samples are scanned from 40 to 500 amu.
q. Chemical compounds of EOs (linalool, eugenol…) are identified by comparison with standard mass spectra obtained in the same conditions, and compared with the mass spectra from the library. The rest of the compounds are identified by comparison with the mass spectra from the library, and by their linear retention index.
r. Finally, a calibration curve is required to quantify chemical compounds of EOs.

References

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods-a review. Int. J. Food. Microbiol, 94, 223-253.

Dima, C., Dima, S., 2015. Essential oils in foods: extraction, stabilization, and toxicity. Curr. Opin. Food. Sci. 5, 29-35.

Fornari, T., Vicent, G., Vázquez, E., García-Risco, M.R., Reglero, G., 2012. Isolation of essential oil from different plants and herbs by supercritical fluid extraction. J. Chromatogr. A. 1250, 34-48.

Hong, K., Park, S. 1999. Melamine resin microcapsule containing fragant oil: synthesis and characterization. Mater. Chem. Phys. 58, 128-131.

Martins, I.M., Barreiro, M.F., Coelho, M., Rodrigues, A.E., 2014. Microencapsulation of essential oils with biodegradable polymeric carriers for cosmetic applications. Chem. Eng. J. 245, 191-200.

Munshi, P., Bhaduri, S., 2009. Supercritical CO_2: a twenty-first century solvent for the chemical industry. Curr. Sci. 97, 63-72.

Sovová, H., 2012. Modeling the supercritical fluid extraction of essential oils from plant materials. J. Chromatogr. A. 1250, 27-33.

1.2.9. Sugar content

Cord-H. Treseler

Weingut Dr. Frey (WDF)

Importance and applications

During maturity the sugar content of grape juice increases. Measuring the sugar content is one main method to define the maturity stage and quality of the grape juice.

Principle

The sugar content in grape juice can be measured as the density of the juice, which is mainly influenced by the content of sugar (must scales) or by refraction through a liquid (refractometer). Must scales are not usually used in the field – a higher quantity of juice is needed.

Reagents

- Water for cleaning
- Grape juice

Materials and equipment

- Refractometer (handheld for field observations)
- Paper towel or tissue

Procedure

a. Observed vine plants (6 per variant) will be tested weekly when softening of berries begins (BBCH 85)
b. Select a single berry (6 berries per vine plant)
c. Open refractometer (Fig. 32.1) and squeeze some drops of grape juice on the prism
d. Close the lid and look through optical lens and read the relevant scale (Brix, °Oechsle, …)
e. Open the lid for cleaning with a moist towel and dry back before testing the next berry

Calculations

The output data are - depending on the scaling – to be converted to the unit Brix (1g saccharose per 100g must at 20°C; %mas) (Meidinger et al., 2000).

References

Meidinger, F., Blankenhorn, D., Funk, E., 2000. Der Winzer. Eugen Ulmer Verlag, Stuttgart. ISBN 3-8001-1175-6, 96.

http://www.enotecnochimica.it/tabelle/hydrometer-scale.htm, 18.04.2018

1.2.10. Thousand kernel weight

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Importance and applications

The Thousand Kernel Weight (TKW) is one of the wheat quality parameters because it gives important information about the wheat's millability potential (i.e. extraction rate) (Raggiri et al., 2016; Posner et al., 1997).
In fact, wheat kernels with a similar size distribution but different TKW indicates that the heavier kernels have a higher percentage of endosperm than the lighter ones (Posner et al., 1997).

Principle

The TKW is determined using a semiautomatic counting instrument to count 1000 wheat kernels and then weigh their mass.

Reagents

- Not required

Materials and equipment

- Electronic kernel counter
- Two-decimal place precision scale

Procedure

a. Sample preparation
   The grain to be measured must be clean, free from impurities and broken kernels.
   Weigh a pre-sample of wheat of about 30 g.

b. Determination
   Select 1000 kernels from the pre-sample of wheat by the electronic kernel counter.
   Weigh the mass (P) of the selected 1000 kernels.

Calculations

The 1000 kernel weight (P) is expressed as g.

Remarks

- The TKW can be corrected to a dry basis or any moisture basis.

References

Raggiri, V., Barron, C., Abecassis, J., Lullien-Pellerin, V., 2016. In-Depth Study of Durum Wheat Grain Tissue Distribution at Milling. Cereal Chem., Vol. 93, N° 3, 219 – 255.
Posner, E.S, Hibbs, A.N., 1997. In Wheat Flour Milling. American Association of Cereal Chemists (AACC, 3340 Pilot Knob Road, St. Paul, MN 55121, U.S.A.

1.2.11. Test weight

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Importance and applications

Test weight (TW) is a rough measure of the density of wheat (i.e. bulk density) (Pomeranz, 1988). This value is one of the factors of the market value of wheat, because in general it is directly related to the extraction rate of the milling products (i.e. semolina). Sound, clean, vitreous wheats with low moisture content tend to give the highest TW (Pomeranz, 1988). One of the big disadvantages of using TW in the trade and processing today is that the determined weight cannot be corrected to a dry or a fixed-moisture basis (Posner & Hibbs, 1997).

Principle

The TW is the weight of a mass of wheat placed in a container (Fig. 34.1) with a defined volume and expressed as kilograms per hectolitre (kg hL^{-1}). The measurement is performed using a grain scale (Fig. 34.2).

Figure 34.1. Test weight equipment.

The determination is affected by several conditions such as the levels of foreign material as well deformed or broken wheat, moisture level of the wheat, wheat shape and roughness, ambient condition, operator efficiency, and equipment conditions (Posner et al., 1994; Madurei, 1995).

Figure 34.2. Test weight grain scale.

Reagents

- Not required

Materials and equipment

- Scale for determining mass per storage volume.
- Technical scales.

Procedure

a. Sample preparation

The grain to be measured must be free from impurities, and must be at around the same temperature as the room in which the measurements will be taking place. It must be air dried, or in other words, must be in hygroscopic equilibrium with the air in the space in which the measurements will be taking place. The relative humidity of the air in this space must not exceed 60%.

b. Instrument preparation

Before beginning the analysis, check that the instrument is in full working order: screw the stem which supports the arm onto the tank, which should be placed in a level position, then check that the central bar is not touching the sides. Place the 100 g weight (supplied) in the hanging grain container, and set the sliding weight on the graduated scale to 100. In this position, the needle of the scale must be perfectly vertical.

If this is not the case, turn the screw, positioned at the top of the graduated shaft, to lighten or weigh down the shaft itself.

c. Determination

Position the hopper over the weighing basket and inspect it to ensure that it is vertical. Place the scraper blade in the starting position.

Pour at least 3 litres of grain into the filling container and through the tube, which should be closed at one end, empty this completely, pouring it into the filling hopper, after ensuring that the hinged door has blocked off the tapered outflow connector.

Pull the bolt to open the door and allow the grain to flow out into the capacity measure. The collar is designed to prevent external factors from affecting the grain outflow.

When the capacity measure is full, activate the scraper blade. This instrument features sharp edges, in order to cut through the grains that have become stuck on the edges of the capacity measure which could prevent the scraping from being completed in a uniform manner.

When the scraper blade has reached its end position, the hopper should be removed from the capacity measure basket, and excess product left over above the scraper blade should be removed and placed on the scale.

The contents of this should be weighed to within ± 5 g.

The same weighing operation can also be performed using a technical scale, ensuring that the

container has been calibrated (i.e. tared).

If proceeding with another measurement of the same sample, the grain from the capacity measure should be thoroughly mixed with that from the holding tank.

Calculations

Using the tables attached to the instrument, which refer to the types of grain being measured (i.e. wheat, barley or rye), read the mass per storage volume value in kg hL^{-1} for the grain in question.
The result is expressed in kilograms per hectolitre.

Remarks

- The result is expressed to two decimal places.

References

Pomeranz, Y., 1988. Wheat, Chemistry and Technology. 3rd Edition. Vol I and II. American Association of Cereal Chemists, USA.
Posner, E.S, Hibbs, A.N., 1997. Wheat Flour Milling. American Association of Cereal Chemists, USA.
Madureri E. Determinazione del peso ettolitrico nei cereali. Tecnica Molitoria. Dicembre 1995, 1335 – 1340.

1.2.12. Grain moisture

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Importance and applications

Like other factors of wheat quality (i.e. protein, ash, falling number) the moisture is also greatly influenced by the growing and harvesting conditions (Pomeranz, 1988). The original moisture of the wheat after the harvest affects its storability at the elevator (5). Normally, water is added to the wheat (i.e. tempering) before milling in order to bring the moisture to between 14% and 17% (5). The water addition enhances the difference (i.e. toughness and friability) of the wheat's parts (i.e. endosperm, bran and germ) making milling possible (Posner & Hibbs, 1997).

Principle

The moisture is the loss in weight, expressed as a percentage, of a product as a result of evaporation in the oven at a defined temperature. This product is dried in a thermostatic oven at 130°C at atmospheric pressure until a constant weight is obtained.

Reagents

- Not required

Materials and equipment

- Thermostatic oven, natural air convection
- Analytical scales, accuracy 0.1 mg or 1 mg
- Laboratory miller
- Mortar, grater
- Perforated porcelain plate dryer containing a dehydrating product
- Glass weighing bottle
- Sieve 1000 µm, 500 µm.
- Clamp
- Jar with cap

Procedure

a. Sample preparation

a.1. - Cereal in grains

Coarsely grind the sample so that the 0.5 mm sieve retains approx. 50-60% of the product and the 1 mm sieve no more than 10% of the product.

Take care not to overheat the product; it is advisable not to grind more than 100 g at a time.

The ground product must be immediately collected in a jar closed with a cap and may only be used after allowing 30 seconds for it to return to room temperature.

a.2. – Semolina

Coarsely grind the sample so that the 0.5 mm sieve retains approx. 35-50% of the product and the

1 mm sieve no more than 10% of the product.
Take care not to overheat the product; it is advisable not to grind more than 100 gr at a time.
The ground product must be immediately collected in a jar closed with a cap and may only be used after allowing 30 seconds for it to return to room temperature.

b. Determination

Weigh the weighing bottle (P_0) previously calibrated on analytical scales.
Weigh the following quantities of ground product (P_1): 10 gr approx.
Place the weighing bottle containing the sample and with open lid in the thermostatic oven at 130°C.
Introduce the weighing bottle as quickly as possible to prevent the oven temperature dropping too much.
Leave to dry in the oven for the minimum times listed below and in any case until constant weight is achieved: 90 minutes
Remove the weighing bottle from the oven using the clamp.
Close the lid of the weighing bottle.
Place in a drying unit to cool for at least 30 minutes and in any case to room temperature.
Weigh the sample after drying (P_2).

Calculations

Calculate the moisture content per 100 g of substance with the following equation:

$$Moisture\ \% = [(P_1 - P_2)/ (P_1 - P_0)] \times 100$$

where:
P_0 = the total weight (g) of the dish and lid after calibration
P_1 = the total weight (g) of the sample, capsule and lid before oven drying
P_2 = the total weight (g) of the sample, capsule and lid after oven drying

Measurement uncertainty for cereals in grain and their flours (semolina, flour), pasta
Repeatability
The difference between the values obtained from two consecutive determinations performed simultaneously or in rapid succession by the same analyst should not exceed:
0.15 g of moisture per 100 g of sample

Remarks

- The result is expressed to 2 decimal places.

References

Pomeranz, Y., 1988. Wheat, Chemistry and Technology. 3rd Edition. Vol I and II. American Association of Cereal Chemists, USA.
Posner, E.S, Hibbs, A.N., 1997. Wheat Flour Milling. American Association of Cereal Chemists, USA.

1.2.13. Grain protein

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Importance and applications

The quantity of protein is, together with its quality, one of the basic parameters for defining wheat's commercial value and the intended use of its relative milling products. The average content of the durum wheat protein is around 12 – 14% (dry matter basis). The protein level is mainly genetically controlled (i.e. variety) but it is also influenced by the environmental conditions and the agronomic practices applied (i.e. nitrogen fertilisers dosage) (Pomeranz, 1988).

Principle

The method describes the determination of the nitrogenous substances. Nitrogenous substances are the content of organic nitrogenous compounds in the product analysed, calculated by multiplying the corresponding nitrogen content by a conventional factor. The procedure uses the technique described by J.B. Dumas: the sample is burnt in a suitable high-temperature system in the presence of oxygen and a catalyst to reduce nitrogen oxides to molecular nitrogen. The resulting gases are selectively removed by passing them in a flow of helium through traps for residual oxygen, water and carbon dioxide. The nitrogen generated by the sample passes intact and reaches a gas chromatographic thermal conductivity detector.

Reagents

- Helium grade 5.0 (99.999% pure) in cylinders.
- Technical compressed air (99.95%).
- Oxygen grade 5.0 (99.999% pure) in cylinders.
- Quartz wool (wear latex gloves and do not inhale the powder).
- Reduced copper.
- Copper oxide.
- Chromosorb (or alumina) for use with liquid samples.
- VHT combustion catalyst.
- VLT combustion catalyst.
- Molecular sieves.
- Sicapent, with water absorber indicator; comparable to phosphorus pentoxide.
- Standard with a known nitrogen content: EDTA (ethylenediaminetetraacetic acid).

Materials and equipment

- Laboratory miller (for solid samples).
- Sieve with 1 mm mesh.
- Technical scales - precision: 0.1 g.

- "Ultraturrax" homogeniser (for liquid samples)
- Miller with plate with holes of diameter no more than 4 mm (for meat)
- NDA 701 Dumas unit VELP.
- NDA 701 software for data management and acquisition.
- Spare parts for NDA 701 system (quartz shuttles, quartz and glass tubes, seals, ...) used as described instruction manual.
- Tin or aluminium crucibles.

Procedure

a. Sample preparation

a.1. - Cereal in grains

Grind the sample roughly and, if necessary, grind it again so that at least 90% of the product passes through the sieve.

Carefully homogenise this material.

a.2. - Semolina

Grind the sample finely so that at least 90% of the ground material passes through the sieve.

Homogenise thoroughly.

b. Determination

b 1.1. - Weight of the sample (P)

The test rate involves quantities varying between 40 to 70 mg for solids and between 100 to 150 for liquids. Methods are pre-set in the software for various types of food product indicating the maximum weighing limit.

The test rate must be as representative as possible of the sample, therefore it is recommended to homogenise it well and repeat the analysis at least three times.

For products that are not very homogeneous, the rate should be more substantial, generally more than 100 mg.

Weigh the sample with analytical scales using a metal crucible, which must then be closed tightly, taking great care not to break it.

b 1.2. - Calibration curve construction

The calibration curve is obtained by weighing 7-8 levels of EDTA in tin crucibles, from 0 mg up to about 80 mg, proceeding as described in detail in the instrument manual.

Good practice indicates that every time the instrument is used after a long period of non-use it is advisable to perform a series of blank tests to control the baseline and then re-check response factors using the EDTA standard before starting, during (every 10-12 samples) and at the end of the analytical sequence. Compared to the theoretical value of nitrogen of 9.57% data between 9.47% and 9.67% are acceptable.

If otherwise, repeat the determination of the standard and identify and eliminate potential sources of error.

Calculations

The result is expressed as nitrogen content out of 100 g of substance as such, to two decimal places. Alternatively, the result can be expressed as the content of nitrogenous substances out of 100 g of substance as such by multiplying the nitrogen value by an appropriate factor.

a. Calculation method

The calculation is carried out directly by the NDA 701 software, after creating the calibration curve using the EDTA standard.

b. Assessment of results

Close attention must always be paid to status of the columns and the ash collection insert.
Maintenance is relatively simple and is very clearly described in the instrument's manual; maintenance must be performed very strictly, otherwise there is a risk of obtaining incorrect results.
A number of indicators provide assistance in this context; the first is the form of the nitrogen peak, which must be symmetrical; the second is the reference substance (EDTA), which is frequently inserted during an analytical sequence (start, middle, end) precisely in order to ensure the validity of the results obtained.
A counter will indicate when the insert, catalyst, copper column or Sicapent column need to be replaced; however, the information provided by the software is merely an outline indication, since the wear of these parts depends on the type of samples measured and their quantities.

c. Measurement uncertainty

c 1.1 - Repeatability

The difference between the results of 2 determinations carried out simultaneously or in rapid succession by the same analyst shall be:
Cereals and cereal-based products
(Ministerial Decree 23 July 1994 - Approval of "Official methods for the analysis of cereals and cereal-based products" – Supplement No. 4)
≤ 0.03 in absolute value for N < 3%
≤ 1.0 % in relative value for 3 < N < 6%
≤ 0.06 in absolute value for N > 6%
UNI 10274 - Durum and soft wheat, semolina and flour, foodstuff pasta. Determination of nitrogenous substances. Reference method
≤0.03 per N < 3%
≤0.05 per 3 < N < 6%

c 1.2. - Reproducibility (from BIPEA)

Durum wheat: 0.30% in absolute value
Semolina: 0.25% in absolute value
Soft wheat and flour: 2.8% of the reference value.

Remarks

None

References

Pomeranz, Y., 1988. Wheat, Chemistry and Technology. 3rd Edition. Vol I and II. American Association of Cereal Chemists, USA.

1.2.14. Grain ash

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Importance and applications

The ash content in wheat mainly refers to the presence of minerals in the grain. Wheat typically has an ash content of about 1.5% (Delcour & Hoseney, 2010). The ash level is influenced more by the environment (i.e. geographic area, soil type, climatic conditions during the growth etc.) than by the genetic background (Pomeranz, 1988). However, the ash is not distributed uniformLy in the grain: the inner endosperm is relatively low in ash (about 0.3%), whereas the outer layers (i.e. the bran) may contain as much as about 6% (Pomeranz, 1988).

Several countries have regulations concerning the ash presence of the wheat milling products (i.e. flour and semolina) for food use. Therefore, the ash content in wheat is a very important factor for the milling industries in terms of purchasing specifications (Posner, 1997). Since semolina's ash is correlated with that of the whole kernel, the amount of semolina with a fixed ash content that can be obtained from a given quantity of wheat (i.e. the extraction rate) directly depends on the ash content of the wheat before milling (Posner, 1997).

The curves of the ash extraction (Fig. 37.1) are useful in determining the maximum yield one can obtain in compliance with the ash specifications.

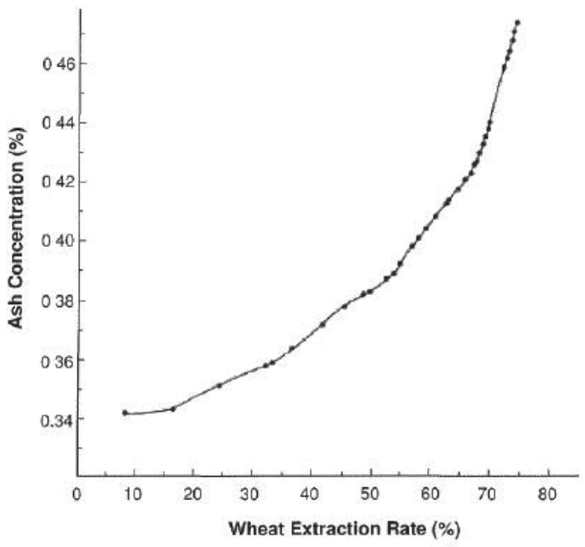

Figure 37.1. Curve showing the ash concentration in a milled product as a function of a total extraction rate (adapted from Delcour et al., 2010)

Principle

The ash content is defined as the residue obtained after ignition in an oxidant atmosphere at 550 ± 10°C under described conditions, until the organic substances are completely combusted to obtain a uniform mass.

It consists of both the original wheat minerals and those chemical elements (i.e. mainly phosphorus and sulphur) initially present in combined forms (i.e. phytic acid, phospholipids, nucleic acids, amino acids) transformed into incombustible compounds (i.e. phosphates and sulphates) through the operative conditions of the analysis (8).

Reagents

- Distilled water or water of at least equivalent quality.

Materials and equipment

- Furnace at 550 ± 10°C
- Furnace at 300°C
- Hot plate or gas burner ring
- Analytical balance, precision 0.1 mg
- Laboratory mill
- Desiccator: containing silica gel or anhydrous calcium chloride
- Capsule: porcelain, platinum, quartz
- Sieve with a nominal mesh opening of 1, 0.5, and 0.3 mm
- Metal tongs
- Heat-resistant metal plate
- Thermostatic oven at 103±2°C
- Pasteur pipette
- Extractor hood

Procedure

a. Sample preparation

Cereals in grain - Mill the sample roughly. If required, mill again without causing heat so that at least 50% of the milled material passes through the 0.5 mm sieve and no more than 10% remains in the 1 mm mesh sieve. Thoroughly mix the sample to a homogenous state.

b. Sample weight

Weigh the capsule which has been calibrated in advance.

Distribute the sample in the capsule, without pressing it, so that it forms a uniform layer.

Weigh about 5 to 10 g of sample (P_1)

The sample amount depends on the estimated amount of ash and on the volume of the sample: for durum wheat and common wheat: 10 g.

c. Determination

c.1. - Pre-ignition: this may be carried out as follows

c.1.1. - Furnace at 300°C

Place the capsule containing the sample in the furnace.

Leave it until completely combusted, this takes approximately 30 minutes.

c.1.2. - Cold furnace

Turn ON the Furnace

Place the capsule containing the sample in the furnace maintaining the hatch open.

As the temperature rises, move the capsule gradually inside the furnace until it is at the back.

Keep the hatch open at all times.

Close the hatch when combustion is complete.

c.1.3. - Hot plate or gas burner ring

Heat the capsule carefully until the material catches fire on the hot plate or gas burner ring.

Do not combust too quickly or material particles will come out.

c.2. - Ignition

At the end of pre-ignition, place the capsule containing the ignited sample in the furnace at 550°C.

If you have carried out pre-ignition in a cold furnace, close the hatch.

Keep igniting until the sample is completely combusted, including all the carbon particles in the residue.

Leave the capsule in the furnace for at least 4 hours and until the weight is constant.

Remove the capsule from the furnace using the metal tongs.

To improve the ignition, when there is a little residue on the bottom of the capsule, remove the capsule from the furnace after about 3 hours.

Place the capsule on a heat-resistant plate and let it cool slightly.

Dampen the content of the capsule with a few drops of distilled water using a Pasteur pipette.

Place the carbon particles in the solution and shake the capsule by hand.

Evaporate the water in an oven at about 100°C.

Place the capsule in the furnace for a further hour.

Remove the capsule from the furnace using the metal tongs.

Place the capsule in the desiccator and cool it for at least 30 minutes.

Weigh the capsule as soon as room temperature is attained (P_2).

Calculations

The ash content, expressed as %, is:

$$Ash\ \% = (P_2 / P_1) * 100$$

$$Ash\ (dry\ matter)\ \% = [(P_2 / P_1)\ x100]* (100/100-U)$$

where:

P_1 = the sample weight, in grams

P_2 = the sample weight after ignition, in grams

U = the % sample moisture

Uncertainty

- Repeatability

  The difference between the results of two single determinations carried out simultaneously or in rapid succession by the same analyst shall not exceed the following value:

  Cereals, legumes and derivate (UNI 2171):

  r = 0.025 (absolute value) for ash content below 1%

  r = 0.034 (absolute value) for ash content between 1.00 and 2.53 %

- Reproducibility

  Differences between values obtained in different laboratories should not be more than:

  Cereals, legumes and derivate (UNI 2171):

  R = 0.064 (absolute value) for ash content below 1%

  R = 0.074 (absolute value) for ash content between 1.00 and 2.53 %

Remarks

- The weights are measured to 4 decimal places.
- The final value is expressed with two decimal places.

References

Delcour J.A., Hoseney R.C., 2010. Principles of Cereals Science and Technology, American Association of Cereal Chemists, USA.

Pomeranz, Y., 1988. Wheat, Chemistry and Technology. 3rd Edition. Vol I and II. American Association of Cereal Chemists, USA.

Posner, E.S, Hibbs, A.N., 1997. Wheat Flour Milling. American Association of Cereal Chemists, USA.

1.2.15. Grain gluten

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Importance and applications

Gluten is a general name for the proteins found in wheat, rye, barley and other cereals. Actually, gluten is a composite of storage proteins termed prolamins and glutelins and stored together with starch in the endosperm (which nourishes the embryonic plant during germination) of various cereal (grass) grains. Gluten gives elasticity to dough, helping it rise and keep its shape and often gives the final product a chewy texture. The wet gluten test provides information on the quantity and estimates the quality of gluten in flour samples. Gluten is responsible for the elasticity and extensibility characteristics of flour dough. Wet gluten reflects protein content and is a common flour specification required by end-users in the food industry (Wheat Marketing Center, Inc., 2004).

Principle

Gluten is a water-insoluble protein complex that forms, by flour starch drag by washing, a very extensible gummy mass. This method is applied for the determination of gluten content of wheat flour and semolina (PANREAC QUÍMICA S.A.).

Reagents

- Distilled water
- Potassium dihydrogen phosphate
- Sodium chloride
- Di-sodium hydrogen phosphate 2-hydrate
- Iodine-resublimed pearls
- A 2% solution of sodium chloride (pH 6.2). Dissolve 200 g of sodium chloride in 10 litres of water. Add 7.54 g of potassium dihydrogen phosphate and 1.40 g of di-sodium hydrogen phosphate 2-hydrate of suitable quality for analysis. The solution will be prepared every day that is used.
- An iodine solution approximately 0.001 N. It serves to check the presence of starch. Prepare the solution by diluting iodine-resublimed pearls in water and adjust to the indicated concentration.

Materials and equipment

- 1. Balance with precision of 0.01 g.
- 2. Gluten extractor with eccentric disc and tensioning mechanism for silk gauze; eccentric disc speed 80 r.p.m.

- 3. Container for water with adjustable expense.
- 4. Chronometer.
- 5. Wooden sieve, 30 x 40 cm, with gauze for semolina number 56.
- 6. Frosted glass plate 40 x 40 cm.
- 7. Thin rubber gloves with a smooth surface
- 8. Press for gluten, Berliner system, with distance between plates of 2.4 mm.
- 9. Internally varnished or frosted metal porcelain capsule, 10 to 15 cm in diameter.
- 10. Spatula 18 to 20 cm long.
- 11. Oven.

Procedure

1. Weigh 10 g of flour with an approximation of ± 0.01 g and place it in a porcelain capsule. Add 5.5 mL of sodium chloride solution drop by drop, continuously stirring the flour with the spatula. After adding all the sodium chloride solution to the flour, compress the mix carefully to avoid losing any flour. The mass adhered to the wall of the capsule is added to the ball of dough.
2. Homogenise the dough by rolling it with the palm of the hand on the frosted glass plate until it has a length of 7 to 8 cm, returning it to the ball shape and repeating the kneading in the same way up to a total of five times. The hand that carries out the homogenisation must be covered with a rubber glove to protect the mass from heat and perspiration of the hand.
3. Place the dough ball on the silk gauze, slightly tense, of the gluten extractor. Wet the dough with a few drops of the sodium chloride solution, then put the eccentric disk in place. Wash for 10 minutes, using about 400 mL of sodium chloride solution.
4. When the gluten extractor apparatus is not available, the previous step can be replaced by a hand wash. To do this, add the sodium chloride solution drop by drop onto the palm of the hand, which should have a temperature of 18°C. The pace of drip should be such that approximately 0.75 litres of the solution drain in 8 minutes. During this time alternately roll and press the dough mass and stretch it seven times so that it splits into two pieces that come together right away. The duration of the hand wash depends on the content of the mass in gluten; however, it should always be approximately the same and should not exceed 8 minutes.
5. A hand washing of a duration of no more than 2 minutes, in general, follows the mechanical washing of the gluten. The gluten extraction can be considered finished as soon as the kneading of the gluten ball with a fresh solution of sodium chloride leaves only traces of starch in the drained water. To check the presence of starch in the washing liquid, use a solution of iodine 0.001N.
6. Remove most of the adherent wash solution from the gluten ball by taking the gluten with the fingertips of one hand and shaking it three times briefly, but hard. Then gently stretch the gluten until a thin sheet is formed, holding it between the fingers, and take it to the press and closing it. Open the press after 5 seconds and pass the gluten sheet to position dry, without deforming it. Press it again. Do this operation fifteen times, drying the glass surfaces carefully after each pressing.
7. Weigh the gluten in the balance with accuracy of 0.01 g.

Calculations

1. Wet gluten. The weight obtained multiplied by ten gives the percentage of wet gluten. The duplicate determinations are considered concordant when they do not differ by more than 0.5% of gluten content. If the deviation is greater, perform a third determination and take the average of the three measurements carried out as an expression of the gluten content. If the deviation found between the highest and lowest values in the three trials is greater than 1%, proceed with a fourth determination.

2. Dry gluten. The wet gluten ball obtained in the previous determination is dried in the oven at a temperature of 100 °C up to constant weight. Let it cool and weigh. The weight obtained multiplied by ten gives the percentage of dry gluten contained in the flour.

References

Wheat Marketing Center, Inc., 2004. Wheat and flour testing methods. A Guide to Understanding Wheat and Flour Quality. Wheat Marketing Center, Inc. Portland, Oregon, USA, 71 p. Available at: https://es.scribd.com/document/101146017/Wheat-and-Flour-Testing-Methods-Book

PANREAC QUIMICA, S.A. Cereales (B.O.E. 19-7-1977 y 20-7-1977). Métodos analíticos en alimentaria. Available at: http://www.usc.es/caa/MetAnalisisStgo1/cereales%20y%20derivados.pdf

1.2.16. Grain screening

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Importance and applications

In commercial channels, the wheat is evaluated according to official grades defined by the wheat agency of specific countries. The wheat's screening (i.e. everything is removed from the wheat before it is milled) affects the wheat values in terms of its storability, milling quality (i.e. extraction rate), end usage destination (i.e. human consumption, feed) and food security (i.e. possible presence of contaminants).

Principle

The procedure considers the separation of impurities, foreign bodies, different seeds, damaged grains, etc., which are reported as a percentage after weighing.

Reagents

- Not required

Materials and equipment

- Two-decimal place precision scale
- Slotted screen, 20 x1.9 mm
- Riddle (mechanical screen mounted on a vibrating table)
- Magnifying glass 1. Electronic kernel counter

Procedure

a. Release procedure for durum wheat

Thoroughly mix the cereal sample in question and take 100 g for analysis.

The sample is sifted, the entire sample passing must be collected and weighed on the precision scales. At this stage all material that falls into the pan is considered waste. If the quantity exceeds 10%, make a more accurate analysis (see Section a.1.) before rejecting the batch.

a.1. - Accurate analysis of durum wheat

Impurities relating to grains means shrivelled grains, grains of other cereals, grains attacked by parasites, grains that have discoloration of the germ, mottled grains or those affected by fusariosis and grains overheated during drying.

Thoroughly mix the cereal sample in question and take 20 g for analysis.

• The sample is sifted, the entire sample passing must be collected and weighed on the precision scales. At this stage only shrivelled grains, glumes, seeds with weeds and broken grains pass through the sieve

- Of the remainder on the sieve, grains are selected by type and divided into:
  - Mottled
  - With fusariosis
  - Attacked by parasites (bugs)
  - Whitened
  - Bread wheat present in durum wheat
- Once this selection procedure has been completed using a magnifying glass, weigh individual fractions on the precision scales.

Calculations

Each isolated fraction is weighed and referred to 100 g of wheat as it is, multiplying the weight by 5. The result is expressed in % to one decimal place.

Remarks

- Waste and impurities

Waste means shrivelled grains, grains of other cereals, grains attacked by parasites, grains that have discoloration of the germ, mottled grains or those affected by fusariosis and grains overheated during drying.

Miscellaneous impurities means extraneous seeds, damaged grains, impurities as such, husks, ergot, decayed grains, dead insects and fragments of insects, earth and herbal filaments or straw.

- Broken grains

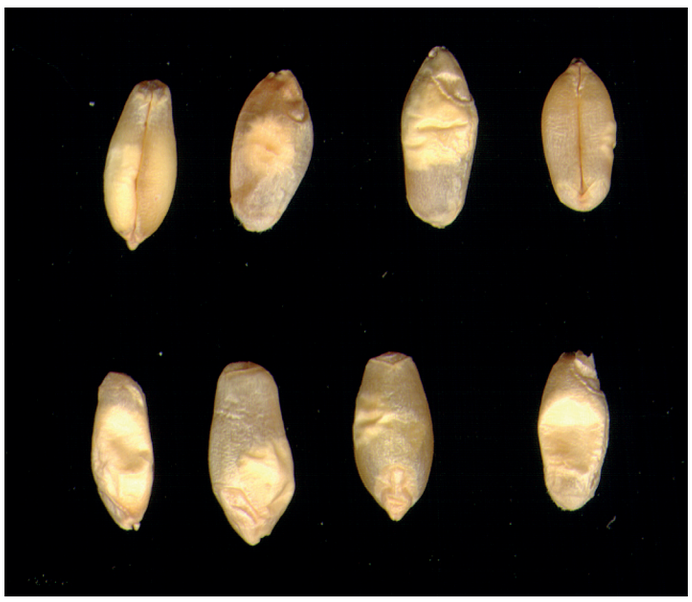
All grains whose endosperm is partially uncovered are considered broken grains. Grains damaged by beating and grains whose germ has been removed also belong to this group.

- Shrivelled grains

Shrivelled grains are considered to be those grains which, after elimination of the other elements of the sample hare referred, pass through sieves with mesh 20x1.9 mm.

- Grains attacked by parasites

Grains attacked by parasites are those with worm holes. Bugged grains also belong to this group.



- Mottled

Those with brown to brownish black coloration on the germ but not elsewhere.

- Grains attacked by fusariosis

Those whose pericarp is contaminated with Fusarium mycelium; these grains are slightly shrivelled, wrinkled, with widespread patches with ill-defined contours, pink or white in colour.

- Glumes

Cereals have flowers gathered in spikes, comprise partial inflorescences or spikelets, each of which is protected by two bracts called glumes. These protections (leaves), not being expelled on threshing, can reach the mill.

- Extraneous seeds

The seeds of plants, cultivated or not, other than cereals. Constituted by worthless and unrecoverable grains, by seeds used for livestock and by noxious seeds.

- Whitened seeds

The grains with powdery, whitish areas.

References

Regulation (EC) No, 824/2000 of the Commission of 19 April 2000 which establishes the procedures for taking charge of cereals by intervention agencies and the methods of analysis for determining the quality. Official Gazette no. L 100 of 20/04/2000 pages 0031 – 0050.

1.2.17. Optical residue

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Consorzio Casalasco del Pomodoro SAC – Strada Provinciale 32 – Rivarolo del Re ed Uniti (CR) – Italy

Importance and applications

This method provides information about the percentage of total soluble substances present in the tomato. A Brix degree (symbol Bx) corresponds to 1 part of solid substance (dry weight) in 99 parts of solution. For example, a 25°Bx solution contains 25 grams of solid substances in 100 grams of total liquid.

Principle

Soluble solids are determined indirectly by deducting them from the value of their refraction index. The refractometer must be equipped with a thermometer as well as a water circulation ultrathermostat that allows measurements to be carried out at a temperature of +20°C with an approximation of + 0.5> C and a lighting device.

Reagents

- Sugary solutions with a known concentration
- Tomato shake

Materials and equipment

- Refractometer (BELLINGAM & STANLEY 90)

Procedure

Every batch of tomato is analysed. The tomato sample is inserted into the Maselli Misure monoblock that chops the tomato before analysis. Before the start of the campaign an official calibration of the instrument is made by a specialised company in charge, which issues a calibration certificate. A calibration is performed every day comparing the Brix value of a shake obtained from the refractometer present at the quality control in input with that of the laboratory refractometer. The tolerance margin considered is ± 0.05°Brix. If the value obtained exceeds the tolerance margin, a calibration with a sugar solution with a known Brix value is carried out.

Calculations

None

Remarks

- The importance of the sample's representativeness
- It is important to turn on the instrument at least 2 hours before

1.2.18. Consistency

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Consorzio Casalasco del Pomodoro SAC – Strada Provinciale 32 – Rivarolo del Re ed Uniti (CR), Italy

Importance and applications

Consistency is a fundamental parameter for the production of tomato pulp and each incoming load is subjected to measurement.

Principle

The consistency mainly depends on the content in insoluble substances and is correlated with the dry residue.

Reagents

- Tomato shake

Materials and equipment

- Bostwich Consistometer

Procedure

Every batch of tomato is analysed. The tomato sample is inserted into the Maselli Misure monoblock that chops the tomato before analysis. The consistency is measured on the tomato shake with a Bostwich Consistometer and is expressed in cm 30 seconds^{-1}. The smoothie is placed in the Bostwich chamber, the instrument levels it with a spatula, the bulkhead is opened and measured in cm exactly after 30 seconds.

Calculations

None

Remarks

- The importance of the sample's representativeness
- It is important to turn on the instrument at least 2 hours before

1.2.19. Colour

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Consorzio Casalasco del Pomodoro SAC – Strada Provinciale 32 – Rivarolo del Re ed Uniti (CR) – Italy

Importance and applications

Colour is a fundamental parameter for the production of concentrates and tomato pulp and each incoming load is subjected to measurement.

Principle

The colour is measured thanks to the different sensitivity of photoelectric cells operating at a given standard angle. A tungsten lamp is used to illuminate the sample. The Lab colour space mathematically describes all perceivable colours in the three dimensions L for lightness and a and b for the colour components green–red and blue–yellow.
One of the most important attributes of the Lab model is device independence. This means that the colours are defined independent of their nature of creation or the device they are displayed on. The space itself is a three-dimensional real number space, which contains an infinite number of possible representations of colours. However, in practice, the space is usually mapped onto a three-dimensional integer space for device-independent digital representation, and for these reasons, the L*, a*, and b* values are usually absolute, with a pre-defined range. The lightness, L*, represents the darkest black at L* = 0, and the brightest white at L* = 100. The colour channels, a* and b*, will represent true neutral grey values at a* = 0 and b* = 0. The red/green opponent colours are represented along the a* axis, with green at negative a* values and red at positive a* values. The yellow/blue opponent colours are represented along the b* axis, with blue at negative b* values and yellow at positive b* values. The scaling and limits of the a* and b* axes will depend on the specific implementation of Lab colour, as described below, but they often run in the range of ±100 or –128 to +127 (signed 8-bit integer).

Reagents

- Tomato shake

Materials and equipment

- Gardner colorimeter

Procedure

Every batch of tomato is analysed. The tomato sample is inserted into the Maselli Misure monoblock that chops the tomato before analysis. The colour is measured with a colorimeter (calibrated at the beginning of the campaign by a specialised company), is expressed in °Gardner, with the value of 2.00 being considered the reference value.

Calculations

None

Remarks

- The importance of the sample's representativeness
- It is important to turn on the instrument at least 2 hours before

1.2.20. Lycopene

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Importance and applications

Lycopene belongs to the class of carotenoids, or the vast class of liposoluble organic pigments. It has a series of positive properties of an antioxidant nature because it is a molecule rich in unsaturated bonds and is responsible for the red colour of the tomato.
Lycopene is not synthesized by the body and its assimilation occurs through the intake of plant foods, first of all the tomato. It is soluble in oil and insoluble in water and is easily assimilated by the human body. Its presence in the tomato is high at the level of the peel.

Principle

The following procedure is used to determine lycopene in food samples by analytical technique HPLC-UV. The concentration range is greater than 2mg / kg.

Reagents

- Hexane (reagent grade)
- Ethanol (reagent grade)
- Acetone (reagent grade)
- Methanol (reagent grade)
- MTBE (reagent grade)

Materials and equipment

- Lycopene (tomatoes)
- HPLC-UV system
- YMC-Pack YMC C30 150 * 4.6 column or equivalent

Procedure

Laboratory practices require that each analyst be perfectly aware of the potential risks of the reagents, products and solvents before starting work. In any case it is better to read the safety data sheets. Even if there are no indications on the dangerousness or toxicity of the used reagents, in agreement with laboratory practices, it is advisable to handle these reagents with caution, avoiding any possible contact.
The elimination of reagents, reagents and solvents must comply with the internal operating procedure P-GS-21.

a. Preparation of solutions

100 mg / L standard solution: weigh 5 mg in a 50 mL flask, dissolve with hexane. This solution

should be stored in the freezer (ideally at -70°C).
The concentration of the stock solution is verified spectrophotometrically in the following way: record the absorbance at 475 nm of a diluted 0.1ppm against a white hexane.
The coefficient (1%) in hexane at 475 nm is 3450.
The concentration is obtained from the formula: C (g $100mL^{-1}$) = A / e (1%) * L
Then prepare diluted standard solutions from 20 to 0.1 mg L^{-1} by dilution with hexane.

b. Preparation of the sample
0.8 g of finely homogenised sample is inserted into a falcon, 0.7 mL of water Q1 are added and extracted vortexing with 10 mL of extracting solution (Hexane / Acetone / Ethanol 2: 1: 1). It is centrifuged at 4000 rpm for 5 minutes as the way to separate the hexane. The operation is repeated 3 times, adding 2.5 mL of water Q1 between the second and third time. The collected hexane (15 mL) is diluted appropriately and stored in the freezer before analysis.

c. Instrumental determination
Chromatographic column: YMC-Pack YMC C30 150 * 4.6
Mobile phase: Isocratic
Flow: 0.51 mL / min
Column temperature: 30°C
l: 470 nm
Stop time (Autosampler and HPLC): 16 min
Post run time: 0 min
Injection volume: 10 µL
Eluent: MeOH / MTBE 30/70

Elution order:
Cis 1
Cis 2 + 3
All trans
Cis 4

Calculations

The concentration is calculated using the calibration line built with the response factor of the isomer trans and expressed in mg / kg according to the calculation below:

$$Conc\ all\text{-}trans\ (mg/Kg) = (C\ all\text{-}trans\ *V) / P$$

C all-trans = the concentration in mg / l calculated from the calibration line
V = the final volume to which the sample was taken in mL
P = the weight of samples, in g

$$Conc\ all\ cis\ (mg/Kg) = [(C\ cis1 + C\ cis2+3 + C\ cis4)* V] / P$$

C cis1 = the concentration in mg / l calculated from the tarature line
C cis2 + 3 = the concentration in mg / l calculated from the calibration line
C cis4 = the concentration in mg / l calculated from the calibration line
V = the final volume to which the sample expressed in mL has been taken
P = weight of samples, in g

$$Conc\ lycopene\ total\ (mg/Kg) = Conc\ all\text{-}trans\ (mg/Kg) + Conc\ all\ cis\ (mg/Kg)$$

If the concentration found in the sample exceeds the highest point on the calibration line, provide an appropriate sample dilution.

Remarks

The importance of the sample's representativeness.

1.2.21. Pesticides

Belotti Enio

Water & Life SRL – Via Enrico Mattei 37 – Entratico (BG) - Italy

Importance and applications

All the tomato conferred must be produced according to the criteria established by the Integrated Production Regulation of the region it belongs to, and in any case in compliance with the minimum requirements set by the Emilia Romagna Region Regulations for the QC mark.

Principle

The method allows any pesticides in fresh tomato to be found and to verify that these pesticides are under the LMR.

Reagents

- Fresh tomato sample

Materials and equipment

- LC/MS/MS Technique
- LC/MS/MD Technique
- LC/MS/MS – UPLC - UV Technique
- GC/ECD Technique
- GC/MS Technique
- GC/MS/MS Technique
- Ionic Chromatography Technique

Procedure

a. At least one sampling in the fresh tomato field is carried out every 1,000 tons of product or fraction (as per EU Regulations 891/2017 and 892/2017), with a minimum of one multi-residual analysis per holding company. The sampling is carried out about ten days prior to the start of the conferment of the field under control. Sampling can be done on fruits and / or on parts of plants at the discretion of the technician. Tomato picking in the field is carried out by the Casalasco Pomodoro agronomic office technician in the presence of the farm representative. The sample, about 6 kg, is obtained, after careful mixing, from the mixture of the fruits taken at 5-10 points, according to the cross pattern, excluding an edge area that may have undergone a non-homogeneous treatment.

b. The sample is then divided into 3 equivalent aliquots: - One that is kept by the farm; - One that is sent to an accredited laboratory; - One is stored by CCDP in the event of a counter analysis.

c. Each aliquot is collected in a sealed bag which is identified, numbered and signed by the technician and the d. The pesticides sought are as follows:

Technique	Pesticide
GC/MS/MS	2,4 Dimethilaniline
GC/MS/MS	2,4,6-Trichlorophenol
GC/MS/MS	2,4'-DDD
GC/MS/MS	2,4'-DDE
GC/MS/MS	2,4'-DDT
GC/MS/MS	2,6 dichloro 4 methyl phenol
GC/MS/MS	2,6 Dimethilaniline
GC/MS/MS	2-Nitroaniline
GC/MS/MS	2-Phenylphenol
GC/MS/MS	3,4 Dichloroaniline
GC/MS/MS	3,5 Dichloroaniline
GC/MS/MS	3-Chloroaniline
GC/MS/MS	4 bromo 2 chlorophenol
GC/MS/MS	4,4 -Dibromobenzophenone
GC/MS/MS	4,4'-DDD
GC/MS/MS	4,4'-DDE
GC/MS/MS	4,4'-DDT
GC/MS/MS	4-phenylphenol
GC/MS/MS	Acequinocyl
GC/MS/MS	Aclonifen
GC/MS/MS	Aldrin
GC/MS/MS	Allethrin mixture of stereo isomers
GC/MS/MS	Benfluralin
GC/MS/MS	Beta-cyfluthrin
GC/MS/MS	Binapacryl
GC/MS/MS	Biphenyl
GC/MS/MS	Bromocyclen
GC/MS/MS	Bromophos-Ethyl
GC/MS/MS	Bromophos-Methyl
GC/MS/MS	Bromoxynil heptanoate
GC/MS/MS	Bromoxynil octanoate
GC/MS/MS	Captafol
GC/MS/MS	Captan
GC/MS/MS	Carbophenothion
GC/MS/MS	Chlordane technical mixture
GC/MS/MS	Chlorfenapyr
GC/MS/MS	Chlorfenson
GC/MS/MS	Chlormephos

GC/MS/MS	Chlorobenzilate
GC/MS/MS	Chloroneb
GC/MS/MS	Chloropropylate
GC/MS/MS	Chlorothalonil
GC/MS/MS	Chlorpropham
GC/MS/MS	Chlorthal-Dimethyl
GC/MS/MS	Chlorthion
GC/MS/MS	Cletodim
GC/MS/MS	Cyfluthrin (mixture of isomers)
GC/MS/MS	Cypermethrin
GC/MS/MS	Cypermethrin Alpha
GC/MS/MS	Deltamethrin
GC/MS/MS	Dialifos
GC/MS/MS	Dichlobenil
GC/MS/MS	Dicloran
GC/MS/MS	Dicofol
GC/MS/MS	Dieldrin
GC/MS/MS	Endosulfan (SUM)
GC/MS/MS	Endosulfan alpha
GC/MS/MS	Endosulfan beta
GC/MS/MS	Endosulfan sulfate
GC/MS/MS	Endrin
GC/MS/MS	Endrin aldehyde
GC/MS/MS	Esfenvalerate
GC/MS/MS	Etridiazole
GC/MS/MS	Fenchlorphos
GC/MS/MS	Fenvalerate
GC/MS/MS	Flutriafol
GC/MS/MS	Folpet
GC/MS/MS	Formothion
GC/MS/MS	HCH alpha
GC/MS/MS	HCH beta
GC/MS/MS	HCH delta
GC/MS/MS	HCH gamma (Lindane)
GC/MS/MS	Heptachlor
GC/MS/MS	Heptachlor epoxide
GC/MS/MS	Heptachlor-exo-epoxide (cis- isomer B)
GC/MS/MS	Hexachlorobenzene
GC/MS/MS	Iprodione
GC/MS/MS	Isodrione

GC/MS/MS	Isopropalin
GC/MS/MS	Lambda-cyhalothrin
GC/MS/MS	Methoxychlor
GC/MS/MS	Mirex
GC/MS/MS	Nitrofen
GC/MS/MS	Nitrothal-Isopropyl
GC/MS/MS	Oxyfluorfen
GC/MS/MS	Parathion
GC/MS/MS	Parathion-Methyl
GC/MS/MS	Pentachloroaniline
GC/MS/MS	Pentachloroanisole
GC/MS/MS	Permethrin
GC/MS/MS	Perthane
GC/MS/MS	Phtalimide
GC/MS/MS	Procymidone
GC/MS/MS	Propachlor
GC/MS/MS	Prothiofos
GC/MS/MS	Pyrethrins
GC/MS/MS	Quintozene
GC/MS/MS	S421
GC/MS/MS	Sulfallate
GC/MS/MS	Sulphur
GC/MS/MS	Tecnazene
GC/MS/MS	Tefluthrin (mixture of isomers)
GC/MS/MS	Tetradifon
GC/MS/MS	Tetrahydrophtalimide(cis- 1,2,3,6)
GC/MS/MS	Tetrasul
GC/MS/MS	Tiocarbazil
GC/MS/MS	Tralomethrin
GC/MS/MS	Transfluthrin
GC/MS/MS	Triallate+Diallate
GC/MS/MS	Trifluralin
GC/MS/MS	Vinclozolin
GC/MS/MS	Zeta-cypermethrin
LC/MS/MS	2,6-Dichlorobenzamide
LC/MS/MS	2,4 D
LC/MS/MS	2,4 DB
LC/MS/MS	2,4,5 TP
LC/MS/MS	2,4,5 Trichlorophenol
LC/MS/MS	2,4,5-T

LC/MS/MS	2,4,5-T methyl ester
LC/MS/MS	2,4-D methyl ester
LC/MS/MS	2,4-DB methyl ester
LC/MS/MS	2-Naphtoxyacetic acid
LC/MS/MS	3 Hidroxycarbofuran
LC/MS/MS	4-CPA
LC/MS/MS	4-Iodophenoxy acetic acid
LC/MS/MS	6-Benzylaminopurine
LC/MS/MS	6-Chloronicotinic acid
LC/MS/MS	Abamectin
LC/MS/MS	Abamectin B1A
LC/MS/MS	Abamectin B1B
LC/MS/MS	Acephate
LC/MS/MS	Acetamiprid
LC/MS/MS	Acetochlor
LC/MS/MS	Acibenzolar-S-methyl
LC/MS/MS	Acifluorfen
LC/MS/MS	Acrinathrin
LC/MS/MS	Alachlor
LC/MS/MS	Aldicarb
LC/MS/MS	Aldicarb-Sulfon
LC/MS/MS	Aldicarbsulfoxid
LC/MS/MS	Aldoxycarb
LC/MS/MS	Ametoctradin
LC/MS/MS	Ametryn
LC/MS/MS	Aminocarb
LC/MS/MS	Amisulbrom
LC/MS/MS	Amitraz
LC/MS/MS	Anilazine
LC/MS/MS	Asulam
LC/MS/MS	Atrazine
LC/MS/MS	Atrazine Desethyl
LC/MS/MS	Atrazine Desisopropyl
LC/MS/MS	Atrazine-Desethyl-Desisopropyl
LC/MS/MS	Azaconazole
LC/MS/MS	Azadirachtin
LC/MS/MS	Azametiphos
LC/MS/MS	Azinphos-ethyl
LC/MS/MS	Azinphos-methyl
LC/MS/MS	Azocyclotin

LC/MS/MS	Azoxystrobin
LC/MS/MS	Barban
LC/MS/MS	Benalaxyl + Benalaxyl-M
LC/MS/MS	Bendiocarb
LC/MS/MS	Benfuracarb
LC/MS/MS	Benomyl
LC/MS/MS	Bensulfuron-Methyl
LC/MS/MS	Bentazone
LC/MS/MS	Bentazone-6-Hydroxy
LC/MS/MS	Bentazone-8-Hydroxy
LC/MS/MS	Benthiavalicarb-isopropyl
LC/MS/MS	Benzoximate
LC/MS/MS	Benzoylprop-ethyl
LC/MS/MS	Benzthiazuron
LC/MS/MS	Bifenazate
LC/MS/MS	Bifenox
LC/MS/MS	Bifenthrin
LC/MS/MS	Bitertanol (mixture of diastereoisomers)
LC/MS/MS	Boscalid
LC/MS/MS	Bromacil
LC/MS/MS	Bromfenvinfos
LC/MS/MS	Bromoxynil
LC/MS/MS	Brompropylate
LC/MS/MS	Bromuconazole
LC/MS/MS	Bupirimate
LC/MS/MS	Buprofezin
LC/MS/MS	Butafenacil
LC/MS/MS	Butocarboxim
LC/MS/MS	Butoxycarboxim
LC/MS/MS	Buturon
LC/MS/MS	Butylate
LC/MS/MS	Cadusafos
LC/MS/MS	Carbaryl
LC/MS/MS	Carbendazim
LC/MS/MS	Carbetamide
LC/MS/MS	Carbofuran
LC/MS/MS	Carbosulfan
LC/MS/MS	Carboxin
LC/MS/MS	Chinomethionat
LC/MS/MS	Chloprop

LC/MS/MS	Chlorantraniliprole
LC/MS/MS	Chlorbufam
LC/MS/MS	Chlorfenvinphos (mixture of Z and E isomers)
LC/MS/MS	Chlorfluazuron
LC/MS/MS	Chloridazon
LC/MS/MS	Chlorobromuron
LC/MS/MS	Chlorotoluron
LC/MS/MS	Chloroxuron
LC/MS/MS	Chlorpyrifos
LC/MS/MS	Chlorpyrifos-methyl
LC/MS/MS	Chlortiamid
LC/MS/MS	Chlorthiophos
LC/MS/MS	Chlozolinate
LC/MS/MS	Cinosulfuron
LC/MS/MS	cis-Mevinphos
LC/MS/MS	Clodinafop free acid
LC/MS/MS	Clodinafop-propargyl
LC/MS/MS	Clofentezine
LC/MS/MS	Clomazone
LC/MS/MS	Clomeprop
LC/MS/MS	Clopyralid
LC/MS/MS	Clothianidin
LC/MS/MS	Coumaphos
LC/MS/MS	Cyanazine
LC/MS/MS	Cyanofenphos
LC/MS/MS	Cyantraniliprole
LC/MS/MS	Cyazofamid
LC/MS/MS	Cycloate
LC/MS/MS	Cycloxydim
LC/MS/MS	Cycluron
LC/MS/MS	Cyflufenamid
LC/MS/MS	Cyhalofop
LC/MS/MS	Cyhalofop-butyl
LC/MS/MS	Cyhexatin
LC/MS/MS	Cymiazol
LC/MS/MS	Cymoxanil
LC/MS/MS	Cyproconazole
LC/MS/MS	Cyprodinil
LC/MS/MS	Cyprosulphamid
LC/MS/MS	Cyromazine

LC/MS/MS	Daminozide
LC/MS/MS	Dazomet
LC/MS/MS	DEET
LC/MS/MS	Demeton-S-methyl
LC/MS/MS	Demeton-S-methylsulfone
LC/MS/MS	Desmedipham
LC/MS/MS	Desmetryn
LC/MS/MS	Diafenthiuron
LC/MS/MS	Diallate
LC/MS/MS	Diazinon
LC/MS/MS	Dibrom
LC/MS/MS	Dicamba
LC/MS/MS	Dichlofenthion
LC/MS/MS	Dichlofluanid
LC/MS/MS	Dichloroacetic Acid
LC/MS/MS	Dichlorprop
LC/MS/MS	Dichlorvos
LC/MS/MS	Diclobutrazol
LC/MS/MS	Diclofop-free acid
LC/MS/MS	Diclofop-methyl
LC/MS/MS	Dicrotophos
LC/MS/MS	Diethofencarb
LC/MS/MS	Difenoconazole
LC/MS/MS	Difenoxuron
LC/MS/MS	Diflubenzuron
LC/MS/MS	Diflufenican
LC/MS/MS	Dimefox
LC/MS/MS	Dimepiperate
LC/MS/MS	Dimethoate
LC/MS/MS	Dimethomorph (mixture of E, Z isomers)
LC/MS/MS	Dimoxystrobin
LC/MS/MS	Diniconazole (mixture of E, Z isomers)
LC/MS/MS	Dinitramine
LC/MS/MS	Dinocap
LC/MS/MS	Dinoterb
LC/MS/MS	Dioxacarb
LC/MS/MS	Dioxathion
LC/MS/MS	Diphenamid
LC/MS/MS	Diphenylamine
LC/MS/MS	Disulfoton

LC/MS/MS	Disulfoton sulfone
LC/MS/MS	Disulfoton sulfoxide
LC/MS/MS	Ditalimfos
LC/MS/MS	Dithianon
LC/MS/MS	Diuron
LC/MS/MS	DMST
LC/MS/MS	Dodine
LC/MS/MS	Edifenphos
LC/MS/MS	Emamectin-benzoate
LC/MS/MS	EPN
LC/MS/MS	Epoxiconazole
LC/MS/MS	EPTC
LC/MS/MS	Etaconazole
LC/MS/MS	Ethiofencarb
LC/MS/MS	Ethiofencarb-sulfon
LC/MS/MS	Ethiofencarb-sulfoxide
LC/MS/MS	Ethion
LC/MS/MS	Ethirimol
LC/MS/MS	Ethofumesate
LC/MS/MS	Ethoprophos
LC/MS/MS	Ethoxyquin
LC/MS/MS	Etofenprox
LC/MS/MS	Etoxazole
LC/MS/MS	Etrimfos
LC/MS/MS	Famoxadone
LC/MS/MS	Famphur
LC/MS/MS	Fenamidone
LC/MS/MS	Fenamiphos
LC/MS/MS	Fenamiphos-sulfone
LC/MS/MS	Fenamiphos-sulfoxide
LC/MS/MS	Fenarimol
LC/MS/MS	Fenazaquin
LC/MS/MS	Fenbuconazole
LC/MS/MS	Fenbutatin-oxide
LC/MS/MS	Fenhexamid
LC/MS/MS	Fenitrothion
LC/MS/MS	Fenothiocarb
LC/MS/MS	Fenoxaprop racemate
LC/MS/MS	Fenoxaprop-P
LC/MS/MS	Fenoxaprop-P-ethyl

LC/MS/MS	Fenoxycarb
LC/MS/MS	Fenpirazamina
LC/MS/MS	Fenpropathrin
LC/MS/MS	Fenpropatrin
LC/MS/MS	Fenpropidin
LC/MS/MS	Fenpropimorph
LC/MS/MS	Fenpyroximate
LC/MS/MS	Fenson
LC/MS/MS	Fensulfothion
LC/MS/MS	Fensulfothion oxon
LC/MS/MS	Fensulfothion oxon sulfone
LC/MS/MS	Fensulfothion PO solfone
LC/MS/MS	Fenthion
LC/MS/MS	Fenthion sulfone
LC/MS/MS	Fenthion sulfoxide
LC/MS/MS	Fenthion-oxon
LC/MS/MS	Fenthion-Oxon-Sulfone
LC/MS/MS	Fenthion-Oxon-Sulfoxide
LC/MS/MS	Fentin acetate
LC/MS/MS	Fentin hydroxide
LC/MS/MS	Fenuron
LC/MS/MS	Fipronil
LC/MS/MS	Fipronil Sulfide
LC/MS/MS	Fipronil Sulfone
LC/MS/MS	Fipronil-desulfinyl
LC/MS/MS	Flamprop free acid
LC/MS/MS	Flamprop-isopropyl
LC/MS/MS	Flamprop-M-isopropyl
LC/MS/MS	Flamprop-M-methyl
LC/MS/MS	Flonicamid
LC/MS/MS	Fluazifop
LC/MS/MS	Fluazifop-p
LC/MS/MS	Fluazifop-butyl
LC/MS/MS	Fluazifop-p-butyl
LC/MS/MS	Fluazifop-P-Butyl (free and conjugate)
LC/MS/MS	Fluazinam
LC/MS/MS	Fluazuron
LC/MS/MS	Flubenzimine
LC/MS/MS	Flucycloxuron
LC/MS/MS	Flucythrinate

LC/MS/MS	Fludioxonil
LC/MS/MS	Flufenacet
LC/MS/MS	Flufenoxuron
LC/MS/MS	Fluometuron
LC/MS/MS	Fluopicolide
LC/MS/MS	Fluopyradifurone
LC/MS/MS	Fluopyram
LC/MS/MS	Fluoxastrobin
LC/MS/MS	Fluquinconazole
LC/MS/MS	Flurochloridone
LC/MS/MS	Fluroxypyr
LC/MS/MS	Fluroxypyr-1-methylheptyl ester
LC/MS/MS	Flusilazole
LC/MS/MS	Flutolanil
LC/MS/MS	Fluxapyroxad
LC/MS/MS	Fonofos
LC/MS/MS	Forchlorfenuron
LC/MS/MS	Formetanate-hydrochloride
LC/MS/MS	Fosthiazate
LC/MS/MS	Fuberidazole
LC/MS/MS	Furalaxyl
LC/MS/MS	Furathiocarb
LC/MS/MS	Giberellic Acid
LC/MS/MS	Haloxyfop
LC/MS/MS	Haloxyfop 2 ethoxyethyl
LC/MS/MS	Haloxyfop methyl
LC/MS/MS	Haloxyfop p-methyl
LC/MS/MS	Heptenophos
LC/MS/MS	Hexaconazole
LC/MS/MS	Hexaflumuron
LC/MS/MS	Hexazinone
LC/MS/MS	Hexythiazox
LC/MS/MS	Imazalil
LC/MS/MS	Imazamox
LC/MS/MS	Imibenconazole
LC/MS/MS	Imidacloprid
LC/MS/MS	Indoxacarb
LC/MS/MS	Iodfenphos
LC/MS/MS	Ioxynil
LC/MS/MS	Iprobenphos

LC/MS/MS	Iprovalicarb
LC/MS/MS	Isazofos
LC/MS/MS	Isocarbophos
LC/MS/MS	Isofenphos
LC/MS/MS	Isofenphos Oxone
LC/MS/MS	Isofenphos-methyl
LC/MS/MS	Isoprocarb
LC/MS/MS	Isoprothiolane
LC/MS/MS	Isoproturon
LC/MS/MS	Isopyrazam
LC/MS/MS	Isoxaben
LC/MS/MS	Isoxaflutole
LC/MS/MS	Isoxathion
LC/MS/MS	Kresoxim-Methyl
LC/MS/MS	Lenacil
LC/MS/MS	Leptophos
LC/MS/MS	Linuron
LC/MS/MS	Lufenuron
LC/MS/MS	Malaoxon
LC/MS/MS	Malathion
LC/MS/MS	Mandipropamid
LC/MS/MS	MCPA
LC/MS/MS	MCPA methyl ester
LC/MS/MS	MCPA-2-ethylhexyl ester
LC/MS/MS	MCPA-butoxyethyl ester
LC/MS/MS	MCPB
LC/MS/MS	MCPP (Mecoprop)
LC/MS/MS	MCPP-P (Mecoprop-P)
LC/MS/MS	Mecarbam
LC/MS/MS	Mepanipyrim
LC/MS/MS	Mephosfolan
LC/MS/MS	Mepronil
LC/MS/MS	Mesotrione
LC/MS/MS	Metaflumizone
LC/MS/MS	Metalaxyl + Metalaxyl-M
LC/MS/MS	Metamifop
LC/MS/MS	Metamitron
LC/MS/MS	Metazachlor
LC/MS/MS	Metconazole
LC/MS/MS	Methabenzthiazuron

LC/MS/MS	Methacrifos
LC/MS/MS	Methamidophos
LC/MS/MS	Methidathion
LC/MS/MS	Methiocarb
LC/MS/MS	Methiocarb-Sulfone
LC/MS/MS	Methiocarb-Sulfoxide
LC/MS/MS	Methomyl
LC/MS/MS	Methomyl-oxyme
LC/MS/MS	Methoxyfenozide
LC/MS/MS	Methyl N-(3-hydroxyphenyl) carbamate
LC/MS/MS	Methyldymron
LC/MS/MS	Metobromuron
LC/MS/MS	Metolachlor + S-Metolachlor
LC/MS/MS	Metolcarb
LC/MS/MS	Metoxuron
LC/MS/MS	Metrafenone
LC/MS/MS	Metribuzin
LC/MS/MS	Metsulfuron-Methyl
LC/MS/MS	Mevinphos
LC/MS/MS	Milbemectina
LC/MS/MS	Molinate
LC/MS/MS	Monocrotophos
LC/MS/MS	Monolinuron
LC/MS/MS	Monuron
LC/MS/MS	Myclobutanil
LC/MS/MS	N(2,4-DimethylPhenyl) Formamide Amitraz metabolite
LC/MS/MS	N(2,4-DimethylPheny-N'-Formamidine Amitraz metabolite
LC/MS/MS	NAA
LC/MS/MS	NAD
LC/MS/MS	Naphthol,1
LC/MS/MS	Napropamide
LC/MS/MS	Naptalam
LC/MS/MS	Neburon
LC/MS/MS	Nicosulfuron
LC/MS/MS	Nitenpyram
LC/MS/MS	NN Dimethylhydrazide
LC/MS/MS	NN Dimethylsulphamide
LC/MS/MS	Norfluazuron
LC/MS/MS	Novaluron
LC/MS/MS	Nuarimol

LC/MS/MS	Omethoate
LC/MS/MS	Oxadiargyl
LC/MS/MS	Oxadiazon
LC/MS/MS	Oxadixyl
LC/MS/MS	Oxamyl
LC/MS/MS	Oxamyl Oxyme
LC/MS/MS	Oxycarboxine
LC/MS/MS	Oxydemeton-Methyl
LC/MS/MS	Paclobutrazol
LC/MS/MS	Paraoxon
LC/MS/MS	Paraoxon-Methyl
LC/MS/MS	Penconazole
LC/MS/MS	Pencycuron
LC/MS/MS	Pendimethalin
LC/MS/MS	Pentachloroanisole
LC/MS/MS	Pentachlorophenol
LC/MS/MS	Penthiopyrad
LC/MS/MS	Phenkapton
LC/MS/MS	Phenmedipham
LC/MS/MS	Phenthoate
LC/MS/MS	Phorate
LC/MS/MS	Phorate Oxon
LC/MS/MS	Phorate Sulfone
LC/MS/MS	Phorate Sulfoxide
LC/MS/MS	Phorate-Oxonsulfone
LC/MS/MS	Phorate-Oxonsulfoxide
LC/MS/MS	Phosalone
LC/MS/MS	Phosmet
LC/MS/MS	Phosmet Oxone
LC/MS/MS	Phosphamidon (mixture of E+D isomers)
LC/MS/MS	Phoxim
LC/MS/MS	Phtalimide
LC/MS/MS	Picloram
LC/MS/MS	Picoxystrobin
LC/MS/MS	Pinoxaden
LC/MS/MS	Piperonyl butoxide
LC/MS/MS	Piperophos
LC/MS/MS	Pirimicarb
LC/MS/MS	Pirimicarb desmethyl
LC/MS/MS	Pirimicarb-desmethyl-formamido

LC/MS/MS	Pirimiphos-Ethyl
LC/MS/MS	Pirimiphos-Methyl
LC/MS/MS	Prochloraz
LC/MS/MS	Profenofos
LC/MS/MS	Profluralin
LC/MS/MS	Prohexadone calcium
LC/MS/MS	Promecarb
LC/MS/MS	Prometon
LC/MS/MS	Prometryn
LC/MS/MS	Propamocarb
LC/MS/MS	Propanil
LC/MS/MS	Propaquizafop
LC/MS/MS	Propargite
LC/MS/MS	Propazine
LC/MS/MS	Propetamphos
LC/MS/MS	Propham
LC/MS/MS	Propiconazole (mixture of stereo isomers)
LC/MS/MS	Propoxur
LC/MS/MS	Propyzamide
LC/MS/MS	Proquinazid
LC/MS/MS	Prosulfocarb
LC/MS/MS	Prothioconazole
LC/MS/MS	Prothioconazole-desthio
LC/MS/MS	Prothoate
LC/MS/MS	Pymetrozine
LC/MS/MS	Pyraclostrobin
LC/MS/MS	Pyraflufen Ethyl
LC/MS/MS	Pyrazophos
LC/MS/MS	Pyridaben
LC/MS/MS	Pyridafol
LC/MS/MS	Pyridaphenthion
LC/MS/MS	Pyridat
LC/MS/MS	Pyrifenox
LC/MS/MS	Pyrimethanil
LC/MS/MS	Pyriproxyfen
LC/MS/MS	Quinalphos
LC/MS/MS	Quinclorac
LC/MS/MS	Quinoxyfen
LC/MS/MS	Quizalofop p-Ethyl
LC/MS/MS	Rimsulfuron

LC/MS/MS	Rotenone
LC/MS/MS	Sethoxydim
LC/MS/MS	Simazine
LC/MS/MS	Simetryn
LC/MS/MS	Spinosad (mixture of Spinosyn A&D)
LC/MS/MS	Spinosad A
LC/MS/MS	Spinosad D
LC/MS/MS	Spirodiclofen
LC/MS/MS	Spiromesifen
LC/MS/MS	Spirotetramat
LC/MS/MS	Spirotetramat-enol
LC/MS/MS	Spirotetramat-enolglucoside
LC/MS/MS	Spirotetramat-Ketohydroxy
LC/MS/MS	Spirotetramat-monohydroxy
LC/MS/MS	Spiroxamine
LC/MS/MS	Sulcotrione
LC/MS/MS	Sulfotep
LC/MS/MS	Sulfoxaflor
LC/MS/MS	Sulprofos
LC/MS/MS	Tau-fluvalinate
LC/MS/MS	TBTO
LC/MS/MS	Tebuconazole
LC/MS/MS	Tebufenozide
LC/MS/MS	Tebufenpyrad
LC/MS/MS	Tebupirimfos
LC/MS/MS	Teflubenzuron
LC/MS/MS	Temephos
LC/MS/MS	Tepraloxydim
LC/MS/MS	Terbufos
LC/MS/MS	Terbufos sulfon
LC/MS/MS	Terbufos sulfoxide
LC/MS/MS	Terbumeton
LC/MS/MS	Terburol
LC/MS/MS	Terbuthylazine
LC/MS/MS	Terbuthylazine-desethyl
LC/MS/MS	Terbutryn
LC/MS/MS	Tetrachlorvinphos
LC/MS/MS	Tetraconazole
LC/MS/MS	Tetramethrin
LC/MS/MS	TFNA

LC/MS/MS	TFNG
LC/MS/MS	Thiabendazole
LC/MS/MS	Thiacloprid
LC/MS/MS	Thiametoxam
LC/MS/MS	Thidiazuron
LC/MS/MS	Thiencarbazone methyl
LC/MS/MS	Thiobencarb
LC/MS/MS	Thiodicarb
LC/MS/MS	Thiometon
LC/MS/MS	Thionazin
LC/MS/MS	Thiophanate-Methyl
LC/MS/MS	Thiram
LC/MS/MS	Tolclofos-Methyl
LC/MS/MS	Tolylfluanid
LC/MS/MS	Tralkoxidym
LC/MS/MS	Triadimefon
LC/MS/MS	Triadimenol (mixture of the diastereoisomers A & B)
LC/MS/MS	Triallat
LC/MS/MS	Triamiphos
LC/MS/MS	Triasulfuron
LC/MS/MS	Triazamate
LC/MS/MS	Triazophos
LC/MS/MS	Tribenuron methyl
LC/MS/MS	Trichlorfon
LC/MS/MS	Trichloronat
LC/MS/MS	Triclopyr
LC/MS/MS	Tricyclazole
LC/MS/MS	Tridemorph
LC/MS/MS	Trietazine
LC/MS/MS	Trifloxystrobin
LC/MS/MS	Triflumizole
LC/MS/MS	Triflumuron
LC/MS/MS	Triforine
LC/MS/MS	Trinexapac-ethyl
LC/MS/MS	Triticonazole
LC/MS/MS	Valifenalate
LC/MS/MS	Vamidothion
LC/MS/MS	Zoxamide
GC/MS	Dithiocarbamates as CS2
LCMSMS	Chlormequat

LCMSMS	Perchlorate
LCMSMS	Chlorate
LCMSMS	Etephon
LCMSMS	Etilenthiurea (ETU)
LCMSMS	Fosetyl-Aluminium
LCMSMS	Glufosinate Ammonio
LCMSMS	Glyphosate
LCMSMS	Idrazide maleica
LCMSMS	Mepiquat
LCMSMS	Paraquat-Diquat
LCMSMS	Phosphonic Acid
LCMSMS	Propilenthiurea (PTU)
Ionic Chromatography	Nitrate
Ionic Chromatography	Nitrite
GC/ECD	Bromide
GCMS	Furane
LCMSMS - UPLC-UV	4-HMF
LCMSMD	Ergosterol

Calculations

None

Remarks

- The importance of the sample's representativeness
- Sampling must be done about ten days prior to the start of the conferment of the field under control.

1.2.22. Mineral composition

Belotti Enio

Water & Life SRL – Via Enrico Mattei 37 – Entratico (BG), Italy

Importance and applications

All the tomato conferred must be produced according to the criteria established by the Integrated Production Regulation of the region it belongs to and in any case in compliance with the minimum requirements set by the Emilia Romagna Region Regulations for the QC mark.

Principle

The method allows any minerals in fresh tomato to be found and to verify that these minerals are under the LMR.

Reagents

- Fresh tomato sample

Materials and equipment

- IPC-MC Technique
- QuPP Technique and TANDEM MASS SPECTROMETRY

Procedure

At least one sampling in the fresh tomato field is carried out every 1,000 tons of product or fraction (as per EU Regulations 891/2017 and 892/2017), with a minimum of one multi-residual analysis per holding company. The sampling is carried out about ten days prior to the start of the conferment of the field under control.

Sampling can be done on fruits and/or on parts of plants at the discretion of the technician. Tomato picking in the field is carried out by the Casalasco Pomodoro agronomic office technician in the presence of the farm representative.

The sample, about 6 kg, is obtained, after careful mixing, from the mixture of the fruits taken at 5-10 points, according to the cross pattern, excluding an edge area that may have undergone a non-homogeneous treatment.

The sample is then divided into 3 equivalent aliquots: - One that is kept by the farm; - One that is sent to an accredited laboratory; - One is stored by CCDP in the event of a counter analysis.

Each aliquot is collected in a sealed bag which is identified, numbered and signed by the technician and the supplier.

The minerals sought are as follows:

Technique	Minerals
ICP-MS	Cadmium
ICP-MS	Lead
QuPP and TANDEM MASS SPECTROMETRY	Chlorine
ICP-MS	Copper
ICP-MS	Arsenic
ICP-MS	Nickel
ICP-MS	Mercury
ICP-MS	Natrium

Calculations

None

Remarks

- The importance of the sample's representativeness
- Sampling must be done about ten days prior to the start of the conferment of the field under control.

PART 2. SOIL PHYSICOCHEMICAL ANALYSES

2.1. SOIL PHYSICAL ANALYSES

2.1.1 Profile location

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Importance and applications

Geographical latitude loosely correlates with the position in bioms (physico-geographical zones), longitude often gives an idea about the distance from the ocean, and altitude informs about the location in lowland, hill, mountain or plateau.

Principle

The 3-D geographical locations of soil profiles must be unambiguously defined by geographical coordinates: latitude, longitude and elevation above sea level. The grid reference number (Universal Transverse Mercator, UTM) can be read directly from the topographic map. The latitude and longitude of the site should be given as accurately as possible (in degrees, minutes, seconds and decimal seconds) (FAO, 2006).

At present, the only acceptable method for the precise allocation of soil profiles is the application of a global navigation satellite system (GNSS). The commonly-used GNSS is the Global Positioning System (GPS) (Hofmann-Wellenhof et al., 1994), which is operated by the United States Department of Defense (DoD) and consists of a network of 24 NAVSTAR satellites orbiting the Earth on six different paths. Two complete orbits take just under 24 hours. A major benefit is that GPS works in all weather conditions.

The three main methods currently used for enhancing data accuracy are real-time differential correction, reprocessing real-time data and post-processing. To improve accuracy, GPS data are differentially corrected (Steede-Terry, 2000). Differential GPS (DGPS) is based on the assumption that any two receivers placed relatively close to each other will experience similar atmospheric errors. DGPS requires a GPS receiver (base or reference station) to be set up at a precisely known location. Using an atomic clock, timing stability is ensured within one-millionth of a second. Integrating Doppler-derived speed with time signal reliability, an extraordinarily accurate distance measurement is achieved. The difference between the base station and the rover receiver is applied in real time in the field (Fig. 2.1.1.1).

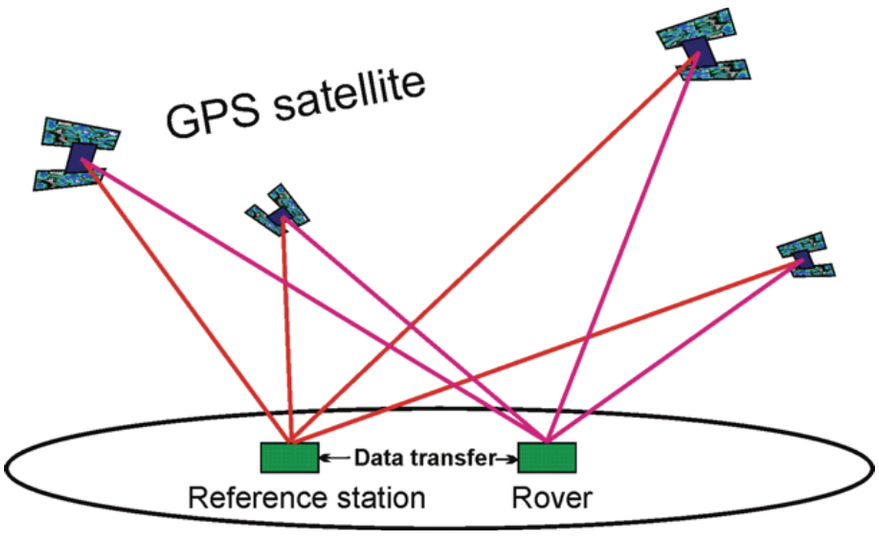

Figure 2.1.1.1 Differential global positioning

With real-time DGPS the base station calculates and broadcasts corrections for each satellite. The correction is received by the rover via a radio signal if the source is land based.
Some GPS devices have a built-in altimeter, which can give quite accurate (to within 3 m) readings of altitude above sea level.

Reagents

- None

Equipment

- Topographic map at 1:25,000 or 1:10,000 scale.
- The most widely used Trimble GPS devices:
- Juno 5 Series – The Juno 5 Series offers smartphone-like operation and compatibility with Trimble mapping and GIS software.
- Geo 7 Series – Equipped with cutting-edge Trimble Flightwave remote positioning technology.
- Yuma 2 – a solidly constructed tablet specifically designed for field applications.
- TDC100 – works like a smartphone, but with enhanced GNSS capabilities.

Procedure

- Check settings for coordinate system (NRC, 1993; EPA, 2015),
- Set accuracy threshold (required accuracy),
- Read and save latitude and longitude coordinates in dd.dddddd format.
- Convert coordinates into degrees/minutes/seconds format.

Remarks

- Now a series of modern smartphones (including Huawei Honor 7X Smartphone Android 7.0, Huawei Honor 9 Lite Smartphone Android 8.0, Huawei Honor V10 Smartphone Android 8.0, Samsung Galaxy Mega 5.8 GT-I9152, Samsung Galaxy S6 SM-G920V, Samsung Galaxy S7 G930V, Samsung Galaxy S7 EDGE G935V, Sony Ericsson Xperia Z3 Compact, Sony Xperia Z5 Compact E5823 etc.) are also capable of site positioning with satisfactory accuracy.
- The Galileo system, now under development, will be operated by the European Global Navigation Satellite Systems Agency (GSA) of the European Union. It will be a new alternative global navigation satellite system, which will remain under civilian control. In the near future, further satellites will be launched to enlarge the constellation, gradually improving Galileo availability worldwide. Its full operational capacity (FOC) of 30 satellites (24 satellites plus six orbital spares) is expected to be accomplished by 2020.

References

EPA, 2015. Global Positioning System. SESDPROC-110-R4. US Environmental Protection Agency, Science and Ecosystem Support Division, Athens, GA. 21 p.
https://www.epa.gov/sites/production/files/2015-10/documents/global_positioning_system110_af.r4.pdf

FAO, 2006. Soil formation factors. Chapter 3 in: Guidelines for soil description. Food and Agriculture Organization of the United Nations, Rome. 9–19.

Hofmann-Wellenhof, B., Lichtenegger, H., Collins, J., 1994. Global positioning systems: theory and practice. Springer Verlag, Heidelberg – Berlin. doi: 10.1007/978-3-7091-3311-8

NRC, 1993. GPS Positioning Guide. Natural Resources Canada, Geomatics Canada, Geodetic Survey Division, Ottawa, ON, Canada. 111 p.
http://www.ceri.memphis.edu/~smalley/ESCI7355/GPS_Guide_e.pdf

Steede-Terry, K., 2000. Integrating GIS and the Global Positioning System. ESRI, Redlands, CA. 150 p.

2.1.2 Major landform

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Importance and applications

The relief of hilly terrains influences soil distribution, water availability and crop growth in a complex way (FAO, 2006). The landform units identified are used for site description in soil and vegetation mapping and in landscape ecology (landscape pattern). For sustainable agriculture, it is important that the typical geometry of the landform where the plot is situated influences land management opportunities and erosion hazard. Particularly, the DEM-based delineation and classification of landforms can assist in land use optimisation and farming practices design (Seif, 2014).

Principle

1. The dominant criteria of major landforms are general slope and relative relief (relief intensity) (FAO, 2006). The relief intensity (expressed in m km^{-1}) is the difference between the highest and lowest point within the terrain unit per distance specified for the actual purpose of study (Table 2.1.2.1).

Table 2.1.2.1 Identification of major landforms from topographic parameters (FAO, 2006)

1st level	2nd level	Gradient (%)	Relief intensity (m km^{-1})	Potential drainage density
L level land	LP plain	< 10	< 50	0-25
	LL plateau	< 10	< 50	0-25
	LD depression	< 10	< 50	16-25
	LV valley floor	< 10	< 50	6-15
S sloping land	SE medium-gradient escarpment zone	10-30	50-100	< 6
	SH medium-gradient hill	10-30	100-150	0-15
	SM medium-gradient mountain	15-30	150-300	0-15
	SP dissected plain	10-30	50-100	0-15
	SV medium-gradient valley	10-30	100-150	6-15
T steep land	TE high-gradient escarpment zone	> 30	150-300	< 6
	TH high-gradient hill	> 30	150-300	0-15
	TM high-gradient mountain	> 30	> 300	0-15
	TV high-gradient valley	> 30	> 150	6-15

Note: Potential drainage density is given in number of “receiving” pixels within a 10 × 10 pixels window.

2. In the simplest way, the geomorphological environment of the agricultural plot, i.e. the landform type on which the plot is located, can be described from a geomorphological map. Depending on the source

of elevation data (resolution), geomorphological maps based on DEMs can be very accurate and allow classification observing subtle changes in elevation (Smith et al., 2011). The geomorphon approach is a grid method to identify landforms at different scales (Józsa & Fábián, 2016).

3. Recently, automated techniques of landform classification have been developed (Blaszczynski, 1997). Most methods are based on the Topographic Position Index (TPI), which is an ArcView GIS application. It shows the difference in elevation between a given cell and the cells in its vicinity (Jenness, 2006):

$$TPI_i = M_0 - \sum_{n-1} M_n \, n^{-1} \quad [-] \qquad \text{(Eq. 2.1.2.1)}$$

where

M_0 is the elevation of the model point (cell) under evaluation,
M_n is the elevation of the grid cell,
n is the total number of surrounding points employed in the evaluation.

Landform classification can be made more accurate through the addition of further topographic metrics, such as elevation, slope gradient or exposure. Combined with slope gradient, the TPI index allows the differentiation of six classes: ridge, upper slope, middle slope, lower slope, flat slope and valley. Altering the diameter of the cell vicinity, Weiss (2001) obtained increased accuracy of landform classification. The landforms can be analysed statistically and correlated with agricultural land-use classes.

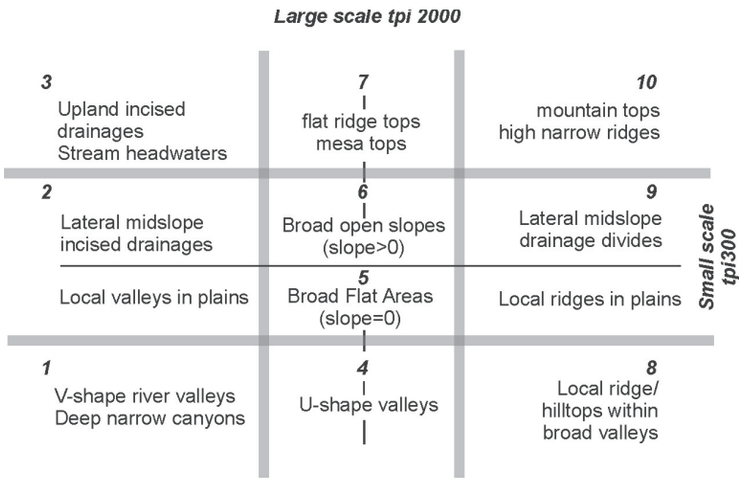


Figure 2.1.2.1 Combining TPI at two scales to identify landform classes, numbers are landform values (after Weiss, 2001)

A well-known algorithm of landform classification (Jenness, 2006) uses a multi-scale approach by fitting a quadratic polynomial to a given window size applying least squares. A product of the method is shown in Fig. 2.1.2.2. A great advantage of this approach is the fact that the definition of classification criteria can be flexibly modified by the user.

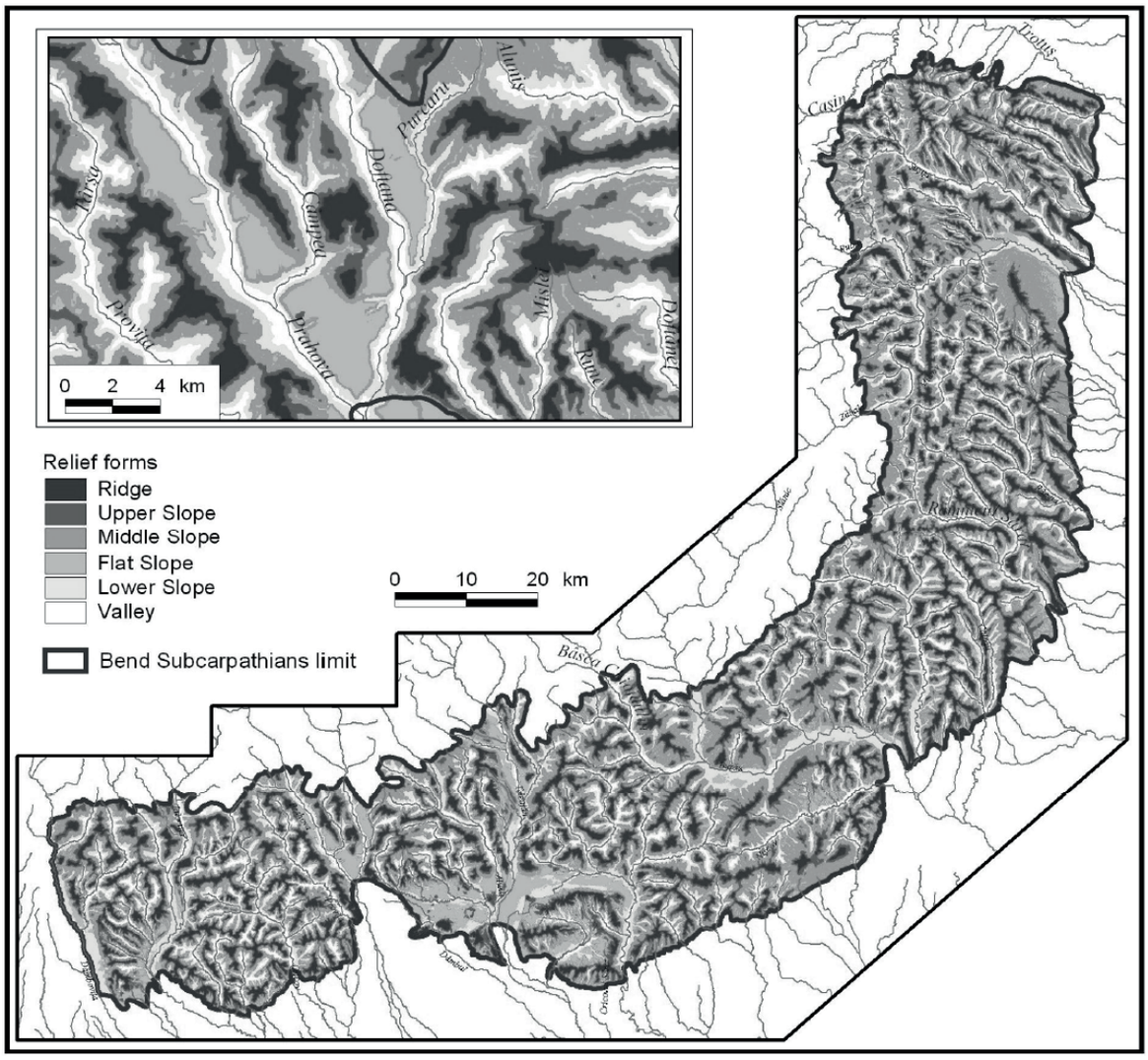
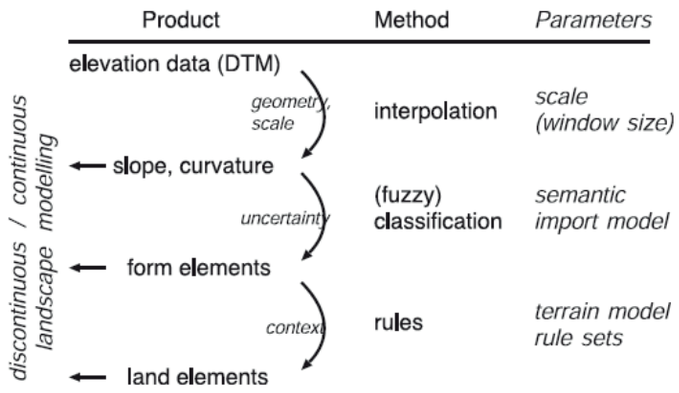


Figure 2.1.2.2 Landform types in the Curvature Subcarpathians, Romania, generated from Digital Elevation Model using the TPI index (after Chendeş et al., 2009)

4. Geometric fuzzy land element classification (Schmidt & Hewitt, 2004) is also based on the fundamental properties of land elements, i.e. local geometry and scale. Since there is a high degree of uncertainty in their delineation and semantic descriptions, land elements have to be fuzzified. An advantage of the model is that it requires a relatively limited number of parameters. 'Object-based image analysis' tools have the ability to segment and classify DEMs into representative objects arranged in a multi-level hierarchy. Ambiguities in landforms both in attribute and geographical space are properly reflected in the fuzzy classification (Gerçek et al., 2011). The methodology for modelling land elements is implemented as a two-step process (Schmidt & Hewitt, 2004): first, form elements are classified based on local geometry, and second, land elements are



derived by evaluating the form elements in their landscape context.

Figure 2.1.2.3 The principles of the fuzzy delineation of landscape elements (after Schmidt & Hewitt, 2004)

5. Remote sensing approaches. The application of radar inferometry for landform classification (Widyatmanti et al., 2016) is based on the combination of multispectral imaging, DEM and radar interferometry (Fig. 2.1.2.4). The classes obtained through DEM segmentation using InSAR Imagery (MacMillan & Shary, 2009) are volcanic, structural, fluvial and karst landforms differentiated by elevation, slope gradient, relief dissection and curvature ("toposhape" features). An advantage of this classification is that it also provides genetic information from morphometric parameters.

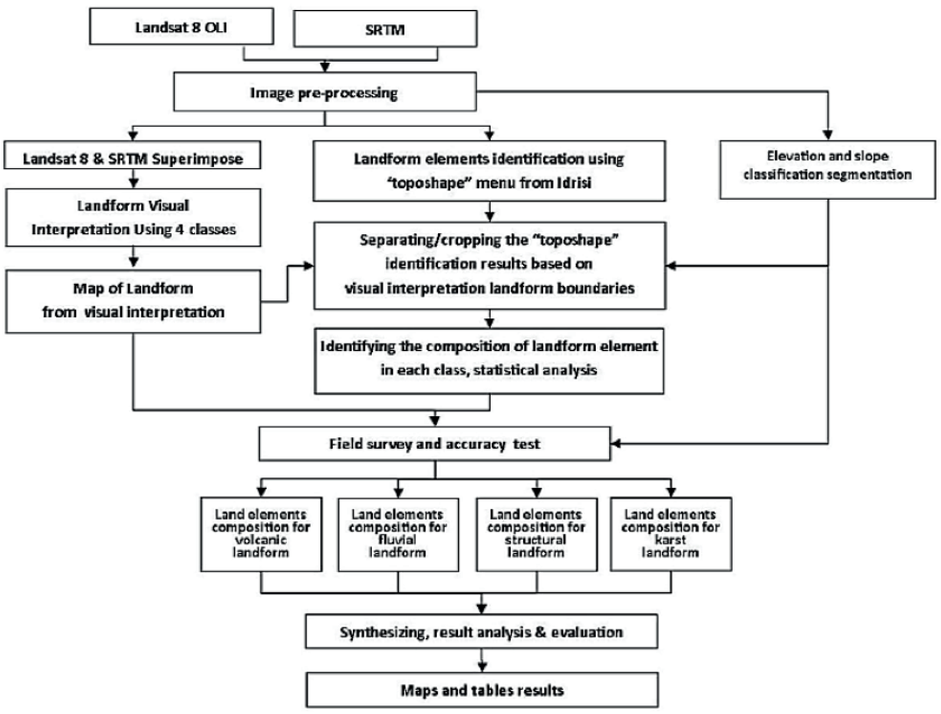


Figure 2.1.2.4 Flow chart of land elements classification (Widyatmanti et al., 2016)

Procedure

1. Classification from topographic map (FAO method):
    1.a. Classify terrain as level, sloping or steep land visually from a topographic map (1st level) (FAO, 2006)
    1.b. Distinguish classes based on relief intensity and potential drainage density (2nd level)
2. Classification by parameters derived from DEM:
    2.a. Define the lookup distance (maximum scale of mapping), the topographic grain, establish the characteristic local ridgeline-to-channel spacing (Pike et al., 1989)
    2.b. Manually find a break-point where the increase rate of local relief is significantly reducing
    2.c. Use a supervised classification algorithm to identify and map physiographic units at the appropriate scale (Józsa & Fábián, 2016)
3. Automated classification based on TPI:
    3.a. Add the elevation or surface model grid to Spatial Analyst for ArcView 3.x software (Jenness, 2006)
    3.b. Select neighbourhood type and radius
    3.c. Generate Slope Position Classification
    3.d. Select the themes and the classification criteria in the Slope Position Analysis dialogue
    3.e. Select and define a classification regime
    3.f. Load criteria sets
    3.g. Confirm your selected classification criteria in the Landform Analysis dialogue
3. Fuzzy land element classification:
    4.a. Create a generalised terrain and parameterise it by scaled derivatives (slope gradient and curvature). These scale-dependent derivatives parameters are calculated at varying window sizes (Wood, 1998)
    4.b. Fuzzify local landform geometry. Slope gradient is continuous, and curvature is referred into three classes: concave, straight, and convex
    4.c. Generate membership-value maps for each of the 15 form elements
    4.d. Identify two moving window sizes for the horizontal spatial scales and specify an elevation threshold to model landforms in terrain context (Schmidt & Hewitt, 2004)
    4.e. Reclassify landforms within the terrain context, using a set of rules; combine the higher scale landscape position model with the form element model
5. Classification using satellite imagery:
    5.a. Interpret Landsat 8 imagery (multi-band composites) visually to draw the boundaries of landform types and to identify topographic elements
    5.b. Use the multi-level landform mapping approach by Van Asselen and Seijmonsbergen (2006) to obtain more accurate landform interpretation
    5.c. Define training sites in the study area for supervised classification
    5.d. Segment the DEM into high and low elevation classes and by slope gradient and elevation (Table 2.1.2.2)
    5.e. Analyse landforms by “toposhape” (using Idrisi package) and make the segmentation (using

eCognition package) per unit area based on landform boundaries

5.f. Overlay drainage map and establish landform types relative to drainage lines (Saadat et al., 2008 – Table 2.1.2.3)

Table 2.1.2.2 DEM segmentation by elevation and slope gradient (Widyatmanti et al. 2016)

Class	Elevation – relative height (m)	Slope (%)
1	< 50 : lowlands	0-2 : flat or almost flat
2	50-200 : low hills	3-7 : gently sloping
3	200-500 : hills	8-13 : sloping
4	500-1000 : high hills	14-20 : moderately steep
5	> 1000 : mountains	21-55 : steep
6	-	56-140 : very steep
7	-	> 140 : extremely steep

Table 2.1.2.3 Landform classification scheme according to Dessaunettes et al. (1971)

Landform	Slope class	Elevation range	Specific characteristics	Land use
River Alluvial Plains (RP)	< 1%	< 150 m	a, These landforms are close to a river	Usually used for irrigated farming
			b, General slope direction is parallel to that of the river. The general shape of this landform is an elongated eclipse with the major axis parallel to the slope of the river	
			c, These landforms are usually next to a meandering river	
Piedmont Plains (PD)	0–5%	n.a.	a, Since RP is really a subset of PD, then RP must be isolated first	Usually used for irrigated or dryland farming
			b, The shape of PD is normally one of a trip with the long dimension parallel to the mountain range front. The transverse elevation cross-section of PDs is normally quite flat	
			c, PDs are restricted to areas with non- or slightly gravelly soils	

Gravelly Talus Fans (GFc)	0–5%	n.a.	a, GFc and GFr are as a result of a major water course or a number of smaller water courses running down to the foot of mountain range fronts	Rarely used for irrigated or dryland farmed
Gravelly River Fans (GFr)	Mostly 0–2%, occasionally 2–5% in the higher parts	n.a.	b, The shape of an individual GFc and GFr is usually triangular-shell shape. A group of GFc/GFr is normally one of a strip with the long dimension parallel to the mountain range front. The transverse elevation cross-section of these fans is clearly convex	
			c, GFc and GFr always have highly gravelly soils	
Plateaux and Upper Terraces (TR)	0–12%, with local relief intensity feature slopes of up to 25%	> 500 m		Tops usually used for dryland farming
Hills (H)	Mostly 8–25%	50–500 m		Usually grazing and/or forestry
Mountains (M)	Over 25%	Mostly 500–1500 m		Usually grazing and/or forestry
Lowlands (LL)	Usually < 1%	< 150 m	a, These landforms are located at the lowest elevations of watersheds	
			b, The transverse cross-section of LL is nearly level and often concave	
			c, The water-table level is usually above the ground surface. The ground and surface water tends to accumulate with subsequent accumulation of fine sediment and salts	
Floodplains (FP)	Usually < 1%	<150 m	a, These landforms are usually next to a river known to flood frequently	
			b, The transverse cross-section of FP is nearly level	
			c, FPs are affected by incoming surface water flow	

Remarks

- Although crop yield variability is largely explained by soil and terrain properties, most agricultural land-use types can also be characterised by landforms identified by a topographic index derived from DEM. This index is relevant in assessing the variability induced by topography on climatic conditions and crop management.
- Landform classification approaches are very similar to each other, but their products may differ greatly

in scale. Therefore, multi-scale techniques are preferred.

- Unsupervised automated landform classifications reflect the frequency distributions of the input variables rather than pre-set criteria. The results must be analysed and calibrated empirically.

References

Blaszczynski, J.S., 1997. Landform characterization with geographic information systems. Photogramm. Eng. Remote Sens., 63, 183–191.

Chendeş, V., Dumitru, S., Simota, C., 2009. Analyzing the landforms – agricultural land-use types relationship using a DTM-based indicator. Scientific Papers, USAMV Bucharest, Series A, Vol. LII. 135–140.

Dessaunettes, J.R., Ochtman, L.H.J., Van de Weg, R.F., 1971. Soil survey, classification and correlation guide for Iran. Food and Agriculture Organization of the United Nations, Rome, and Pilot Development Project of Soil Institute, Tehran, Iran. 177 p.

Draguţ, L., Blaschke, T., 2006. Automated classification of landform elements using object-based image analysis. Geomorphology, 81, 3–4, 330–344. doi: 10.1016/j.geomorph.2006.04.013

Evans, D.A., Williard, K.W.J., Schoonover, J.E., 2016. Comparison of terrain indices and landform classification procedures in low-relief agricultural fields. J. Geospat. Appl. Nat. Res. 1, 1, 1–17. http://scholarworks.sfasu.edu/j_of_geospatial_applications_in_natural_resources/vol1/iss1/1

FAO, 2006. Soil formation factors. Chapter 3 in: Guidelines for soil description. Food and Agriculture Organization of the United Nations, Rome. 9–19.

Florinsky, V., Eilers, R.G., Manning, G., Fuller, L.G., 2002. Prediction of soil properties by digital terrain modelling. Environ. Model. Softw., 17, 295–311. doi: 10.1016/S1364-8152(01)00067-6

Gerçek, D., Toprak, V., Stribl, J., 2011. Object-based classification of landforms based on their local geometry and geomorphometric context. Int. J. Geogr. Inform. Sci., 25, 1011–1023. doi: 10.1080/13658816.2011.558845

Iwahashi J., Pike R.J., 2007. Automated classifications of topography from DEMs by an unsupervised nested-means algorithm and a three-part geometric signature. Geomorphology, 86, 409–440. doi: 10.1016/j.geomorph.2006.09.012

Jenness, J., 2006. Topographic Position Index (TPI) v. 1.3a. Jenness enterprises, Flagstaff, AZ. http://www.jennessent.com/arcview/tpi.htm

Józsa, E., Fábián, Sz.Á., 2016. Mapping landforms and geomorphological landscapes of Hungary using GIS techniques. Stud. Geomorph. Carp-Balc., 50, 19–31.

MacMillan, R.A., Shary, P.A., 2009. Chapter 9: Landforms and topographic elements in geomorphometry. In: Hengl, T., Reuter, H.I., (eds), Geomorphometry—Concepts, Software, Applications. Develop. Soil Sci. 33, Elsevier, Amsterdam. 227–254.

Pike, R.J., Acevedo, W., Card, D.H., 1989. Topographic grain automated from digital elevation models. Proceedings, Auto-Carto 9, ASPRS/ACSM Baltimore MD, 2–7 April 1989. 128–137.

Saadat, H., Bonnell, R., Sharifi, F., Mehuys, G., Namdar, M., Ale-Ebrahim, S., 2008. Landform classification from a digital elevation model and satellite imagery. Geomorphology, 100, 453–464. doi: 10.1016/j.geomorph.2008.01.011

Seif, A., 2014. Using Topography Position Index and relationship between land use AP and geology for

determination of quaternary landform in Zagros Mountain. Bull. Environ. Pharmacol. Life Sci., 3, 11, 70–85.
Schmidt, J., Hewitt, A., 2004. Fuzzy land element classification from DTMs based on geometry and terrain position. Geoderma, 121, 243–256. doi: 10.1016/j.geoderma.2003.10.008
Smith, M.J., Paron, P., Griffiths, J.S., 2011. Geomorphological mapping: methods and applications. Elsevier, Amsterdam – Boston. 612 p.
Van Asselen, S., Seijmonsbergen, A.C., 2006. Expert-driven semi-automated geomorphological mapping for mountainous area using a laser DTM. Geomorphology, 78, 3–4, 309–320. doi: 10.1016/j.geomorph.2006.01.037
Weiss A.D., 2001. Topographic Position and Landforms Analysis. Poster, ESRI User Conference, San Diego, CA.
Widyatmanti, W., Wicaksono, I., Rahma Syam, P.D., 2016. Identification of topographic elements composition based on landform boundaries from radar interferometry segmentation (preliminary study on digital landform mapping). Earth Environ. Sci., 37, 012008. doi:10.1088/1755-1315/37/1/012008
Wood, J., 1998. Modelling the continuity of surface form using digital elevation models. In: Proceedings of the 8th International Symposium on Spatial Data Handling, Burnaby, BC, Canada (Poiker, T., Chrisman, N. Eds). 725–736.

2.1.3 Position in landscape

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Importance and applications

Position on the landform influences soil-water relationships, microclimate and vegetation. Straight slopes allow maximum slope length and slope wash can shape their surface unimpeded. In contrast, on complex slopes localised breakpoints appear and reduce slope length and, thus, the generation of sheet wash and concentrated forms of soil erosion emerge (rills and gullies) (Sensoy & Kara, 2014).

Principle

According to the catena principle, soil types follow one another along slopes in a more or less predictable sequence (Gerrard, 1992). The automatic extraction of landforms through discretising a DEM begins with defining a simple succession of peak – slope – horizontal surface – depression. Further refinement of landform classification is possible by applying the Topographic Position Index (TPI) (Chendeş et al., 2009) (see Chapter 2.1.2 on Major landform) or the nine-unit slope model (Fig. 2.1.3.1 – Dalrymple et al., 1968) or vertical and horizontal slope curvature (Young, 1972).

Reagents

- None

Materials and equipment

- None

Procedure

1. The locations of study plots are defined on the 9-unit slope model (Fig. 2.1.3.1 – Dalrymple et al., 1968).
    1.a. Stratify by scale into landscapes, landforms and microfeatures
    1.b. Discretise slope by gradient classes
    1.c. Identify predominant geomorphic processes (fluvial, eolian, etc.)
    1.d. Distinguish between landform elements: interfluve, valley shoulder (seepage slope), convex upper slope, fall face, midslope, colluvial footslope, alluvial toeslope, channel bank, riverbed
2. Alternatively, vertical and horizontal slope curvature is used to establish the types of slopes where the plot is located (Fig. 2.1.3.2 – Young, 1972).
    2.a. Establish straight (S), convex (V) and concave (C) slopes in planform
    2.b. Establish the same types in lateral view
    2.c. Identify terraced and other complex (irregular) slope shapes

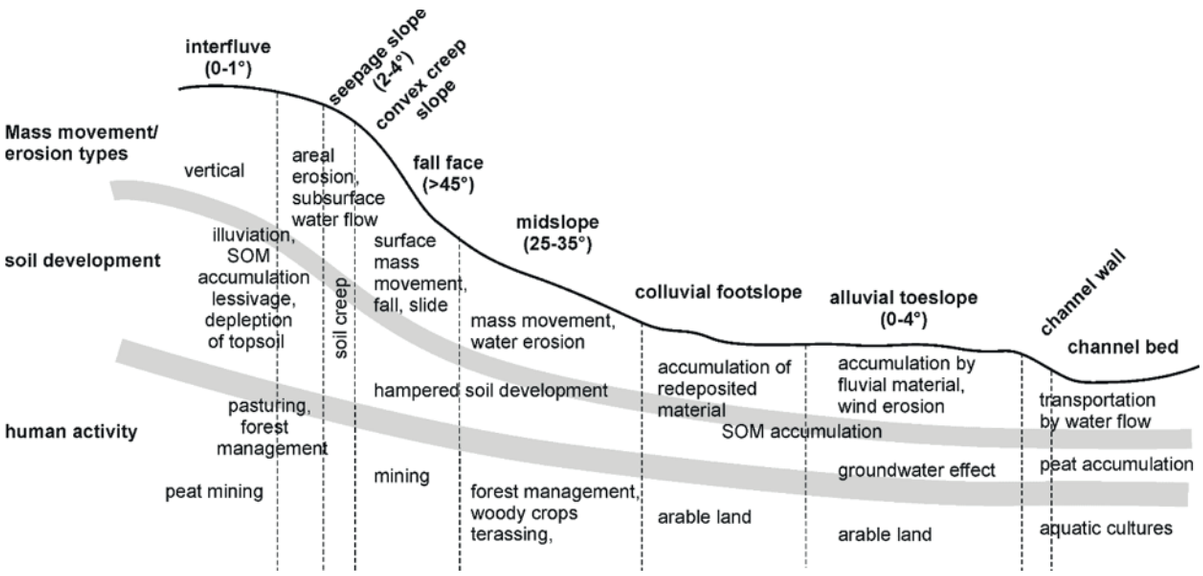

Figure 2.1.3.1 The nine-unit slope model (after Dalrymple et al., 1968), soil development and human activity

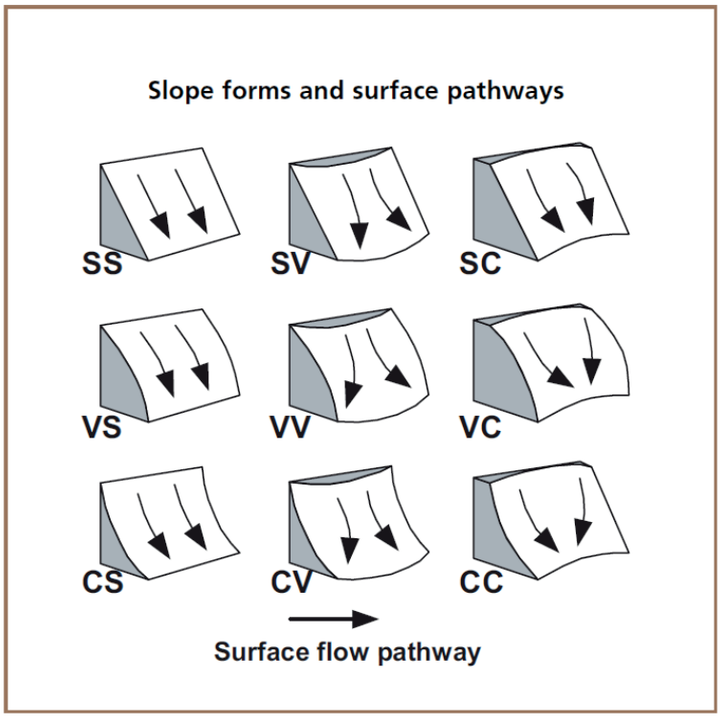

Figure 2.1.3.2 Slope curvature types influencing runoff

Calculations

- None

Remarks

- None

References

Chendeş, V., Dumitru, S., Simota, C., 2009. Analyzing the landforms – agricultural land-use types relationship using a DTM-based indicator. Scientific Papers, USAMV Bucharest, Series A, Vol. LII. 135–140.

Dalrymple, J.B., Blong, R.J., Conacher, A.J., 1968. An hypothetical nine-unit land surface model. Z. Geomorphol., 12, 60–76.

FAO, 2006. Soil formation factors. Chapter 3 in: Guidelines for soil description. Food and Agriculture Organization of the United Nations, Rome. 9–19.

Gerrard, A.J., 1992. The catena concept. Chapter 3 in: Soil Geomorphology. Springer, Dordrecht. 29–50.

Sensoy, H. and Kara, O., 2014. Slope shape effect on runoff and soil erosion under natural rainfall conditions. Biogeosci. Forestry, 7, 2, 110–114. doi: 10.3832/ifor0845-007

USDA, 2017. Soil Survey Manual. Soil Science Division Staff, United States Department of Agriculture, Washington, DC. 639 p.

Young, A., 1972. Slopes. Oliver and Boyd, Edinburgh. 288 p.

2.1.4 Slope gradient

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Importance and applications

The topography of an agricultural field determines how susceptible the soil is to erosion by water. Among the topographic factors the slope gradient is the most important in governing the rate of soil erosion (Zingg, 1940; Assouline & Ben-Hur, 2006). In general, the steeper and longer the slopes are in a field, the greater the soil erosion potential (OMAFRA, 2016). The close relationship between slope gradient and soil erosion must be taken into account in planning land use in hilly areas (Marsh, 2014). The relationship between slope gradient and soil erosion, neverthless, varies considerably with different land use classes (Fig. 2.1.4.1) and land management practices (Liu et al., 1994).

Through soil erosion, slope gradient also controls the loss of nutrients from plots. For instance, a study in Poland (Chowaniak et al., 2016) found that annual average losses of calcium and magnesium were the highest from plots with gradients above 16% (on average, 25% higher for Ca and 26% higher for Mg than losses measured on plots with a 9% slope gradient).

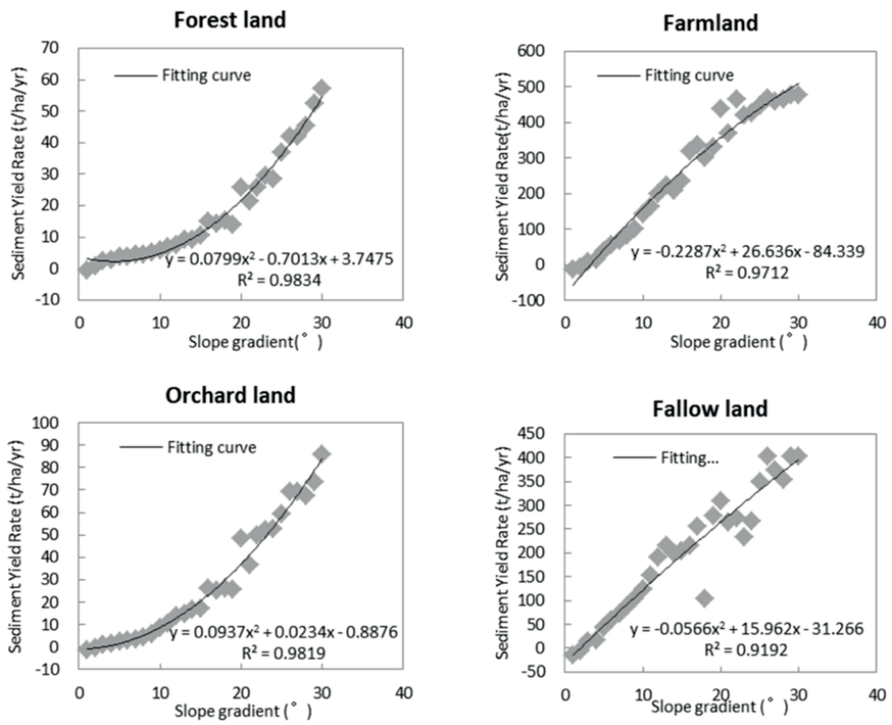

Figure 2.1.4.1 Dependence of sediment yield on slope gradient by land use classes in Jiangxi province, Southern China (Zhang et al., 2015)

Principle

The slope along a line in the surface is expressed either
as a percentage, or the number of metres of change in elevation over a horizontal distance of 100 m, or
as an angle in degrees, as the measurement of the vertical angle made by the slope and the horizontal plane, or
as a ratio of vertical distance to horizontal distance (used to express the slope for the sides of dykes and canals).
Without a map available, slope gradient is to be determined by direct field measurements using an Abney level, a clinometer (OMAFRA, 2016) or a theodolite. Applications for slope measurement exist for smartphones. To ensure accuracy, however, smartphone results must be tested and compared to those from standard measuring procedures. Slope length can simply be measured with a measuring tape.

Reagents

- None

Equipment

- Abney level: a protractor is coupled to a sighting tube, with a bubble level and a mirror prism (OMAFRA, 2016). Sliding the eyepiece forward or backward the bubble image is focused. The scale plate (protractor) has both percent and degree scale graduations. The indicator or scale-pointing arm has a vernier scale. The bubble level on the main body is used to level the instrument.
- Indian pattern clinometer: consists of a base plate with a small bubble tube and a leveling screw; the eye vane has a peep hole on the base plate; the object vane has graduations in degrees on one side and the tangent of the angles on the other (OMAFRA, 2016).
- Buriel hand level: consists of a frame with a mirror and a plain glass. The principle is that a horizontal ray of light is reflected back from a vertical mirror. With the instrument at eye level, the image of the eye is visible at the edge, while the objects appearing opposite the image of the eye are at the observer's eye level.
- Foot rule clinometer: consists of a box wood rule with two arms hinged to each other, both supplied with a small bubble tube, a pair of sights and a graduated arc.
- Fennel's clinometer: consists of a telescope, two plate levels, a vertical arc which rotates or tilts with the telescope, and a holding staff with a target.
- De Lisle's clinometer: consists of a simple frame with a mirror (vertical reference line) and a semicircular graduated arc with a moveable radial arm.
- Sextant: The arrangement of two mirrors enables the observer to sight at two different objects simultaneously. The angle between the mirrors is equal to half the actual angle between two objects. Slope angle can be measured in a single observation.
- Theodolite: A movable telescope mounted within two perpendicular axes: the horizontal or trunnion axis and the zenith axis. For measuring vertical angles between the zenith and the ground surface.

Procedure

1. Measurement of vertical angle using Abney level:
    1.a. Set the instrument at eye level
    1.b. Direct it to the object
    1.c. Bring the bubble to the centre
    1.d. Read angle on the arc by means of the vernier scale

2. Measurement using the Indian pattern clinometer:
    2.a. Set the plane table over the station and keep the clinometer on it
    2.b. Use the levelling screw to level the clinometer
    2.c. Look through the peep hole, move the slide of the object vane until it bisects the signal
    2.d. Read the tangent of the angle
    2.e. Calculate the angle from distance x tangent of vertical angle (d tanα)

3. Measurement with the foot rule clinometer:
    3.a. Hold the instrument firmLy against a rod, with the bubble centred in the lower arm
    3.b. Raise the upper arm until the sight line passes through the object
    3.c. Take the reading on the arc

4. Measurement with Fennel's clinometer:
    4.a. Incline the telescope towards the sighted object
    4.b. Make the reading on the diaphragm with stadia lines

5. Measurement with De Lisle's clinometer:
    5.a. Slide the weight to the inner stop of the arm
    5.b. Turn the arc forward for rising gradients and backwards for falling gradients
    5.c. Suspend the instrument and hold it at arm's length to see the reflected image of one's eye at the edge of the mirror
    5.d. Move the radial arm until the object sighted is coincident with the reflection of the eye
    5.e. Make the reading on the arc

6. Measurement of vertical angle with theodolite:
    6.a. Rotate the theodolite until the arrow in the rough sights is lined up with the point to measure
    6.b. Look through the small eyepiece, and adjust the knob to obtain a precise horizontal lined up with your object
    6.c. Read the slope angle through the small eyepiece, do the vertical measurement

Calculations

- None

References

Assouline, S., Ben-Hur, A., 2006. Effects of rainfall intensity and slope gradient on the dynamics of interrill erosion during soil surface sealing. Catena, 66, 211–220. doi: 10.1016/j.catena.2006.02.005

Chowaniak, M., Klima, K., Niemiec, M., 2016. Impact of slope gradient, tillage system, and plant cover on soil losses of calcium and magnesium. J. Elem., 21, 2, 361–372. doi: 10.5601/jelem.2015.20.2.873

FAO, 1998. A Manual for the Planning, Design and Construction of Forest Roads in Steep Terrain. 6. Surveying. United Nations Food and Agriculture Organization, Rome.
http://www.fao.org/docrep/w8297e/w8297e05.htm

Liu, B.Y., Nearing, M.A., Risse, L.M., 1994. Slope gradient effects on soil loss for steep slopes. Trans. ASAE, 37, 1835–1840.

Marsh, W.M., 2014. Framing the Land use Plan: A Systems Approach. In: The Ecological Design and Planning Reader (Ndubisi, F.O. Ed.) Island Press, Washington, DC. 396–403.

OMAFRA, 2016. Measuring field slopes to estimate soil erosion. Ontario Ministry of Agriculture, Food and Rural Affairs, Guelph http://www.omafra.gov.on.ca/english/engineer/rusle2/fieldslope.htm

Zhang, Zh.-Y., Sheng, L.-T., Yang, J., Chen, X.-A., Kong, L.-L., Wagan, B., 2015. Effects of Land Use and Slope Gradient on Soil Erosion in a Red Soil Hilly Watershed of Southern China. Sustainability, 7, 14309–14325. doi: 10.3390/su71014309

Zingg A.W., 1940. Degree and length of land slope as it affects soil loss in runoff. Agric. Engng., 21, 59–64.

2.1.5 Slope exposure

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Importance and applications

Although slope exposure or aspect is of minor importance in the tropics, it is a major factor influencing the distribution of solar radiation at middle and high latitudes. Direct insolation is a function of slope exposure. The indirect effects of slope exposure are associated with the position relative to the direction of the prevailing wind. Soils on windward slopes will typically be shallower, while on leeward slopes winds promote soil formation through depositing fine air-borne particles. At mid-latitudes southwestern slopes (which receive the highest amount of insolation in early afternoon) usually show the lowest soil moisture and lowest soil organic matter content. Slope exposure also influences seasonal temperature-dependent soil biological processes (Bardelli et al., 2017).

For agricultural cultivation, northern slopes should be avoided; southerly slopes (S, SE and SW), where heat accumulation is at its maximum, are preferred. For instance, in northern regions vineyards are located on south/southwest facing slopes (Stafne, 2015). Under climates with warm or hot summers and cold winters, eastern, northern, and northeastern slopes are the preferred sites for crop cultivation. Southern and western exposures are warmer than eastern and northern exposures. Southern exposures warm earlier in the spring and can slightly advance bud break, thus increasing the potential for frost damage (Stafne, 2015). Eastern slopes are exposed to the morning sun; vinestocks and fruit trees there will dry (from dew or rain) sooner than those on a western slope, potentially reducing disease risk. Management costs can be reduced if the optimal slopes are selected for cultivation.

Principle

Slope exposure/aspect is defined as the directional component of the slope gradient vector, i.e. the direction of maximum gradient of the surface at a given point (FAO, 2006). It is expressed as the compass direction the slope faces (north, south, east or west). It is also often expedient to distinguish secondary directions (northeast, southeast, southwest, northwest). As a GIS derivative, ArcGIS uses Horn's 8-point formula (Burrough & McDonell, 1998; De Smith et al., 2018) and slope exposure/aspect is calculated counterclockwise from east (Fig. 2.1.5.1).

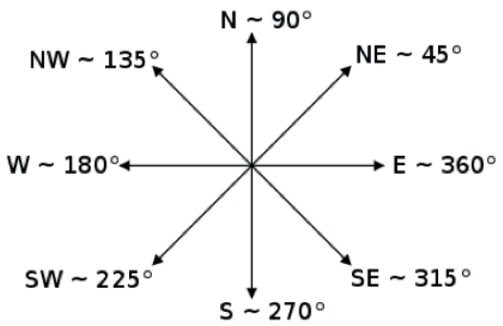


Figure 2.1.5.1 Slope aspects (after Shapiro & Waupotitsch, 2015)

Reagents

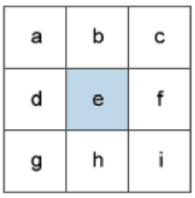
- None

Materials and equipment

- None

Procedure

a. Set up a 3 x 3 moving window grid over the studied surface



b. Calculate gradient in different directions for different cells (see Calculations)

Calculations

a. Calculate the rate of change in the **x** direction for cell **e**:

$$[dz/dx] = ((c + 2f + i) - (a + 2d + g)) / 8 \quad \text{(Eq. 2.1.5.1)}$$

b. Calculate the rate of change in the **y** direction for cell **e**:

$$[dz/dy] = ((g + 2h + i) - (a + 2b + c)) / 8 \quad \text{(Eq. 2.1.5.2)}$$

c. Calculate the aspect for cell **e**:

$$aspect = 57.29578 * atan2 ([dz/dy], -[dz/dx]) \quad \text{(Eq. 2.1.5.3)}$$

Remark

- Exposure values can be transformed to azimuth (0 is north, 90 is east, etc)

References

Bardelli, T., Gómez-Brandón, M., Ascher-Jenull, J., Fornasier, F., Arfaioli, P., Francioli, D., Egli, M., Sartori, G., Insam, H., Pietramellara, G., 2017. Effects of slope exposure on soil physico-chemical and microbiological properties along an altitudinal climosequence in the Italian Alps. Sci. Total Environ., 575, 1041–1055. doi: 10.1016/j.scitotenv.2016.09.176

Burrough, P.A., McDonell, R.A., 1998. Principles of Geographical Information Systems. Oxford University Press, New York. 190 p.

De Smith, M.J., Goodchild, M.F., Longley, P.A., 2018. Geospatial Analysis: A Comprehensive Guide to Principles, Techniques and Software Tools Hardcover. Sixth Edition. Troubadour Publishing, Leicester

FAO, 2006. Soil formation factors. Chapter 3 in: Guidelines for soil description. Food and Agriculture Organization of the United Nations, Rome. 9–19.

Shapiro, M., Waupotitsch, O., 2015. GRASS GIS 7.4.1svn Reference Manual. U.S. Army Construction Engineering Research Laboratory, Champaign, IL.
https://grass.osgeo.org/grass74/manuals/r.slope.aspect.htmL

Stafne, E., 2015. Vineyard Site Selection. eXtension Foundation, Kansas City, MO
http://articles.extension.org/pages/31027/vineyard-site-selection

2.1.6 Parent material

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Importance and applications

Parent material, composed of primary minerals, is among the five factors of soil development. It determines soil physical properties such as texture, structure, water holding capacity and partly or totally the way of weathering, clay content and, thus, influences soil workability and the opportunities for an alternative cropping system (like minimum or no tillage).

The mineralogy of the parent material is mirrored in the soil, it affects the processes of weathering and natural vegetation growth (Anderson, 1998, Augusto et al., 2017, Rafael et al., 2018). Soil parent material is also the basic source of nutrients for microbial life (Sun et al., 2015).

Parent material also affects the chemical properties of soils. Particularly in the case of young soils, releasing nutrients (e.g. phosphorous and potassium) by weathering and controlling rooting depth by rock resistance, the parent material has a major influence on soil fertility. Alluvial soils derived from fluvial deposits, for instance, are rich in plant nutrients but deficient in organic C and N. Vineyards and orchards are cultivated on steep slopes on the exposed rock, with a lack of any soil cover, and, thus, parent sediments have a decisive role.

Principle

The task is to reveal to what extent soil parent material determines the nutrient supply and its limitations. Concerning phosphorus, the relationships between actual P pools of soils and physico-chemical properties (acidity, P richness) of the parent material must be quantified (Rafael et al., 2018).

Therefore, the geological rock classification system is not suitable for pedological purposes (FAO, 2006). For instance, the amounts of exchangeable cations in the soil deriving from the parent material also depend on climate (weathering intensity) (Fig. 2.1.6.1).

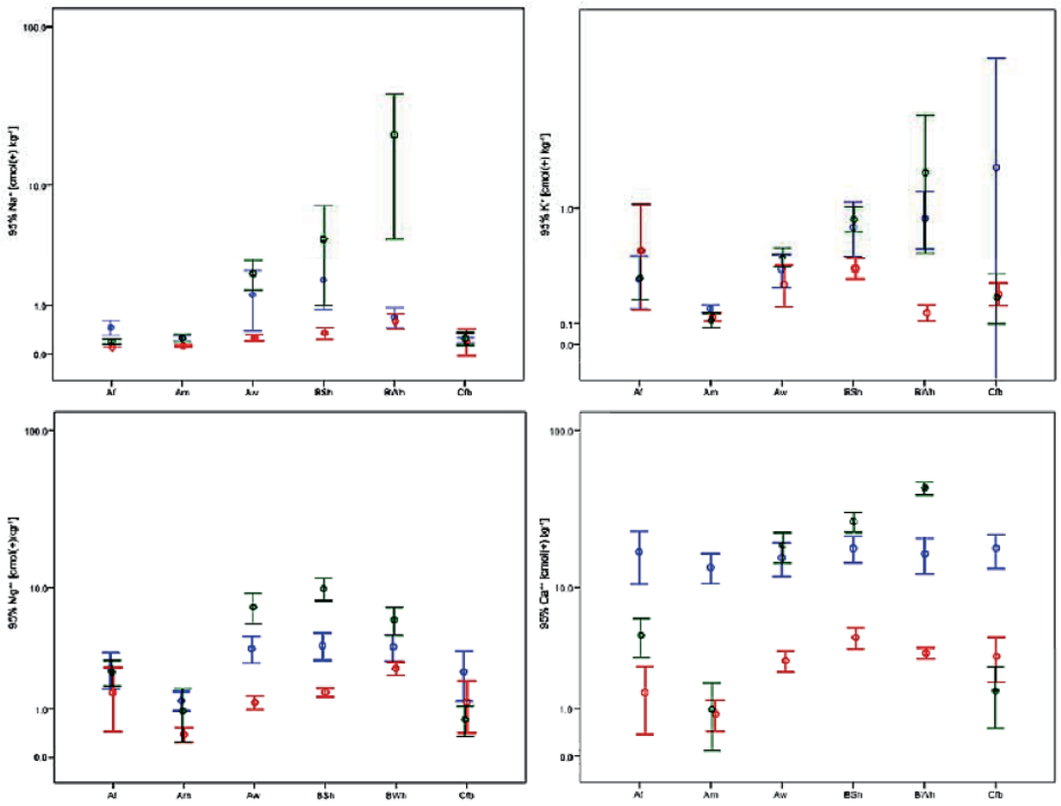

Figure 2.1.6.1 The 95% confidence interval of exchangeable cations (Na, K, Mg and Ca) in soils from granite, basalt and limestone for different Köppen-Geiger climates (WISE database processed by Batjes, 2008). Af = equatorial, humid; Am = tropical monsoon; Aw = equatorial, winter dry; BSh = hot arid, steppe; BWh = hot arid, desert; Cfb = warm temperate, humid, warm summer

Reagents

- None

Materials and equipment

- None

Procedure

The parent material classification is based on the FAO SOTER system (FAO, 2006), modified by Schuler et al. (2010). The revised classification system consists of several levels with weighted properties (Table 2.1.6.1):

a. level 1: consolidated or unconsolidated
b. level 2: geochemical properties (e.g. siliceous, carbonaceous, saline)
c. level 3: expression strength of geochemical properties
d. level 4: genetic type (e.g. igneous, metamorphic)
e. level 5: rock name according to IUGS terminology or traditional terms.

Table 2.1.6.1 The revised system of soil parent material classification (after Schuler et al., 2010)

Level2	Level 3	Level 4	Level 5
Level 1: C consolidated			
CS siliceous	**CSA** acid (> 66% SiO2)	**CSAI** igneous	**CSAI1** quartz rich granitic rock, quartzolite
			CSAI2 aplite (75% SiO_2), rhyolite (74% SiO_2), rhyolitic tuff, alkali feldspar rhyolite (73% SiO_2), quartz latite (73% SiO_2), granite (72% SiO_2), monzogranite (72% SiO_2), syenogranite (72% SiO_2), pegmatite (71% SiO_2), alkali feldspar granite (70% SiO_2)
			CSAI3 dacite (68% SiO_2), granodiorite (68% SiO_2), quartz syenite (67% SiO_2)
		CSAM metamorphic	**CSAM1** quartzite (81% SiO_2), siliceous shale, siliceous schist
			CSAM2 spilite (71% SiO_2), migmatite (70% SiO_2), gneiss (69% SiO_2), paragneiss, orthogneiss, psammite (69% SiO_2), meta-felsic rock
			CSAM3 semipelite
		CSAS sedimentary rock	**CSAS1** chert (77% SiO_2), flint, radiolarite, spiculite
			CSAS2 quartz arenite, quartz wacke, sandstone (76% SiO_2), conglomerate (73% SiO_2), breccias consisting of acid rock fragments, fanglomerate, arkose (71% SiO_2), arkosic arenite
			CSAS3 greywacke (66% SiO_2), feldspathic greywacke, arkosic wacke
	CSI intermediate (52-66% SiO_2)	**CSII** igneous	**CSII1** tonalite (65% SiO_2), latite (65% SiO_2), obsidian (65% SiO_2), quartz monzonite (64% SiO_2), syenite (63% SiO_2), trachyte (63% SiO_2), quartz alkali feldspar syenite, quartz alkali fedspar trachyte, quartz diorite, quartz gabbro, quartz anorthosite, foid-bearing syenite/alkali feldspar syenite/trachyte
			CSII2 monzonite (59% SiO_2), monzodiorite (59% SiO_2), benmoreite (58% SiO_2), andesite 58% SiO_2), boninite, diorite (57% SiO_2), monzogabbro (56% SiO_2), keratophyre[2] (56% SiO_2), phonolite (55% SiO_2), kersantite (55% SiO_2), foid-bearing monzonite/diorite/ monzodiorite/monzogabbro
			CSII3 alkali feldspar syenite (54% SiO_2), alkali feldspar trachyte, trachyandesite (52% SiO_2),

CS siliceous	**CSI** intermediate (52-66% SiO_2)	**CSIM** metamorphic	**CSIM1** pelite (63% SiO_2), slate (63% SiO_2), phyllite (62% SiO_2), hornfels (61% SiO_2), schist (60% SiO_2), mica schist, metamudstone
			CSIM2 granofels (56% SiO_2)
			CSIM3 granulite (53% SiO_2)
		CSIS sedimentary rock	**CSIS1** diamictite (61% SiO_2), tillite
			CSIS2 siltstone (61% SiO_2), claystone (61% SiO_2), mudstone (60 SiO_2)
	CSB basic (45-52% SiO_2)	**CSBI** igneous	**CSBI1** basalt (50% SiO_2), dolerite (50% SiO_2), gabbro (49% SiO_2), anorthosite (49% SiO_2), lamprophyre (48% SiO_2), alkali basalt, tholeiite, diabase, foid-bearing gabbro/ anorthosite
			CSBI2 theralite (46% SiO_2), basanite (46% SiO_2), limburgite (46% SiO_2), pyroxenite (46% SiO_2), tephrite (45% SiO_2), basanite (45% SiO_2)
		CSBM metamorphic	**CSBM1** amphibolite (50% SiO_2)
			CSBM2 meta-basic rock, meta-mafic rock, greenstone, greenschist, blueschist, spillite
			CSBM3 eclogite (50% SiO_2)
			CSBM4 calc-silicate rock (49% SiO_2)
		CSBS sedimentary rock	**CSBS1** breccia (51% SiO_2)
		CSBA artificial	**CSBA1** acid slag (45-50% SiO_2)
	CSU ultrabasic (<45% SiO_2)	**CSUI** igneous	**CSUI1** foid syenite, foid monzonite, foid monzodiorite, foid monzogabbro, foid diorite, foid gabbro
			CSUI2 leucitite (44% SiO_2), nephelinite (44% SiO_2), foidolite, foidite
			CSUI3 picrite (43% SiO_2), komatiite (41% SiO_2), meimechite
			CSUI4 hornblendite (41% SiO_2)
			CSUI5 peridotite (39% SiO_2)
			CSUI6 melilitite (37% SiO_2)
			CSUI6 kimberlite (29% SiO_2)
		CSUM metamorphic	**CSUM1** serpentinite (43% SiO_2), meta-ultramafic rock
			CSUM2 skarn (42% SiO_2)
		CSUA artificial	**CSUA1** basic slag (25-30% SiO_2)

CS siliceous	**CSX** unspecified	**CSXI** igneous	**CSXIx** igneous rock (unspecified)
			CSXI1 agglomerate, pyroclastic breccia, scoria
			CSXI2 tuff-breccia
			CSXI3 lapilli-stone
			CSXI4 lapilli-tuff
			CSXI5 tuff, consolidated ignimbrite (welded tuff)
		CSXM metamorphic	**CSXMx** metamorphic rock (unspecified)
			CSXM1 suevite, impactite, impact-melt breccias, impact-melt rock
			CSXM2 cataclasite, mylonite
		CSXS sedimentary rock	**CSXSx** sedimentary rock (unspecified)
			CSXS1 tuffaceous-sedimentary rock, tuffite
CC carbonatic	**CCP** pure	**CCPM** metamorphic	**CCPM1** marble
		CCPS sedimentary rock	**CCPS1** limestone, travertine
			CCPS2 dolomite
	CCI impure	**CCIS** sedimentary rock	**CCIS1** impure limestone, impure, dolomite, marlstone
	CCX unspecified	**CCXI** igneous	**CCXI1** carbonatite
		CCXM metamorphic	**CCXMx** metacarbonate rock
		CCXS sedimentary rock	**CCXSx** carbonatic sedimentary rock (unspecified)
CY saltic	**CYX** unspecified	**CYXS** sedimentary rock	**CYXS1** halite, sylvite
CG gypsic	**CGX** unspecified	**CGXS** sedimentary rock	**CGXS1** gypsum, anhydrite
CP phosphatic	**CPX** unspecified	**CPXS** sedimentary rock	**CPXS1** phosphorite, guano
CO organic	**COX** unspecified	**COXS** sedimentary rock	**COXS1** bituminous coal, anthracite, graphite
CF fealic	**CFX** unspecified	**CFXS** sedimentary rock	**CFXS1** ironstone, iron ore
Level 1: S semi-consolidated			
SS siliceous	**SSA** acid	**SSAR** residual deposit	**SSAR1** kaolin
SC carbonatic	**SCX** unspecified	**SCXS** sedimentary rock	**SCXS1** chalk
			SCXS2 tufa
SF fealic	**SFX** unspecified	**SFXS** sedimentary rock	**SFXS1** laterite, bauxite

SO organic	**SOX** unspecified	**SOXS** sedimentary rock	**SOXS1** lignite
			SOXS1 asphalt
Level 1: U unconsolidated			
US siliceous	**USA** acid (>66% SiO_2)	**USAI** igneous	**SOXS1** pumice
		USAS sediment	**USAS1** sand (77% SiO_2)
			USAS2 gravel (67% SiO_2)
	USI intermediate (52-66% SiO_2)	**USIS** sediment	**USIS1** silt (57% SiO_2)
			USIS2 clay (59% SiO_2)
	USX unspecified	**USXI** igneous	**USXIx** igneous unconsolidated (unspecified)
			USXI1 block-tephra, bomb-tephra
			USXI2 ash-breccia
			USXI3 lapilli-tephra
			USXI4 lapilli-ash
			USXI5 ash, unconsolidated ignimbrite (non-welded sillar)
		USXS sediment	**USXSx** sediment (unspecified)
			USXS1 breccia
			USXS2 loess
			USXS3 loam
			USXS4 mud, siliceous ooze
			USXS5 diamicton, till
		USXA anthropogenic	**USXA1** waste
			USXA2 heap material
			USXA3 ash (anthropogenic)
			USXA4 brick
			USXA5 mud
UC carbonatic	**UCX** unspecified	**UCXS** sediment	**UCXS1** carbonate sand
			UCXS2 carbonate mud, carbonate ooze
			UCXS3 carbonatic diamicton
			UCXS4 carbonatic sediment, marl
		UCXA anthropogenic	**UCXA1** lime plaster, cement plaster
			UCXA2 concrete
			UCXA3 waste combustion ash
UO organic	**UOX** unspecified	**UOXS** sediment	**UOXS1** half-bog
			UOXS2 peat
			UOXS3 sapropel
		UOXA anthropogenic	**UOXA1** plaggen
			UOXS2 coal/coke dump material
			UOXS3 road construction material: tar, asphalt, bitumen)

UY saltic	**UYX** unspecified	**UYXS** sediment	**UYXS1** salt mud
		UYXA anthropogenic	**UYXS2** saline material
UG gypsic	**UGX** unspecified	**UGXS** sediment	**UGXS1** gypsum-mud
		UGXA anthropogenic	**UGXA1** gypsum plaster
UP phosphatic	**UPX** unspecified	**UPXS** sediment	**UPXS1** phosphoric mud
UF fealic	**UFX** unspecified	**UFXS** sediment	**UFXS1** iron sediment
		UFXA anthropogenic	**UFXA1** red mud
			UFXA2 metal-sludge
UR radioactive	**URX** unspecified	**URXA** anthropogenic	**URXA1** nuclear waste
X unspecified	**X** unspecified	**X** unspecified	**X** unspecified

E	**X**	**X**	**x** evaporitic rock sequence
K	**X**	**X**	**x** carbonatic rock sequence
L	**X**	**X**	**x** organic rock sequence
M	**X**	**X**	**x** iron ore sequence

Remarks

- The influence of parent material on soil properties is usually indirect and, therefore, difficult to detect.
- Multiple parent materials can be found in many soil profiles. For instance, the conditions of soil formation will be fundamentally different in those with thin slope deposits or loess covering the volcanic bedrock, from those on bare bedrock.

References

Anderson, D.W., 1998. The effect of parent material and soil development on nutrient cycling in temperate ecosystems. Biogeochem., 5, 1, 71–97. doi: 10.1007/BF02180318

Augusto, L., Achat, D.L., Jonard, M., Vidal, D., Ringeval, B., 2017. Soil parent material – A major driver of plant nutrient limitations in terrestrial ecosystems. Glob. Chang. Biol., 23, 9, 3808–3824. doi: 10.1111/gcb.13691

Batjes, N.H., 2008. ISRIC-WISE Harmonized Global Soil Profile Dataset (Ver. 3.1). Report 2008/02, ISRIC – World Soil Information, Wageningen (with dataset)

FAO, 2006. Guidelines for soil description. Fourth edition. Food and Agriculture Organization of the United Nations, Rome. 16–17.

Rafael, R.B.A., Fernández-Marcos, M. L., Cocco, S., Ruello, M. L., Weindorf, D. C., Cardelli, V., Corti, G., 2018. Assessment of potential nutrient release from phosphate rock and dolostone for application in acid soils. Pedosphere, 28, 1, 44–58.

Schuler, U., Baritz, R., Willer, J., Dill, H., 2010. A revised approach to classify parent material for soil mapping. Federal Institute for Geosciences and Natural Resources (BGR), Hannover

Sun, L., Gao, J., Huang, T., Kendall, J.R., Shen, Q., Zhang, R., 2015. Parental material and cultivation determine soil bacterial community structure and fertility. FEMS Microbiol. Ecol., 91, 1, 1–10. doi: 10.1093/femsec/fiu010

2.1.7 Type of soil horizon

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Importance and applications

The diagnostic horizons of soil taxonomy are not the same as those used in the genetic soil classification: the designations of genetic horizons are based on a qualitative judgement on soil formation, diagnostic horizons are quantitatively defined. A diagnostic horizon may encompass several genetic horizons (Soil Survey Staff, 2014). In the World Reference Base (WRB) such diagnostic horizons serve as a basis for classification (FAO, 2015). The WRB soil classification is widely applied in designing sustainable soil management techniques (Lal & Stewart, 2013; Stolte et al., 2016).

Principle

Soil horizons must be clearly defined and designated in any soil classification. The classification of soils is based on diagnostic horizons, diagnostic properties and diagnostic materials (FAO, 2015 – Table 2.1.7.1). A soil horizon is a unit of mineral or organic soil material approximately parallel to the land surface with properties altered by soil formation processes, different from adjacent horizons in colour, texture, structure, consistency as well as chemical, biological or mineralogical composition (Agriculture Canada, 2013). The major mineral horizons are A, B, and C. The major organic horizons are L, F, and H (decomposed forest litter) and O (wetland organic matter). Subclasses are identified in the field or in the laboratory by adding lower-case suffixes to the main horizon symbols.

Table 2.1.7.1 The diagnostic horizons, properties and materials of the WRB (FAO, 2015)

1. Anthropogenic diagnostic horizons (all are mineral)

anthraquic horizon in paddy soils: the layer comprising the puddled layer and the plough pan, both showing a reduced matrix and oxidised root channels
hortic horizon: dark, high content of organic matter and P, high animal activity, high base saturation; resulting from long-term cultivation, fertilisation and application of organic residues
hydragric horizon in paddy soils: the layer below the anthraquic horizon showing redoximorphic features and/or an accumulation of Fe and/or Mn
irragric horizon: uniformLy structured, at least moderate content of organic matter, high animal activity; gradually built up by sediment-rich irrigation water
plaggic horizon: dark, at least moderate content of organic matter, sandy or loamy; resulting from application of sods and excrements
pretic horizon: dark, high content of organic matter and P, low animal activity, high contents of exchangeable Ca and Mg, with remnants of charcoal and/or artefacts; including Amazonian Dark Earths
terric horizon: showing a colour related the source material, high base saturation; resulting from adding

mineral material (with or without organic residues) and deep cultivation

2. Diagnostic horizons that may be organic or mineral

cryic horizon: perennially frozen (visible ice or, if not enough water, ≤ 0°C)
calcic horizon: accumulation of secondary carbonates, non-cemented
fulvic horizon: andic properties, highly humified organic matter, higher ratio of fulvic acids to humic acids
melanic horizon: andic properties, highly humified organic matter, lower ratio of fulvic acids to humic acids, blackish
salic horizon: high amounts of readily soluble salts
thionic horizon: with sulphuric acid and a very low pH

3. Organic diagnostic horizons

folic horizon: organic layer, not water-saturated and not drained
histic horizon: organic layer, water-saturated or drained

4. Surface mineral diagnostic horizons

chernic horizon: thick, very dark-coloured, high base saturation, moderate to high content of organic matter, well-structured, high biological activity (special case of the mollic horizon)
mollic horizon: thick, dark-coloured, high base saturation, moderate to high content of organic matter, not massive and hard when dry
umbric horizon: thick, dark-coloured, low base saturation, moderate to high content of organic matter, not massive and hard when dry

5. Other mineral diagnostic horizons related to the accumulation of substances due to (vertical or lateral) migration processes

argic horizon: subsurface layer with distinctly higher clay content than the overlying layer and/or presence of illuvial clay
duric horizon: concretions or nodules, cemented or indurated by silica
ferric horizon: ≥ 5% reddish to blackish concretions and/or nodules or ≥15 % reddish to blackish coarse mottles, with accumulation of Fe (and Mn) oxides
gypsic horizon: accumulation of secondary gypsum, non-cemented
natric horizon: subsurface layer with distinctly higher clay content than the overlying layer and/or presence of illuvial clay; high content of exchangeable Na
petrocalcic horizon: accumulation of secondary carbonates, relatively continuously cemented or indurated
petroduric horizon: accumulation of secondary silica, relatively continuously cemented or indurated
petrogypsic horizon: accumulation of secondary gypsum, relatively continuously cemented or indurated
petroplinthic horizon: sheet of connected yellowish, reddish and/or blackish concretions and/or nodules or of concentrations in platy, polygonal or reticulate patterns; high contents of Fe oxides at least in the concretions, nodules or concentrations; relatively continuously cemented or indurated
pisoplinthic horizon: ≥ 40% strongly cemented to indurated, yellowish, reddish, and/or blackish concretions

and/or nodules, with accumulation of Fe oxides
plinthic horizon: ≥ 15% (single or in combination) of reddish concretions and/or nodules or of concentrations in platy, polygonal or reticulate patterns; high contents of Fe oxides, at least in the concretions, nodules or concentrations
sombric horizon: subsurface accumulation of organic matter other than in spodic or natric horizons
spodic horizon: subsurface accumulation of organic matter and/or Fe and Al

6. Other mineral diagnostic horizons

cambic horizon: evidence of pedogenic alteration; not meeting the criteria of diagnostic horizons that indicate stronger alteration or accumulation processes
ferralic horizon: strongly weathered; dominated by kaolinites and oxides
fragic horizon: structure compact to the extent that roots and percolating water penetrate only along interped faces; non-cemented
nitic horizon: rich in clay and Fe oxides, moderate to strong structure, shiny aggregate faces
protovertic horizon: influenced by swelling and shrinking clays
vertic horizon: dominated by swelling and shrinking clays

7. Diagnostic properties related to surface characteristics

aridic properties: surface layer characteristics of soils under arid conditions
takyric properties: heavy-textured surface layers under arid conditions in periodically flooded soils (special case of aridic properties)
yermic properties: pavement and/or vesicular layer in soils under arid conditions (special case of aridic properties)

8. Diagnostic properties defining the relationship between two layers

abrupt textural difference
very sharp increase in clay content within a limited depth range
albeluvic glossae interfingering of coarser-textured and lighter coloured material into an argic horizon forming vertically continuous tongues (special case of retic properties)
lithic discontinuity differences in parent material
retic properties interfingering of coarser-textured and lighter coloured material into an argic or natric horizon

9. Other diagnostic properties

andic properties: short-range-order minerals and/or organo-metallic complexes
anthric properties: applying to soils with mollic or umbric horizons, if the mollic or umbric horizon is created or substantially transformed by humans
continuous rock consolidated material (excluding cemented or indurated pedogenetic horizons)
geric properties: very low effective CEC and/or acting as anion exchanger
gleyic properties: saturated with groundwater (or upwards moving gases) long enough for reducing conditions to occur
protocalcic properties: carbonates derived from the soil solution and precipitated in the soil (secondary

carbonates), less pronounced than in calcic or petrocalcic horizons
reducing conditions: low pH value and/or presence of sulphide, methane or reduced Fe
shrink-swell cracks open and close due to swelling and shrinking of clay minerals
sideralic properties: relatively low CEC
stagnic properties: saturated with surface water (or intruding liquids), at least temporarily, long enough for reducing conditions to occur, vitric properties ≥ 5% (by grain count) of volcanic glass and related materials and containing a limited amount of short-range-order minerals and/or organo-metallic complexes

10. Diagnostic materials related to the concentration of organic carbon

mineral material: < 20% soil organic carbon
organic material: ≥ 20% soil organic carbon
soil organic carbon: organic carbon that does not meet the diagnostic criteria of artefacts

11. Diagnostic material related to colour

albic material: light-coloured fine earth, expressed by high Munsell value and low chroma

12. Technogenic diagnostic materials (predominantly understood as parent materials)

artefacts created, substantially modified or brought to the surface by humans; no subsequent substantial change of chemical or mineralogical properties
technic hard material: consolidated and relatively continuous material resulting from an industrial process

13. Other diagnostic materials (predominantly understood as parent materials)

calcaric material: ≥ 2% calcium carbonate equivalent, inherited from the parent material
colluvic material: heterogeneous mixture that has moved down a slope
dolomitic material: ≥ 2% of a mineral that has a ratio $CaCO_3/MgCO_3 < 1.5$
fluvic material: fluviatile, marine or lacustrine deposits with evident stratification
gypsiric material: ≥ 5% gypsum, at least partially inherited from the parent material
hypersulphidic material: sulphidic material capable of severe acidification
hyposulphidic material: sulphidic material not capable of severe acidification
limnic material: deposited in water by precipitation or through action of aquatic organisms
ornithogenic material: remnants of birds or bird activity
sulphidic material: containing detectable inorganic sulphides
tephric material: ≥ 30% (by grain count) of volcanic glass and related materials

Master soil horizons mostly have genetic connotations (Fig. 2.1.7.1):
A horizon: at or near the surface with maximum in situ accumulation of organic matter, which makes it darker. If the organic matter is removed, the soil colour is lighter. If clay is removed from the upper part of the solum, the soil texture is coarser. If iron is leached, it is paler.
B horizon: characterised by enrichment in organic matter, sesquioxides or clay; or by distinct soil structure (prismatic or columnar); or by a change of colour denoting hydrolysis, reduction or oxidation. Clay

accumulation is indicated by finer soil textures and by clay cutans coating peds and lining pores.
C horizon: comparatively unaffected by pedogenic processes, except gleying (Cg), carbonate and other soluble salt accumulations.
E horizon: a light coloured, leached horizon, mainly in forest soils
O horizon: organic horizon developed mainly from mosses, rushes, and woody materials
L horizon: accumulation of organic matter with original structures easily discernible
F horizon: accumulation of partly decomposed organic matter
H horizon: accumulation of decomposed organic matter with original structures indiscernible
R horizon: consolidated bedrock layer hard to break with the hands.
W horizon: water layer in gley, organic or cryosols (segregated ice).
For transitional horizons upper-case letters are used, e.g. AB, BC etc.

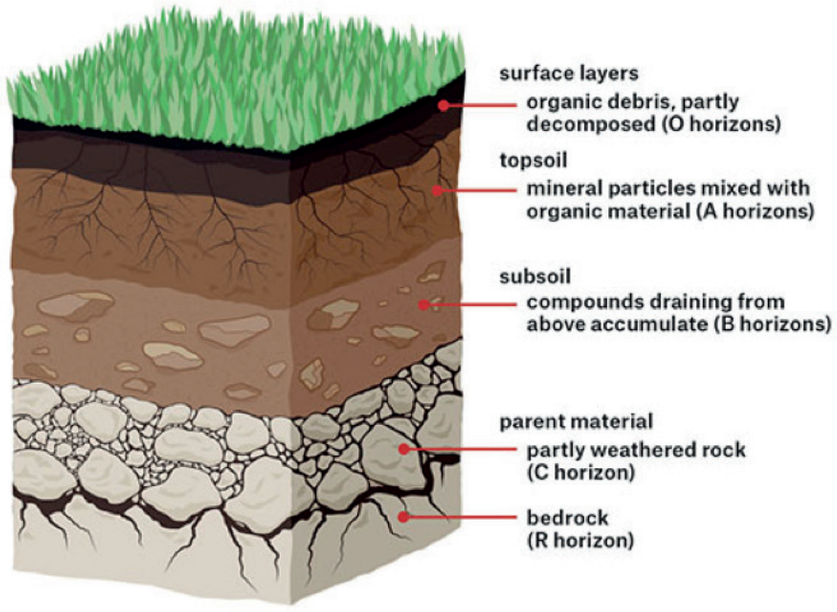

Figure 2.1.7.1 Master soil horizons

Within organic soil horizons, the fibric, mesic and humic materials are usually distinguished (Table 2.1.7.2).

Table 2.1.7.2 Typical physical properties of fibric, mesic and humic materials (after Boelter, 1969)

	Fibric material	Mesic material	Humic material
bulk density (Mg m^{-3})	< 0.075	0.075–0.195	> 0.195
total porosity (% volume)	> 90	90–85	< 85
0.01 MPa H_2O content (% volume)	< 48	48–70	> 70
hydraulic conductivity (cm h^{-1})	> 6	6–0.1	< 0.1

Lower-case suffixes added to the above-listed master horizons (Agriculture Canada, 2013):
b: buried; **c:** cemented; **ca:** secondary carbonate enrichment; **cc:** cemented concretions; **e:** clay eluviation; **f:** amorphous (Al, Fe, organic matter); **g:** greyish or mottled; **h:** organic matter enrichment; **j:** modifier (weak expression of property); **k:** effervescent to HCl; **m:** slightly altered by hydrolysis, oxidation or solution; **n:** exhcangeable Ca to exchangeable Na < 10; **p:** disturbed by human activity; **s:** saline; **sa**: secondary enrichment of salts; **ss**: with slickensides; **t:** illuvial with silicate enrichment; **u:** disrupted by bioturbation; **v:** disrupted by shrinking/swelling; **x:** fragipan; **y:** cryoturbated; **z:** frozen

Material and equipment

The identification of soil horizons is based on bulk density, particle size distribution, Munsell colour, soil reaction, organic matter and carbonate contents etc., for materials see the relevant chapters.

Procedure

a. Determine bulk density, particle size distribution, Munsell colour, soil reaction, organic matter and carbonate contents etc. for the soil horizon
b. Determine master soil horizon
c. Use Table 2.1.7.1 to identify diagnostic horizon type

Remarks

- All horizons may be vertically subdivided by consecutive numeral suffixes, e.g. Ae_1 and Ae_2.
- The upper-case horizon designations A, B and O are always accompanied by lower-case specifications, e.g. A_h, B_w, O_m.
- In some cases, such as B_{gf} and B_{hf}, the combination of suffixes does not simply show the sum of the two suffixes used singly.

References

Agriculture Canada, 2013. Canadian System of Soil Classification. Third Edition. Agriculture Canada, Ottawa. http://sis.agr.gc.ca/cansis/taxa/cssc3/chpt02_a.htmL

Boelter, D.H., 1969. Physical properties of peats as related to degree of decomposition. Soil Sci. Soc. Am. Proc., 33, 606–609.

FAO, 2015. Diagnostic horizons, properties and materials. In: World reference base for soil resources 2014. International soil classification system for naming soils and creating legends for soil maps. Third Edition. Update 2015. Food and Agriculture Organization of the United Nations, Rome. 22–84. (World Soil Resources Report, 106)

Lal, R., Stewart, B.A., 2013. Principles of Sustainable Soil Management in Agroecosystems. CRC Press, Boca Raton, FL. 568 p.

Soil Survey Staff, 2014. Designations for horizons and layers. In: Keys to Soil Taxonomy. Twelfth Edition. USDA Natural Resources Conservation Service, Washington, DC. 335–342.

Stolte, J., Tesfai, M., Øygarden, L., Kværnø, S., Keizer, J., Verheijen, F., Panagos, P., Ballabio, C., Hessel, R. (Eds.), 2016. Soil threats in Europe. EUR 27607 EN. 207 p. doi:10.2788/488054 (print), doi:10.2788/828742 (online)

2.1.8 Depth and thickness of horizon

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Importance and applications

A soil horizon is distinguished from other horizons by texture, colour and structure, which result from soil-forming processes. The individual horizons of a soil profile are of variable thickness. Along with its designation, the depth to and thickness of the horizon should also be recorded because this informs about important ecological properties, such as water-holding and filtering capacity, and rooting depth (Cousin et al., 2009). Although soil horizons are usually conceived as homogeneous, the structure needs to be characterised in 3D at the horizon scale to describe soil hydraulic functioning.

Principle

The description of pedons is essential for soil surveys. A pedon is a three-dimensional body of soil that has sufficient area (roughly 1 to 10 m^2) and depth (up to 200 cm) (USDA no date). When describing the sequence of horizons, quantitative and qualitative data are equally used for the description of the individual soil horizons. The thickness of horizons varies with soil type (Fig. 2.1.8.1).
It is often observed that the depth to a horizon (or layer) boundary differs within short distances, even within a pedon (Knotters et al., 1995). Therefore, the most typical and representative part of the pedon should be described, but variations should also be recorded (Gastaldi et al., 2002). The designation of the horizon is

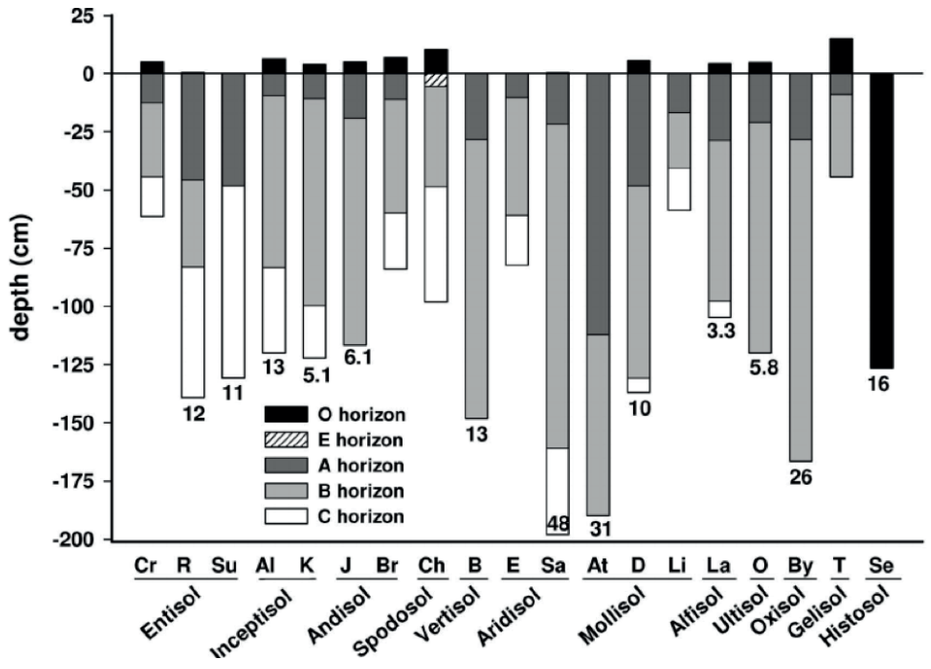

Figure 2.1.8.1 Average thickness of soil horizons for various North-American soil types (Zabowski et al., 2011)

followed by the values that express the depths from the ground surface to the upper and lower boundaries (e.g., Bt1 – 8 to 20 cm). The depth to the lower boundary of a horizon is always the depth to the upper boundary of the underlying horizon. The thickness of each horizon or layer is the vertical distance between the upper and lower boundaries.

In some soils, the variations in depths to boundaries are so complex that the usual terms used to describe the boundary topography are inadequate. These irregularities (e.g. tongues extending to greater depths) are described separately.

Reagents

- None

Materials and equipment

- Measuring tape

Procedure

a. Dig soil pit
b. Clean all sides of the pit of all loose material disturbed by digging
c. Measure the upper and lower boundaries of each horizon on exposed vertical faces
d. Take photographs of all horizons identified (USDA no date)

Calculations

- None

Remarks

- The accuracy required for the determination of soil horizon depth and thickness does not usually exceed 5-10 cm.

References

Cousin, I., Frison, A., Giot, G., Bourennane, H., Guérin, R., Richard, G., 2009. Three-dimensional structure of a highly heterogeneous soil horizon derived by Electrical Resistivity Tomography. Revue d'Archéometrie, 33 (Suppl.), 279–281.

Gastaldi, G., Minasny, B., McBratney, A.B., 2012. Mapping the occurrence and thickness of soil horizons within soil profiles. In: Digital Soil Assessments and Beyond: Proceedings of the 5th Global Workshop on Digital Soil Mapping 2012, Sydney, Australia (Minasny, B., Malone, B.P. and McBratney, A.B. Eds.). 145–148.

Knotters, M., Brus, D.J., Voshaar, J.H.O., 1995. A comparison of kriging, co-kriging and kriging combined with regression for spatial interpolation of horizon depth with censored observations. Geoderma, 67, 3–4, 227–246.

USDA no date. Examination and Description of Soil Profiles. In: Soil Survey Manual. United States Department of Agriculture, Natural Resources Conservation Service, Texas, Temple, TX. https://www.nrcs.usda.gov/wps/portal/nrcs/detail/tx/home/?cid=nrcs142p2_054253

Zabowski, D., Whitney, N., Gurung, J., Hatten, J., 2011. Total soil carbon in the coarse fraction and at depth. Forest Sci., 57, 1, 11–18. doi: 10.1093/forestscience/57.1.11

2.1.9 Bulk density

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Importance and applications

Bulk density (D_b) is important both for soil description (evaluation of soil fertility) and the provision of ecosystem services. Measurements of physical properties such as bulk density and pore size distribution are relevant to assess soil compaction at the macroscopic scale (Gupta et al., 1989). High bulk density is a limiting factor of root penetration (Table 2.1.9.1), soil aeration and water infiltration (FAO, 2006).

Resulting from bad management practices, soil compaction causes an increase in soil bulk density. Porosity is reduced in the compacted layer which loses its ability to transmit water. The compaction layer may result in perched water tables and waterlogging. In a dry state, the compacted layer is a physical barrier to root growth, restricts rooting depth and limits the availability of water and nutrients to the crop (Moody & Cong, 2008).

Table 2.1.9.1 Relationship between bulk density and root growth

Soil texture	Ideal bulk density (g cm^{-3})	Marginal bulk density (g cm^{-3})	Root restricting bulk density (g cm^{-3})
Sands, loamy sands	< 1.60	1.69	> 1.80
Sandy loams, loams	< 1.40	1.63	> 1.80
Sandy clay loams, clay loams	< 1.40	1.60	> 1.75
Silts, silt loams	< 1.40	1.60	> 1.75
Silt loams, silty clay loams	< 1.40	1.55	> 1.65
Sandy clays, silty clays, loams, clay loams	< 1.10	1.49	> 1.58
Clays (> 45% clay)	< 1.10	1.39	> 1.47

Principle

Bulk density is defined as the mass of soil (M_{solids}) of unit volume (V_{soil}) in a dry state, i.e. at 105°C temperature. Thus, the bulk density reflects total soil porosity (FAO, 2006). Total soil volume is the volume of solids and pores together: both pore air (V_{air}) and water volume (V_{water}). Bulk density classes (1 to 5) depend on texture (clay content) (Fig. 2.1.9.1). If values are low (generally below the threshold of 1.3–1.6 g cm^{-3}), porosity is high.

Packing density is an integrated single measure of soil compactness, combining bulk density, structure, organic matter and clay content (Gupta & Larson, 1979; van Ranst et al., 1995):

$$PD = BD + 0.009 \qquad \text{(Eq. 2.1.9.1)}$$

where

PD is the packing density [t m^{-3}],

BD is the actual bulk density [t m^{-3}],

C is the clay content [%]

There are three classes of packing density:

low: < 1.40 t m^{-3},

medium 1.40-1.75 t m^{-3} (soils are prone to compaction), and

high > 1.75 t m^{-3} (soils are not very susceptible to further compaction).

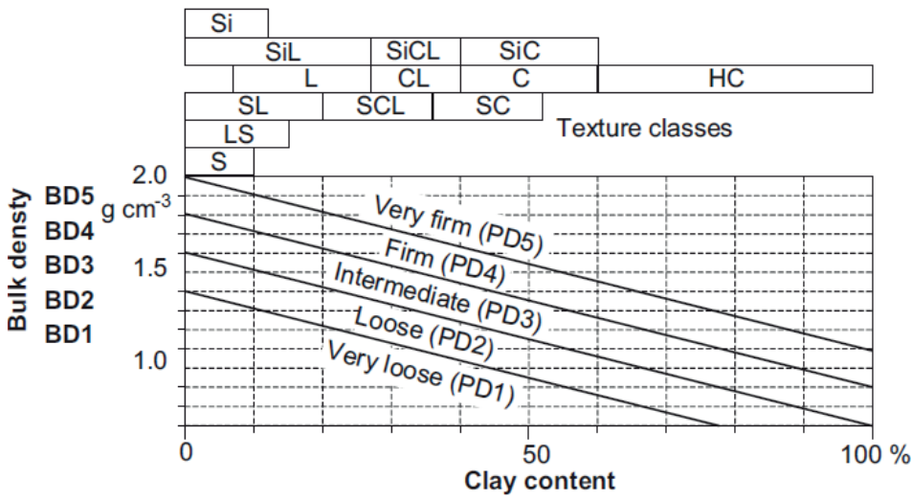

Figure 2.1.9.1 Assessment of bulk density (Ad-hoc-AG-Boden, 2005). PD = packing density. Texture classes: HC = heavy clay; C = clay; L = loam; Si = silt; S = sand

Reagents

- None

Materials and equipment

- Bulk Density Sampler Cup and Cap
- Cylinder, metal ring (100-500 cm^3)
- Oven
- Scale (accuracy: 0.01 g)
- (For the coat clod method): paraffin or other water-repellent substance

Procedure

There are several methods for determining soil bulk density. Field estimation of bulk density refers to the force required to push a knife into a soil horizon exposed at a field moist pit wall.

Three sampling methods are common: the core, the excavation and the clod method (ISO, 2017).

The core method uses a special coring instrument (cylindrical metal device) to determine the dry mass of an undisturbed sample

1.a. Remove the cup of ring
1.b. Dry undisturbed soil sample, drying at 105°C 24 h
1.c. Scale m_2 (metal ring + soil)
1.d. For surface horizons, a simple excavation method is applied:
1.e. Dig a soil pit
1.f. Fill it completely with a measured volume of sand
1.g. The clod method is used in cases of large soil aggregates, with the help of paraffin or other water-repellent substance coatings (Hirmas & Furquim, 2006)
1.h. Weigh the coated clod in air
1.i. Measure the volume of water displaced by the clod in a graduated cylinder
1.j. Wash the paraffin-coated clod in boiling water to separate the paraffin from gravel and hardened soil aggregates
1.k. Weigh the clod in water to determine its volume

Calculations

$$\rho_b = (m_2\text{-}m_1)v^{-1} \quad [M\ L^{-3};\ g\ cm^{-3};\ kg\ m^{-3};\ Mg\ m^{-3}] \qquad \text{(Eq: 2.1.9.2)}$$

where

ρb is the bulk density of the soil [g m^{-3}],
m_1 is the weight of the metal ring [g],
m_2 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, Mixed Anthrosol) 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

References

Ad-hoc-AG-Boden, 2005. Bodenkundliche Kartieranleitung. 5. Auflage. Hannover. 438 p.

Blake, G.R., Hartge, K.H., 1986. Bulk density. In: Methods of Soil Analysis. Part 1. (Klute, A. Ed.) American Society of Agronomy, Madison, WI. 374–390.

Carter, M. R., 1990. Relative measures of soil bulk density to characterise compaction in tillage studies on fine sandy loams. Can. J. Soil Sci., 70, 425–433.

FAO, 2006. Guidelines for soil description. Food and Agriculture Organization of the United Nations, Rome. 51–52.

Gupta, S.C., Larson, W.E., 1979. A model for packing density of soils using particle size distribution. Soil

Sci. Soc. Am. J., 43, 758–764.
Gupta, S.C., Sharma, P.P., DeFranchi, S.A., 1989. Compaction effects on soil structure. Adv. Agron., 422, 311–338.
Hirmas, D.R., Furquim, S.A.C., 2006. Simple modification of the clod method for determining bulk density of very gravelly soils. Comm. Soil Sci. Plant Anal., 37, 7–8, 899–906. doi: 10.1080/00103620600588579
ISO, 2017. Soil quality – Determination of dry bulk density. International Organization for Standardization, Geneva ISO/TC 190/SC 3
Moody, P.W., Cong, P.T., 2008. Soil Constraints and Management Package (SCAMP): guidelines for sustainable management of tropical upland soils. Australian Centre for International Agricultural Research (ACIAR), Canberra, Australia. ACIAR Monograph No. 130. 86 p.
Van Ranst, E., Thomasson, A.J., Daroussin, J., Hollis, J.M., Jones, R.J.A., Jamagne, M., King, D., Vanmechelen, L., 1995. Elaboration of an extended knowledge database to interpret the 1:1,000,000 EU Soil Map for environmental purposes. In: European Land Information Systems for Agro-environmental Monitoring (King, D., Jones, R.J.A. and Thomasson, A.J. Eds.) EUR 16232 EN. Office for Official Publications of the European Communities, Luxembourg. 71–84.

2.1.10 Particle size distribution

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Importance and applications

Important soil properties are associated with particle size distribution (PSD), among others: porosity, permeability, infiltration, shrinking-swelling, water-holding capacity, susceptibility to erosion and compaction, organic matter dynamics (Table 2.1.10.1). PSD controls the rate of drainage of a saturated soil. Water percolates relatively freely through sandy soils. At field capacity it influences water availability to plants. Clayey soils have higher water-holding capacities than sandy soils. Well-drained soils show good aeration and contain air similar to atmospheric air. Soil textures also differ in their susceptibility to erosion (erodibility): those with a high percentage of silt and clay are of higher erodibility than sandy soils. Organic matter breaks down more rapidly in sandy soils if environmental conditions are otherwise the same. Tillage and soil management are also influenced by particle size proportions: in lighter-textured soils more oxygen is available for decomposition. The cation exchange capacity of the soil grows with increased clay and organic matter percentage. The pH buffering capacity of a soil is also closely associated with its clay content (Berry *et al*., 2007).

The complex interrelationships among all these soil properties should be considered when land management decisions are made. Loamy soils are a mix of sand, silt, and clay that optimises these properties and, thus, agricultural productivity.

Soil texture, identified by PSD determination (also called granulometric analysis), refers to the proportion of the various particle-size classes (fractions) in a given soil volume and is described as soil textural class (Table 2.1.10.1). In addition to the textural class, a field estimate of the percentage of clay is given. This estimate is useful for indicating increases or decreases in clay content within textural classes, and for comparing field estimates with analytical results. The relationship between the basic textural classes and the percentages of clay, silt and sand is indicated in a triangular form in Fig. 2.1.10.1.

Table 2.1.10.1 Properties of the main soil textural classes

Property/Behaviour	Sand	Silt	Clay
Water holding capacity	Low	Medium to high	High
Aeration	Good	Medium	Poor
Organic matter decomposition	Fast	Medium	Slow
Water erosion potential	Low	High	Low
Compactability	Low	Medium	High
Sealing	Poor	Poor	Good
Nutrient supply	Poor	Medium to high	High
Pollutant leaching	High	Medium	Low

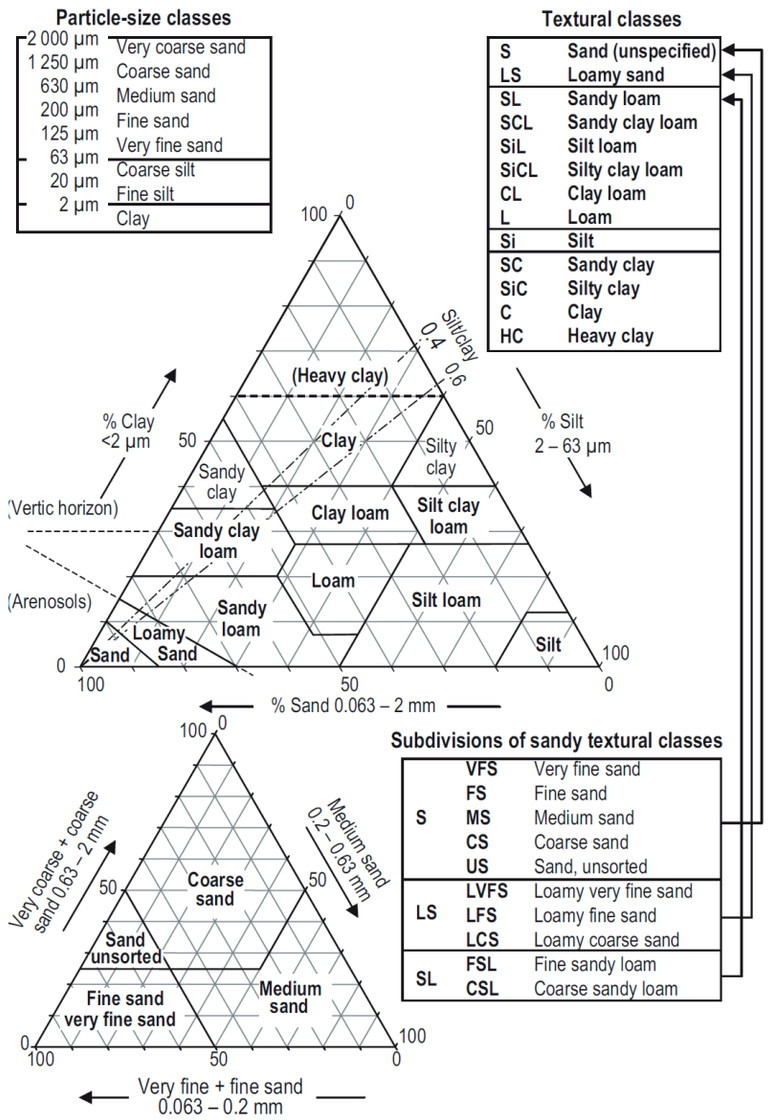

Figure 2.1.10.1 Assessment of textural classes (FAO, 2006). HC = heavy clay; C = clay; L = loam; Si = silt; S = sand

Principle

PSD involves the determination of the percentages of different grain size classes in a soil. There are two principal approaches (Taubner et al., 2009): mechanical or sieve analysis (for the coarser, larger-sized particles) and the hydrometer and/or laser-beam based methods (for the finer particles).

Sieving is the first step to determine PSD if the soil contains coarse sands, gravels or pebbles. Depending on soil structure and aggregates stability, the mechanical sieve analysis has two (dry and wet) techniques. Dry sieving is commonly used if the soil is structureless and the particles do not adhere to one another (sandy soils, regosol etc.). Wet sieving is useful for direct PSD for aggregates with high fine (clay) contents. In the system of the United States Department of Agriculture (Soil Survey Staff, 1975) and that of the FAO

(2006), the particle-size classes are named similarly to the common standard terminology in sedimentology (Table 2.1.10.2. The phi scale is widely used in the statistical analyses: $\Phi = \log_2 D$ [D in mm] (Krumbein, 1938).

Table 2.1.10.2 Particle size classes (after Friedman and Sanders, 1978 and FAO, 2006) and the methods used

<table>
<tr><th>Phi</th><th>Sieve/particle diameter [µm]</th><th>Range [mm-mm]</th><th>Categories</th><th>FAO 2006</th><th colspan="3">Method</th></tr>
<tr><td>-6</td><td>64,000</td><td>> 64</td><td>Very coarse pebbles</td><td>Stones (60.00–200.00 mm)</td><td colspan="3" rowspan="5">Sieving</td></tr>
<tr><td>-5</td><td>32,000</td><td>32–64</td><td>Very coarse pebbles</td><td rowspan="2">Coarse gravel (20.00-60.00 mm)</td></tr>
<tr><td>-4</td><td>16,000</td><td>16–32</td><td>Coarse pebbles</td></tr>
<tr><td>-3</td><td>8,000</td><td>8–16</td><td>Medium pebbles</td><td>Medium gravel (6.00-20.00 mm)</td></tr>
<tr><td>-2</td><td>4,000</td><td>4–8</td><td>Fine pebbles</td><td rowspan="2">Fine gravel (2.00-6.00 mm)</td></tr>
<tr><td>-1</td><td>2,000</td><td>2–4</td><td>Very fine pebbles</td><td colspan="3" rowspan="2">Sieving</td></tr>
<tr><td>0</td><td>1,000</td><td>1–2</td><td>Very coarse sand</td><td>Very coarse sand (1.25-2.00 mm)</td></tr>
<tr><td>1</td><td>500</td><td>0.50–1</td><td>Coarse sand</td><td>Coarse sand (0.630-1.250 mm)</td><td rowspan="2"></td><td rowspan="15">Laser (MALVERN Mastersizer 3000 (200 nm–3,000 µm))</td></tr>
<tr><td>2</td><td>250</td><td>0.250–0.50</td><td>Medium sand</td><td rowspan="2">Medium sand (0.200-0.630 mm)</td><td rowspan="13">Pipette and hydrometer</td></tr>
<tr><td>3</td><td>0.125</td><td>0.125–0.250</td><td>Fine sand</td><td rowspan="2">Laser (FRITSCH Analysette A22-32)</td></tr>
<tr><td>4</td><td>0.063</td><td>0.063–0.125</td><td>Very fine sand</td><td>Fine sand (0.125-0.200)</td></tr>
<tr><td>5</td><td>0.031</td><td>0.031–0.063</td><td>Very coarse silt</td><td>Very fine sand</td></tr>
<tr><td>6</td><td>0.016</td><td>0.016–0.031</td><td>Coarse silt</td><td rowspan="2">Coarse silt (0.020-0.063)</td></tr>
<tr><td>7</td><td>0.08</td><td>0.008–0.016</td><td>Medium silt</td><td rowspan="4">Fine silt (0.002-0.020)</td></tr>
<tr><td>8</td><td>0.04</td><td>0.004–008</td><td>Fine silt</td></tr>
<tr><td>9</td><td>0.002</td><td>0.002–0.004</td><td>Very fine silt</td></tr>
<tr><td>10</td><td>0.001</td><td>0.001–0.002</td><td>Clay</td><td rowspan="2">Clay (0.000-0.002)</td></tr>
<tr><td>11</td><td>0.000-0.001</td><td>0.000–0.001</td><td>Fine clay + colloids</td></tr>
</table>

The fine material of the soil is separated by ***sedimentation***, two main methods of which should be distinguished: the pipette and hydrometric methods, fundamentally based on Stokes' Law (Khön, 1928; Elonen, 1971; Bouyoucos 1962):

$$v = g(\rho_p - \rho_l)D^2/(18\eta) \qquad \text{(Eq. 2.1.10.1)}$$

where

v is the velocity of the falling particle [m s^{-1}]	-
g is the gravitional acceleration [m s^{-2}]	9.81m s^{-2}
ρ_p is the particle density [m L^{-3}; g cm^{-3}; kg m^{-3}]	in practice: 2,600–2,650 kg m^{-3}
ρ_l is the liquid density [m L^{-3}; g cm^{-3}; kg m^{-3}]	for water at 20°C: 998 kg m^{-3}
D is the particle diameter [L, m]	-
η is the fluid viscosity	for water at 20°C: 0.001 Pas

Stokes proposed a general equation for the fall velocity of small particles (< 0.1 mm diameter) by first considering the frictional resistance which the fluid offers to movement of a settling sphere.

The *pipette metho*d depends on the assumption that sedimentation eliminates from the depth L [m], in a time t [sec], all the particles having settling velocities greater than L t^{-1}, while retaining at that depth the original concentration of particles with settling velocities less than L t^{-1}.

In the hydrometer method an aerometer is used to measure the density of the suspension (soil sample + water mixture) loss over time.

Reagents

- 30% H_2O_2, to remove organic material
- 10% HCl to remove carbonates
- Dithionite-Citrate system with 1M $NaHCO_3$ to remove iron-oxides (Mehra & Jackson, 1960)
- Sodium-acetate (1M), or sodium-hexametaphosphate (Calgon) to remove/eliminate the calcium and magnesium ions from the solution

Materials and equipment

For sieving:

- Scale
- Set of sieves (according to the estimated range of particle size),
- Automatic sieve shaker

For pipette method:

- Electric mixer and cup
- Sedimentation cylinder (1,000 mL)
- 20-mL pipette with attached suction bulb
- Oven
- Glass beakers

For sedimentation:

- Electric mixer and cup
- Sedimentation cylinder (1,000 mL)
- Hydrometer (Bouyoucos Scale, 5 to 60 g range)
- Tape (mm)
- Dispersant graduated cylinder
- Scale
- Stopwatch
- Thermometer

For laser particle sizer:

- Laser-beam based equipment

Procedure

For sieving:

1. Sieve Analysis

1.a. Weigh 100 g (clay); 200 g (silt), 500 g (coarse sand), 1,000 g (gravel), 2,000 g

1.b. Heat the soil sample to 105°C in an oven and keep for 24 hours

1.c. Fix the sieve series on the sieve shaker. The size of the sieves (sieve column) depends on the range of particle distribution and the predictable number of sieves (see section on sieve sizes and classes)

1.2. Wet sieving

1.2.a. Treat the soil sample with 30% H_2O_2, to remove organic material; 10% HCl, Sodium Pyrophosphate ($Na_4P_2O_7$) or Calgon to remove carbonates, Dithionite-Citrate-Sodium Bicarbonate to remove iron-oxides if needed. Continue the treatment until the soil particles reach a suspended phase

1.2.b. Pour it on the sieve series and close the series with a cap to fix the sieve column

1.2.c. Set the appropriate vibration intensity and time, launch the sieve-shaker. If the sieve-shaker is constructed by a pump for circulating the sieved material, set and control the pump yield. Check fouling in case of a high percentage of fine material

1.2.d. At the end of sieving take the sieve series apart

1.2.e. Remove the soil fractions from the sieves. Do so cautiously to avoid loss of material

1.2.f. Put the wet material into the oven and dry it at 105°C for 24 hours

1.2.g. For reporting, use Table 2.1.10.3

1.3. Dry sieving

1.3.a. Take the dried sample on the sieve column

1.3.b. Set the appropriate vibration intensity and time, launch the sieve-shaker

1.3.c. At the end of sieving take the sieve series apart

1.3.d. Remove the soil fractions from the sieves

1.3.e. Measure the soil fractions

2. For the pipette method:

2.a. Transfer a prepared sample to a 1,000 cm^3 graduated cylinder

2.b. Mix the sample with a plunger or by inversion for one minute or until homogenised

2.c. About 20 seconds are allowed to pass before drawing the initial aliquot to permit a reduction in turbulence

2.d. Remove the initial aliquot using a 20-mL pipette with attached suction bulb

2.e. Proceed in a similar fashion for the remainder of the aliquots at times and depths

2.f. Dry the aliquots to a constant weight in an oven at 90°C

3. For the hydrometer method:

3.a. Place sieved and dried soil (20–50 g or 100 g if sandy)

3.b. Fill cup to within 6 cm of the top with 20°C distilled water

3.c. Add 5 mL of 1N sodium hexametaphosphate (Calgon)

3.d. Mix for 5 minutes for sandy soils, 15 minutes for fine-textured soils

3.e. Transfer suspension to sedimentation cylinder and fill it to 1000 mL

3.f. Carefully mix suspension with plunger

3.g. Remove plunger and start timing

3.h. Place hydrometer in the suspension, take readings at 40 seconds and repeat them several times to increase accuracy. Check temperature (mixing raises the temperature)

3.i. Mix suspension again and begin timing for the two-hour reading

4. Bouyoucos hydrometer method:

4.a. Place an air-dried sample (50 g of soil) in a shaker bottle. Weigh the sample and record its weight before placing the sample in the bottle. Use 100 g of soil if the sample is sand.

4.b. Add 2.0 grams of sodium metaphosphate

4.c. Add distilled water until the bottle is two-thirds full

4.d. Cap the bottle and shake it in a mechanical shaker for at least 4 hours

4.e. Alternatively, agitate for 5 minutes using a stirrer (Malt Mixer type)

4.f. Transfer soil from the bottle into a settling cylinder. Rinse the remaining soil from the bottle and cap into the cylinder using distilled water from a wash bottle

4.g. For cylinders marked at 1,130 mL and 1,205 mL, respectively, add distilled water to approx. 5 cm of the lower graduation on the cylinder. Insert the hydrometer, bulb-end down. Filling the cylinder:

1,130-mL line 50-g sample

1,205-mL line 100-g sample

4.h. After filling to the desired mark, remove the hydrometer from the cylinder

4.i. For cylinders marked at 1,000 mL only, fill the cylinder to the mark with distilled water without inserting the hydrometer

4.j. Stopper the cylinder, turn it end-over-end several times, return it to the upright position, record the time, and place it gently in a selected place. It must remain in the location for at least 2 hours

4.k. Insert the hydrometer into the suspension. Read the hydrometer exactly 40 seconds after the cylinder is returned to the upright position

4.l. Remove the hydrometer and repeat steps 9 and 10 until a consistent hydrometer reading is obtained

4.m. Record the 40-second hydrometer reading in the data table

4.n. Remove the hydrometer. Use a thermometer to measure the temperature of the suspension.

4.o. Record the temperature. Let the cylinder sit undisturbed for 2 hours

4.p. Obtain a hydrometer reading after a settling period of 2 hours. Measure the temperature. Record the temperature and the hydrometer reading in the data table.

5. For laser analyser:

Based on the Mie-Fraunhoffer method. The setting and procedure strongly depends on manual options on the particle sizer.

5.a. Set the calculation parmeters: interpolation values as oversize or undersize

5.b. Set the mode of measurement: wet or dry method

5.c. Interpolation values, fixed particle sizes, oversize or undersize µm

5.d. Calculation form: cummulative and/or differential calculation

5.e. Distribution forms: model independent, monomodal etc.

5.f. Set measuring range and channels which generates the resolution

5.g. Beam obscuration percentage (generally: 8%)

5.h. Number of measurements = “scan” (3 times recommended)

5.i. Set the graphical mode of the result (triangle, Q-distribution, Gauss distribution, etc)

5.j. Set the raw data file type for exporting (text file recommended)

Calculations

1. Sieve Analysis

Calculate the percentage retained on each sieve by dividing the weight retained on each sieve by the original sample mass (Table 2.1.10.3). Calculate the percentage passing (or finer) by starting with 100 percent and subtracting the percentage retained on each sieve as a cumulative procedure.

Table 2.1.10.3 Table for calculation in the case of PSD determination by sieving

Sieve diameter [µm]	Mass of sieve [g]	Sieve + soil retained [g]	Soil retained [g]	Soil retained [%]	Passing [%]
64,000					
32,000					
16,000					
8,000					
4,000					
2,000					
1,000					
500					
250					
125					
Remnants					
(0–250)					
Total:					

2. Calculation for sedimentation:

Table 2.1.10.4 Calculating time-dependent diameter by Stokes' Law

Stokes diameter [µm]	Length of sedimentation [L; m]	Dynamic viscosity [Pas^{-1}]	Gravitational acceleration [ms^{-2}]	Density of particle [kg m^{-3}]	Density of water [kg m^{-3}]	Time of sedimentation [t; sec]	Time of sedimentation [t; hour]
258.28						5	0.0014
182.63						10	0.0028
105.44						30	0.0083
74.56						60	0.0167
52.72						120	0.0333
43.05						180	0.0500
37.28						240	0.0667
33.34						300	0.0833
23.58						600	0.1667
19.25	0.30	0.00101	9.81	2650	998	900	0.250
16.67						1200	0.333
13.61						1800	0.500
9.63						3600	1.000
6.81						7200	2.000
4.81						14400	4.000
3.40						28800	8.000
1.96						86400	24.00
1.39						172800	48.00
1.13						259200	72.00

2.1 Pipette method:

The force (F) of gravity pulling the particle downward is:

$$F = 4/3\pi r^3 \rho_p g \qquad \text{Eq. 2.1.10.2)}$$

The net result of forces acting on the particle is given by:

$$F = 4/3\pi r^3 (\rho_p - \rho_l) g \qquad \text{(Eq. 2.1.10.3)}$$

where

ρ_p is particle density [m L^{-3}; g cm^{-3}; kg m^{-3}]
ρ_l is liquid density [m L^{-3}; g cm^{-3}; kg m^{-3}]
g is gravitional acceleration [m s^{-2}]
η = fluid viscosity [Pas]

If temperature and fluid density are constant and the density of the sphere is known, the equation is:

$$v = Cr^2 \qquad \text{(Eq. 2.1.10.4)}$$

where

$$C = 2\,(\rho_p - \rho_l)g\,(9\,\eta)^{-1} \quad and \quad C = 3.59 \times 104 \quad / if\ T = 20°C;\ \rho_p = 2.65\ g\ cm^{-1}$$

$$v = 3.59 \times 10^4 r^2 \qquad \text{(Eq. 2.1.10.5)}$$

Eq. 2.1.10.5 is used to compute the time required for a particle of a given diameter to settle at a given depth.

2.*2 Hydrometer method:*

The Bouyoucos hydrometer determines the concentration of solids in suspension.

Determine Soil Moisture Correction Factor

Determine weight of air-dried soil (AD) AD = Weight of pan + air-dried soil-pan weight

Determine weight of oven-dried soil (OD) OD = Weight of pan + oven-dried soil-pan weight

Determine soil moisture correction factor (MCF) MCF = 1 – [(AD – OD) ÷ AD)]

Weight of Dry Soil: determined by multiplying the air-dried weight by the moisture correction factor (MCF) Weight of Dry Soil = Air-dried Soil x MCF

Correcting Hydrometer Reading:

For temperatures above 20°C:

Hydrometer reading = Measured reading g/L + [(measured temperature – 20) x 0.36 g/L]

For temperatures below 20°C:

Hydrometer reading = Measured reading g/L – [20 – (measured temperature) x 0.36 g/L]

To correct the hydrometer readings for temperature, add 0.36 gL^{-1} for every 1°C above 20°C; subtract 0.36 g/L^{-1} for every 1°C below 20°C.

Determine the percentages of Sand, Silt and Clay (Fig. 2.1.10.2):

% clay = (Corrected 2-hour hydrometer reading x 100) $(Oven\text{-}dried\ Weight\ of\ Soil)^{-1}$

% silt + clay = (Corrected 40-second hydrometer reading x 100) $(Oven\text{-}dried\ Weight\ of\ Soil)^{-1}$

% sand = 100 – % silt + clay

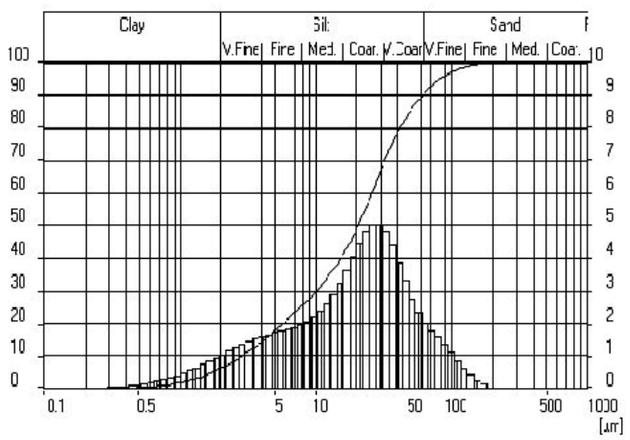

Fig. 2.1.10.2 Example of combined graphical representation (cumulative and frequency distribution) of a Raman brown soil (by FRITSCH Analysette A22-32, Idar-Oberstein, Germany)

Fitting the result of sieve and fine component analysis:

For the total range of PSD (or Particle Distribution Frequency) a percentage calculation is needed, where 100% is the initial weight of the soil sample. The total (100%) is divided into two parts: one is the product of sieving and the other is the fine component.

Remarks

- In the case of soils with gravels and boulders (Regosols, mixed Anthrosol) coarse constituents hinder sampling and increase measurement error.
- The main assumptions used in applying Stokes' Law to sedimenting soil suspensions are:
  1. Terminal velocity is attained as soon as settling begins.
  2. Hydrometer or pipette and the sedimentation-cylinder wall may also influence the settling rate.
  3. Particles are smooth and spherical; therefore, the result is considered as "Stokes-equivalent diameter".
  4. There is no interaction between individual particles in the solution.

References

Berry, W., Ketterings, Q., Antes, S., Page, S., Russell-Anelli, J., Rao, R., DeGloria, S., 2007. Soil texture. Cornell University Cooperative Extension, Ithaca, NY. 2 p. Agronomy Fact Sheet 29.

Bouyoucos, J.B., 1962. Hydrometer method for making particle size analysis of soils. Agron. J., 54, 464–465.

Elonen, P., 1971. Particle-size analysis of soil. Acta Agr. Fenn., 122, 1–122.

Köhn, M., 1928. Beitrage zur Theorie und Praxis der mechanischen Bodenanalyse. Landw. Jahrbuch. 67

Friedman, G.M., Sanders, F.E., 1978. Principles of sedimentology. Wiley, New York. 792 p.

FAO, 2006. Guidelines for soil description. Food and Agriculture Organization of the United Nations, Rome. 26–33.

Gee, G.W., Or, D., 2002. Particle-size analysis. In: Methods of Soil Analysis, Part 4. Physical methods (Dane, J.H., Topp, G.C., Eds.). Soil Science Society of America, Madison, WI. 255–293.

ISO 13317-2:2001(en) Determination of particle size distribution by gravitational liquid sedimentation methods — Part 2: Fixed pipette method

Kohnke, H., 1968. Soil physics. McGraw-Hill Book Company, New York, NY.

Konert, M., Vandenberghe, J., 1997. Comparison of laser grain size analysis with pipette and sieve analysis: a solution for the underestimation of the clay fraction. Sedimentology, 44, 523–535.

Krumbein, W.O., 1938. Size frequency distributions and the normal phi curve. J. Sediment. Petrol. 8, 84–90.

Loizeau J., Arbouille D., Santiago S., Vernet J., 1994. Evaluation of a wide range laser diffraction grain size analyser for use with sediments. Sedimentology, 41, 353–361.

Mehra, O.P., Jackson, M.L., 1960. Iron oxide removal from soils and clay by a dithionite-citrate system buffered with sodium bicarbonate. Clays Clay Miner., 7, 317–327.

Soil Survey Staff, 1975. Soil taxonomy: A basic system of soil classification for making and interpreting soil surveys. USDA-SCA Agric. Handbook 436. U.S. Government Printing Office, Washington, DC.

Taubner, H., Roth, B., Tippkötter, R., 2009. Determination of soil texture: Comparison of the sedimentation method and the laser-diffraction analysis. J. Plant Nutrition Soil Sci., 172, 2, 161–171. doi: 10.1002/jpln.200800085

2.1.11 Munsell colour

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Importance and applications

Soil horizons have different colours, reflecting chemical processes acting on the soil (weathering, oxidation-reduction of minerals, particularly iron and manganese minerals and the decomposition of organic matter). The colour indicates properties that are important for soil management: texture, moisture and organic matter content. For the purposes of soil classification, the Munsell system allows for direct comparison of soils anywhere in the world. The colour of sediments and soils also depends on the colour of their constituent minerals (Table 2.1.11.1 – Lynn and Pearson, 2000).

Some examples of the conclusions which can be drawn from soil colour include: Black soils often have a high organic matter content (peat soils), waterlogging, high acidity and poor workability. White or pale horizons are generally leached of nutrients or of low availability of water. Red colour indicates good drainage, iron oxide content (‘rusty colour’) or high phosphorus fixation. If iron compounds are hydrated, the red colour changes to yellowish brown. Darker brown horizons show moderate organic matter and iron oxides. Greenish grey horizons are gleyed because of poor drainage and waterlogging and may be associated with methane emission hazard (Moody and Than, 2008).

Table 2.1.11.1 Munsell colours of some minerals (differentiated by mineral size) (after Lynn and Pearson, 2000)

Mineral	Formula	Size	Munsell	Colour
goethite	FeOOH	(1–2 mm)	10YR 8/6	yellow
goethite	FeOOH	(~0.2 mm)	7.5YR 5/6	strong brown
hematite	Fe_2O_3	(~0.4 mm)	5R 3/6	red
hematite	Fe_2O_3	(~0.1 mm)	10R 4/8	red
lepidocrocite	FeOOH	(~0.5 mm)	5YR 6/8	reddish-yellow
lepidocrocite	FeOOH	(~0.1 mm)	2.5YR 4/6	red
ferrihydrite	$Fe(OH)_3$		2.5YR 3/6	dark red
glauconite	$K(Si_xAl_{4-x}(Al,Fe,Mg)O_{10}(OH)_2$		5Y 5/1	dark gray
iron sulphide	FeS		10YR 2/1	black
pyrite	FeS_2		10YR 2/1	black (metallic)
jarosite	$KFe_3(OH)_6(SO_4)_2$		5Y 6/4	pale yellow
todorokite	MnO_4		10YR 2/1	black

humus			10YR 2/1	black
calcite	$CaCO_3$		10YR 8/2	white
dolomite	$CaMg(CO_3)_2$		10YR 8/2	white
gypsum	$CaSO_4 \times 2H_2O$		10YR 8/3	very pale brown
quartz	SiO_2		10YR 6/1	light grey

Principle

Striving for a rational and accurate description of colours, Professor Albert H. Munsell (1858–1918), an American artist, created a colour identification system based on the findings of photometry, the science of the measurement of light, i.e. the reflectance of a surface as a function of wavelength of radiation (Munsell Color 2018). He used the principle of 'perceived equidistance' to distinguish colours. He started work on the system in 1898 and published the first version in 1905.

The Munsell system is a colour system that is based on three dimensions: hue, value and chroma (Fig. 2.1.11.1). The **hue** of a color indicates how it relates to the 'pure' colours red, yellow, green, blue and purple, i.e. what the predominant wavelength of the reflected light is. **Value** denotes lightness or darkness. A value of 0 is black and 10 is pure white. **Chroma** marks colour saturation (intensity). A chroma of 0 is neutral grey and the maximum chroma is 20 (but it is never approached). Value becomes successively lighter vertically, from the bottom upward, by visually equal steps. Chroma increases horizontally to the right and becomes greyer to the left.

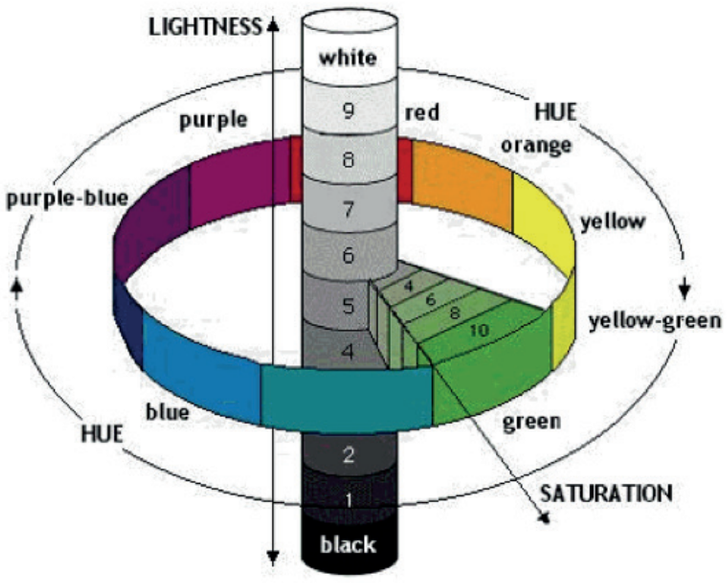

Figure 2.1.11.1 The Munsell colour system

Reagents

- Water for set moist condition of the soil

Materials and equipment

- Munsell Soil Color Charts

Procedure

Create a homogeneous soil aggregate under semi-wet conditions
Fit the aggregate by visual comparison to the Munsell Color Chart
Read hue, value and chroma codes

Calculations

- None

Remarks

- In soils a mottling pattern may refer to soil aeration or drainage. Mottles (spots in the soil matrix) are of genetic importance and differ in colour from the matrix and their colour should be determined separately
- In mixed soil profiles the colours of the matrix and the enclosed clasts have to be determined

References

Lynn, W.C., Pearson, M.J., 2000. The color of soil. The Science Teacher, 67, 5, 20–23.
Moody, P.W., Cong, P.T., 2008. Soil Constraints and Management Package (SCAMP): Guidelines for sustainable management of tropical upland soils. Australian Centre for International Agricultural Research (ACIAR), Canberra, Australia. ACIAR Monograph, 130, 86 p.
Munsell Color, 2018. Official site of Munsell Color Company, Grand Rapids, MI. http://munsell.com/
Munsell Color Company, 2000. Munsell Soil Color Charts. Revised Washable Edition. 50 p.

2.1.12 Soil reaction (pH)

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Importance and applications

Soil reaction is important because it affects nutrient availability, microbial activity and plant growth. Most plant species perform best in a pH range 5.5 to 6.5 or 7.0 although some prefer extremes (Table 2.1.12.1). Soil pH controls the solubility of nutrients and, thus, their availability for plants (Fig. 2.1.12.1). In humid climates the leaching of calcium, magnesium, potassium and sodium ions naturally causes a decrease in pH over time because it leaves the soil clays dominated by H+ and aluminium ions (Al^{3+}) (Ketterings et al., 2005).

Human activity also influences soil pH. Applying nitrogen fertilisers, manure or compost, sources of organic nutrients, nitric acid (HNO_3) and/or sulphuric acid (H_2SO_4), which are strong acids, form soil acidity (Foth & Ellis, 1997).

Table 2.1.12.1 Preferred pH ranges for common crops (Albert, 2018)

Acidophile crops (pH from 4 to 5.5)	Slightly acidophile crops (tolerate pH from 5.5 to 6.5)	Moderately alkalophile crops (tolerate pH from 6.0 to 7.0 or greater)		Crops of great tolerance (tolerate a wide range of soil acidity or alkalinity, from about 5.0 to 7.0)
Blackberry (5.0-6.0)	Apple (5.0-6.5)	Artichoke (6.5-7.5)	Jerusalem Artichoke/ Sunchoke (6.7-7.0)	Alpine strawberry (5.0-7.5)
Blueberry (4.5-5.0)	Basil (5.5-6.5)	Arugula (6.5-7.5)	Kale (6.0-7.5)	Carrot (5.5-7.0)
Cranberry (4.0-5.5)	Carrot (5.5-7.0)	Asparagus (6.0-8.0)	Kohlrabi (6.0-7.5)	Cauliflower (5.5-7.5)
Parsley (5.0-7.0)	Cauliflower (5.5-7.5)	Bean, pole (6.0-7.5)	Leek (6.0-8.0)	Corn (5.5-7.5)
Peanut (5.0-7.5)	Chervil (6.0-6.7)	Bean, lima (6.0-7.0)	Lettuce (6.0-7.0)	Cucumber (5.5-7.0)
Potato (4.5-6.0)	Corn (5.5-7.5)	Beet (6.0-7.5)	Marjoram (6.0-8.0)	Dill (5.5-6.7)
Raspberry (5.5-6.5)	Cucumber (5.5-7.0)	Broccoli (6.0-7.0)	Mizuna (6.5-7.0)	Endive/Escarole (5.8-7.0)
Sweet potato (5.5-6.0)	Dill (5.5-6.5)	Broccoli rabe (6.5-7.5)	Mustard (6.0-7.5)	Garlic (5.5-7.5)
	Eggplant (5.5-6.5)	Brussels sprouts (6.0-7.5)	Okra (6.0-7.5)	Parsley (5.0-7.0)
	Garlic (5.5-7.5)	Cabbage (6.0-7.5)	Onion (6.0-7.0)	Parsnip (5.5-7.5)

	Melon (5.5-6.5)	Cantaloupe (6.0-7.5)	Oregano (6.0-7.0)	Peanut (5.0-6.5)
	Parsley (5.0-7.0)	Cauliflower (6.0-7.5)	Pak choi (6.5-7.0)	Pepper (5.5-7.0)
	Pepper (5.5-7.0)	Celery (6.0-7.0)	Parsnip (5.5-7.5)	Rutabaga (5.5-7.0)
	Pumpkin (6.0-6.5)	Chinese cabbage (6.0-7.5)	Pea (6.0-7.5)	Squash, winter (5.5-7.0)
	Radicchio (6.0-6.7)	Celeriac (6.0-7.0)	Radicchio (6.0-6.7)	Tomato (5.5-7.5)
	Radish (6.0-7.0)	Celery (6.0-7.0)	Radish (6.0-7.0)	Turnip (5.5-7.0)
	Rhubarb (5.5-7.0)	Chinese cabbage (6.0-7.5)	Rhubarb (6.5-7.0)	
	Sorrel (5.5-6.0)	Chive (6.0-7.0)	Sage (6.0–6.7)	
	Squash, winter (5.5-7.0)	Cilantro (6.0-6.7)	Salsify (6.0-7.5)	
	Sweet potato (5.5-6.0)	Claytonia (6.5-7.0)	Spinach (6.0-7.5)	
	Tomato (5.5-7.5)	Collard (6.5-7.5)	Squash, summer (6.0-7.0)	
	Turnip (5.5-7.0)	Cress (6.0-7.0)	Sunflower (6.0-7.5)	
		Endive/escarole (6.0-7.0)	Swiss chard (6.0-7.5)	
		Fennel (6.0-6.7)	Tarragon (6.0-7.5)	
		Gourd (6.5-7.5)	Tomatillo (6.7-7.3)	
		Horseradish (6.0-7.0)	Watermelon (6.0-7.0)	

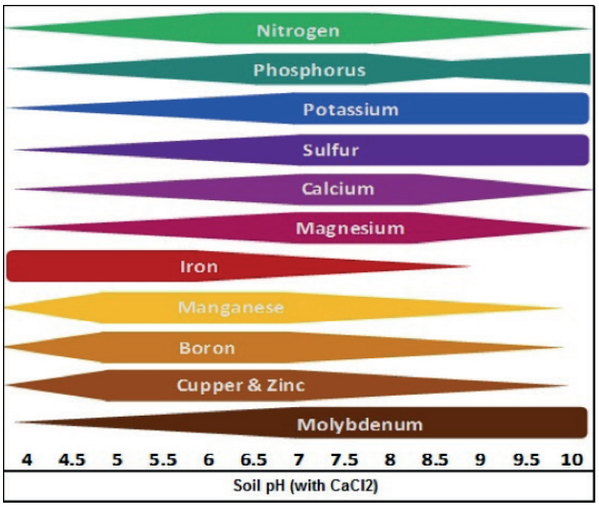

Figure 2.1.12.1 Solubility of plant nutrients as a function of soil pH($CaCl_2$)

Principle

Soil pH is the activity of hydrogen ions (H^+). It is a measure of the acidity/alkalinity of a soil solution on a scale from 0 to 14 (Fig. 2.1.12.2). Acidic solutions have a pH below 7, while basic or alkaline solutions have a pH above 7.

By definition, pH is measured on a negative logarithmic scale of the hydrogen ion activity [H^+], i.e., pH = -log [H^+]. Therefore, as hydrogen ion concentrations (and acidity) rise, pH values drop. Also, because pH is a logarithmic function, each unit on the pH scale is 10 times more acidic than the unit above it. For example, a pH 6 solution has 10 times higher concentration of H^+ ions than a solution with pH 7 and a concentration 100 times higher than a solution with pH 8.

Soil pH is influenced by both acid and baseforming cations (positively charged dissolved ions) in the soil. Common acid-forming cations are hydrogen (H^+), aluminium (Al^{3+}), and iron (Fe^{2+} or Fe^{3+}), whereas common base-forming cations include calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^+) and sodium (Na^+) (McCauley et al., 2017).

Selecting the proper extracting solution, two types of soil acidity can be measured. Extraction by distilled water results in active acidity (potential H^+ concentration of soil solution) Potential acidity can be divided into exchangeable and hydrolytic acidity. To determine exchangeable acidity, neutral KCl solution is used. In this case the proton (H^+ and Al^{3+}) release capacity of soil colloids is measured. When determining hydrolytic acidity using a non-potentiometric method, Ca- and Na-acetates basic solution (pH = 8.2) releases H^+ ions from the radicals (-COOH, phenol -OH, -AlOH etc.) with changeable charges, supplying protons. $CaCl_2$ is also often used as the exchanging solution. The pH($CaCl_2$) reflects biological processes (Čapka et al., 2009).

Instrumental methods are specified for routine pH determination (ISO 10390:2005): a glass electrode in a 1:5 (volume) suspension of soil in water (pH in H_2O), in 1 mol/L potassium chloride solution (pH in KCl) or in 0.01 mol/L calcium-chloride solution (pH in $CaCl_2$).

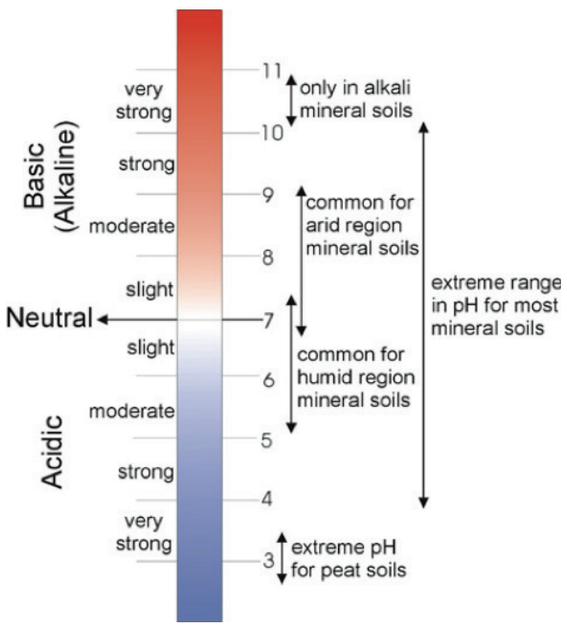

Figure 2.1.12.2 Classification of soils by pH

Reagents

- Distilled H_2O
- a solution of potassium chloride (KCl) in water, c = 1 mol/L
- a solution of calcium chloride ($CaCl_2$) in water, c = 0,01 mol/L

Materials and equipment

- pH meter (based on voltametry)
- pH and reference electrode or combined electrode
- 50 mL beaker
- Scale (0.1g)
- Standard buffers pH4, pH7, pH10

Procedure

Samples should be analysed as soon as possible after being taken.

a. Calibrate the pH meter following the manufacturer's instructions using the buffer depending on the expected values for the soils (Pansu & Gautheyrou, 2006)
b. Prepare 10 g soil (~) and 25 mL solution (FAO, 2006) OR a 1:5 soil: water suspension with 10 g air-dried soil (<2mm) weighed into a bottle and with 50 mL solution added
c. Cover and continuously stir the suspension for 5 min
d. Mechanically shake it for 1 hour at 15 rpm
e. Let the soil suspension stand for about 1 hr to allow most of the suspended clay to settle out from the suspension, or filter or centrifuge
f. Immerse the electrode just deep enough into the clear supernatant solution to establish a good electrical contact through the ground-glass joint or the fiber-capillary hole
g. Insert the electrodes into the sample solution. The pH value can be read when the pH value does not change

Calculations/Evaluation

The pH measured with $CaCl_2$ can be brought into relation with organic matter content (Table 2.1.12.2).

Table 2.1.12.2 Relationship between pH and organic matter content

Classification of pH value	
pH_{CaCl2}	< 5.1, if > 15% OM
	< 4.6, if 4–15% OM
	< 4.2, if < 4% OM
	< 3.6, if > 15% OM
	< 3.4, if 4–15% OM
	< 3.2, if < 4% OM

Remarks

- Field pH measurement should not be a substitute for laboratory determination, but correlated with laboratory analyses where possible.
- A common method for increasing soil pH is to lime soils with calcium carbonate, calcium oxide (CaO), calcium hydroxide ($Ca[OH]_2$) or calcium containing by-products such as sugar-beet lime. The liming material reacts with carbon dioxide and water in the soil to yield bicarbonate (HCO_3^-) and hydroxide (OH^-), which take H^+ and aluminium (acid-forming cations) out of solution, thereby raising the soil pH.

References

Albert, S., 2018. Vegetable crop soil pH tolerances. In: Harvest to Table. https://harvesttotable.com/vegetable-crop-soil-ph-tolerances/

Čapka, D., Kisić, I., Zgorelec, Ž., Mesić, M., Jurišić, A., 2008. Determination of soil pH in dominant soil types in the Republic of Croatia. Agriculturae Conspectus Scientificus, 74, 1, 13–19.

Foth, H.D., Ellis, B.G., 1997. Soil pH management. In: Soil Fertility. 2nd Edition. CRC publishers, Boca Raton, FL. 93–98.

ISO 10390:2005. Soil quality – Determination of pH. American National Standards Institute. 24 p.

Ketterings, Q.M., Albrecht, G., Beckman, J., 2005. Soil pH for field crops. Cornell University Cooperative Extension, Ithaca, NY. 2 p. Agronomy Fact Sheet 5

McCauley, A., Jones, C., Olson-Rutz, K., 2017. Soil pH and organic matter. Land Resources, Montana State University, Bozeman, MT. 16 p. Nutrient Management Module 8
http://landresources.montana.edu/nm/documents/NM8.pdf

Pansu, M., Gautheyrou, J., 2006. pH measurement. In: Handbook of Soil Analysis. Mineralogical, Organic and Inorganic Methods. Springer Verlag, Berlin – Heidelberg. 551–579.

2.1.13 Electrical conductivity

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Importance and applications

The electrical conductivity (EC) of soils correlates with soil properties which influence crop yields (Anderson-Cook et al., 2002). These include: soil texture, clay content or depth to clay-rich layers or hardpans, cation exchange capacity (CEC), drainage conditions, organic matter content, salinity and subsoil character (Sudduth et al., 2005; Grisso et al., 2009). Soil EC varies with the amount of moisture held by soil particles. Sands have low, silts have medium, and clays have high EC values. Soils in the middle range of EC, which are both medium-textured and have medium water-holding capacity, may be the most productive. Soil organic matter content and CEC can be estimated from EC measurements (Kweon et al., 2013).

Dissolved salts in the soil are easily detected by EC. High concentrations of electrolytes in the soil solution can affect plants in multiple ways: Specific toxicity can be due to the abundance of a particular ion (e.g. sodium). Excessive osmotic pressure around the roots prevents an efficient water absorption by the plant. Some crops are more susceptible to salinity than others. Each species has an electrical conductivity threshold, beyond which a reduction in yield must be taken into account.

Electrical conductivity measurements are among the most frequently used tools in precision agriculture research with the purposes of describing soil properties and human activities which influence the crop yield (Corwin & Lesch, 2005).

Principle

The measurement of electrical conductivity is based on the ability of a material to transmit (conduct) an electrical current and is commonly expressed in units of milliSiemens per metre [mS/m]. The EC or specific conductance is the reciprocal of electrical resistivity (Ohm, symbol: the Greek letter omega), Ω^{-1}. Its SI unit is Siemens metre^{-1}.

Reagents

- 0.01 mol potassium chloride reference solution

Materials and equipment

- EC meter
- EC electrode
- 50 mL beaker
- Automatic shaker

Procedure

a. Calibrate the conductivity meter according to the manufacturer's instructions using the KCl reference

solution to obtain the cell constant

b. Prepare a 1:5 soil:water suspension by weighing 10 g air-dried soil into a beaker

c. Add 50 mL deionised water

d. Shake at 15 rpm for 1 hour to dissolve soluble salts

e. Rinse the conductivity cell with the soil suspension

Calculation/Evaluation

The electrical conductivity values can be evaluated for soil salinity (Table 2.1.13.1).

Table 2.1.13.1 Salinity classes of soils based on electrical conductivity (Campbell, 2017)

USDA Class	Conductivity Range dS/m	Salt in Soil g/100g	Osmotic potential kPa	Crop Salt Tolerance	Example Crop
A	0–2	0–0.13	0 to -70	Sensitive	Bean
B	2–4	0.13–0.26	-70 to -140	Moderately Sensitive	Corn
C	4–8	0.26–0.51	-140 to -280	Moderately Tolerant	Wheat
D	8–16	0.51–1.02	-280 to -560	Tolerant	Barley

Remark

- There is no clear relationship between electrical conductivity (1:5 soil:water) and total soluble salts due to the different ionic conductivities of the various salts and the influence of the soil particles

References

Rayment, G.E., Higginson, F.R., 1992. Australian Laboratory Handbook of Soil and Water Chemical Methods. Inkata Press, Melbourne. (Australian Soil and Land Survey Handbooks, vol 3)

Anderson-Cook, C.M., Alley, M.M., Roygard, J.K., Khosla, R., Noble, R.B., Doolittle, J.A., 2002. Differentiating soil types using electromagnetic conductivity and crop yield maps. Soil Sci. Soc. Am. J., 66, 1562–1570. doi: 10.2136/sssaj2002.1562

Campbell, G., 2017. Electrical Conductivity of Soil as a Predictor of Plant Response. Environmental Biophysics http://www.environmentalbiophysics.org/electrical-conductivity-soil-predictor-plant-response/

Corwin, D.L., Lesch, S., 2005. Application of soil electrical conductivity measurements in agriculture. Comput. Electron. Agric., 46, 1–3, 11–43. doi: 10.1016/j.compag.2004.10.005

Grisso, R., Alley, M., Holshouser, D., Thomason, W., 2009. Precision Farming Tools: Soil Electrical Conductivity. Virginia Cooperative Extension, College of Agriculture and Life Sciences, Virginia Polytechnic Institute and State University, Petersburg, VA. 6 p. Publication 442-508

Kweon, G., Lung, E., Maxton, C., 2013. Soil organic matter and cation-exchange capacity sensing with on-the-go electrical conductivity and optical sensors. Geoderma, 199, 80–89. doi: 10.1016/j.geoderma.2012.11.001

Sudduth, K.A., Kitchen, N.R., Wiebold, W.J., Thelen, K.D., 2005. Relating apparent electrical conductivity to soil properties across the North-Central USA. Comput. Electron. Agric., 46, 1–3, 263–283. doi: 10.1016/j.compag.2004.11.010

2.1.14 Aggregate stability and size distribution

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Importance and applications

This method provides information about aggregate stability and size distribution. It can be used as an indicator for soil structure and how soil structure changes under different management and land use changes. It has also allowed detailed study into how soil organic matter is transformed and stabilised under different management regimes (Six et al., 1998). Lastly, it has been used to identify and study the dynamics of soil microenvironments and their hosted microbial community.

Principle

This method analyses the aggregate stability and size distribution based on a wet sieving method of air-dried soil. (Elliott et al., 1986). The wet sieving of air-dried soil induces the process of slaking (i.e. break-up of non-stable structures in the soil due to a build-up of air pressure within pores upon wetting) and thereby isolates only stable aggregates. In contrast, when the air-dried soil is rewetted, then the process of slaking is minimised and hence less stable aggregates are isolated. In practical terms, this means that field-moist soil is first gently broken up to pass an 8-mm sieve and then air-dried. Subsequently, two pre-treatments are applied before wet sieving: (i) air-dried soil is rapidly immersed in water (slaked treatment) and (ii) air-dried soil is capillary-rewetted before immersion in water (rewetted treatment). For capillary rewetting, air-dried soil is placed on filter paper that is slowly moistened until a water content of 1.05 times field capacity is reached (Six et al., 2000a). Three sieve sizes (2 mm, 0.250 mm, and 0.053 mm) are used to separate the soil into four different aggregate size classes.

Reagents

- No reagents are needed, but deionised water should be used if available

Materials and equipment

- 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
- Aluminium pans (large and small) for drying of samples
- Air-forced drying oven (60°C)
- Spatula and brush
- Rinsing bottle
- Balance (2 significant digits)

Optional:

- Erlenmeyer flask with tube and pipette tip
- Vacuum pump
- Convection drying oven (105°C)

Procedure

Part A: Wet-sieving of whole soil:

a. Take 80 g (or between 50 and 100 g) subsample from air-dried or rewetted whole soil (weigh on digital balance and record weight; two significant numbers).
b. Fill up white basin (30 cm diameter; 8 cm deep) with water until water level is approximately 1 cm above 2000 µm sieve-mesh.
c. Spray soil evenly out on sieve and wait for 5 minutes (to allow the slaking process).
d. After the 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.
e. Depending on the soil, carry out steps 5–10 or instead steps 11–14. Put the sieve down and rinse off the insides g. Aspirate off the floating litter into the first flask attached to the vacuum line.
h. When all floating material is aspirated, empty the flask into the waste basket.
i. 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.
j. Put the sieve with aggregates into the 105°C convection oven (for 30 min).
k. 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.
l. Backwash > 2000 µm aggregates (i.e. large macroaggregates) into a pre-weighed small drying pan with sufficient water.
m. Decant off the floating litter into the waste bucket.
n. Put the drying pan with the large macroaggregates into the 60°C forced air oven (overnight).
o. 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).
p. 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.
q. Backwash 250-2000 µm aggregates (i.e. small macroaggregates) into a pre-weighed small drying pan.
r. Put the drying pan with the small macroaggregates into the 60°C forced air oven (overnight).
s. 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 the second 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).

t. 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.
u. Backwash 53–250 µm aggregates (i.e. microaggregates) into a pre-weighed small drying pan.
v. Put the small drying pan with microaggregates into the 105°C forced air oven (overnight).
w. Pour the water + < 53 µm particles (i.e. silt + clay) remaining in the white basin into a pre-weighed large drying pan.
x. Put the large drying pan with silt + clay particles into the 105°C forced air oven (overnight)
y. If steps 5-10 were chosen: Take the > 2000 µm sieve out of the convection oven and transfer the large macroaggregates to the pre-weighed small drying pan (do not use any water in this step, just lightly brush aggregates off the sieve into the pre-weighed small drying pan
z. The following day weigh all fractions

Calculations

The results are generally expressed as bar graphs showing the proportions of the different aggregate size classes or are expressed as the mean weight diameter (MWD):

$$MWD = \Sigma A_i * P_i \qquad \text{(Eq. 2.1.14.1)}$$

where
Ai is the mean size of the aggregate size class,
Pi is the proportion of the respective aggregate size class.

Remarks

- If soils with different textures are compared, a sand correction should be performed (see Six et al., 2000b)
- When both the air-dried and rewetted soils are fractionated into different aggregate size classes and sand-corrected, the Normalised Stability Index (NSI) can be calculated, according to Six et al., 2000b. The NSI is a preferable indicator for soil stability for soils with different textures

References

Elliott, E.T. 1986. Aggregate structure and carbon, nitrogen, and phosphorus in native and cultivated soils. Soil Sci. Soc. Am. J., 50, 627–633.

Six, J., Elliott, E.T., Paustian, K., Doran, J.W., 1998. Aggregation and soil organic matter accumulation in cultivated and native grassland soils. Soil Sci. Soc. Am. J. 62, 1367–1377.

Six, J., Paustian, K., Elliott, E.T., Combrink, C., 2000a. Soil structure and organic matter: I. Distribution of aggregate-size classes and aggregate-associated carbon. Soil Sci. Soc. Am. J., 64, 681–689.

Six, J., Elliott, E.T., Paustian, K., 2000b. Soil structure and soil organic matter: II. A normalised stability index and the effect of mineralogy. Soil Sci. Soc. Am. J., 64, 1042–1049.

2.1.15 Soil structure

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Importance and applications

Structure is not only an important property for soil classification but an indicator of soil conditions. Soil structure strongly influences soil hydraulic and solute transport processes, which can be significantly improved or deteriorated through management practices (Bronick & Lal, 2005). Soil structure deteriorates when structural units are deformed. This happens when pressure is applied to a soft soil in wet conditions. Pressure squeezes the soil units together and reduces pore space within the units. A dry soil can withstand pressure without deforming the soil structure. Practices that increase productivity and decrease soil disruption enhance aggregation and structural development. Environmental changes of natural origin also have an impact on soil structure. All these influences should be taken into account when striving for sustainable farming (Six et al., 1999).

Principle

Soil structure is defined by the way individual particles of sand, silt, and clay are assembled. Single particles when assembled appear as larger particles, called aggregates. During pedogenesis, clay minerals and Fe- and Al-(hydr)oxides are bound together as microaggregates (i.e. compound soil structures smaller than 250 μm), primarily by physicochemical and chemical interactions involving cementing (e.g. carbonates) and gluing agents (Fe, Mn, and Al compounds) (Totsche et al., 2018). The small aggregates build large fractions, macroaggregates, by combining with organic matter. The structure of a soil refers to both the geometric arrangement of the particles or mineral grains, i.e. soil fabric (Holtz & Kovacs, 1981) as well as the pore spaces that are left between them. The processes of root penetration, wetting and drying cycles, freezing and thawing and animal activity combined with inorganic and organic cementing agents produce soil structure (Snyder & Vázquez, 2005).
Soil structure is most usefully described in terms of the degree of aggregation, i.e. grade. Grade expresses the difference between the cohesion within aggregates and the adhesion between aggregates. The class of structure refers to the average size, while type reflects the form of aggregates (see also Chapter 2.1.14). In some soils, different kinds of aggregates are found together and then described separately. The characteristic structure of a soil can be recognised best in a fresh profile.

There are four major grades of structure (FAO, 2006):

1. Structureless soils show no observable aggregation or no definite orderly arrangement of natural lines of weakness.
2. Weak structure is poorly formed from indistinct aggregates that can barely be observed in place. When removed from the profile, the soil material breaks down into a mixture of very few entire aggregates, many broken aggregates and much unaggregated material.
3. Moderate structure is well formed from distinct aggregates that are moderately durable and evident but not distinct in undisturbed soil. When removed from the profile, the soil material breaks down into a mixture of many distinct entire aggregates, some broken aggregates and little unaggregated material.

4. Strong structure is well formed from distinct aggregates that are durable and quite evident in undisturbed soil. When removed from the profile, the soil material consists very largely of entire aggregates and includes few broken ones and little or no non-aggregated material.

The type of structure describes the form or shape of individual aggregates (or the lack of structure):
1. *Structureless categories* (Fig. 2.1.15.1): no aggregation when the soil is dry. Massive structure (coherent): where the entire soil horizon appears cemented in one great mass or single-grain structure (non-coherent) where the individual soil particles show no tendency to cling together, such as pure sand.

Figure 2.1.15.1 Structureless soils. a. single grain; b. massive

2. *Granular and crumb structures* (Fig. 2.1.15.2) are individual particles of sand, silt and clay grouped together in small, nearly spherical grains. They are commonly found in the A horizon of the soil profile. Both granular and crumb structures have rounded surfaces, but crumb structures are larger.

Figure 2.1.15.2 Granular and crumb-structured soils

3. *Blocky and subangular structures* (Fig. 2.1.15.3) cling together in angular clumps with sharp edges. Blocky structures are cubes with flattened surfaces, sharp edges, while subangular blocky structures are more rounded.

Figure 2.1.15.3 Coarse (Ø 30-50 mm) angular blocky soils

4. *Prismatic and columnar structures* (Fig. 2.1.15.4) are soil particles separated into vertical columns or pillars by miniature, but definite, vertical cracks. Prismatic aggregates are rectangular, elongated with a flattened top, while in columnar structure they have a rounded top.

Figure 2.1.15.4 Prismatic and columnar soils

5. *Platy and lenticular structure* (Fig. 2.1.15.5) is made up of soil particles aggregated into thin plates or sheets piled horizontally on one another. Plates often overlap to a large extent impairing water percolation.

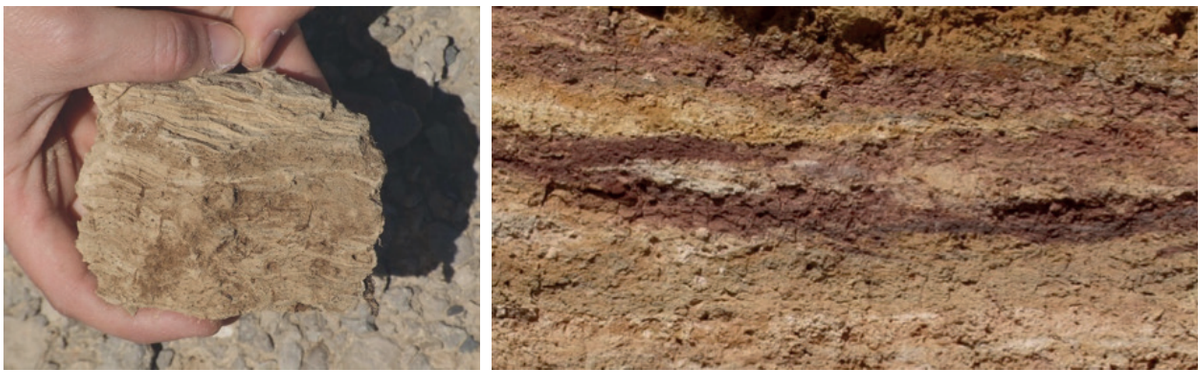

Figure 2.1.15.5 Platy and lenticular soils

Reagents

- None

Materials and equipment

- Shovel or soil core sampler
- Measuring tape
- Plates with photographs for visual comparison

Procedure

a. Dig a soil pit and prepare the soil profile
b. Take an undisturbed sample using a shovel or soil core sampler
c. Carefully tease the soil apart along lines of natural weakness and breaking the soil into structural units
d. Measure the size and describe the shape of structural units
e. Determine soil strength by applying pressure to a 3 cm cube of soil using your forefinger and thumb (Environment Agency, 2010)

Calculations/Evaluation

Table 2.1.15.1 is helpful for the identification of classes and types of soil structure.

Table 2.1.15.1 Occurrence of classes and types of soil aggregates

Structure	Appearance	Size of individual aggregates	Soil type examples
Massive and single grain structureless	all horizons	x	Sandy soils, Fluvisols
Granular and crumb	A	Fine (< 2 mm)	Phaeozem, Chernozem
		Medium (2-5 mm)	
		Coarse (> 5 mm)	

Blocky and subangular	B	Fine (< 10 mm)	Brown forest soils
		Medium (10-50 mm)	
		Coarse (> 50 mm)	
Prismatic and columnar	B	Fine (< 20 mm)	Umbrisols, Vertisols
		Medium (20-50 mm)	
		Coarse (> 50 mm)	
Platy	all	Foliated (< 1 mm)	Antroposols, Forest soil (A horizon)
		Platy (1-3 mm)	
		Tabular (3-5 mm)	

Remarks

- Optimal soil structure for plant growth has stable aggregates between 0.5 and 2 mm in diameter
- Relatively large blocks indicate that the soil resists penetration and allows the movement of water. In soils with prismatic and columnar structures water circulation is hindered and drainage is poor
- In soils with sandy texture aggregate stability is often difficult to maintain due to low organic matter and clay content and weak cementing. In clay soils, however, there is insufficient pore space between aggregates for hair root growth
- Plates occur both on the surface and in the subsoil, while single grains occur mostly in the C horizon.

References

Bronick, C.J., Lal, R., 2005. Soil structure and management: a review. Geoderma, 124, 1–2, 3–22. doi: 10.1016/j.geoderma.2004.03.005

Díaz-Zorita, M., Perfect, E., Grove, J.H., 2002. Disruptive methods for assessing soil structure. Soil and Tillage Res., 64, 3–22.

Environment Agency, 2010. Examining soil structure. Environmental Agency, Bristol, UK. 32 p. https://ahdb.org.uk/projects/documents/ThinkSoils_001.pdf

FAO, 2005. Organization of soil constituents. In: Guidelines for Soil Description. Fourth Edition. Food and Agriculture Organization of the United Nations, Rome. 44–50. http://www.fao.org/3/a-a0541e.pdf

Holtz, R.D., Kovacs, W.D., 1981. An Introduction to Geotechnical Engineering. Prentice-Hall, Eaglewood Cliffs, NJ.

Six, J., Elliott, E.T., Paustian, K., 1999. Aggregate and soil organic matter dynamics under conventional and no-tillage systems. Soil Sci. Soc. Am. J., 63, 1350–1358.

Snyder, V.A., Vázquez, M.A., 2005. Structure. In: Encyclopedia of Soils in the Environment. Elsevier, Amsterdam. 54–68.

Totsche, K.-U., Amelung, W., Gerzabek, M.H., Guggenberger, G., Klumpp, E., Knief, C., Lehndorff, E., Mikutta, R., Peth, S., Prechtel, A., Ray, N., Kögel-Knabner, I., 2018. Microaggregates in soils. J. Plant Nutr. Soil Sci., 181, 104–136. doi: 10.1002/jpln.201600451

2.1.16 Soil micromorphology

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Importance and applications

Soil micromorphology is defined as 'a method of studying undisturbed soil and regolith samples with the aid of microscope and ultramicroscopic techniques in order to identify the different constituents and to determine the mutual relations, in space and time, as far as the latter is possible' (Encyclopaedia of Soil Science, 2008). Soil micromorphology covers the description, measurement and interpretation of pedofeatures at microscopic level (Bullock et al., 1985). The micromorphological features well reflect short-term changes during field experiments. The evaluation of organic components is important for the study of the impacts of low-input management practices.

Principle

Micropedofeatures classification and evaluation were developed by Kubiena (1938), Brewer (1976), Bullock et al. (1985) and FitzPatrick (1993). The characteristic difference between the classifications lies in the definition of constituents. The following descriptions, illustrated by photographs, are not a perfect but useful key for interpreting microscopic pedological features.

The basic components are **mineral and organic components** as the simplest fabric units of the soil. Mineral components are well described by handbooks of petrography and are not detailed here.

At the microscopic scale, soils and paleosols consist of a fine-grained **soil matrix (S-matrix)** and the following pedological features related to soil-forming processes (Brewer, 1976):

- *plasma:* mainly fine clay-sized clay mineral particles, organic material of colloid size
- *skeleton grains:* chiefly silicate sand and silt grains embedded in the plasma
- *soil voids:* macropores (> 1-2 μm diameter, up to several cm) and matrix pores (< 1-2 μm diameter) in the soil matrix

The **organic components** are

- Coarse fragments: roots and tissue residues (Fig. 2.1.16.1)

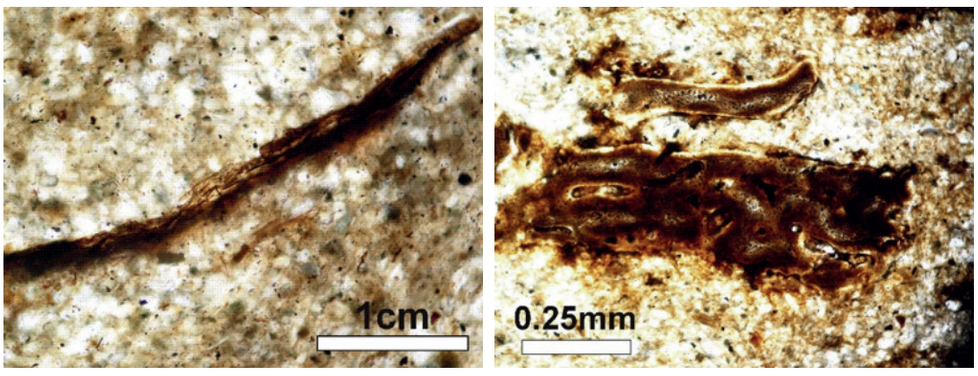


Figure 2.1.16.1 Root fibre and tissue residue

- Organic fine materials: cells and cell residues (Fig. 2.1.16.2) and amorphous organic materials (mono- and polymorphic, punctuations, organic pigments) (Fig. 2.1.16.3)

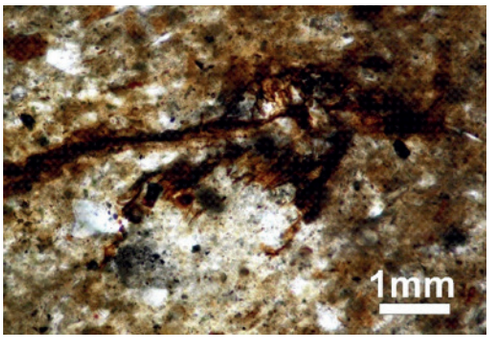


Figure 2.1.16.2 Root fibre and tissue residue

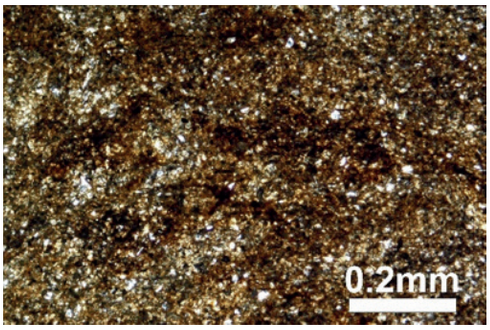


Figure 2.1.16.3 Opaque organic material with organic pigments

The soil material is composed by groundmass and pedofeatures. **Groundmass** is the coarse and fine base material. Pedofeatures are discrete fabric units recognisable by a different concentration of one or more components or by a difference in the internal fabric (Bullock et al., 2005).

A. Voids are pores filled with air and water. Simple voids are found between skeletal grains (Fig. 2.1.16.4). Compound voids are located between aggregates and their faces do not accommodate each other (Fig. 2.1.16.5), while complex packing voids are between single grains and aggregates (Fig. 2.1.16.6).

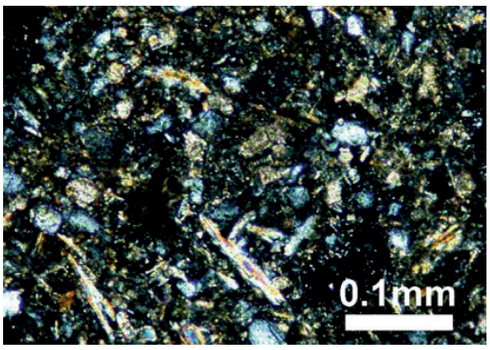


Figure 2.1.16.4 Depleted microstructure with simple pores (voids)

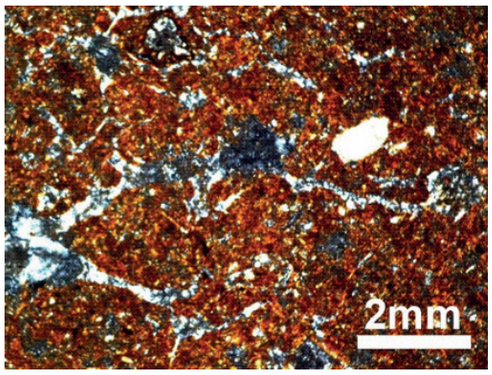


Figure 2.1.16.5 Compound packing voids between aggregates

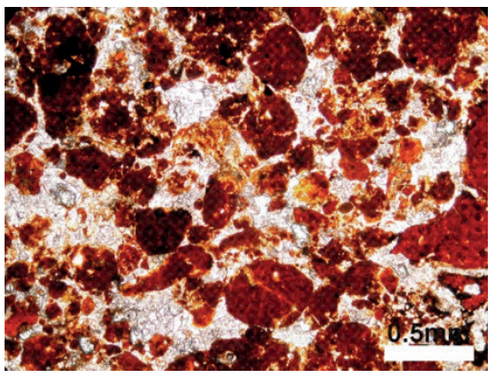


Figure 2.1.16.5 Complex voids in deposited material

Vughs are irregular voids whose origin cannot be attributed to a simple packing of units (Fig. 2.1.16.6). Vesicles are independent, separate features with spherical or elliptical shapes and smooth walls (Fig. 2.1.16.7). Channels are tubular forms developed by roots. Chambers are of spherical shape, partly or totally connected with pores or vughs (Fig. 2.1.16.8). Planes are fissures, frequently due to soil desiccation (Fig. 2.1.16.9).

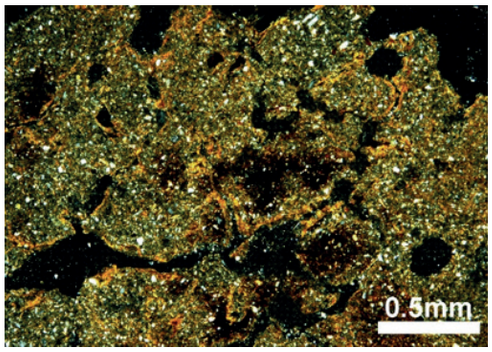


Figure 2.1.16.6 Vughs with birefrigrent clay coatings

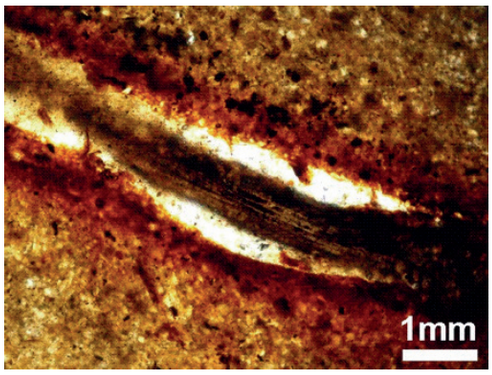


Figure 2.1.16.7 Root residue in channel with red hypo-coating and opaque dark organic material

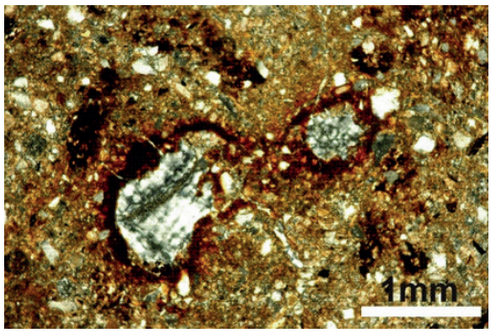


Figure 2.1.16.8 Chambers with coatings

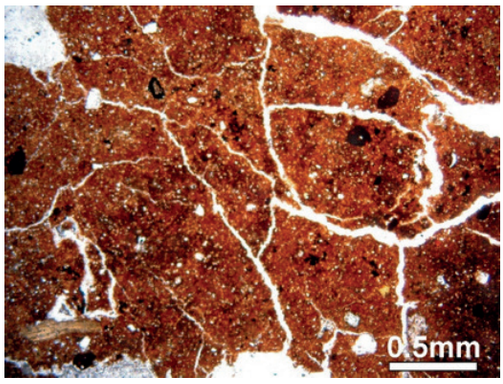


Figure 2.1.16.9 Fissures between angular blocks

B. Aggregates include crumbs, which are porous aggregates with a spheroidal shape (Fig. 2.1.16.10); granular, non-porous, semi-spheroidal aggregates (Fig. 2.1.16.11); angular blocks with irregular polyhedral shapes (Fig. 2.1.16.12); prismatic angular blocks (Fig. 2.1.16.13); platy, leaf-shaped aggregates (Fig. 2.1.16.14).

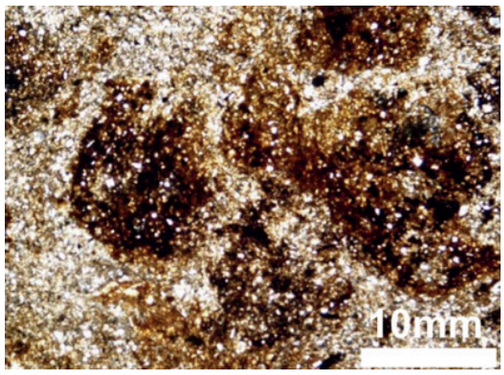


Figure 2.1.16.10 Porous spheroidal crumbs

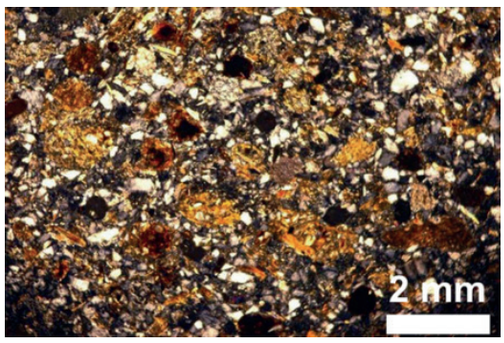


Figure 2.1.16.11 Granular, semi-spheroidal crumbs

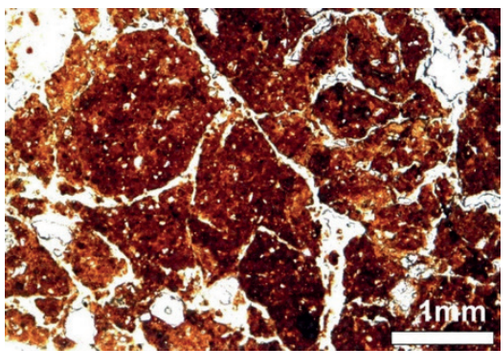


Figure 2.1.16.12 Angular blocks

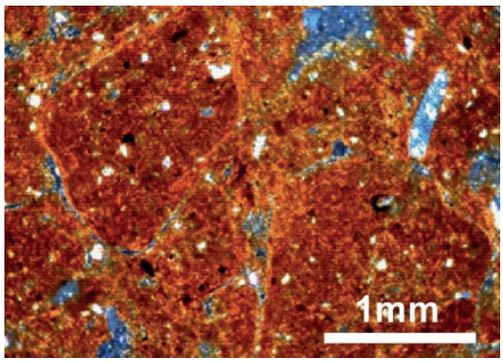


Figure 2.1.16.13 Prismatic angular blocks with birefringent coatings

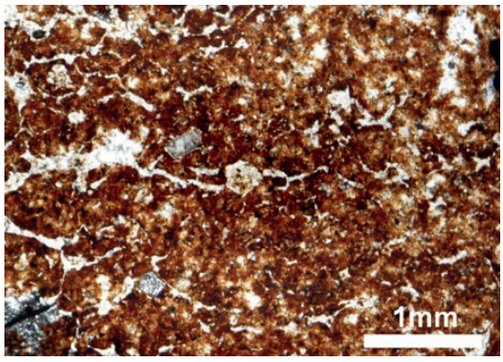


Figure 2.1.16.14 Aggregates in platy structure

C. Relationship between coarse and fine constituents (after Stoops & Jongerius, 1975): monic ('single population'), applicable for amorphous and uniform size particles (Fig. 2.1.16.15); *gefuric* ('linked and coated'), bridges and braces of fine material (Fig. 2.1.16.16); *chitonic* ('coated'), where the fine material partially or entirely coats the coarser particles (Fig. 2.1.16.17); *enaulic* ('intergrain aggregate'), where the finer material partially fills irregular spaces between the coarse particles (Fig. 2.1.16.18); *porphyric* ('embedded'), where coarser material 'swims' in finer material.

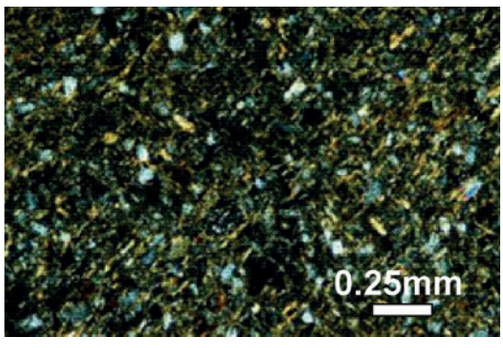


Figure 2.1.16.15 Uniform size particles

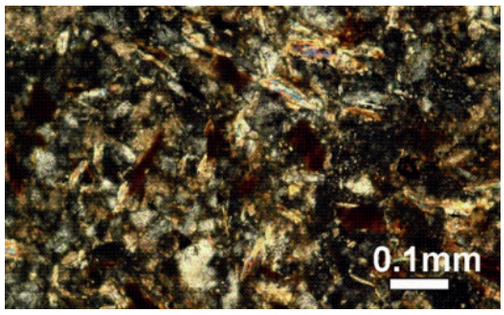


Figure 2.1.16.16 Gefuric microstructure

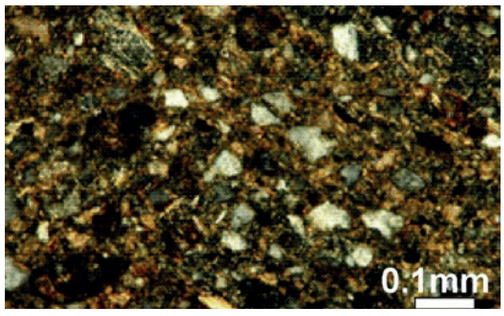
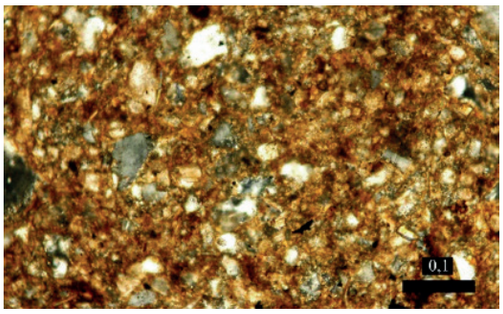


Figure 2.1.16.17 Chitonic microstructure



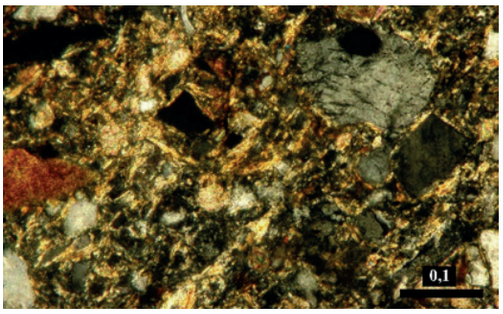


Figure 2.1.16.17 Enaulic microstructure

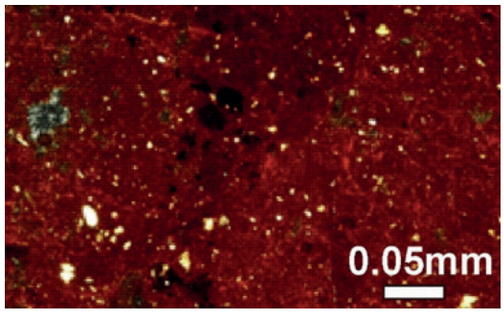


Figure 2.1.16.18 Porphyric microstructure with birefringent fine material

D. Birefringence (b-)fabric is usually recognised under crossed nicols. Fine materials (clay or fine organic matter) appear through the interference of colours. By the orientation of the fine matrix, random (*speckled b-fabric*) or *elongated zones* with parallel extinction (*striated b-fabric*) are distinguished. The *granostriated fabric* means striations around grains (Fig. 2.1.16.19). In the *monostriated* microstructure striations are isolated lines (Fig. 2.1.16.20). *Parallel* or subparallel striations also occur (Fig. 2.1.16.21). Cross-striated if striations intersect and are inclined (Fig. 2.1.16.22). *Crystallitic* if (micro) crystallites or small mineral fragments are present (Fig. 2.1.16.23). *Total striated* if micromass has a total parallel orientation (Fig. 2.1.16.24).

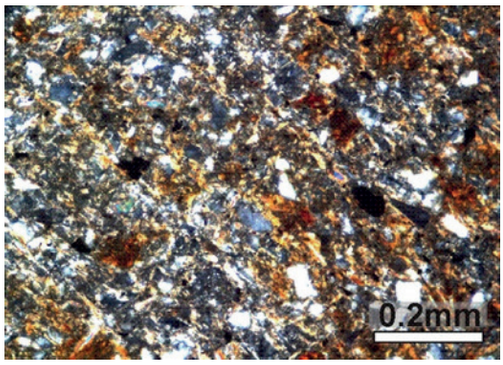


Figure 2.1.16.19 Granostriated microstructure with striated material

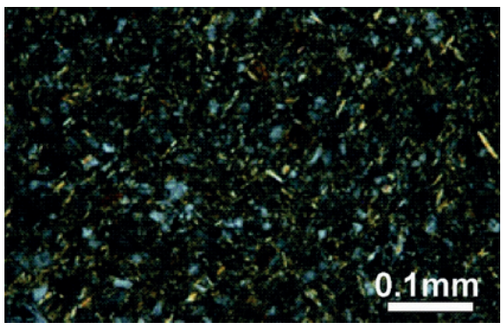


Figure 2.1.16.20 Monostriated microstructure

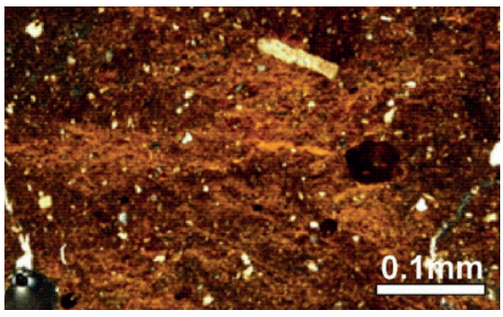


Figure 2.1.16.21 Parallel striated, embedded microstructure

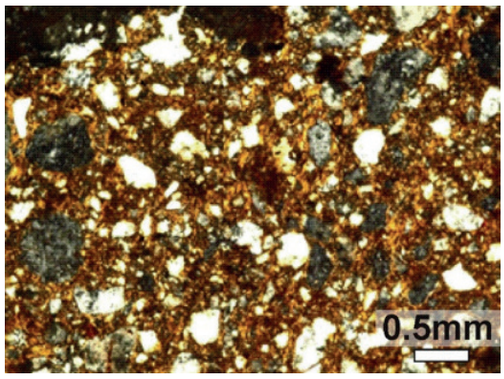


Figure 2.1.16.22 Cross striated, enaulic microstructure

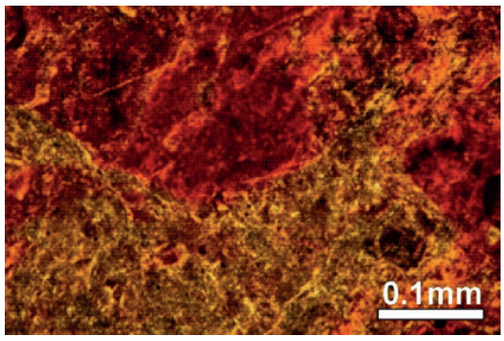


Figure 2.1.16.23 Microcrystallitic structure

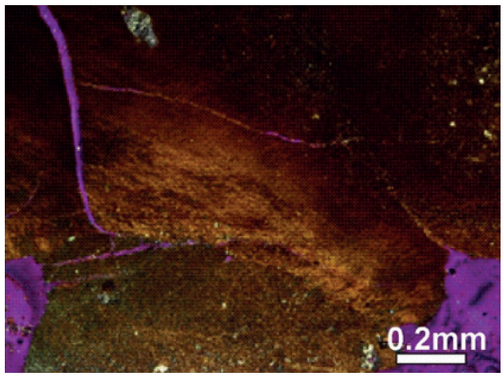


Figure 2.1.16.24 Total, parallel striated structure

Pedological features, distinguishable from the enclosing soil S-matrix, are defined as (after FitzPatrick 1993):

- *Fabric:* mutual arrangement of soil particles within the soil as a whole and within the various features
- *Structure:* type and degree of aggregation
- *Ensemble (assemblage):* the totality of all features in a specimen

The morphology of pedofeatures (related to groundmass) includes

- *Coatings:* defined by their composition and by the surface of their coats (clay, carbonates, gypsum, Fe, Mn and organic compouds; they are not impregnations) (Fig. 2.1.16.25)

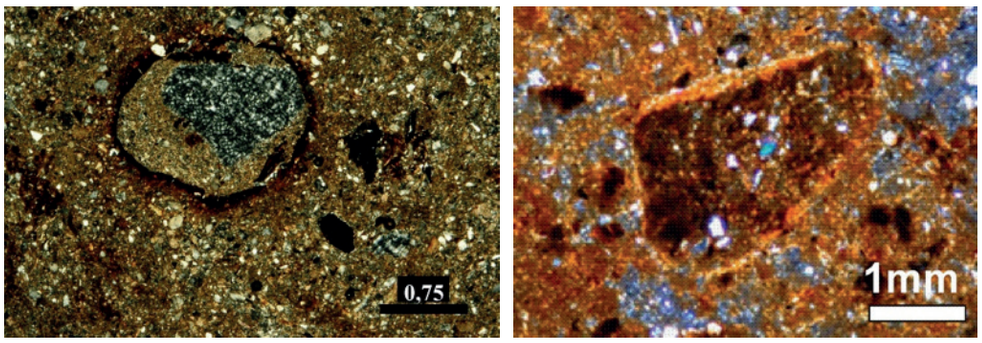


Figure 2.1.16.25 Fe- clayey coatings

- *Hypo-coatings* occur in the matrix, adjacent to natural surfaces (carbonate, Fe, Mn, Fe/Mn compouds; can be of impregnative, depletion or fabric type) (Fig. 2.1.16.26)

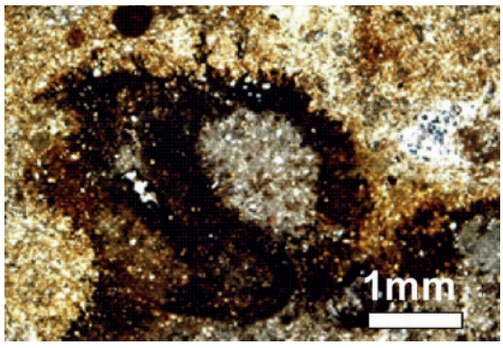


Figure 2.1.16.26 Mn-dendrital hypocoatings around crystallic infilling

- *Quasi-coatings* are not immediately adjacent to the surfaces (Fig. 2.1.16.27)

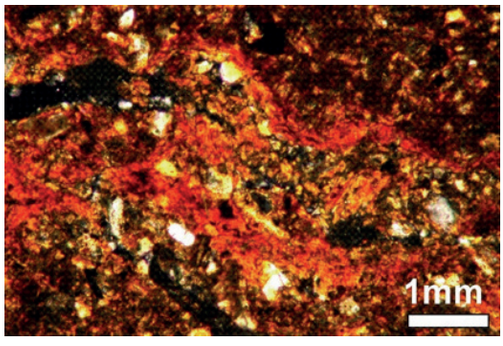


Figure 2.1.16.27 Clayey quasi-coatings

• *Infillings* are voids (partially) filled with soil or some fraction (fine material, clay, gypsum, carbonates); totally filled (Fig. 2.1.16.28); continuous infilling with some empty spaces (Fig. 2.1.16.29); infilling without continuity, consisting of grains, aggregates, crystals or excrements regularly distributed throughout the entire void (Fig. 2.1.16.30);

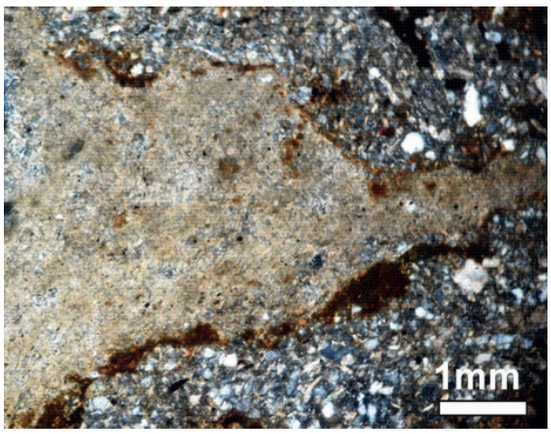


Figure 2.1.16.28 Irregular void with Fe coating filled by carbonate

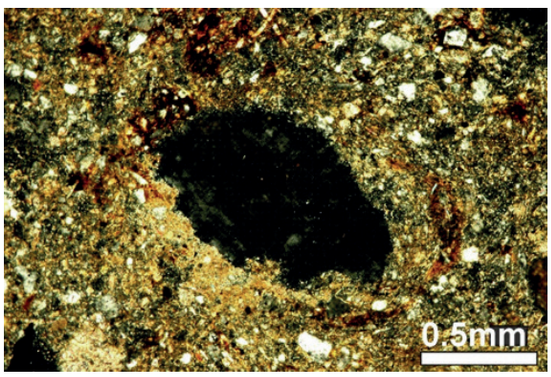


Figure 2.1.16.29 Partly degraded infilling

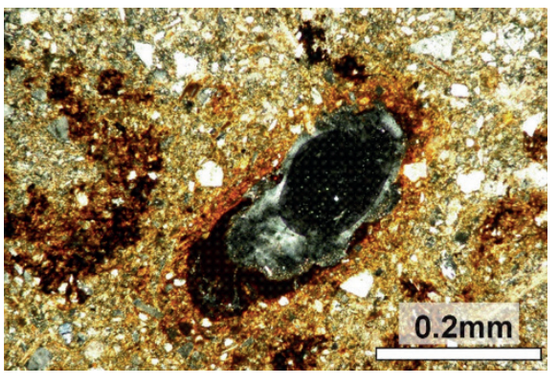


Figure 2.1.16.30 Partly degraded Fe-infilling accumulated in the lower part of channel

Pedofeatures unrelated to groundmass include crystals and crystal-intergrowths formed in situ (> 20 microns), embedded in the groundmass (Fig. 2.1.16.31); nodules, i.e. unrelated to natural surfaces and not consisting of single crystals (organic material, Fe/Mn compouds, carbonates) (Fig. 2.1.16.32);

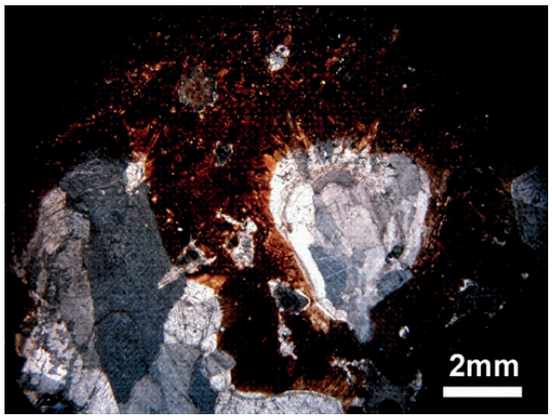


Figure 2.1.16.31 Calcite crystals growing in the fine material

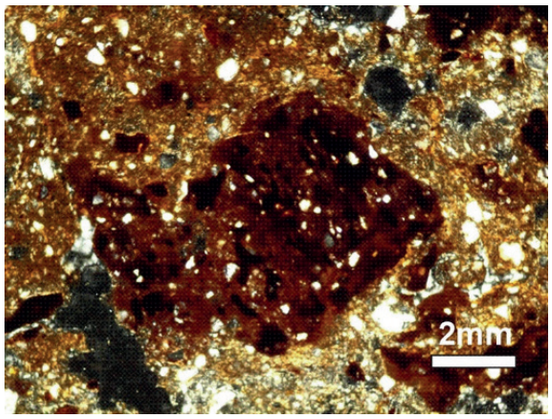


Figure 2.1.16.32 Nodule built up of several components

Reagents

- Resin
- Acetone
- Oil
- Grinding powder (0.125, 0.050, 0.010, 0.002 mm)
- Diamant paste (< 0.001 mm)

Materials and equipment

- Vacuum chamber
- Grinding machine
- Polishing machine

Procedure

a. Take the undisturbed sample and put it into boxes with double lids to avoid disturbance
b. Use of synthetic resins for improved impregnation
c. Apply acetone as a solvent of the resins to remove water from the samples (reducing shrinkage)
d. Add acetone to the resin to increase viscosity (the amount depends on the density, structure, and composition of the material
e. Use accelerator, hardener, depending on the official instructions for the use of the resin
f. Allow samples to be saturated with resin by capillary rise
g. Place samples into the vacuum chamber, slowly bring to 12 to 28 mercury vacuum to eliminate air bubbles; leave samples under pressure for an initial 24-hour period
h. Add resin and repeat this process twice or three times
i. Final curing in the oven at 50°C for a 24 to 48-hour period
j. Cut the impregnated solid soil blocks into 4-5 mm thin slices
k. Fix the slices on the glass (6 x 4, 8 x 6, 12 x 8 cm respectively)
l. Put the material in the grinding machine and grind to 0.1–0.05 mm thickness
m. Hand-finish using 0.01–0.002 mm grinding-powder and diamant paste,
n. Finish the polishing by the finest polish-powder at 20-50 µm (depending on the subject)

Calculations/evaluation

The thin section is usually described for

- *fabric:* spatial arrangement of material which, formed by particles, constituents
- *colour:* recorded by plane polarised light (PPL), crossed polarised light (XPL), oblique incident light (OIL) settings at different magnification, and/or computerised image analysis
- *grain size:* measured by micrometer. The ‘coarse’ and ‘fine’ limit, calculating or estimating coarse:fine (C:F) ratio is not a fixed value, it depends on the investigated material and aim; 5–15 micrometer (using optical microscope)
- *composition:* single and/or compound mineral grains, fragments; organic materials, residues; inorganic residues of biological origin; human articrafts
- *abundance:* relative percentage of particles
- *shape:* as circularity in two dimensions
- *roundness:* evaluating two-dimensional silhouettes shape (angular, subangular, subrounded or rounded)
- *sorting:* degree of variability (well-sorted, moderately sorted, unsorted bimodal [in the case of two component groups])
- *feature and pattern:* predicted change in the size of microaggregates, abudance of opaque organic materials, distribution of roots, fillings or depletions of channels, cracks, compaction or lack of bioturbation, etc.

Remarks

- The interpretation of features and patterns is based on experience combined with aims. The recognition of individual features is a complex task. Experts have to focus on soil micromorphological features which are able to alter within a short period (3 years) of research or will change with treatments

References

Bullock, P., Fedoroff, N., Jongerius, A., Stoops, G., Tursina, T., 1985. Handbook for Soil Thin Section Description. Waine Research Publications, Wolverhampton. 152 p.

Brewer, R., 1976. Fabric and Mineral Analysis of Soils. Krieger, New York. 482 p.

FitzPatrick, E.A., 1993. Soil Microscopy and Micromorphology. John Wiley, Chichester. 304 p.

Kubiena, W.L., 1938. Micropedology. Collegiate Press, Amsterdam. 243 p.

Stoops, G., Jongerius, A., 1975. Proposal for micromorphological classification in soil materials. I. A classification of the related distributions of coarse and fine particles. Geoderma, 13, 189–200.

2.1.17 Runoff coefficient and infiltration rate

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Importance and applications

The occurrence of extreme rainfall events severely affects society and can have a significant economic impact (de Lima et al., 2013; Kovats et al., 2014). High runoff rates may lead to increased soil erosion, especially when runoff concentration occurs and thus, linear erosion increases. On the other hand, reduced infiltration rates may lead to reduced water retention in the soil; they may also be indicators for crusting and compaction of the soil.

Principle

The runoff coefficient (%) interrelates the amount of runoff to the amount of precipitation received. It has a higher value for areas with low infiltration and high runoff and lower for areas with high infiltration rates. The infiltration rate (mm h^{-1}) is the amount of water entering the soil within a certain time interval. As runoff and infiltration are highly variable at temporal and spatial scales, in-situ measurements are difficult.
By means of rainfall simulation experiments, a calibrated uniform and reproducible rainfall with a defined intensity is sprayed on a delimited plot (Iserloh et al., 2013). The total runoff produced during a defined duration is collected at plot outlet. Runoff coefficient and infiltration rate are calculated afterwards.

Reagents

- Water

Materials and equipment

A small portable rainfall simulator (described in detail by Iserloh et al. 2012) equipped with:

- Plot
- Framework
- Nozzle holder with nozzle
- Rubber tarpaulin as cover against wind influence
- Water barrel
- A rod to mount flow control
- Flow control
- Bilge pump with battery and battery charger
- Hose connecting pump and flow control,
- Hose connecting flow rate meter and nozzle

Tools:

- Geometer (yardstick)
- Rubber hammer to drive in the plot

- Small shovel
- Screwdrivers (normal and phillips)
- Level (to align the irrigation setup horizontally and vertically)
- Pipe wrench

Other requirements:

- Clinometer including a compass (to measure plot inclination and orientation)
- Camera (for documentation)
- Plumb bob (on a string)
- Water canister
- Wide-neck plastic bottles (250, 500 mL)
- Graduated beakers
- Board with chalk
- Data recording sheets
- Silicon band
- Stopwatch

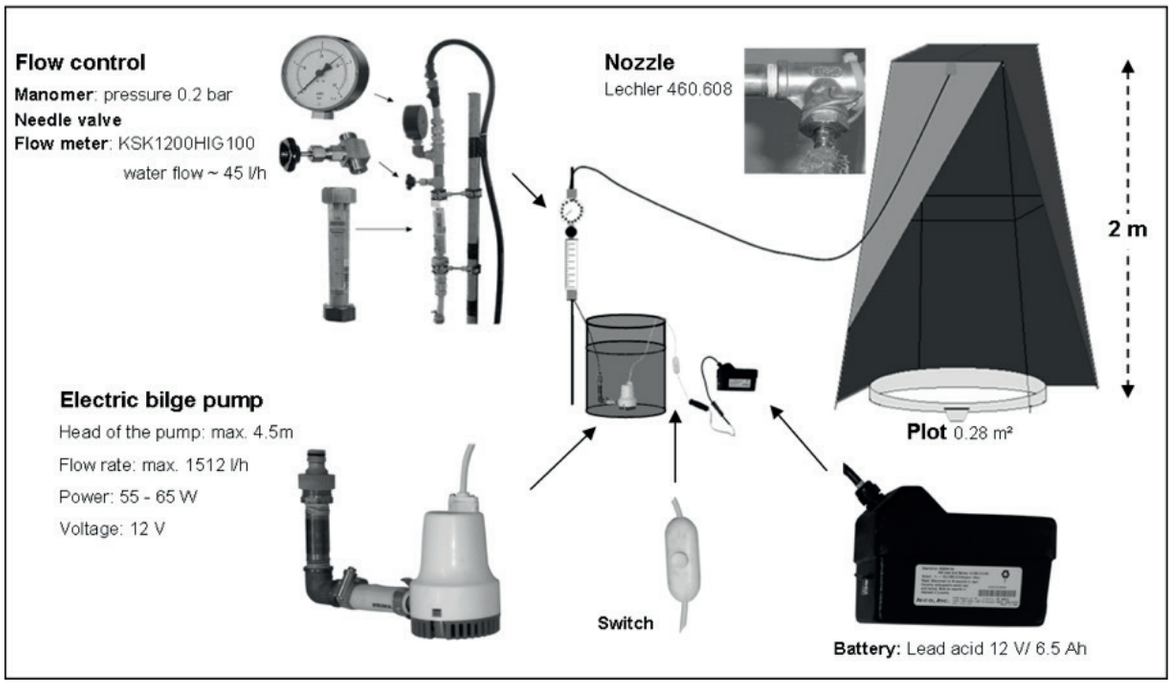

Figure 2.1.17.1 Small portable rainfall simulator (Iserloh et al., 2012).

Procedure

a. Place the plot on the soil and ensure that the runoff shield is oriented to point downhill

b. Carefully drive the plot into the soil with a rubber hammer

c. In order to position the collection containers, dig a small hole underneath the runoff shield

d. Map and photograph the soil surface

e. Place the calibration plate on the plot (thus, the plot is protected)

f. Before installing the framework, check hoses, connectors, flow meter and especially the nozzle for dirt (and clean if necessary). Check battery load

g. Set up the rainfall simulator:

h. Install the framework with wind protection

i. Orient and fix the simulator: Lock the nozzle in place at the correct position over the plot surface by using the plumb lead and arrange the nozzle holder vertically using a level

j. Place the flow meter on the rod close to the plot

k. Place the water barrel close to the irrigation setup and fill with water (at least 50 L)

l. Connect the bilge pump to the flux meter and this to the nozzle with the hoses

m. Place the pump into the water barrel and connect to the battery

n. Turn on pump and regulate to desired flow

o. Calibrate rainfall intensity by irrigating for 2.5 minutes on a calibration plate covering the whole plot and collect the runoff in a calibration vessel

Experimental procedure:

p. Start the measurement immediately after successful calibration without interruption by removing the calibration plate from the plot

q. After runoff starts, collect runoff water in plastic bottles of 250 mL and 500 mL capacity (depending on the amount of runoff, several bottles will be needed)

r. Record runoff start and time for every change of bottle

Calculations

a. Calibration of rainfall intensity on the plot (mm h-1):

$$I\ [mm\ h^{-1}] = \frac{Water\ volume\ [L]}{Time\ [h] \times Plot\ area\ [m^2]} \qquad \text{(Eq. 2.1.17.1)}$$

Runoff coefficient $[\%]: RC\ [\%] = \frac{Runoff\ [L]}{Precipitation\ [L]} \times 100$ (Eq. 2.1.17.2)

b. Infiltration rate (mm h-1):

$$I\ [mm\ h^{-1}] = Precipitation\ [mm\ h^{-1}] - Runoff\ [mm\ h^{-1}] \qquad \text{(Eq. 2.1.17.3)}$$

Remarks

- none

References

Iserloh, T., Fister, W., Seeger, M., Willger, H., Ries, J.B., 2012. A small portable rainfall simulator for reproducible experiments on soil erosion. Soil Till. Res., 124, 131–137.

Iserloh, T., Ries, J.B., Arnáez, J., Boix-Fayos, C., Butzen, V., Cerdà, A., Echeverría, M.T., Fernández-Gálvez, J., Fister, W., Geißler, C., Gómez, J.A., Gómez-Macpherson, H., Kuhn, N.J., Lázaro, R., León, F.J., Martínez-Mena, M., Martínez-Murillo, J.F., Marzen, M., Mingorance, M.D., Ortigosa, L., Peters, P., Regüés, D., Ruiz-Sinoga, J.D., Scholten, T., Seeger, M., Solé-Benet, A., Wengel, R., Wirtz, S., 2013. European small portable rainfall simulators: A comparison of rainfall characteristics. Catena, 110, 100–112.

de Lima, J.L.M.P., Carvalho, S.C.P., P. de Lima, M.I., 2013. Rainfall simulator experiments on the importance o of when rainfall burst occurs during storm events on runoff and soil loss. Z. Geomorphol., Supp. 57, 91–109.

Kovats, R.S., Valentini, R., Bouwer, L.M., Georgopoulou, E., Jacob, D., Martin, E., Rounsevell, M., Soussana, J.-F., 2014. Europe. in: Barros, V.R., Field, C.B., Dokken, D.J., Mastrandrea, M.D., Mach, K.J., Bilir, T.E., Chatterjee, M., Ebi, K.L., Estrada, Y.O., Genova, R.C., Girma, B., Kissel, E.S., Levy, A.N., MacCracken, S., Mastrandrea, P.R., White, L.L. (Eds.) 2014. Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part B: Regional Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1267–1326.

2.1.18 Sediment load and concentration

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Importance and applications

Soil erosion is now being recognised as a severe threat to socio-ecological security and stability. The manifold issues concerning soil health involve aspects as fundamental as food security, resilience to climate change and geosocial stability (Marzen et al., 2017). Soil erosion by water is generally expressed by sediment output (total sediment load and sediment concentration).

Principle

Gerlach troughs are built, installed and utilised as sediment collectors (Gerlach, 1967). Amounts of soil loss, surface flow and sediment concentration are calculated in g, L and g L^{-1}, respectively. Open soil erosion plots give information about the soil (g) and water losses (L), but the contributing area is not defined and may be variable. Consequently, soil erosion or overland flow are shown in g m^{-1} and L m^{-1} of slope width, respectively. Sediment output of a definable field section is measured under real agricultural conditions. The collected material will provide basic data on the transported grain sizes and nutrients of the particular field (Schmidt, 1979).

Reagents

- Water

Materials and equipment

Gerlach troughs are located at the bottom of each crop field studied. They are equipped with a slanted front edge to prevent scouring or undercutting of the trough. Additionally, they can be connected to collecting tanks to be prepared for extreme rainfall events, which can exceed the total storage capacity of the collector.
Material:

- Gerlach trough. Custom construction from galvanised sheet metal (1mm wall thickness). The dimensions are shown in Figure 2.1.18.1.

Equipment:

- Trowel
- Windscreen cleaner
- Scraper
- Brush
- Wash bottle
- Buckets with lids
- Funnels and filters

- Drying cabinet
- Precision scale

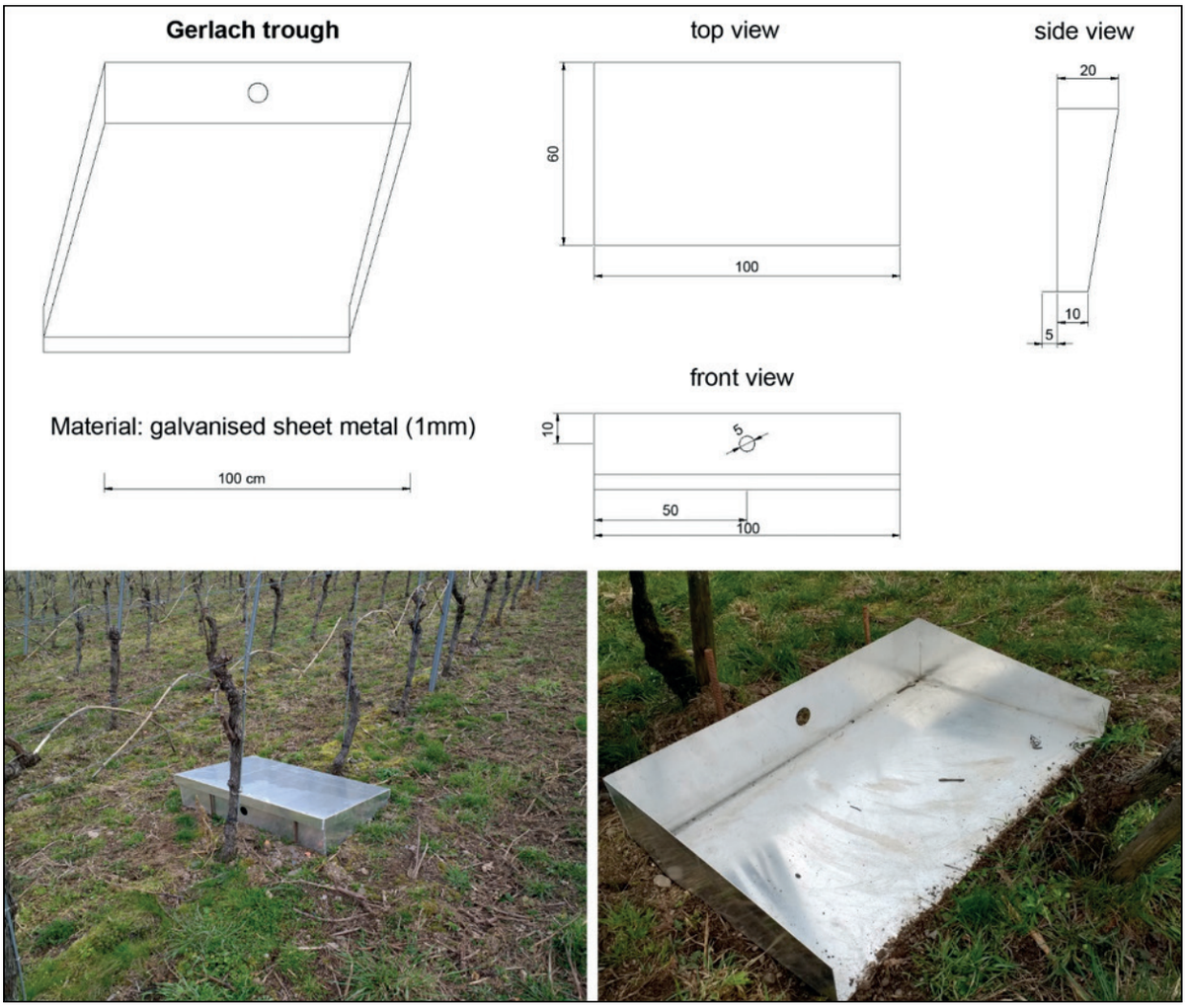

Figure 2.1.18.1. Gerlach trough

Procedure

a. Install the Gerlach troughs at the lower field edges. The field must be uniformLy inclined, best stretched out and exposed in one direction only.
b. Clearly delimit the catchment area.
c. Ensure a smooth transition between soil surface and Gerlach trough.
d. Empty the Gerlach troughs after every heavy rainfall event or after constant rain, at least once a week.
e. Collect total surface flow and soil loss by means of trowel, windscreen cleaner, scraper, brush and wash bottle until Gerlach trough is completely clean.
f. Fill total surface flow and soil loss and transport them in buckets with lids.

g. Determine total runoff by measuring the amount of water, in measuring cups.
h. Determine total soil loss by filtration of the samples in the laboratory. Weigh the amount of eroded material after filtering and air-drying (to allow for further investigations, e.g. particle size analysis).
i. Calculate sediment concentration by dividing the total soil loss by the total runoff.

Calculations

a. Sediment load (g m^{-1}):

$$SSL\ (g\ m^{-1}) = \frac{Sediment\ load\ (g)}{trough\ width\ (m)} \qquad \text{(Eq. 2.1.18.1)}$$

b. Sediment concentration (g L^{-1}):

$$SSC\ (g\ L^{-1}) = \frac{Sediment\ load\ (g)}{Runoff\ (L)} \qquad \text{(Eq. 2.1.18.2)}$$

Remarks

- none

References

Gerlach, T., 1967. Hillslope troughs for measuring sediment movement. Rev. Geomorphol. Dyn., 17, 173.
Marzen, M., Iserloh, T., De Lima, J.L.M.P., Fister, W., Ries, J.B., 2017. Impact of severe rain storms on soil erosion: Experimental evaluation of wind-driven rain and its implications for natural hazard management. Sci. Total Environ., 590–591, 502–513.
Schmidt, R.-G., 1979. Probleme der Erfassung und Quantifizierung von Ausmass und Prozessen der aktuellen Bodenerosion (Abspülung) auf Ackerflächen. Physiogeographica, 1, 240 p.

2.1.19 Rill and ephemeral gully density, cross-sectional profile

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Importance and applications

The erosion caused by concentrated flows generates rills (depth: 0.02 m–0.1 m) and ephemeral gullies (depth: 0.1 m–0.8 m) of small dimensions that can reach several tens of metres in length. The development of these rills and ephemeral gullies may increase erosion rates to an order of magnitude higher than erosion caused by non-concentrated surface flows (Cerdan et al., 2002; Merz and Bryan, 1993; Nouwakpo et al., 2016a; Poesen, 1987; Wirtz et al., 2012). Because of their highly erratic appearance and their easy removal by soil management, such erosion processes are difficult to quantify (Casalí et al., 2006; Giménez et al., 2009; Nouwakpo et al., 2016b; Wells et al., 2016).

Principle

Mapping in the field by visual identification and recording on the prepared mapping basis (DVWK, 1996). Characterisation by reference to their size (width and depth) as well as form. Measurement of rill and ephemeral gully length and building up relation between rill and ephemeral gully length and mapped area (rill and ephemeral gully density). Rill and ephemeral gullies may be also mapped by interpretation of high-resolution aerial photographs taken by unmanned aerial vehicle (UAV) (Aber et al., 2010). Cross-sectional profiles of rills and ephemeral gullies are determined after methods from Casalí et al. (2006) and Giménez et al. (2009). Simplified estimation of rill and ephemeral gully volume is possible (see below).

Reagents

- none

Materials and equipment

- Detailed map and (recent) aerial photography.
- Field mapping equipment:
- Scale and yardstick
- Compass, inclination measuring device

Alternatively:

- UAV with optical camera
- Computer with SfM (Structure from motion) software (e.g. Agisoft Photoscan, Visual SFM)

Procedure

a. Identify linear erosion features in the field
b. Record on the mapping base with appropriate symbols

c. Classify features by
- size (rill, ephemeral gully)
- form (v-shaped, rectangular, u-shaped).

d. Record their
- length [m]
- depth and width [m] at representative cross-sections of the feature.

e. Optional: digitise into GIS (geographic information system).

Alternative procedure:

a. Take aerial photography by UAV
- Overlap 80%, lateral overlap > 60%, if possible also oblique images
- Ground resolution ~0.1 m
- Point cloud and DEM (digital elevation model) generation by means of appropriate SfM-software
- Orthophoto as basis for visual rill and ephemeral gully identification
- (Volume calculations are until now a subject for scientific development).

Calculations

a. Rill and ephemeral gully density:

$$\textit{Rill and ephemeral gully density } [m/m^2] = \frac{\textit{Rill and ephemeral gully length } [m]}{\textit{Research area } [m^2]} \quad \text{(Eq. 2.1.19.1)}$$

b. Cross-sectional area:

$$V - \textit{shaped area } [m^2] = 1/2 \times \textit{depth } [m] \times \textit{width } [m] \quad \text{(Eq. 2.1.19.2)}$$

$$U - \textit{shaped area } [m^2] = \textit{depth}[m] \times \textit{width}[m] \times \pi \quad \text{(Eq. 2.1.19.3)}$$

$$\textit{Rectangular} - \textit{shaped area } [m^2] = \textit{depth}[m] \times \textit{width } [m] \quad \text{(Eq. 2.1.19.4)}$$

c. Estimation of rill and ephemeral gully volume:

$$\textit{Volume } [m^3] = \textit{area } [m^2] \times \textit{length } [m] \quad \text{(Eq. 2.1.19.5)}$$

Remarks

- none

References

Aber, J.S., Marzolff, I., Ries, J., 2010. Small-Format Aerial Photography: Principles, Techniques and Applications. Elsevier.

Casalí, J., Loizu, J., Campo, M.A., De Santisteban, L.M., Álvarez-Mozos, J., 2006. Accuracy of methods for field assessment of rill and ephemeral gully erosion. Catena, 67, 128–138.

Giménez, R., Marzolff, I., Campo, M.A., Seeger, M., Ries, J.B., Casalí, J., Álvarez-Mozos, J., 2009. Accuracy of high-resolution photogrammetric measurements of gullies with contrasting morphology. Earth Surf. Proc. Land., 34, 1915–1926.

Casalí, J., Loizu, J., Campo, M.A., De Santisteban, L.M., Álvarez-Mozos, J., 2006. Accuracy of methods for field assessment of rill and ephemeral gully erosion. Catena, 67, 128–138.

Cerdan, O., Le Bissonnais, Y., Couturier, A., Bourennane, H., Souchère, V., 2002. Rill erosion on cultivated hillslopes during two extreme rainfall events in Normandy, France. Soil Till. Res., 67, 99–108.

DVWK (1996): Bodenerosion durch Wasser – Kartieranleitung zur Erfassung aktueller Erosionsformen. - DVWK- Merkblätter zur Wasserwirtschaft 239, Fachausschuss Bodenerosion, Bonn, 62 S.

Merz, W., Bryan, R.B., 1993. Critical conditions for rill initiation on sandy loam Brunisols: laboratory and field experiments in southern Ontario, Canada. Geoderma, 57, 357–385.

Nouwakpo, S.K., Williams, C.J., Al-Hamdan, O.Z., Weltz, M.A., Pierson, F., Nearing, M., 2016a. A review of concentrated flow erosion processes on rangelands: Fundamental understanding and knowledge gaps. Int. Soil Water Conserv. Res., 4, 75–86.

Nouwakpo, S.K., Weltz, M.A., and McGwire, K., 2016b. Assessing the performance of structure-from-motion photogrammetry and terrestrial LiDAR for reconstructing soil surface microtopography of naturally vegetated plots. Earth Surf. Proc. Land., 41, 308–322.

Poesen, J., 1987. Transport of rock fragments by rill flow – a field study. Catena Suppl., 8, 35–54.

Wells, R.R., Momm, H.G., Bennett, S.J., Gesch, K.R., Dabney, S.M., Cruse, R., and Wilson, G.V., 2016. A Measurement Method for Rill and Ephemeral Gully Erosion Assessments. Soil Sci. Soc. Am. J., 80, 203–214.

Wirtz, S., Iserloh, T., Rock, G., Hansen, R., Marzen, M., Seeger, M., Betz, S., Remke, A., Wengel, R., Butzen, V., and Ries, J.B., 2012a. Soil Erosion on Abandoned Land in Andalusia: A Comparison of Interrill and Rill Erosion Rates. ISRN Soil Sci., 2012, 1–16.

2.1.20 Gully depth, gully growth rate

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Importance and applications

Gullies (depth: > 0.8 m) are three-dimensional erosion forms that may appear in various shapes, sizes and complexities (Poesen et al., 2003). Gully erosion destroys agricultural land and is difficult to quantify because of the size and complexity of the forms as well as the tempo-spatial variability of their development (Castillo & Gómez, 2016). Moreover, gully growth is the result of many different processes, such as headwall retreat, lateral collapses and incision (Marzolff & Ries, 2007).

Principle

For the quantification of gully-erosion processes, detailed aerial photographic monitoring has proved to be the most efficient method (d'Oleire-Oltmanns et al., 2012; Eltner et al., 2016; Marzolff & Poesen, 2009; Ries & Marzolff, 2003; Stöcker et al., 2015; Wang et al., 2016). Growth rates are determined either by measurement of gully volume changes or by measurement of headcut retreat rates (Marzolff et al., 2011).

Reagents

- none

Materials and equipment

- UAV (unmanned aerial vehicle) with optical camera
- Computer with SfM (Structure from motion) software (e.g. Agisoft Photoscan, Visual SFM)

Procedure

a. Gully depth: Take aerial photography by UAV
   - Overlap 80%, lateral overlap > 60%, if possible also oblique images
   - Ground resolution ~0.25 m
   - Point cloud and DEM (digital elevation model) generation by means of appropriate SfM-software
   - Orthophoto as basis for visual delineation of the gully edge
   - Identification of the uppermost gully headcut point
   - (volume calculations are until now a subject for scientific development)

b. Gully growth rate: As a simple and common measure of gully development, linear retreat rates reflect the average annual backward migration of gully heads in the upslope direction of the drainage line and thus the increase in gully length (Marzolff et al., 2011).

Calculations

a. Gully depth: Calculation from DEM

b. Gully growth rate:

$$\textit{Gully growth rate [m/a]} = \frac{\textit{headcut retreat [m]}}{\textit{year [a]}} \qquad \text{(Eq. 2.1.20.1)}$$

Remarks

- none

References

Castillo, C., Gómez, J.A., 2016. A century of gully erosion research: urgency, complexity and study approaches. Earth Sci. Rev., 160, 300–319.

d'Oleire-Oltmanns, S., Marzolff, I., Peter, K., Ries, J., 2012. Unmanned Aerial Vehicle (UAV) for Monitoring Soil Erosion in Morocco. Remote Sens., 4, 3390–3416.

Eltner, A., Kaiser, A., Castillo, C., Rock, G., Neugirg, F., Abellán, A., 2016. Image-based surface reconstruction in geomorphometry – merits, limits and developments. Earth Surf. Dynam., 4 (2), 359–389.

Marzolff, I., Ries, J.B., Poesen, J., 2011. Short-term versus medium-term monitoring for detecting gully-erosion variability in a Mediterranean environment. Earth Surf. Proc. Land., 36, 1604–1623.

Marzolff, I., Poesen, J., 2009. The potential of 3D gully monitoring with GIS using high-resolution aerial photography and a digital photogrammetry system. Geomorphology, 111, 48–60.

Marzolff, I., Ries, J.B., 2007. Gully erosion monitoring in semi-arid landscapes. Z. Geomorphol., 51, 405–425.

Poesen, J., Nachtergaele, J., Verstraeten, G., Valentin, C., 2003. Gully erosion and environmental change: Importance and research needs. Catena, 50, 91–133.

Ries, J.B., Marzolff, I., 2003. Monitoring of gully erosion in the Central Ebro Basin by large-scale aerial photography taken from a remotely controlled blimp. Catena, 50, 309–328.

Stöcker, C., Eltner, A., Karrasch, P., 2015. Measuring gullies by synergetic application of UAV and close range photogrammetry – A case study from Andalusia, Spain. Catena, 132, 1–11.

Wang, R., Zhang, S., Pu, L., Yang, J., Yang, C., Chen, J., Guan, C., Wang, Q., Chen, D., Fu, B., Sang, X., 2016. Gully Erosion Mapping and Monitoring at Multiple Scales based on Multi-Source Remote Sensing Data of the Sancha River Catchment, Northeast China. Int. J. Geo-Inf., 5, 11, 200.

2.1.21 Soil water content at field capacity and wilting point

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Importance and applications

Soil moisture conditions are closely linked to pore volume, pore size distribution, capillary rise capacity and the groundwater table. Water tension controls the germination time of seeds. In orchards and for horticultural crops, irrigation design is based on the prediction of the dynamics of soil moisture status.

Actual soil moisture content (m m^{-1}% or V V^{-1}%), however, only provides limited information on soil-plant system hydrodynamics. The available water (AW) for plant uptake is closely associated with the soil water budget (WB) (Kirkham, 2014). This is a changeable parameter, which must be investigated within the total range of water capacity. This range covers two characteristic points: the field capacity and the wilting point.

Principle

Field capacity (FC) corresponds to the upper limit of AW and represents the soil moisture content left behind after the water contained in the macropores is drained by gravity (Assouline & Or, 2014). Wilting point (WP) refers to the water content when the soil becomes dry and plants can no longer take up water. Both FC and WP are agreement-based thresholds and differ with plants. FC ‘‘is that water content at which the soil water flux out of the rooting zone becomes negligible and no significant change in water content occurs with time’’ (Cassel & Nielsen, 1986). The theory of non-limiting water range (NLWR) points out that water may not be equally available to plants between FC and the permanent WP (Letey, 1985).

For the determination of FC, soil samples are dried by raising the air pressure in an extractor with a porous ceramic plate. The pores of the plate are filled with water and prevent high-pressure air from flowing through. The smaller the pore size, the higher the pressure. Soil moisture will flow around the individual soil particles, through the ceramic plate and an outflow tube until equilibrium is reached (i.e. air pressure in the extractor equals water tension in the samples) (UGT, 2018).

The soil water potential is defined as “the work that would have to be supplied to a unit of water linked to the soil to take it from this state of union to a state of reference, corresponding to that of pure water at the same temperature and atmospheric pressure (Azcón-Bieto & Talón, 2000). The total water potential can be expressed as the sum of the individual contributions of several factors:

$$\Psi t = \Psi g + \Psi o + \Psi m \qquad \text{(Eq. 2.1.21.1)}$$

where Ψg, is the gravitational potential; Ψo, the osmotic potential and Ψm, the matric potential.

The matric potential determines the energy that the plant must apply to extract the water from the soil and is defined as “the force by which the water is retained due to the interactions with soil matrices” (Smith &

Mullins, 2001).

The moisture status of the soil is expressed in terms of a volumetric moisture content (Θ) and the capillary potential (ψ) of pore water. The relationship between both parameters is given by the water retention curve (WRC). The function of the water retention curve was proposed by van Genuchten (1980).

$$\Theta(\psi) = \Theta r+(\Theta s - \Theta r)/(1+(\alpha \left|\psi\right| n)^{1-(1/n)} \quad \text{(Eq. 2.1.21.2)}$$

where

Θ(ψ) is the WRC [L^3L^{-3}],

$\left|\psi\right|$ is the suction pressure [L; kPa; H_2Ocm],

Θs is the saturated water content [L^3L^{-3}],

Θr is the residual water content [L^3L^{-3}],

α is a coefficient related to the inverse of the air entry suction, α > 0 [L^-1; cm^{-1}],

n is a measure of pore size distribution, n > 1 [dimensionless].

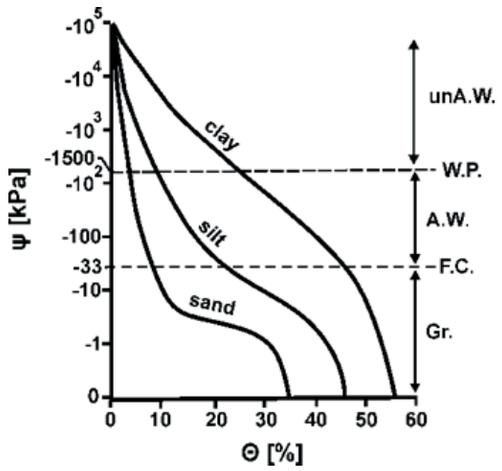

Figure 2.1.23.1 Θ - ψ water retention curves for the main textural groups of soils. Gr. = vol% released by gravity; FC = field capacity (-33 kPa); AW = vol% available water; WP = wilting point (-1500 kPa); unAW = vol% unavailable water

FC is determined by the retention curve method in the laboratory. FC moisture is represented by the balance moisture with tension of 6–33 kPa, depending on soil texture, structure and organic matter content. Richards and Weaver (1949) found that water content held by soil at a potential of −33 kPa correlate closely with FC (−10 kPa for sandy soils). Permanent WP is commonly approximated as the soil water content at –1500 kPa.

The working range of the vacuum gauge tensiometers is limited (ψm from 0 to -80 kPa) as opposed to the -200 kPa of the WATERMARK-type resistance sensor. Such values suppose no limitation to scheduling localised irrigation, since they can be useful in the application of deficit irrigation strategies, in order to avoid promoting severe water stress to the crop (Pérez-Pastor et al., 2009). The values of ψm obtained with tensiometers have been widely used in woody crops (Pérez-Pastor et al., 2016). Kaufmann and Elfving (1972) found a good correlation between the readings of the tensiometers and the leaf water potential

before dawn (ψa).

The suction value ψ is typically expressed with pressure units (kPa), pressure head (m of water), or centimetric logarithmic head (pF); at 20°C, 9.8 kPa Ξ 100 cm of water Ξ pF of 2 (Aubertin et al., 2003) (Table 2.1.21.1).

Table 2.1.21.1 Conversion between different suction values

kPa	Bar	H_2Ocm	pF	Meaning
0	0	0	1	Saturated soil
-33	-0.333	-336.50	2.5	Field Capacity (FC)
-1500	-15	-15849.00	4.20	Wilting Point (WP)

The sensors that measure the matric potential are based on the direct measurement of the soil water tension (tensiometers), and indirectly of the electrical resistance of the soil (granular matric sensors) and the dielectric permeability of the soil matrix (porous ceramic disc sensors) (Smith & Mullins, 2001).

In-situ capillary potential may be measured using a tensiometer consisting of a water-filled porous cup connected to a manometer or pressure transducer or, alternatively, by the scattering of neutrons or absorption of gamma rays from a radioactive source (Vaz et al., 2013). The essential part of the instrument is a pipe with a small volume of water reservoir and the tensiometer or irrometer (above ground) ending in a ceramic tip (Fig. 2.1.21.2). The leaking generates vacuum in the pipe according to soil moisture conditions. This rapid field method is applicable between 0 and 30 kPa.

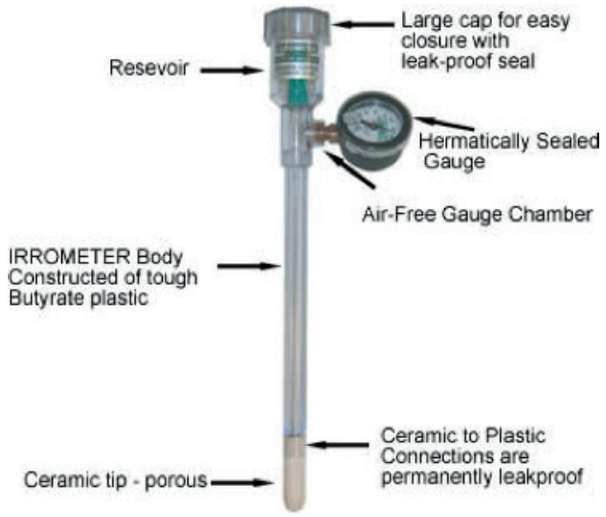

Figure 2.1.21.2 Irrometer (source: Calafrica, 2018)

Reagents

- Distilled water

Materials and equipment

- *Auger for undisturbed soil sampling*
- *Equipment for determinating actual (in situ) FC:*
  - Auger to prepare the hole for the tensiometer
  - Tensiometer
  - Matric potential sensors (MPS-6, now called Teros 21, METER GROUP)
- *Equipment for determining FC, WP by pressure ceramic plate exactors at different vacuum levels in the laboratory*
  - Pressure control panel equipped with two manometers 0-2 MPa and 0-0.4 MPa
  - Pressure vessel(s)
  - Compressor (220 V/50 Hz); maximum pressure 2.0 MPa,
  - Pressure ceramic plates, 0.1, 0.3, 0.5 and 1.5 kPa standards

Procedure

Determination of actual FC by tensiometer (water-filled porous cup) in the field:

- Prepare a perfect hole in the soil horizon
- Fit the tensiometer into the hole
- Check if fitting is tight between the soil surface and ceramic tip
- Wait 10-30 minutes for the balance between the internal and external part of the ceramic tip (depending on soil textural properties)

Determination of FC and WP by pressure plate exactor at different pressure levels in the laboratory:

- Saturate the undisturbed soil sample with distilled water
- Place the samples on the ceramic plate of 0.1 kPa type.
- Set the compressor to the adequate pressure level at 33 kPa (for FC)
- Measure the soil water content (SWC) of FC when equilibrium is reached
- Change the ceramic plate to 1.5 kPa type
- Set the compressor to the pressure 1500 kPa (for WP)
- Measure the soil water content (SWC) of WP when equilibrium is reached

Calculations

The amount of water held by the root zone of the soil between FC and WP (available for plants):

$$AW = FC - WP$$

Remarks

- When establishing the WRC curve, hysteresis (difference in the rates of saturation and desiccation) may result in up to 20% variation.
- When determining FC and WP it is difficult to know how much time is needed for the wet soil sample to reach the moisture content appropriate at actual pressure (i.e. suction).

References

Aubertin, M., Mbonimpa, M., Bussière, B., Chapuis, R.P., 2003. A model to predict the water retention curve from basic geotechnical properties. Can. Geotech. J., 40, 6, 1104–1122. doi: 10.1139/t03-054

Azcón-Bieto, J., Talón, M., 2000. Fundamentos de Fisiología Vegetal. Mc Graw Hill/Interamericana de España SAU Madrid. 522 p

Assouline, S., Or, D., 2014. The concept of field capacity revisited: Defining intrinsic static and dynamic criteria for soil internal drainage dynamics. Water Resour. Res., 50, 4787–4802. doi: 10.1002/2014WR015475.

Calafrica, 2018. Soil moisture measurements. Irrometers Calafrica SA., Nelspruit, South Africa. http://www.calafricasa.co.za/soil_moisture_measurement.htm

Cassel, D.K., Nielsen, D.R., 1986. Field capacity and available water capacity In: Methods of Soil Analysis: Part 1. Physical and Mineralogical Methods, Agron. Monogr. Ser. 9 (Klute, A. Ed.) American Society of Agronomy and Soil Science Society of America, Madison, WI. 901–926.

Kirkham, M.B., 2014. Field Capacity, Wilting Point, Available Water and the Nonlimiting Water Range. Chapter 10 in: Principles of Soil and Plant Water Relations. Second Edition. Elsevier, Amsterdam. 153–170. doi: 10.1016/B978-0-12-420022-7.00010-0

Letey, J., 1985. Relationship between soil physical properties and crop production. In: Advances in Soil Science Vol. 1. (Stewart, B.A. Ed.). Springer Verlag, New York. 277–294.

Pérez-Pastor A., Domingo R., Torrecillas A., Ruiz-Sánchez M.C., 2009. Response of apricot trees to deficit irrigation strategies. Irrig. Sci 27, 231–242.

Pérez-Pastor, A., Ruiz-Sánchez, M.C., Conesa, M.R., 2016. Drought stress effect on woody tree yield. Water Stress and Crop Plants: A Sustainable Approach, Volume 2, First Edition. John Wiley & Sons, Ltd. pp. 356-374.

Richards, L.A., Weaver, L.R., 1944. Moisture retention by some irrigated soils as related to soil moisture tension. J. Agr. Res., 69, 215–235.

Smith, K.A., Mullins, C.E., 2001. Soil and Environmental Analysis. 2a ed, New York, USA. 1-64 p.

Sun, H., Yang, J., 2013. Modified numerical approach to estimate field capacity. J. Hydrol. Eng., 18, 431–438.

UGT, 2018. UGT Products. Soil science. UGT, Müncheberg, Germany. http://www.ugt-online.de/en/products/soil-science/

van Genuchten, M.Th., 1980. A closed-form equation for predicting the hydraulic conductivity of unsaturated soils. Soil Sci. Soc. Am. J., 44, 892–898.

Vaz, C.M.P., Calbo, A.G., Porto, L.F., Porto, L.H., 2013. Principles and applications of a new class of soil water matric potential sensors: The Dihedral Tensiometer. Procedia Environ. Sci., 19, 484–493. doi: 10.1016/j.proenv.2013.06.055

2.1.22 Saturated Hydraulic Conductivity

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Importance and applications

Saturated hydraulic conductivity measured in the field (K_s) is one of the most important hydrological properties of soil. In agro-ecosystems this property provides information about the internal drainage of soils, highlighting good soil structure or compactness/saturation that may hinder proper water flow in the soil profile. This is important for understanding and characterising the hydrological cycle and the transfer of contaminants transported by water (Lassabatère et al., 2006). This property also informs about possible water logging problems and runoff after intense rainfall events. In irrigated agriculture, the K_s can be used in designing water application rates for drip and sprinkler systems, and thus avoid water-logging and runoff (Mbagwu, 1995).

Principle

The method proposed here to determine K_s was developed by Bagarello et al., (2012). For this, a simple annular ring is inserted at a short depth into the soil, to produce minimal disturbance of the porous medium, and the infiltration time is measured of a few small volumes of water repeatedly applied at the surface of the confined soil. The acronym SBI was suggested by Bagarello et al., (2012) to denote this method, given that it is a 'Simplified method based on a Beerkan Infiltration run'.

Reagents

- Water

Materials and equipment

- A metal ring of internal radius r = 0.075 m (15 cm diameter). Its height can vary, but should be at least 10 cm
- Hammer
- Plastic measuring cylinders (150 mL)
- Plastic beakers (200 mL)
- Timer

Procedure

a. Remove surface vegetation and litter

b. Insert the ring to a depth (d) of about 1 cm into the soil surface with the help of a hammer to avoid lateral loss of the pondered water at the soil surface

c. Pour a known volume of water (0.150 L) into the ring using a cylinder. Immediately when the amount of water is totally infiltrated, measure and record the elapsed infiltration time (s), and pour a second

identical amount of water into the ring. Again, record the time (s) needed for the water to infiltrate (cumulative time)

d. Repeat step c. until the difference in infiltration time between five consecutive trials becomes negligible, indicating a practically steady state of infiltration

e. Record the number of water additions (N)

Calculations

a. Calculate the experimental water infiltration "*I* " as follows:

$$I\ (L\ m^{-2} = mm) = \frac{Poured\ water\ volume\ (L)\ \ at\ each\ time}{\pi r^2\ (m)} \qquad \text{(Eq. 2.1.22.1)}$$

b. Calculate the experimental cumulative infiltration "*I* " as follows at each cumulative time t (s):

$$Cumulative\ I\ (mm) = \sum^{N}_{t=1} I \qquad \text{(Eq. 2.1.22.2)}$$

c. Make a plot of the cumulative infiltration, *I*, vs time, *t*, such as that shown in Fig. 2.1.22.1:

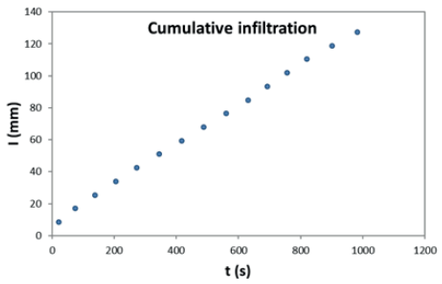

Figure 2.1.22.1. Cumulative infiltration plot

d. Calculate the **infiltration rate**, *IR* (mm s^{-1}) by the slope of the linearised cumulative infiltration curve (Figure 2.1.22.1), estimated by a linear regression analysis of the ($I / \sqrt{t}$, $\sqrt{t}$) data collected during the steady-state phase of the infiltration run.

e. Calculate K_s as follows:

$$K_s\ (mm\ s^{-1}) = \frac{IR}{0.467\left(1+\frac{2.92}{r\,\alpha^*}\right)} \qquad \text{(Eq. 2.1.22.3)}$$

where

IR (mm s^{-1}): is the infiltration rate

r (m): is the radius of the ring

α* (mm-1): 0.0262 + 0.0035 x ln(IR) (Eq. 2.1.22.4)

Table 1.22.1. Saturated Hydraulic Conductivity classes according to the Natural Resources Conservation Service of the USDA

K_s class	Ks rate (mm s^{-1})
Very rapid	141.14 10^{-3}
Rapid	42.34 10^{-3}–141.14 10^{-3}
Moderately rapid	14.11 10^{-3}–42.34 10^{-3}
Moderate	4.23 10^{-3}–14.11 10^{-3}
Moderately slow	1.41 10^{-3}–4.23 10^{-3}
Slow	0.42 10^{-3}–1.41 10^{-3}
Very slow or impermeable	0.00–0.42 10^{-3}

Remarks

- According to the literature, ***α**** can be estimated on the basis of a general description of soil textural and structural characteristics. However, Bagarello et al., (2012) developed the explained relationship between ***α**** and IR, working with 149 infiltration curves collected on Burundian soils. These authors suggested that the infiltration rate contains the necessary information to estimate ***α****.

References

Bagarello, V., Di Prima, S., Iovino, M., Provenzano, G., 2012. Estimating field-saturated soil hydraulic conductivity by a simplified Beerkan infiltration experiment. Hydrol. Process., 28, 1095–1103.

Lassabatère, L., Angulo-Jaramillo, R., Soria Ugalde, J.M., Cuenca, R., Braud, I., Haverkamp, R., 2006. Beerkan estimation of soil transfer parameters though infiltration experiments – BEST. Soil Sci. Soc. Am. J., 70, 521–532.

Mbagwu, J.S.C., 1995. Saturated hydraulic conductivity in relation to physical properties of soils in the Nsukka Plains, southeastern Nigeria. Geoderma, 68, 51–66.

2.1.23 Actual field soil moisture

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Importance and applications

The measurement of soil moisture content is fundamental for many studies in agriculture; it measures the amount of water that is retained among the solid particles of the soil and can be expressed as an absolute amount, although it is usually expressed as a fraction of a determined base constant (Jones, 2006). Basically, soil moisture (Soil Water Concentration, SWC) is the water that is held in the spaces between soil particles. Soil moisture is a changeable parameter which governs a whole range of soil processes. It is a major control on the water availability of cultivated crops.
Soil moisture content impacts on many fundamental biophysical processes (Bittelli, 2011): on germination, plant growth, microbial decomposition of the soil organic matter, and nutrient transformations in the root zone. Heat and water transfer at the land–atmosphere interface is also dependent on moisture content. As a major reservoir for water within a catchment, soil moisture directly influences susceptibility to soil erosion and slope stability.

Principle

A wide range of focuses and instrumentation are available for the direct and indirect measurement of the soil moisture content (Smith & Mullins, 2001), of which the most notable are taking soil samples (gravimetric and volumetric methods), neutron probes, Time-Domain Reflectometry (TDR), and Frequency-Domain Reflectometry, (FDR) (Smith & Mullins, 2001). The FDR sensors present several advantages over the other techniques used, amongst which one can highlight: low cost, robustness, that the salt content of the soil does not affect them nor do temperature variations (Paltineanu & Starr, 1997) and that they are easily automated (Martí et al., 2013; Starr & Paltineanu, 1998).
Actual soil moisture content (SWC_a) is often measured *gravimetrically* by drying a soil sample under controlled conditions (Reynolds, 1970a,b,c). For soil moisture monitoring, Time Domain Reflectrometry (TDR) sensors are also used. *Permittivity*, ε (Greek letter epsilon) is the measure of a material's ability to resist an electric field (Davood et al., 2012). By definition, perfect vacuum has a relative permittivity of exactly 1. The difference in permittivity between vacuum and air can often be considered negligible, as κ_{air} = 1.0006. and for water: κ_{water} = 80.

$$K = \frac{\varepsilon}{\varepsilon_0} \qquad \text{(Eq. 2.1.23.1)}$$

The lowest possible permittivity is that of a vacuum. Vacuum permittivity (or electrical constant) is represented by ε_0 and has a value of approximately 8.85×10^{-12} F/m (Faraday/metre). Due to the relative permittivity of materials, an electromagnetic wave travelling through them will experience an increase in the characteristic velocity

$$v: v = c\ \sqrt{\mu} = (c\ t\ 2L)^2 \qquad \text{(Eq. 2.1.23.2)}$$

where

c is the speed of light,
μ is the relative magnetic permeability of the soil (~1),
t is the travel time of the Time Domain Reflectometry (TDR) pulse,
2**L** is the travel path of the wave.

Since the travel time of electromagnetic waves through soil is a function of the effective moisture, and the relative contribution of water is a factor 20 times larger than all other (in)organic soil parts, the travel time is mainly a function of the water content.
Other alternative techniques such as satellite measurements became the practice in the event of regional or global investigations (Little et al., 1998).

Reagents

- None

Materials and equipment

For the gravimetric method:

- Oven with 105°C temperature
- Precision balance (±0.01 g)
- Aluminium tins
- Auger for soil sampling

TDR-based equipment for the in-situ determination of actual field capacity

- Portable or fixed equipment/data logger with TDR sensor(s)

Procedure

For the gravimetric method:

a. Weigh an aluminum tin, and record its weight (tare).
b. Place a soil sample of about 10g in the tin and record this weight as (wet soil + tare).
c. Place the sample in the oven at 105°C, and dry for 24 hours.
d. Weigh the sample, and record this weight as (dry soil + tare).
e. Return the sample to the oven and dry for several hours, and determine the weight of (dry soil + tare).
f. Repeat step 5 until there is no difference between any two consecutive measurements of the weight of the dry soil and the tare (de Angelis, 2007).

For the in-situ TDR-based method:

a. Calibrate the TDR sensors for the soil types in the laboratory
b. Create soil sample series which have different moisture contents (e.g. 5, 10, 30, 40 %) gravimetrically determined
c. Push the sensor into undisturbed samples
d. Plot soil moisture (%) against the measured values (%) and determine its accurancy
e. (In the field) push the TDR-sensor into the surface of the soil horizon

Calculations

The soil moisture content (SWC) in dry weight basis may be calculated using the following formula:

$$SWC=((W_2-W_3)\ (W_3-W_1)^{-1})\ 100 \quad [\%] \qquad \text{(Eq. 2.1.23.3)}$$

where

$\mathbf{W}_1$ is the weight of the tin [M; g]
$\mathbf{W}_2$ is the weight of the moist soil + tin [M; g]
$\mathbf{W}_3$ is the weight of the dry soil + tin [M; g]

The TDR equipment shows the results as percentages.

Remarks

- Methodological problems: the site is partially destroyed by the gravimetric method; the method itself modifies soil moisture distribution in time and space.
- Sample size is influenced by mean moisture content, the level of saturation, and the amount of insolation.
- Water content is not uniform throughout the profile.
- In the case of the in-situ TDR method, the soil water content will be measured using the different sensors installed at differing depths in the soil. A borer will be used in the placing of the sensors, seeking to make close contact between the sensor and the soil. Once the sensors have been installed they are connected to the Datalogger, with the corresponding program.

References

Aubertin, M., Mbonimpa, M., Bussière, B., Chapuis, R.P., 2003. A model to predict the water retention curve from basic geotechnical properties. Can. Geotech. J., 40, 6, 1104–1122. doi: 10.1139/t03-054
Bittelli, M., 2011. Measuring Soil Water Content: A Review. Hort. Techn., 21, 3, 293–300.
Davood, N.-K., Shorafa, M., Heidari, A., 2012. Estimating Soil Water Content from Permittivity for Different Mineralogies and Bulk Densities. Soil Sci. Soc. Am. J., 76, 4, 1149–1158. doi: 10.2136/sssaj2011.0144
de Angelis, K.M., 2007. Measurement of soil moisture content by gravimetric method. Berkeley University nature.berkeley.edu/soilmicro/methods/Soil%20moisture%20content.pdf
Jones, H.G., 2006. Monitoring plant and soil water status: established and novel methods revisited and their relevance to studies of drought tolerance. J. Exp. Bot. 58, 119-130.

Little, K.M., Metelerkamp, B., Smith, C.W., 1998. A comparison of three methods of soil water content determination. South Afr. J. Plant Soil, 15, 2, 80–89. doi: 10.1080/02571862.1998.10635121

Martí, P., Gasque,M., González-Altozano, P., 2013. An artificial neural network approach to the estimation of stem water potential from frequency domain reflectometry soil moisture measurements and meteorological data. Comput. Electron. Agr. 91, 75-86.

Paltineanu, I.C., Starr, J.L., 1997. Real-time soil water dynamics using multisensor capacitance probes: laboratory calibration. Soil Sci. Soc. Am. J. 61, 1576-1585.

Smith, K.A., Mullins, C.E., 2001. Soil and Environmental Analysis. 2a ed, New York, USA. 1-64 p.

Starr, J.L., Paltineanu, I.C., 1998. Soil water dynamics using multisensory capacitance probes in nontraffic interrows of corn. Soil Sci. Soc. Am. J. 62, 114-122.

Reynolds, S.G., 1970a. The gravimetric method of soil moisture determination. Part I A study of equipment, and methodological problems. J. Hydr., 11, 3, 258–273. doi: 10.1016/0022-1694(70)90066-1

Reynolds, S.G., 1970b. The gravimetric method of soil moisture determination. Part II Typical required sample sizes and methods of reducing variability. J. Hydr., 11, 3, pp. 274–287. doi: 10.1016/0022-1694(70)90067-3

Reynolds, S.G., 1970c. The gravimetric method of soil moisture determination. Part III An examination of factors influencing soil moisture variability. J. Hydr., 11, 3, pp. 288–300. doi: 10.1016/0022-1694(70)90068-5

Richards, L.A., Weaver, L.R., 1944. Moisture retention by some irrigated soils as related to soil moisture tension. J. Agr. Res. 69, 215–235.

van Genuchten, M.Th., 1980. A closed-form equation for predicting the hydraulic conductivity of unsaturated soils. Soil Sci. Soc. Am. J., 44, 892–898.

Vaz, C.M.P., Calbo, A.G., Porto, L.F., Porto, L.H., 2013. Principles and Applications of a New Class of Soil Water Matric Potential Sensors: The Dihedral Tensiometer. Procedia Environ. Sci., 19, 484–493. doi: 10.1016/j.proenv.2013.06.055

2.1.24 Abundance and size of roots

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Importance and applications

Soil mechanical constraints can restrict the development of plant roots. High soil compaction by increasing machinery intensity can increase soil penetration resistance and bulk density, and as a consequence, decrease crop yields (Botta et al., 2004). The diagnosis of soil physical constraints by root development is mainly based on soil relative compaction, soil pore volume and soil penetration resistance (Micucci & Taboada, 2006). Thus, the assessment of the size and abundance of roots in the soils can help elucidate soil physical constraints for plant growth and development. The abundance of roots is also indicative of soil biological activity, and thus of a healthy soil.

Principle

The method proposed here is adapted from FAO, (2006). The size and abundance of plant roots is determined using a 10 cm x 10 cm transparent grid subdivided into 1 cm x 1 cm, as shown in Fig. 2.1.25.1. Soil is spread on a flat surface, preferably of a light colour, and the grid is placed over it to assess the abundance of roots. After this, roots are separated from the soil and the diameter measured with a ruler with the help of a magnifier, or with a magnifier with graticule for small roots.

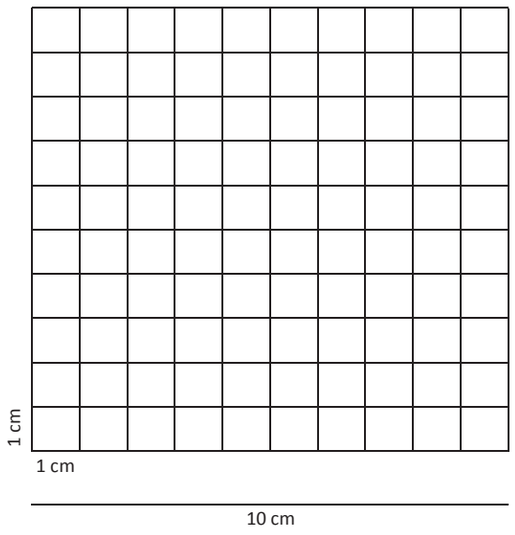

Figure 2.1.25.1. Grid for estimating abundance and size of roots

Reagents

- None

Materials and equipment

- 10 cm x 10 cm transparent grid subdivided into 1 cm x 1 cm. Material can be glass or plastic.
- Magnifier
- Magnifier with graticule for small roots
- Ruler for coarse roots

Procedure

a. Collect a soil sample from the layer or horizon to be characterised
b. Place the soil on a flat surface (preferably of a light colour). Spread the soil to cover all the surface
c. Put the 10 cm x 10 cm transparent grid on the soil
d. Record the number of roots present within the 10 cm x 10 cm grid. Record the number of roots according to two different categories for the classification of abundance: < 2 mm of diameter and > 2 mm diameter. A magnifier can be used to identify fine roots
e. Once the number of roots observed in the grid is recorded, separate the different roots from the soil and measure and record the size of the roots. For coarse roots, a ruler can be used with the help of a magnifier. For small roots, use a magnifier with graticule

Calculations

a. Abundance of roots: Indicate the number of roots < 2 mm diameter and the number of roots > 2 mm diameter and express this per 100 cm² (number or roots / 100 cm²). Classify the abundance of roots according to Table 2.1.25.1:

Table 2.1.25.1. Classification of the abundance of roots (FAO, 2006)

Classification	Size of roots	
	< 2 mm diameter	> 2 mm diameter
None	0	0
Very few	1–20	1–2
Few	20–50	2–5
Common	50–200	5–20
Many	> 200	> 20

b. Classify the roots into four different size categories according to Table 2.1.25.2 (very fine, fine, medium, coarse). Calculate the percentage of roots belonging to each size category with relation to the total number of roots identified and measured.

Table 2.1.25.2 Classification of the size of roots (FAO, 2006)

Classification	Diameter (mm)
Very fine	< 0.5
Fine	0.5 – 2
Medium	2 - 5
Coarse	> 5

Remarks

- It is advisable to repeat the procedure at least three times per soil layer/horizon to obtain representative conditions regarding the layer/horizon.

References

Botta, G.F., Jorajuria, D., Balbuena, R., Rosatto, H., 2004. Mechanical and cropping behavior of direct drilled soil under different traffic intensities: effect on soybean (Glycine max L.) yields. Soil Till. Res., 78, 53–58.

FAO, 2006. Guidelines for Soil Description, 4th Edition. FAO, Rome.

Micucci, F.G., Taboada, M.A., 2006. Soil physical properties of soybean (Glycine max, Merrill) root abundance in conventionally- and zero-tilled soils in the humid Pampas of Argentina. Soil Till. Res., 86, 152–162.

2.2. SOIL CHEMICAL ANALYSES

2.2.1 NH_4-Nitrogen

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Importance and applications

In the soil, the exchangeable ammonia (NH_4^+) is adsorbed on the exchange complex, while the nitrate form circulates in the liquid phase of the soil. Both the soil nitrate and the ammonia are mineral N forms contributing to crop nutrition, simultaneously present in a dynamic equilibrium: merely by virtue of this balance, they are under constant transformation from one form to another, as mediated by soil microflora (*Nitrobacter* and *Nitrosomonas* bacteria). The pool of exchangeable NH_4^+ is easily extractable from the soil by $CaCl_2$ or KCl extraction. It differs from the fixed NH_4^+, which is the pool of immobilised ammonia by the clay minerals, detectable only after soil treatment with fluoride acid.

The determination of ammonia (NH_4^+) is a key analysis on the assessment of soil fertility. Several methods are available for determination of this ion in soil extracts (Fig. 2.2.1.1). Among them, colorimetric methodologies by the Berthelot reaction (sometimes called the indophenol reaction) present the advantages of being quick, simple, and sensitive, and have been widely employed in the design of automated analyser systems (continuous flow analysis) (Keeney & Nelson, 1982; Mulvaney, 1996; Rhine et al., 1998). In agricultural sciences, the application of colorimetric methodologies is commonly found as a primary reaction methodology for the determination of NH_4^+ in plant materials (Davidson et al., 1970), soil and its extracts (Nelson, 1983), as well as fertilisers (Seely et al., 1967). It is used also in food, water, pharmaceuticals, and many others (Searle, 1984). As extraction solution, potassium chloride (KCl) is frequently adopted, because of its high determination coefficient.

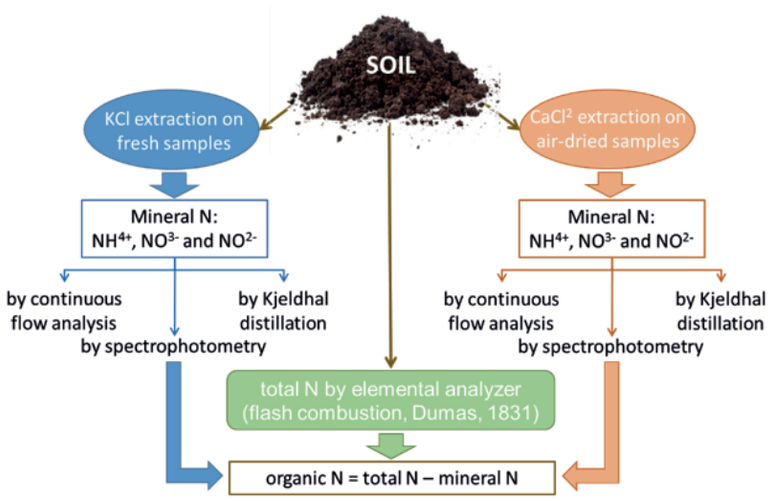

Figure 2.2.1.1 Diagram for the determination of the different soil nitrogen pools

Principle

The soil sample is treated with a solution of potassium chloride (2 mol x L^{-1}); the extract is then analysed by continuous flow colorimetric system. The ammonia nitrogen content is determined by the Berthelot reaction (sometimes called the "indophenol reaction"), discovered in 1859, in which sodium salicylate forms an indophenol in the presence of ammonia and hypochlorite. When NH_3, phenol, and hypochlorite were mixed in sequence, the reaction produced a blue or blue-green coloured solution, whose intensity is correlated to the concentration of NH_4^+ in the soil extract.

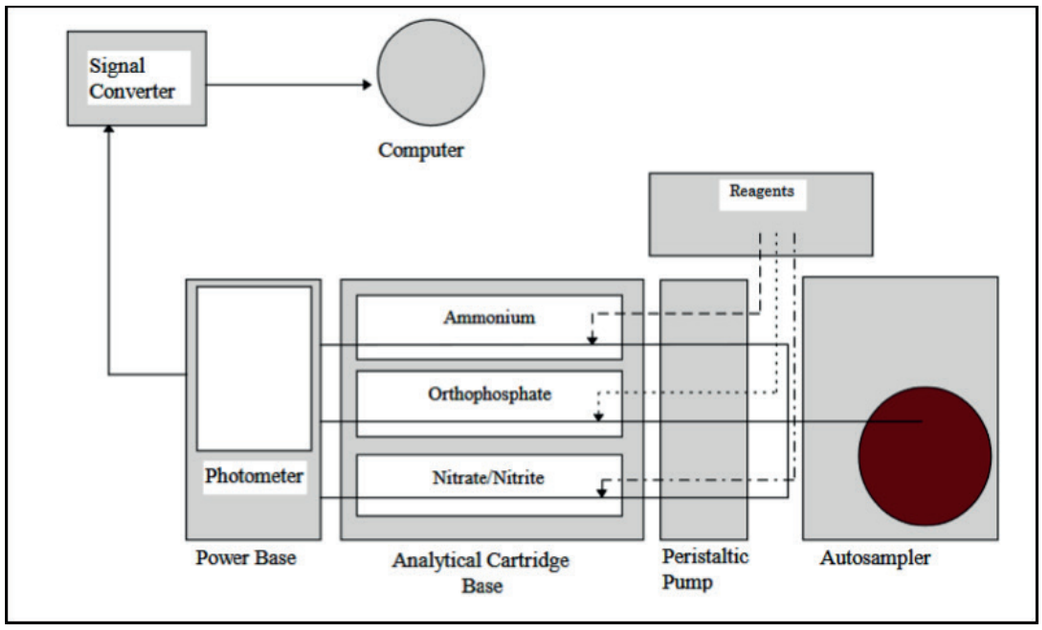

Figure 2.2.1.2 Operating scheme of the AutoAnalyzer Unit

Reagents

- Solution of potassium chloride (2 mol x L^{-1} KCl) (R0): dissolve 149 g of potassium chloride (KCl) in a 1000 mL graduated flask containing approximately 800 mL of H_2O. Bring to volume
- Buffer solution pH 5.2 (R1): dissolve 24 g of sodium citrate ($C_6H_5Na_3O_7$) and 33 g of sodium and potassium tartrate ($C_4H_4KNaO_6$) in H_2O in a 1000 mL graduated flask. Bring to volume
- Colorimetric reagent (R2): dissolve 80 g of sodium salicylate ($C_7H_5NaO_3$) and 25 g of sodium hydroxide (NaOH) in H_2O in a 1000 mL graduated flask. Bring to volume
- Sodium nitroprusside solution (R3): dissolve 1g of sodium nitroprusside dihydrate [Na_2Fe (CN) 5NO] x $2H_2O$ in a little water in a 1000 mL graduated flask. Bring to volume
- Sodium dichloroisocyanurate solution (R4): dissolve 2 g of sodium dichloroisocyanurate dihydrate ($C_3Cl_2N_3NaO_3$ x $2H_2O$ and 25 g of sodium hydroxide (NaOH) in H_2O in a 1000 mL graduated flask. Bring to volume
- Solution (200 mg x L^{-1}) of ammonia nitrogen (N-NH_4+) (R5): dissolve 0.9439 g of ammonia sulphate [$(NH_4)_2SO_4$] in H_2O in a 1000 mL graduated flask. Bring to volume. This solution can be stored for 1

month at a temperature of 4°C

- Standard working solutions of ammonia nitrogen (R6): prepare from R5 a series of standards containing from 0 to 1.6 mg x L^{-1} of $N\text{-}NH_4$+ in 100 mL calibrated flasks. Bring to volume with potassium chloride (KCl) solution (2 mol x L^{-1}). Standard work solutions must be prepared at the time of use

Materials and equipment

- AutoAnalyzer Unit consisting of sampler, manifold or analytical cartridge, proportioning pump, heating cell, colorimeter equipped with tubular flow cell and 630-660 nm filters, recorder (Fig. 2.2.1.1)
- Plastic containers that do not absorb or release ammonia or nitrite ions
- Rotary agitator (40 rpm x min^{-1}) or oscillating agitator (120-140 cycles x min^{-1})
- Common laboratory equipment

Procedure

a. Homogenise the soil sample, either manually or mechanically. The soil sample must be transferred to the laboratory in a refrigerator container. If the sample is analysed within three days from sampling, it can be stored at 4°C. Otherwise, to avoid possible loss in mineral nitrogen, it is necessary to freeze it at -20°C. Temperature and duration of the defrosting process must be controlled: the samples should at room temperature, if they are analysed within 4 hours after removal from the freezer. It is also possible to maintain the samples at 4°C, in which case the defrosting time must not exceed 48 hours.

b. Extraction (Bremner & Keeney, 1966): transfer 20 g of soil sample to a 500 mL plastic container. Add 200 mL of R0 solution kept at a temperature of 20°C (the ratio must be 1:10). Keep stirring for 1 hour at 20°C. Centrifuge approximately 60 mL of the suspension for 10 min at approximately 3000 rpm min^{-1}. Transfer the supernatant into an Erlenmeyer flask. The content of nitrate, nitrite and ammonia ions should be determined within 24 hours, if not, store the extracts for no more than one week at a temperature not exceeding 4°C. Prepare the blank by following the same operating procedures, omitting the soil sample.

c. Moisture determination: weigh 20 g of the soil sample, set it into a preheated oven at 105°C for at least 16 hours. After cooling in the desiccator, weigh and calculate the moisture content in g x kg^{-1}.

d. Before starting the analysis, solutions (R1-R2-R3-R4) must be injected into the tubular flow cell until the absorbance value at λ = 660 nm becomes constant.

e. Make the calibration curve using the standard working solutions R6 (from 0 to 1.6 mg x L^{-1} of $N\text{-}NH_4^+$).

f. Perform the colorimetric analysis, according to the scheme shown in Figure 2.2.1.1, of the soil extracts in the solution of potassium chloride R0. Check the calibration every 10-20 samples, using the standard working solutions R6. If necessary, make a new calibration curve.

Calculations

The result is generally expressed as the ammonia nitrogen content ($N\text{-}NH_4^+$), expressed in mg x kg^{-1}:

$$C = \frac{(A - B)*D*V}{m} \qquad \text{(Eq. 2.2.1.1)}$$

where

C is the soil ammonia nitrogen content ($N\text{-}NH_4^+$), expressed in mg x kg^{-1};
A is the ammonia nitrogen content of the soil extracts, expressed in mg x L^{-1};
B is the ammonia nitrogen content of the blank sample, expressed in mg x L^{-1};
D is the dilution factor (D = 1, if no dilutions are made)
V is the extracts volume, expressed in millilitres mL
m is the soil mass, expressed in grams g

Remarks

- As reported, the soil ammonia is present in a dynamic equilibrium with nitrate. Thus, it is not useful to refer to a given range of soil $N\text{-}NH_4^+$, since it is affected by pedo-climatic and environmental conditions (temperature and soil moisture), fertilisation mode (mineral, organic), crop phenological phase, etc.
- The extraction temperature is a parameter that must be strictly reported in the analysis report (the amount of extractable ammonia nitrogen is influenced by the temperature). Centrifugation is preferred to filtration given that most paper filters can contain or absorb ammonia ions.
- The reaction is pH-dependent, therefore it is advisable to keep the alkaline reagents in plastic containers, with hermetic seals, to avoid the absorption of atmospheric CO_2.
- The presence of amino acids and proteins in solution can inhibit the reaction because these molecules react with sodium dichloroisocyanurate, by consequently decreasing the concentration of the hypochlorite in solution. Other N organic compounds can also react directly with hypochlorite.
- Copper (Cu) and mercury (Hg) can cause reaction inhibition, but the buffer solution limits their interference. Sulphur (S), selenium (Se) and halogens (X^-), in particular bromine (Br^-), can also interfe.

References

Bremner, J.M., Keeney, D.R., 1966. Determination and Isotope-Ratio Analysis of Different Forms of Nitrogen in Soils: 3. Exchangeable Ammonia, Nitrate, and Nitrite by Extraction-Distillation Methods 1. Soil Sci. Soc. Am. J., 30, 5, 577–582.

Davidson, J., Mathieson, J., Boyne, A.W., 1970. The use of automation Kjeldahl method, in determining nitrogen with final calculations by computer. Analyst, 95, 181–193.

Keeney, D.R., Nelson, D.W., 1982. Nitrogen-Inorganic forms. In: Methods of soil analysis (A.L. Page et al. Ed.). Part 2. 2nd edition. Agron. Monogr., 9. ASA and SSSA, Madison, WI. 643–698.

Mulvaney, R.L., 1996. Nitrogen – Inorganic Forms. In: Methods of Soil Analysis. Part 3. Chemical Methods (D. L. Sparks et al. Eds.). SSSA and ASA, Madison, WI.

Nelson, D.W., 1983. Determination of ammonia in KCl extracts of soils by the salicylate method. Commun. Soil Sci. Plant Anal., 14, 1051–1062.

Italian Official Methods for Soil Analysis, Official Italian Gazette of 21/10/1999, n. 248.

Rhine, E.D., Mulvaney, R.L., Pratt, E.J., Sims, G.K., 1998. Improving the Berthelot reaction for determining ammonia in soil extracts and water. Soil Sci. Soc. Am. J., 62, 2, 473–480.

Searle, P.L., 1984. The Berthelot or indophenol reaction and its use in the analytical chemistry of nitrogen. Analyst, 109, 549–568.

Seely, J.H., Petitelere, J.C., Benoiton, L., 1967. Interference by protein and amines in the determination of ammonia by the isocyanurate method. Clin. Chim. Acta, 18, 85–86.

2.2.2 NO_3- and NO_2-Nitrogen

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Importance and applications

Soil nitrate (NO_3^-) is an indicator of chemical soil fertility, being promptly utilised by plants (Keeney & Nelson, 1982). Due to potential N leaching in some specific pedo-climatic conditions, the use of high mineral N inputs or non-stabilised animal manure could determine an excess of NO_3^- in the soil circulating solution and, thus, consequent water pollution. Moreover, soil nitrite (NO_2^-) is seldom present: its determination is normally unwarranted except in neutral to alkaline soils receiving NH_4^- or NH_4^- -producing fertilisers. When accumulated, or transformed into NO and NO_2, by interaction with other soil constituents, it could cause tropospheric ozone formation, acid rain, the greenhouse effect and the destruction of the stratospheric ozone (Van Cleemput & Samater, 1996; Su et al., 2011).
Several methods are available for the determination of nitrate (NO_3^-) and nitrite (NO_2^-) in soil extracts, such as key analysis on the assessment of soil fertility. Among them, colorimetric methodologies are simple and sensitive, being widely employed in the design of automated analyser systems (continuous flow analysis) (Keeney & Nelson, 1982; Mulvaney, 1996; Rhine et al., 1998).

Principle

The use of colorimetric methodologies for quantification of NO_2^- as NO_3^- and N-NO_2^- + NO_3^- by the reaction of Griess-Ilosvay (Dorich & Nelson, 1984; Keeney & Nelson, 1982; Nelson, 1983) showed the best performance by reducing preliminary the NO_2^- to NO_3^- (Mulvaney, 1996; Shinn 1941). It is recommended to use potassium chloride (KCl) as the extraction solution, which is already widely used in analytical laboratories because of its high determination coefficient (Fig. 2.2.2.1).

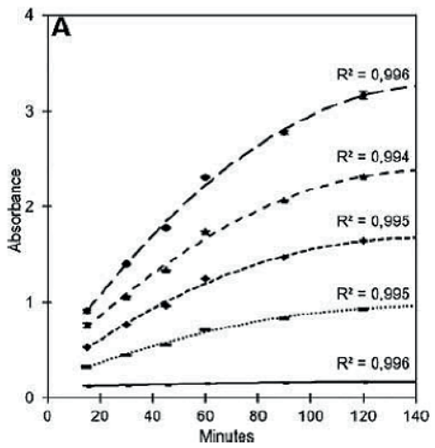

Figure 2.2.2.1 Tendency of N-NO_3^- readings for standard samples (quadratic model) in concentrations of 0.0, 2.5, 5.0, 7.5, and 10.0 mg L^{-1} for different reaction times (15, 30, 45, 60, 90, and 120 min) in 2 mol L^{-1} KCl. Symbols represent mean values, while vertical bars represent the standard error of the mean (n = 18) (after Sattolo et al., 2016)

The soil is treated with a solution of potassium chloride (2 mol x L^{-1}), then the extract is analysed by continuous flow colorimetry: the nitrate and nitrite content is determined by the Griess-Ilosvay reaction, in which nitrous and nitric ions form, by di-azotation with sulfonyl amide and N-(1-naphthyl)-ethylenediamine dihydrochloride, a reddish-purple compound whose intensity is measured at λ = 540 nm.

- Solution of N-(1-naphthyl)-ethylenediamine dihydrochloride (R6): dissolve 1 g of N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{12}H_{14}N_2$ x 2HCl) in H_2O, in a volumetric flask of 1000 mL, bring to volume with H_2O. This solution can be stored in the refrigerator, in a dark glass bottle, for no more than one week.
- Solution (100 µg x mL^{-1}) of nitric nitrogen ($N\text{-}NO_3^-$) (R7): dissolve 0.7218 g of potassium nitrate (KNO_3) in H_2O, in a volumetric flask of 1000 mL, bring to volume with H_2O. Keep the solution in a refrigerator.
- Standard working solutions of nitric nitrogen (R8): take 0, 1, 10, 50 and 100 mL of the solution R7 and transfer to five volumetric flasks of 1000 mL, bring to volume with the solution R0. In each of the four solutions, the nitric nitrogen concentration ($N\text{-}NO_3^-$) is, respectively, 0, 0.1, 1, 5 and 10 µg x mL^{-1}. Standard solutions must be prepared for each series of determinations.

Materials and equipment

a. AutoAnalyzer Unit consisting of sampler, manifold or analytical cartridge, proportioning pump, heating cell, colorimeter equipped with tubular flow cell and 630-660 nm filters, recorder (Fig. 2.2.2.2)
b. Plastic containers that do not absorb or release ammonium or nitrite ions
c. Rotary agitator (40 rpm x min^{-1}) or oscillating agitator (120-140 cycles x min^{-1})
d. Common laboratory equipment

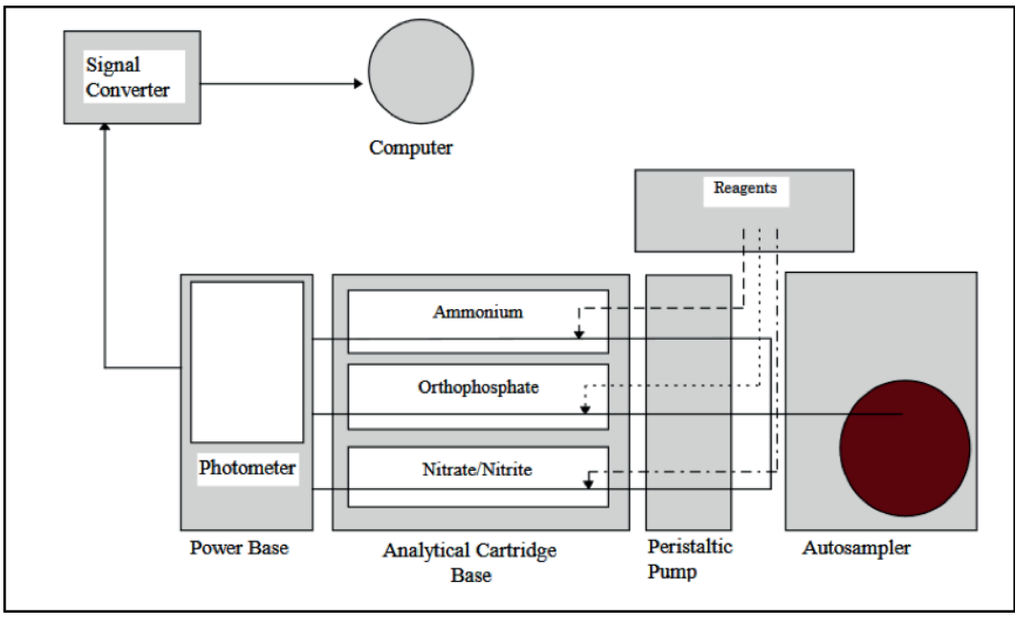

Figure 2.2.2.2 Operating scheme of the AutoAnalyzer Unit

Reagents

- Solution of potassium chloride (2 mol x L^{-1} KCl) (R1): dissolve 149 g of potassium chloride (KCl) in a 1000 mL graduated flask containing approximately 800 mL of H_2O. Bring to volume
- Solution (1: 1 v / v) of hydrochloric acid: add 500 mL of hydrochloric acid (HCl) 37% (R2) to a 1000 mL graduated flask containing approximately 450 mL of H_2O, mix and, after cooling, bring to volume with H_2O
- Ammonium hydroxide solution (100 mL x L^{-1}) (R3): transfer 100 mL of ammonium hydroxide solution (NH_4OH) 30% to a 1000 mL graduated flask containing about 600 mL of H_2O, bring to volume with H_2O
- Buffer solution (R4): dissolve 53.5 g of ammonium chloride (NH_4Cl) in a 1000 mL graduated flask containing about 600 mL of H_2O, bring the pH of the solution to 8.5 by progressively adding R4, bring to volume with H_2O
- Solution of sulfonyl amide (R5): dissolve 10 g of sulfonyl amide ($C_6H_8N_2O_2S$) in a 1000 mL graduated flask containing approximately 300 mL of H_2O and 26 mL of hydrochloric acid (HCl) 37%, bring to volume with H_2O. Keep the solution in a refrigerator

Procedure

a. Homogenise the soil sample, either manually or mechanically. The soil sample must be transferred to the laboratory in a refrigerator container. If the sample is analysed within 3 days from sampling, it can be stored at 4°C. Otherwise, to avoid possible losses in mineral nitrogen, it is necessary to freeze it at -20°C. The temperature and duration of the defrosting process must be controlled: the samples should be thawed at room temperature if they are analysed within 4 hours after removal from the freezer. It is also possible to thaw the samples at 4°C, in which case the defrosting time must not exceed 48 hours

b. Extraction (Bremner & Keeney, 1966): transfer 20 g of soil sample to a 500 mL plastic container. Add 200 mL of R1 solution kept at a temperature of 20°C (the ratio must be 1:10). Keep stirring for 1 hour at 20°C. Centrifuge approximately 60 mL of the suspension for 10 min at approximately 3000 rpm min^{-1}. Transfer the supernatant to an Erlenmeyer flask. The content of nitrate, nitrite and ammonium ions should be determined within 24 hours, if not, store the extracts for no more than one week at a temperature not exceeding 4°C. Prepare the blank by following the same operating procedures, omitting the soil sample

c. Moisture determination: weigh 20 g of the soil sample, set it into a preheated oven at 105°C for at least 16 hours. After cooling in the desiccator, weigh and calculate the moisture content in g x kg^{-1}

d. Before starting the analysis, solutions (R4-R5-R6) must be injected into the tubular flow cell until the absorbance value at λ = 540 nm becomes constant

e. Make the calibration curve using the standard working solutions R8 (from 0 to 1.6 mg x L^{-1} of $N\text{-}NH_4^+$)

f. Perform the colorimetric analysis, according to the scheme shown in 2.2.2.1, of the soil extracts in the solution of potassium chloride R1. Check the calibration every 10-20 samples, using the standard working solutions R8. If necessary, make a new calibration curve

Calculations

The result is generally expressed as the nitrate and nitrite nitrogen content ($N\text{-}NO_2^-$ + $N\text{-}NO_3^-$), expressed in mg x kg^{-1}:

$$C = \frac{(A - B)*D*V}{m} \qquad \text{(Eq. 2.2.1.1)}$$

where

C is the soil nitrate and nitrite nitrogen content ($N\text{-}NO_2^-$ + $N\text{-}NO_3^-$) [mg kg^{-1}],
A is the nitrate and nitrite nitrogen content of the soil extracts [mg L^{-1}],
B is the nitrate and nitrite nitrogen content of the blank sample [mg L^{-1}],
D is the dilution factor (D = 1 if no dilutions are made)
V is the extracts volume [mL]
m is the soil mass [g]

Remarks

- The soil mineral N content, as nitrate, nitrite and ammonia, represents the pool of nitrogen available to the crop. It is useful to refer to a given range of soil $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ related to land use or soil type, with these values being dynamic and strongly affected by pedo-climatic and environmental conditions (temperature and soil moisture), fertilisation mode (mineral, organic), crop phenological phase, etc.
- Interference may be due to the presence of coloured components in the sample, which can absorb at the wavelength used. Other interferences may be related to the presence in the sample of strong oxidants or reducing agents, at high concentrations of aromatic amines, copper (Cu), iodine (I) and humic acids.
- The Griess-Ilosvay method is very sensitive and specific and is not affected by cations and anions interference. The soil extracts in R1 may on occasions be coloured, but this occurrence does not interfere with the analysis, according to the method.
- Colour development is very rapid. At 25°C, the maximum colouring is achieved in 10 minutes and stable for a few hours.

References

Bremner, J.M., Keeney, D.R., 1966. Determination and Isotope-Ratio Analysis of Different Forms of Nitrogen in Soils: 3. Exchangeable Ammonium, Nitrate, and Nitrite by Extraction-Distillation Methods 1. Soil Sci. Soc. Am. J., 30, 5, 577–582.

Dorich, R.A., Nelson, D.W., 1984. Evaluations of manual cadmium reduction methods for determination of nitrate in potassium chloride extracts of soils. Soil Sci. Soc. Am. J., 48, 72–75.

Keeney, D.R., Nelson, D.W., 1982. Nitrogen-Inorganic forms. In: Methods of soil analysis (Page, A.L. et al. Ed.) Part 2. 2nd ed. Agron. Monogr. 9. ASA and SSSA, Madison, WI. 643–698.

Mulvaney, R.L., 1966. Nitrogen – Inorganic Forms. In: Methods of Soil Analysis. Part 3. Chemical Methods (Sparks, D.L. Ed.). SSSA Book Series no. 5. SSSA and ASA Inc. Madison, WI.

Italian Official Methods for Soil Analysis, Official Italian Gazette of 21/10/1999, no. 248.

Sattolo, T.M.S., Otto, R., Mariano, E., Kamogawa, M.Y., 2016. Adaptation and Validation of Colorimetric Methods in Determining Ammonium and Nitrate on Tropical Soils. Commun. Soil Sci. Plant Anal., 47, 22, 2547–2557.

Shinn, M.B., 1941. Colorimetric method for determination of nitrite. Ind. Eng. Chem. Res., 13, 33–35.

Van Cleemput O., Samate A.H., 1996. Nitrite in soils: accumulation and role in the formation of gaseous N compounds. Fertilizer Res., 45, 1, 81–89.

Su H., Cheng Y., Oswald R., Behrendt T., Trebs I., Meixner F.X., Andreae M.O., Cheng P., Zhang Y., Pöschl U., 2011. Soil Nitrite as a Source of Atmospheric HONO and OH Radicals. Science, 333, 6049, 1616–1618.

2.2.3 Available P content

Introduction

As essential element in the life cycle of plants, P constitutes 2 to 3% of the tillable soil profile, where it is strongly bound to calcium, aluminium, iron and other elements as a phosphate anion (PO_4^{3-}). Phosphorus is present in the soil in relatively low quantities, between 0.2 and 5 g Kg^{-1}. From the point of view of plant nutrition, the soil phosphates can be divided into three fractions in equilibrium:

phosphates present in the liquid phase;

phosphates in labile form;

phosphates in non-labile form (Fig. 2.2.3.1).

In the soil solution, P ranges from 0.01 to 0.2 mg / L^{-1}, and is not very mobile. Two different methods for the analysis of soil available P content will be illustrated in the following paragraphs: the Olsen method (Section 2.2.3.1), and the Mehlich 3 method (Section 2.2.3.2).

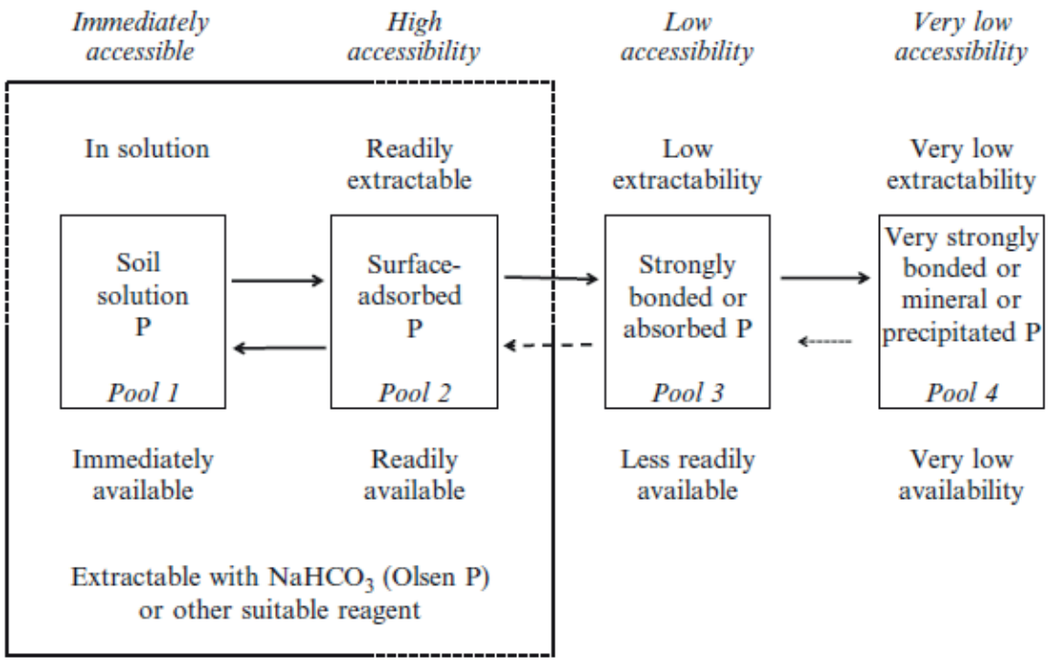

Figure 2.2.3.1 Forms of inorganic P in soils in terms of accessibility, extractability, and plant availability, in relation to the extraction range of the Olsen method (from Johnston et al., 2014)

2.2.3.1 Olsen method

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Importance and applications

The analysis of assimilable P content is critically important to the discussion about the retention of plant-available P in soil. To evaluate the P available for the crops in calcareous / neutral soils, Kamprath and Watson (1980) proposed the extraction with diluted solutions of weak acids and buffered alkaline solutions (Olsen et al., 1954; Soltanpour & Schwab, 1977). The Olsen method is safely suitable for a wide range of soil types and pH values. In acid soils containing Al and Fe phosphate, the P concentration in the solution increases as the pH rises. Precipitation reactions in acid and calcareous soils are reduced to a minimum because the concentrations of Al, Ca and Fe remain at a low level in this extractant.
In long-term experiments (> 40 years at Rothamsted, Woburn and Saxmundham), a linear relationship was demonstrated between the increase in Olsen P and the increase in total soil P (when both are expressed in kg P ha^{-1}) (Fig. 2.2.3.2) (Johnston et al., 2014).

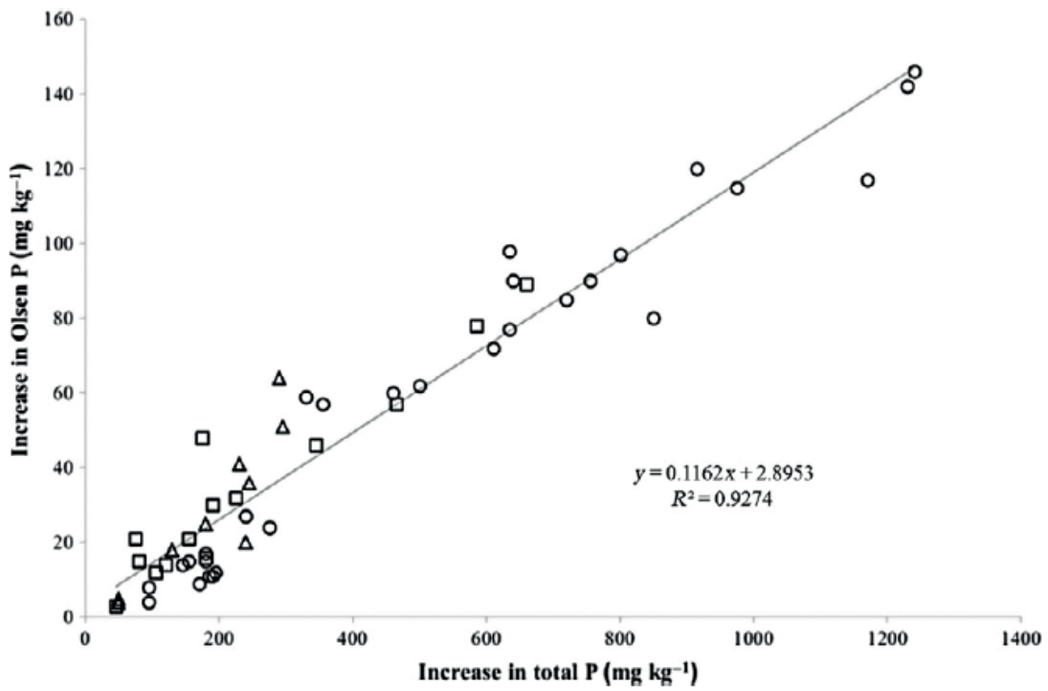

Figure 2.2.3.2 Relationship between the Olsen P and the total P in soils of long-term experiments (>40 years) where P has been applied as both fertiliser and organic manure: silty clay loam (o), sandy loam (□), and sandy clay loam (Δ) (from Johnston et al., 2014).

A relationship between crop yield and Olsen P can be profitably described by an asymptotic regression

equation (Mitscherlich type) and the asymptotic (maximum) yield determined. From this relationship, the critical Olsen P associated with the yield at an arbitrary proportion of the asymptotic yield can be calculated from the parameters of the fitted curve using an appropriate equation.

Principle

According to the method, the soil is extracted with 0.5 mol solution of sodium bicarbonate at pH 8.5. In calcareous, alkaline or neutral soils containing calcium phosphate, this extracting solution decreases the concentration of Ca in solution by precipitating Ca as $CaCO_3$, and the result is an increase of the P concentration in the solution. The concentration of phosphorus in the solutions obtained is then generally determined by the colorimetric method.

Reagents

- Solution of sulphuric acid (2.5 mol x L^{-1}) (R1): carefully add 140 mL of sulphuric acid (H_2SO_4) [96%] to a 1000 mL graduated flask containing approximately 5.00 mL of H_2O. Stir and, after cooling, dilute to volume with H_2O.
- Sodium hydroxide solution (1.0 mol x L^{-1}) (R2): dissolve 40 g of sodium hydroxide (NaOH) in H_2O in a 1000 mL graduated flask. Stir and, after cooling, dilute to volume with H_2O.
- Sodium bicarbonate solution (0.5 mol x L^{-1}) (R3): dissolve 42 g of sodium bicarbonate ($NaHCO_3$) in a beaker containing about 900 mL of H_2O. Bring the pH to 8.5 by adding the R2 solution drop by drop. Transfer to a 1000 mL graduated flask and dilute to volume with H_2O. To avoid direct contact of the solution with atmospheric air, add a layer of mineral oil.
- Activated carbon: check the purity of this reagent by performing an extraction with R3. In the presence of phosphorus, wash several times with R3 up to levels of P that are not detectable by spectrophotometry.
- P-nitrophenol solution (0.25%) (R4): in a 100 mL volumetric flask, dissolve 0.25 g of p-nitrophenol ($NO_2C_6H_4OH$) in H_2O.
- Ammonium molybdate solution (40 g x L^{-1}) (R5): dissolve 40 g of ammonium molybdate [$(NH_4)\cdot 6Mo_7O_{24}$ x $4H_2O$] in H_2O in a 1000 mL graduated flask. Dilute to volume with H_2O and store in a dark glass container.
- Potassium tartrate antimony solution (1 mg of Sb x mL^{-1}) (R6): dissolve 0.2728 g of potassium antimony tartrate [(K (SbO) x $C_4H_4O_6$ x ½ H_2O] in H_2O in a 100 mL graduated flask. Bring to volume with H_2O.
- Solution of ascorbic acid (0.1 moles x L^{-1}) (R7): dissolve 1.76 g of ascorbic acid ($C_6H_4O_6$) in H_2O in a 100 mL graduated flask. Bring to volume with H_2O. Prepare the solution at the time of use.
- Sulphomolybdic Reagent (R8): mix, at the time of use, 50 mL of the R1 solution, 15 mL of the R5 solution, 30 mL of the R7 solution and 5 mL of the R6 solution.
- Standard P solution (1000 mg x V) (R9): transfer 4,3938 g of potassium dihydrogen phosphate (KH_2PO_4) dried in an oven at 40°C to a 1000 mL graduated flask containing approximately 500 mL of H_2O. After dissolving the salt, dilute to volume with H_2O.
- Diluted standard P solution (R10): transfer 10 mL of R9 into a 1000 mL graduated flask. Bring to volume with H_2O. In this solution the phosphorus concentration is 10 mg x L^{-1}.

Materials and equipment

- pH meter with temperature compensator
- Oscillating agitator at 120-140 cycles x minute^{-1}
- 0.45 µm membrane filters
- Spectrophotometer
- Common laboratory equipment

Procedure

a. *Extraction*: transfer 2 g of the sample to a conical flask or a 125 mL plastic container. Add 0.5 g of activated carbon and 40 mL (V1) of the R3 solution. Stir for 30 minutes and filter several times with Whatman n° 42. If necessary, use the 0.45 µm membrane filter. Prepare the blank test by following the same operating procedures, but omitting the soil sample

b. *Colorimetric determinatio*n: transfer an aliquot of the clear, extracted solution containing from 2 to 40 µg of P (V2) to a 50 mL graduated flask. Add 5 drops of the R4 solution and, drop by drop, a quantity of the R1 solution to turn the colour of the indicator to yellow. Dilute with H_2O to approximately 25 mL and add 8 mL of the R8 reagent. Bring to volume with H_2O. After 10 minutes, read the extinction value 882 nm on the spectrophotometer against a blank containing all the reagents excluding the phosphorus solution

c. *Calibration curve*: transfer 0, 5, 10, 15, 20 and 25 mL of the R10 standard solution to six graduated flasks (50 mL volume). Dilute with H2O to approximately 25 mL and add 8 mL of the R8 reagent. Bring to volume with H_2O. In each of the six solutions, the phosphorus concentration is, respectively: 0, 1, 2, 3, 4 and 5 mg x L^{-1}. After 10 minutes, read the extinction value 882 nm on the spectrophotometer against a blank containing all the reagents excluding the phosphorus solution.

Calculations

The result is generally expressed as phosphorus content, expressed in mg x kg^{-1}:

$$C = (A - B)\frac{V_1}{V_2}\frac{50}{m} \qquad \text{(Eq. 2.2.3.1.2)}$$

where

C is the soil extractable P content [mg kg^{-1}],

A is the P concentration in the sample solution [mg L^{-1}],

B is the P concentration in the blank sample solution [mg L^{-1}],

$\mathbf{V}_1$ is the volume of the extract [40 mL],

$\mathbf{V}_2$ is the volume of the sample solution used for colorimetric determination,

m is the soil mass [g]

Table 2.2.3.1 Range of values for P-Olsen data clustered according to major FAO soil groups (FAO, 1988; after Batjes, 2010)

P-Olsen (mg kg^{-1})	pH (in water)	TOC (g kg^{-1})	Ntot (g kg^{-1})	Soil type (0-20 cm)
7.2474	6.9183	2.7425	0.4114	Arenosols
19.528	8.0784	6.1143	1.2413	Calcisols
12.078	7.9485	7.5033	1.9959	Cambisols
13.061	7.9306	7.7798	1.3277	Fluvisols
10.638	7.0000	15.858	0.4100	Gleysols
13.381	7.0114	5.0595	1.6009	Luvisols
16.739	7.5408	4.3386	0.8795	Regosols
8.5943	7.2343	6.5474	0.9522	Vertisols

Remarks

- All the products used must be free of silicon, taking into account the reactivity of this element with the sulphomolybdic reagent. For the same reason, it is preferable to use distilled water since deionised contain silica.
- The presence of sodium bicarbonate, carbonate and hydroxyl ions in the solution lowers the activity of Ca^{2+} and Al^{3+} with a consequent increase in the solubility of phosphorus (P).
- In calcareous soils, the increased solubility of calcium phosphate derives from the decrease in the calcium concentration due to the high presence of carbonate ions and the consequent precipitation of $CaCO_3$.
- At high pH, the increase in negative charges and / or the decrease of the adsorption sites on the surfaces of aluminium and iron oxides can lead to the desorption of the fixed phosphorus.
- In acid or neutral soils, the solubility of aluminium and iron phosphates is increased by the increase in the concentration of hydroxyl ions which induces a decrease in the concentration of Al^{3+} with the formation of aluminate ions, and of Fe^{3+}, with precipitation of oxides.

References

Anderson, J.M., Ingram, J.S.I., (Eds.) (1989). TSBF: A Handbook of Methods of Analysis. CAB International, 38–39.

Batjes, N.H., 2010. Inventory of P-Olsen data in the ISRIC-WISE soil database for use with QUEFTS (No. 2010/06). ISRIC World Soil Information

Bray, R.H., Kurtz, L.T., 1945. Determination of total, organic, and available forms of phosphorus in soils. Soil Sci., 59, 1, 39–46.

FAO, 1988. FAO-UNESCO Soil Map of the World. Revised legend, with corrections and updates. World Soil Resources Report 60, FAO, Rome. Reprinted with updates as Technical Paper 20 by ISRIC, Wageningen, 1997

FAO/IIASA/ISRIC/ISSCAS/JRC, 2009. Harmonized World Soil Database (version 1.1), Food and Agriculture Organization of the United Nations, International Institute for Applied Systems Analysis, ISRIC - World Soil Information, Institute of Soil Science - Chinese Academy of Sciences, Joint Research Centre of the European Commission, Laxenburg

Johnston, A.E., Poulton, P.R., Fixen, P.E., Curtin, D., 2014. Phosphorus: its efficient use in agriculture. Advance. Agron., 123, 177–228.

Kamprath, E.J., Watson, M.E., 1980. Conventional soil and tissue tests for assessing the phosphorus status of soils. In: The role of phosphorus in agriculture. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America. 433–469.

Olsen, S.R., 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. United States Department of Agriculture, Washington, DC

Smit, A.L., Bindraban, P.S., Schröder, J.J., Conijn, J.G., van der Meer, H.G., 2009. Phosphorus in agriculture: global resources, trends and developments: report to the Steering Committee Technology Assessment of the Ministery of Agriculture, Nature and Food Quality, The Netherlands Report. Plant Research International 282. Wageningen

Soltanpour, P.A., Schwab, A.P., 1977. A new soil test for simultaneous extraction of macro-and micro-nutrients in alkaline soils. Commun. Soil Sci. Plant Anal., 8, 3, 195–207.

Thomas, G.W., Peaslee, D.E., 1973. Testing soils for phosphorus. In: Soil testing and plant analysis (Walsh, L.M and Beaton, J.D. Eds.). Revised edition. Soil Sci. Soc. America, Madison, WI. 115–132.

USDA – National Resources Conservation Service, Washington, DC. 2004. Soil Survey Laboratory Manual Soil Survey Investigations Report 42 (ver. 4.0). USDA–National Resources Conservation Service, Washington

Watanabe, F.S., Olsen, S.R., 1965. Test of an ascorbic acid method for determining phosphorus in water and sodium bicarbonate extracts from soils. Soil Sci. Am. Proc., 29, 677–678.

2.2.3.2 Mehlich 3 method and ICP-AES

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Importance and applications

The Mehlich 3 index of phosphorus availability (M3-P) (Mehlich, 1984) measures the readily plant-available P of the soil solution. The M3 method is widely used in North America, Europe, and Australia since it can be applied to determine the nutrient status of soils ranging in reaction from acid to basic, and particularly for assessing available P and K (Jones, 1998; Zbiral & Nemec, 2000a,b; Cox, 2001; Bolland et al., 2003). Many studies in the literature provide a strong correlation between M3-P and crop ecophysiological responses, i.e. plant uptake and plant yield, for a wide range of soils (Tran & Giroux, 1987; Ziadi et al., 2001; Mallarino, 2003). The method has been the only soil test validated through inter-laboratory studies for the extraction of plant-available P, which is usually less than 0.01 – 0.02% of the total P, and has been used as a reference method for testing soils for extractable P (Alvey, 2013 and references therein; Zhang et al., 2009). A comparison of the M3 method with the many other methods developed to determine the soil P content is given in Fig. 2.2.3.3.

Name of Test	Extractant	Reference
AB-DPTA	1M NH_4HCO_3 + 0.005 M DPTA, pH 5	59
Bray I	0.025 N HCl + 0.03 N NH_4F	6
Bray II	0.1 N HCL + 0.03 N NH_4F	6
Citric acid	1% Citric acid	3
EDTA	0.02 M Na_2-EDTA	61
Mehlich 1	0.05 M HCl + 0.0125 M H_2SO_4	224
Mehlich 3	0.015 M NH_4F + 0.2 M CH_3COOH + 0.25 M NH_4NO_3 + 0.013 M HNO_3	56
Morgan[a]	0.54 N HOAc + 0.7 N NaOAc, pH4	5
Olsen	0.5 M $NaHCO_3$, pH 8.5	58
Truog	0.001 M H_2SO_4 + $(NH4)_2SO_4$, pH 3	4
Water[b]	Water	225

[a]A modification of the Morgan by Wolf to include 0.18 g/L DPTA gives better correlations for micronutrients.
[b]From: C.A. Sanchez. Soil Testing and Fertilizer Recommendations for Crop Production on Organic Soils in Florida. University of Florida Agricultural Experiment Station Bulletin 876, Gainesville, 1990.

Chemical	Form of Phosphorus Extracted
Acid (H^+)	Solubilizes all chemical P in the following order Ca-P>Al-P>Fe-P
Bases (OH^-)	Solubilizes Fe-P and Al-P in respective order. Also results in release of some organic P
Fluoride ion	Forms complexes with Al thus releasing Al-P. Also precipitates Ca as CaF_2 and thus will extract more Ca-P as $CaHPO_4$. No effect on basic Ca-P and Fe-P
Bicarbonate ions	Precipitate Ca as $CaCO_3$ thus increasing solubility of Ca-P. Also remove Al-bound P
Acetate ions	Form weak complexes with polyvalent metal ions. Possibly prevents readsorption of P removed by other ions
Sulfate ions	Appear to reduce readsorption of P replaced by H ions

[a]Adapted from G.W. Thomas and D.E. Peaslee, in *Soil Testing and Plant Analysis*. Madison, WI: Soil Sci. Soc. Am. Inc., 1973 and E.J. Kamprath and M.E. Watson, in *The Role of Phosphorus In Agriculture*. American Society of Agronomy Inc. 677 South Segoe Road, Madison WI 53711, 1980.

Figure 2.2.3.3 Some historical and commonly used soil tests and extracting solutions for determining available phosphorus, and forms of phosphorus extracted (modified from Barker et al., 2015)

M3 correlates to Bray P1 (Bray & Kurtz, 1945) on acid soils (R2 = 0.966) and to Olsen (Olsen et al., 1954) on alkaline soils (R_2 = 0.918), and to P extracted by M2, strontium chloride–citric acid, and water (Fig. 2.2.3.4) (Mehlich, 1984; Simard et al., 1991; Mallarino, 1995; Sawyer et al., 1999; Zbiral & Nemec, 2002; Iatrou et al., 2014). The Olsen and M3 tests are generally well correlated across all soils, the relationship can be slightly affected by the inclusion of calcareous soils (the slope of lines and intercepts tends to be

similar for the different pH classes) (Mallarino, 1995). However, none of the three methods (Bray, Olsen and M3) correlate well for calcareous soils (Mallarino, 1995).

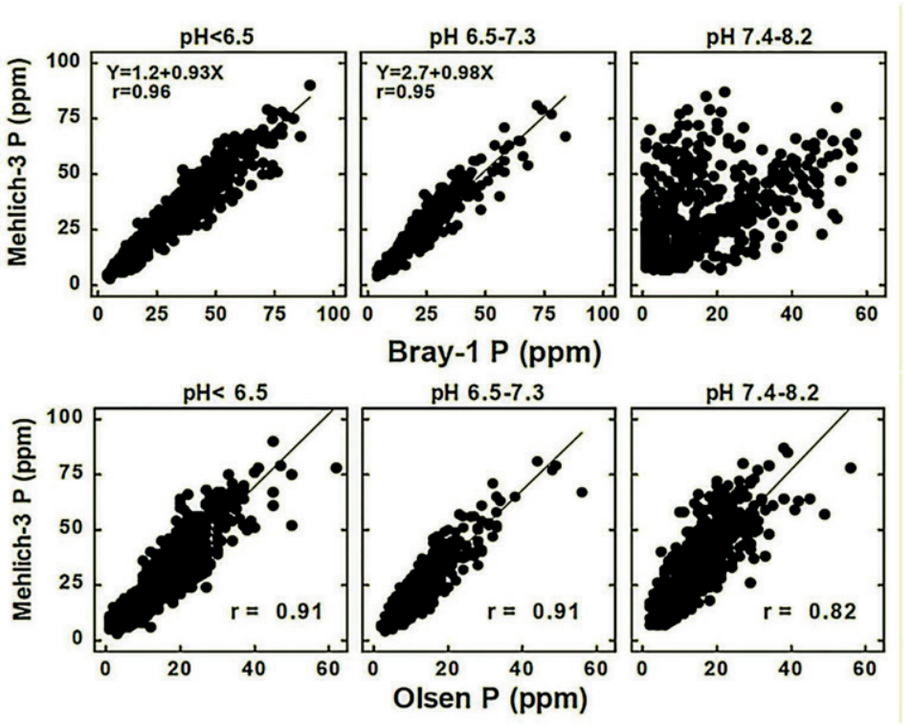

Figure 2.2.3.4 Correlations between amounts of P extracted by Mehlich 3, Olsen and Bray tests for 2925 sample soils across acid, neutral, and high pH. Bray was strongly influenced by soil pH and extracted less P than the M3 in many calcareous soils (data points with Bray values near zero but higher M3 values). Olsen and M3 are well correlated across all soils and the correlation was highly independent of soil pH. The Olsen, as expected, extracts less P than the other tests (modified from Sawyer & Mallarino, 1999)

In addition to P, the extraction with M3 solution showed significant correlations with different currently used methods for K, Ca, Mg, Na, Cu, Zn, Mn, B, Al, and Fe (for a more detailed review, see Ziadi et al., 1993), it is therefore being widely used as the 'universal extractant' to evaluate the soil macro- and micro-nutrient status (Zhang et al., 2009, Schroder et al., 2010). Unlike the Olsen method, M3 extracts can be analysed by inductively coupled plasma emission spectroscopy (ICP), reducing the analysis time and also providing the advantage of measuring the P content simultaneously with the other nutrients in the same soil extract (Sawyer & Mallarino, 1999; Iatrou et al., 2014). Compared with the original colorimetric determination of M3 extracts (ascorbic acid method, considered specific for the orthophosphate form of P), the ICP usually measures higher values of M3-P (Mallarino, 2003; Sikora et al., 2005; Iatrou et al., 2014), since the instrument reads all P forms in the sample (the orthophosphate P form and also other small amounts of inorganic and simple organic P forms). However, several studies showed highly significant relationships between M3 ICP and colorimetric M3 for both acidic and alkaline soils (Figure 2.2.3.5) (Mallarino, 2003; Sikora et al., 2005; Pittman et al., 2005; Iatrou et al., 2014 and references therein). The reading difference

between the colorimetric determination and the ICP analysis does not apply for the other nutrients that can be measured in M3 soil extracts (Mallarino, 2013).

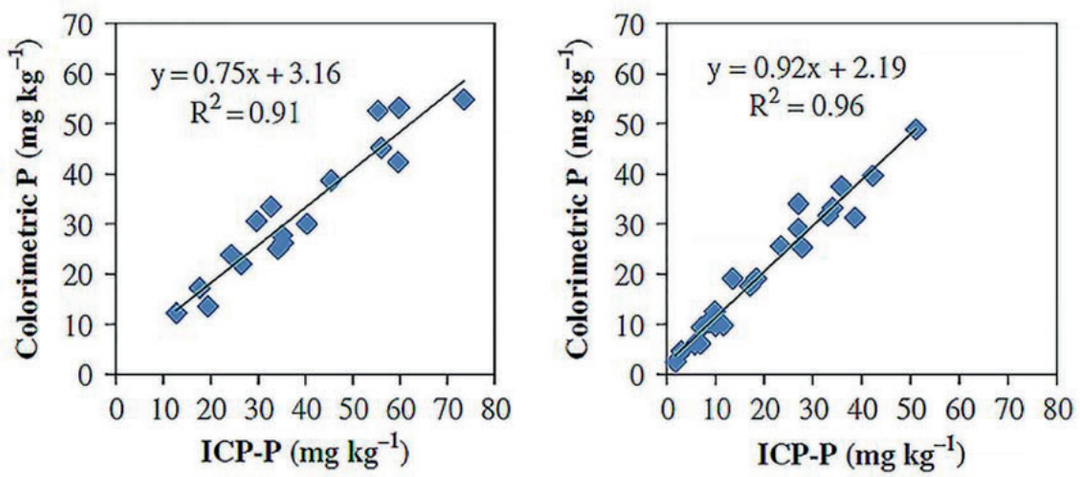

Figure 2.2.3.5 Relationships for extractable P content between M3 ICP test and colorimetric M3 test, for acidic soils (pH from 4.30 to 6.75, on the left) and for alkaline soils (pH from 7.12 to 7.98, on the right) (from Iatrou et al., 2014)

Principle

In the Mehlich 3 procedure, P extractable phosphorus is obtained by reaction with a dilute acid-fluoride-EDTA solution of pH 2.5. The extracting solution is composed of CH_3COOH (0.2 M), NH_4NO_3 (0.25 M), NH_4F (0.015 M), HNO_3 (0.013 M), and EDTA (ethylene diamine tetra-acetic acid 0.001 M). The phosphorus is solubilised under different mechanisms: nitric and acetic acids increase the solubility of Fe and Al phosphates and extracts Ca phosphates, fluoride increases the quantity of orthophosphate in solution by complexing Al cations, and the acetic acid keeps the solution buffered below pH 2.9 to prevent CaF_2 precipitate. The M3 extractant is less aggressive towards apatite and other calcium phosphates and is neutralised less by carbonate than the Bray extractant. The variety of M3 acids (i.e., acetic and nitric acids) makes it more versatile for soils having high concentrations of calcium. The M3 extracts are then analysed by inductively coupled plasma atomic emission spectrophotometer (ICP-AES) in radial mode, which allows multiple element determinations on the same soil extract. Data on the elemental concentration are reported as mg kg^{-1} soil.

The method is applicable for the determination of extractable K, Ca, Mg, Na and micronutrients, such as Mn, Fe, Cu and Zn. The exchangeable base cations K, Ca, Mg, and Na are removed by the action of ammonium nitrate and nitric acid, with a recovery nearly identical to the ammonium acetate method. The micronutrients are extracted by NH_4^+ and the chelating agent EDTA, their recovery is linearly related to DTPA and 0.1M HCl methods.

The repeatability and reproducibility of M3 for plant available macro- and micro-nutrients were thoroughly evaluated through inter-laboratory studies by Zhang et al., (2009) and Schroder et al., (2009).

Reagents

- Nitric acid 10% v/v: dilute 10 mL concentrated HNO_3 (HNO_3 68-70% ACS grade, CAS 7698-37-2) in 100 mL of deionised water
- Ammonium fluoride (NH_4F) CAS 12125-01-8
- Ethylene diamine tetra-acetic acid (EDTA) CAS 60-00-4
- Ammonium nitrate (NH_4NO_3) CAS 6484-52-2
- Glacial acetic acid (CH_3COOH) CAS 64-19-7
- M3 stock solution: (1.5 M NH_4F + 0.1 M EDTA): dissolve 55.56 g of ammonium fluoride (NH_4F) in 600 mL of deionised water in a 1 L volumetric flask. Add 29.23 g of EDTA to this mixture, dissolve, bring to 1 L volume using deionised water, mix thoroughly, and store in plastic bottle
- M3 extracting solution: dissolve 200.1 g of ammonium nitrate (NH_4NO_3) in a 10 L plastic carboy containing 8 L of deionised water, and add 100 mL of stock solution M3, 115 mL concentrated acetic acid (CH_3COOH), 82 mL of 10% v/v nitric acid, bring to 10 L with deionised water and mix thoroughly. The pH of the extracting solution should be 2.3±0.2. Store in a polyethylene container. Make a fresh solution weekly. Store in a refrigerator

Materials and equipment

- Oscillating shaker, 200 oscillations min^{-1}
- Centrifuge tubes, 50-mL, polyethylene or poly-propylene
- Centrifuge
- Filter paper, Whatman 42, 150 mm
- Pipettes, electronic digital, 1000 µL and 10 mL, with tips
- Inductively coupled plasma atomic emission spectrophotometer (ICP-AES)
- Common laboratory equipment

Procedure

a. Pre-rinse Whatman N42 filters: suspend filter funnels with filters on test-tube racks and fill filters with deionised water, let water drain completely from funnels and repeat using the M3 extracting solution.

b. Extraction: weigh 2.5 g of air-dry soil, 2 mm sieved, into a 50-mL centrifuge tube. Add 25.0 mL of the M3 extracting solution (soil:solution = 1:10). Shake immediately for 5 min at 200 oscillations min^{-1} at room temperature (20°C± 2°C). Centrifuge at 2000 rpm for 10 min or until the solution is free of soil mineral particles. Then decant, filter through pre-rinsed Whatman N42 filter paper until clear extracts are obtained, and collect the extracts into clean centrifuge tubes. A blank of M3 is prepared. Analyse by ICP-AES immediately after the extraction, or store at 4°C and analyse the samples within 72 h. Use the M3 extracting solution to dilute those samples with concentrations greater than the high standard.

c. Calibrations standards: these will vary depending on the expected soil P concentrations. From a 1,000 mg L^{-1} standard solution, prepare 1 L of the standard at the highest P concentrations using the M3 extracting solution for dilution. Then prepare 250 mL of the other calibration standards by diluting

the most concentrated one. In general, make up 0, 10, 25, 50 mg Kg^{-1} calibration standards. Depending on the soil analysed, standards between 0 and 10 mg Kg^{-1} may be required for samples with low P concentrations.

d. Analysis: calibrate the ICP instrument using the calibration standards and following the manufacturer's recommendations, then analyse the samples. If a sample has P concentrations above the highest standard, dilution should be made using the M3 extracting solution.

Calculations

The result is generally expressed as soil M3 extractable P content, expressed in mg kg^{-1} and given directly by the ICP instrument.

Calculation of mg kg^{-1} of P in the soil is as follows:

$$P = \frac{C}{F}\,\frac{V}{W}\,DF \qquad \text{(Eq. 2.2.3.2)}$$

where

P is the soil extractable P content [mg kg^{-1}],
C is the sample P content from the ICP read-out [mg L^{-1} or µg L^{-1} for the ICAP Trace],
F is the concentration unit factor (e.g. 1.00 for ICAP61E, 1000 for ICAP Trace),
V is the final volume of the (undiluted) sample solution [mL],
W is the weight of the sample [g],
DF is dilution factor (DF = 1.00 with no sample dilution).

If dilution of the sample is required, the DF is given by

$$DF = \frac{B + C}{C} \qquad \text{(Eq. 2.2.3.3)}$$

where

B is mL of the acid blank matrix used for dilution,
C is mL of the sample aliquot taken for dilution,
B + C is the volume of (diluted) sample solution.

Soil-test P interpretation classes available in the literature for the Bray, Olsen, colorimetric M3, and M3-ICP tests are reported in Table 2.2.3.2 (Mallarino et al., 2013).

Table 2.2.3.2 Interpretation of M3-P soil test values measured by the Bray-P1, Olsen, colorimetric M3 and M3 ICP tests for most Iowa soils and crops (15-20 cm soil sampling depth) (modified from Mallarino et al., 2013).

Relative Level	P (g kg^{-1}) M3 ICP	P (g kg^{-1}) Bray P1 or M3 P	P (g kg^{-1}) Olsen
Very low	0–15	0–8	0–5
Low	16–25	9–15	6–9
Optimum	26–35	16–20	10–13
High	36–45	21–30	14–18
Very high	46+	31+	19+

Remarks

- Air-dried soils may be stored several months without affecting the M3-P measurement
- During extraction, since the shaking time is so short it is advisable to do the extraction in batches of samples (maybe 10 at a time). The idea is to have all samples in contact with the extracting solution the same amount of time.
- Mehlich (1984) proposed to use 0.2 % $AlCl_3$ as a rinsing solution for all labware, including qualitative filter paper. Ziadi et al., (1993) suggested the use of M3 extracting solution as a rinsing solution for filter paper.
- Because of Zn contamination, Pyrex glassware cannot be used for extraction or storage of the M3 extractant and laboratory standards. Tap water is a major source of Cu and Zn contamination.
- The M3 extract is not stable for long periods of time; the extracting solution should not be used after 10 days.
- The ICP analysis of M3 extracts has been reported to quantify higher P amount than colorimetric methods (Mallarino, 2003; Pittman et al., 2005; Iatrou et al., 2014), therefore caution is needed when comparing results.

References

Alvey, R., 2013. Major Soil P Analytical Methods, Their Responses and Use of Mehlich 3 Multi- element Extractant for North American Crops. Technical Symposium of the Fertilizer. Association of Southern Africa. August 12, 2013, Pretoria, South Africa

Barker, A.V., Pilbeam, D.J. (Eds.), 2015. Handbook of plant nutrition. 2nd edition. CRC Press, Boca Raton, FL.

Bolland, M.D.A., Allen, D.G., Walton, K.S., 2003. Soil testing for phosphorus: comparing the Mehlich 3 and Colwell procedures for soils of south-western Australia. Aust. J. Soil Res., 41, 1185–1200.

Bray, R.H., Kurtz, L.T., 1945. Determination of total, organic, and available forms of phosphorus in soils. Soil Sci., 59, 1, 39–46.

Cox, M.S., 2001. The Lancaster soil test method as an alternative to the Mehlich 3 soil test method. Soil Sci., 166, 484–489.

Iatrou, M., Papadopoulos, A., Papadopoulos, F., Dichala, O., Psoma, P., Bountla, A., 2014. Determination of soil available phosphorus using the Olsen and Mehlich 3 methods for Greek soils having variable amounts of calcium carbonate. Commun. Soil Sci. Plant Anal., 45, 16, 2207–2214.

Jones, J.B. Jr., 1998. Soil test methods: past, present, and future use of soil extractants. Commun. Soil Sci. Plant Anal., 29, 1543–1552.

Mallarino, A., 1995. Comparison of Mehlich - 3, Olsen, and Bray - P1 Procedures for Phosphorus in Calcareous Soils. Proceedings of the 25th North Central Extension – Industry Soil Fertility Conference 11, 96–101.

Mallarino, A., Sawyer, J.E., 2000. Interpreting Mehlich-3 soil test results, SP126. Iowa State University, University Extension, Ames, IW. www.extension.iastate.edu

Mallarino, A.P., 2003. Field calibration for corn of the Mehlich-3 soil phosphorus test with colorimetric and inductively coupled plasma emission spectroscopy determination methods. Soil Sci. Soc. Am. J., 67, 1928–1934.

Mallarino, A.P., Sawyer, J.E., Barnhart, S.K., 2013. A general guide for crop nutrient and limestone recommendations in Iowa. Publ. Pm-1688 (rev. 2013). Iowa State University Extension, Ames, IW.

Mehlich, A., 1984. Mehlich-3 soil test extractant: a modification of Mehlich-2 extractant. Commun. Soil Sci. Plant Anal., 15, 1409–1416.

Olsen, S.R., 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. United States Department of Agriculture, Washington, DC.

Pittman, J., Zhang, H., Schroder, J.L., Payton, M.E., 2005. Differences of phosphorus in Mehlich 3 extracts determined by colorimetric and spectroscopic methods. Commun. Soil Sci. and Plant Anal. 36, 1641–1659.

Sawyer, J.E., Mallarino, A., 1999. Differentiating and Understanding the Mehlich 3, Bray, and Olsen Soil Phosphorus Tests. Agronomy Conference Proceedings and Presentations 12.
https://lib.dr.iastate.edu/agron_conf/12

Simard, R.R., Tran, T.S., Zizka, J., 1991. Strontium chloride–citric acid extraction evaluated as a soil-testing procedure for phosphorus. Soil Sci. Soc. Am. J., 55, 414–421.

Tran, T.S., Giroux, M., 1987. Disponibilité du phosphore dans les sols neutres et calcaires du Québec en relation avec les propriétés chimiques et physiques. Can. J. Soil Sci., 67, 1–16.

Schroder, J.L., Zhang, H., Richards, J.R., Payton, M.E., 2009. Interlaboratory validation of the Mehlich 3 method as a universal extractant for plant nutrients. J. AOAC International, 92, 4, 995–1008.

Zbiral, J., 2000a. Determination of phosphorus in calcareous soils by Mehlich 3, Mehlich 2, CAL, and Egner extractants. Commun. Soil Sci. Plant Anal., 31, 3037–3048.

Zbiral, J., 2000b. Analysis of Mehlich III soil extracts by ICP-AES. Rostlinna-Vyroba, 46, 4, 141–146.

Zbiral, J., Nemec, P., 2002. Comparison of Mehlich 2, Mehlich 3, CAL, Egner, Olsen, and 0:01M CaCl2 extractants for determination of phosphorus in soils. Commun. Soil Sci. Plant Anal., 33, 3405–3417.

Zhang, H., Kariuki, S., Schroder, J., Payton, M., Focht, C., 2009. Inter-laboratory validation of the Mehlich 3 for extraction of plant-available phosphorus. J. AOAC International, 92, 1, 91–102.

Zhang, H., Hardy, D.H., Mylavarapu, R., Wang, J.J., 2014. Mehlich-3. In: Soil Test Methods from the Southeastern United States. Southern Cooperative Series Bulletin No. 419. USDA-SERA-IEG-6.

Ziadi, N., Tran, T.S., Simard, R.R., 1993. Mehlich III-extractable elements. In: Soil sampling and methods of analysis (Carter, M.R. Ed.). Can. Soc. Soil Sci. Lewis Publ., Boca Raton, FL. 43–50.

Ziadi, N., Simard, R.R., Tran, T.S., Allard, A., 2001. Evaluation of soil-available phosphorus for grasses with Electro-Ultrafiltration technique and some chemical extractions. Can. J. Soil Sci., 81, 167–174.

2.2.4 Potential and effective cation exchange capacity

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Importance and applications

The cation exchange capacity (CEC) of soils is a chemical soil parameter of overall relevance. To a great extent, it determines the ability of soils to retain nutrients and toxic compounds as well as the ability to provide plants with nutrients. Thus, CEC is a key parameter of soil fertility as a major ecosystem service of soils. The CEC depends on the types and content of clay minerals and pedogenic oxides as well as soil organic matter and its quality. The contribution of functional groups to the CEC varies with pH so the potential CEC, measured at high pH >7, deviates more and more from the effective CEC (ECEC, measured at original soil pH) the more acidic a soil is. The potential CEC is furthermore needed to determine the soil base saturation. Numerous methods to determine the potential CEC and the ECEC can be found in the literature.

Two methods have been standardised (ISO 13536 and ISO 11260) to determine potential CEC and ECEC, and are reported here. These are ISO 1160 “Soil quality - Determination of effective cation exchange capacity and base saturation level using barium chloride solution” and ISO 13536 “Soil quality - Determination of the potential cation exchange capacity and exchangeable cations using barium chloride solution buffered at pH = 8.1” (ISO, 1995, 2018). The latter method is also needed to determine the base saturation.

Both guidelines were published several years ago, so not all details refer to the latest state of the art. Hence, some comments have been added to the text. These are suggestions for alternative realisation of the ISO guidelines.

Principle

The methods described here are largely based on the International Standard ISO 13536 (Section 2.2.4.1) and on the ISO 11260 (Section 2.2.4.2). The former is a modification of the method according to Mehlich (1938) and Mehlich (1942). The CEC of soil is determined using a barium chloride ($BaCl_2$) solution, buffered with triethanolamine at pH 8.1. With the latter method, the effective cation exchange capacity (ECEC; 2.2.4.2) of the soil is determined at the original pH and at a low total ionic strength (about 0.01 mol L^{-1}). Additionally, the content of exchangeable sodium (Na), potassium (K), calcium (Ca) and magnesium (Mg) in soil is determined.

In both methods, the soil’s exchange sites are saturated with Ba, either using a $BaCl_2$ solution buffered at pH 8.1 (Section 2.2.4.1) or unbuffered solution (Section 2.2.4.2). Subsequently, Ba is replaced and precipitated by the addition of magnesium sulphate ($MgSO_4$). The potential CEC (at pH 8.1) or the effective CEC (ECEC) is determined by analysis of excess Mg in the second, re-exchange solution. Acidified lanthanum solution is used to determine excess magnesium using flame atomic absorption spectrometry (FAAS) in an air/acetylene flame. Lanthanum inhibits the formation of incombustible compounds of magnesium with phosphate, aluminium etc.

Additionally, the sum of the exchangeable cations, i.e. Na, K, Ca and Mg can be quantified in the Ba exchange solution and represents the exchangeable bases (method in 2.2.4.1) or the relative contribution

of the bases to the ECEC is termed as the base saturation level (method in 2.2.4.2).
Both methods are applicable to all types of air-dried soil samples; pre-treatment according to ISO 11464 is recommended. A generalised flow chart for both methods is shown in Fig. 2.2.4.1.

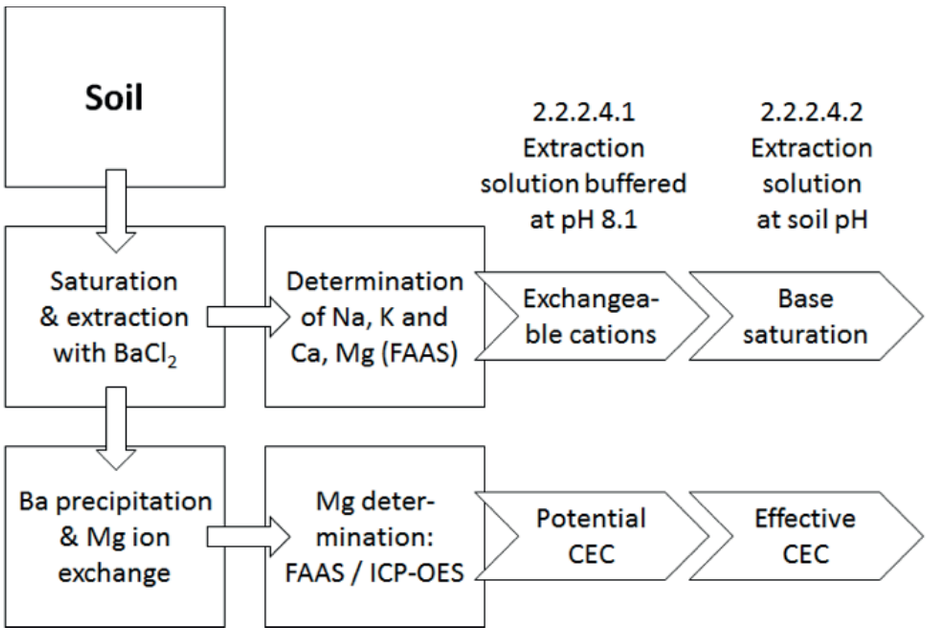

Figure 2.2.4.1 Flow chart of the two-step extraction scheme for the determination of the exchangeable cations and the potential cation exchange capacity and effective cation exchange capacity, respectively.

Soil samples are air-dried, sieved <2 mm and additionally microaggregates are carefully destroyed using a mortar and pestle. Soils should not contain higher amounts of soluble salts, calcite and/or gypsum, which is indicated by a higher electric conductivity. Determination of electric conductivity (EC), e.g. using ISO 11265, indicates possible salt affection of soils. Cations released from these compounds will distort the exchangeable cation content. In that case, soil samples must be treated in parallel using water. The exchangeable cation contents determined in this water extract are subtracted from the contents in the $BaCl_2$ extract.
For soils with high sulphate content it might be advisable to determine the CEC with the help of methods using ammonium solutions, e.g. ammonium acetate, instead of Ba.

Materials and equipment

- 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

2.2.4.1 Determination of potential CEC and exchangeable cations using a pH 8.1 buffered barium chloride solution

Reagents

Reagents of recognised analytical grade shall be used, including

- Deionised water (electric conductivity < 0.2 mS m^{-1} at 25 °C)
- Barium chloride ($BaCl_2$) solution; c($BaCl_2$) = 1 mol L^{-1}. Preparation: Dissolve 224 g of $BaCl_2 \times 2\ H_2O$ in 1000 mL of water (use volumetric flask)
- Hydrochloric acid, c(HCl) = 2 mol L^{-1}. Preparation: Dissolve 166 mL of concentrated HCl (ρ = 1.19 g/cm^3) in 1000 mL of water (use volumetric flask)
- Triethanolamine solution, pH 8.1. Preparation: Dissolve 90 mL triethanolamine in water in a total volume of 1000 mL. Adjust the pH to 8.1 using about 140 to 150 mL of hydrochloric acid. Fill up with water to 2 L
- Extraction solution. Add equal volume fractions of solutions 1 mol L^{-1} $BaCl_2$ and triethanolamine. Protect the solution during storage from contact with CO_2 and/or prepare fresh solution any time when needed
- Magnesium sulphate solution; c($MgSO_4$) = 0.020 mol L^{-1}. Preparation: Dissolve 4.930 g magnesium sulphate heptahydrate ($MgSO_4 \times 7\ H_2O$) in 1000 mL of water (use volumetric flask). Prepare a fresh solution. Magnesium sulphate can lose crystal water during storage. Protect from that by wrapping the flask in an additional PE bag and storing the chemical in a refrigerator
- Hydrochloric acid, c(HCl) = 12 mol L^{-1} (ρ = 1.19 g cm^{-3})
- Magnesium standard solution; c($MgSO_4$) = 0.0010 mol L^{-1}. Preparation: Add 50 mL of magnesium sulphate solution in a 1000 mL volumetric flask and fill up with water to 1 L. See Remarks
- Acidified lanthanum solution: c(La) = 10 g L^{-1}. Preparation: Add 15.6 g lanthanum nitrate hexahydrate [$La(NO_3)_3 \times 6\ H_2O$] in a 500 mL volumetric flask, add 42 mL hydrochloric acid, and fill up with water to 500 mL
- Sodium and potassium stock solution: c(Na) = 400 mg L^{-1}, c(K) = 1000 mg L^{-1}. 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 Na and K in 250 mL volumetric flask and fill up with water to the mark. See Remarks
- Hydrochloric acid, c(HCl) = 1 mol/L^{-1}. Add 83 mL of concentrated HCl (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) = 100 mg L^{-1}. Dissolve 1.0168 g sodium chloride and 0.836 g magnesium chloride hexahydrate ($MgCl_2 \times 6\ H_2O$) in water. Transfer to 1000 mL volumetric flask and fill up to the mark with water. See Remarks

Procedure

Extraction:

a. Weigh air-dried soil in a tightly locking polyethylene centrifuge tube (ca. 50 mL). Use 2.50 g of clayey and/or humus rich soil and 5.00 g of sandy and/or humus poor soil. Note down the total weight of soil plus centrifuge tube (m_1)
b. Add 30 mL of extraction solution and shake for 1 h. Centrifuge for 10 min at 3000 g
c. Decant the supernatant into a 100 mL volumetric flask. Repeat this procedure (extraction, shaking, centrifugation, decanting) twice more. Collect all three supernatants in the same volumetric flask. Finally, fill up to the mark with fresh extraction solution
d. Shake well before filtering the whole solution. Save filtrate for the analysis of the exchangeable bases (Na, K, Ca, Mg)
e. Add 40 mL of water to the precipitate in the centrifugation tube and shake for 1 to 2 min to resuspend. Centrifuge for 10 min at 3000 g. Decant and discard the supernatant
f. Weigh the centrifuge tube together with the remaining content (m_2). Add 30 mL of $MgSO_4$ solution f) and shake overnight. Decant the solution and filter into PE bottles. Store filtrate II for analysis of excess magnesium
g. Prepare blank samples in parallel

Determination of CEC:

a. Pipette 0.20 mL from the filtrates II of the samples and blank samples into 100 mL volumetric flasks. Add 10 mL of acidified lanthanum solution i), fill up with water to the mark and mix. Determine the concentration of magnesium using the diluted filtrates II
b. For calibration, use dilutions of the magnesium standard solution h). Pipette 0 mL, 1 mL, 2 mL, 3 mL, 4 mL, and 5 mL into a series of 100 mL volumetric flasks. Add 10 mL of acidified lanthanum solution to each flask. Final concentration of the calibration solutions: 0, 0.01, 0.02, 0.03, 0.04, and 0.05 mmol L^{-1}, respectively
c. Analyse magnesium using FAAS (or with ICP-OES) at a wavelength of 285.2 nm. Use instrumentation settings following the manufacturer's instructions for optimum performance of the instrument

Calculation

The magnesium concentration is measured in filtrate II. This must be corrected for the liquid remaining after decanting in the soil pellet:

$$c_2 = [c_1 \times (30 + m_2 - m_1)] \times 30^{-1} \quad \text{(Eq. 2.2.4.1.1)}$$

where

$\boldsymbol{c_1}$ is the magnesium concentration in the diluted filtrate II [mmol L^{-1}],
$\boldsymbol{c_2}$ is the corrected magnesium concentration in the diluted filtrate II [mmol L^{-1}],
$\boldsymbol{m_1}$ is the mass of the centrifuge tube plus air-dried soil [g],
$\boldsymbol{m_2}$ is the mass of the centrifuge tube plus moist soil [g].

The cation exchange capacity (CEC) of the soil is calculated with the following equation:

$$CEC = 3000 \times (c_{b1} - c_2)] \times m^{-1}\ [cmol_c\ kg^{-1}] \quad \text{(Eq. 2.2.4.1.2)}$$

where

c_2 is the corrected magnesium concentration in the diluted filtrate II [mmol L^{-1}],

c_{b1} is the magnesium concentration in the diluted filtrate II of the blank sample [mmol L^{-1}],

m is the mass of the air-dried soil sample [g].

If the CEC exceeds 40 $cmol_c$ kg^{-1}, the determination should be repeated with less weight of soil sample taken. Adjust all calculations appropriately.

Determination of the exchangeable sodium and potassium:

a. Determine sodium (Na) and potassium (K) in acidified barium chloride/triethanolamine extract of the soil samples using FAAS
b. *Calibration:* Prepare solutions with 0 mL, 5 mL, 10 mL, 15 mL, 20 mL and 25 mL of the diluted stock in 50 mL volumetric flasks. Add 10.0 mL of extraction solution e) and 5.0 mL of 1 mol L^{-1} hydrochloric acid. 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}
c. *Analysis*: Fill 2.0 mL of the filtrate I and of the blank sample, respectively, into reaction tubes. Add 1.0 mL of 1 mol L^{-1} hydrochloric acid l) and 7.0 mL of water and mix (see also Remarks). Determine Na and K with FAAS (or ICP-OES) at wavelength 589 nm and 766 nm, respectively, with the instrument set according to the manufacturer's instructions for optimum performance
d. *Calculations*: The content of exchangeable Na and K in soil samples is calculated as follows:

$$c(Na, exchangeable) = 2.1749 \times (c_{sample} - c_{blank}) / m \quad [cmol_c\ kg^{-1}] \qquad \text{(Eq. 2.2.4.1.3)}$$

$$c(K, exchangeable) = 1.2788 \times (c_{sample} - c_{blank}) / m \quad [cmol_c\ kg^{-1}] \qquad \text{(Eq. 2.2.4.1.4)}$$

with the measured concentrations in the diluted sample (c_{sample}) and the diluted blank sample (c_{blank}), respectively, and m is the soil mass in g.

Determination of the exchangeable calcium and magnesium:

a. Determine calcium (Ca) and magnesium (Mg) in acidified barium chloride/triethanolamine extract of soil samples using FAAS
b. *Calibration*: Prepare solutions with 0 mL, 2 mL, 4 mL, 6 mL, 8 mL and 10 mL of the mixed stock solution in 100 mL volumetric flasks. Add 10.0 mL of extraction solution and 10.0 mL of 1 mol L^{-1} HCl. Fill up to the mark with water. 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}
c. *Analysis*: Fill 1.0 mL of the filtrate I and of the blank sample, respectively, into reaction tubes. Add 1.0 mL of hydrochloric acid l) and 8.0 mL of water and mix (see also above comment). Determine Ca and Mg with FAAS at wavelength 422.7 nm and 285.2 nm, respectively, with the instrument set according to the manufacturer's instructions for optimum performance
d. *Calculations*: The content of exchangeable Ca and Mg in soil samples is calculated as follows:

$$c(Ca, exchangeable) = 8.2288 \times (c_{sample} - c_{blank}) / m \quad [cmol_c\ kg^{-1}] \qquad \text{(Eq. 2.2.4.1.5)}$$

$$c(Mg, exchangeable) = 4.9903 \times (c_{sample} - c_{blank}) / m \quad [cmol_c\ kg^{-1}] \qquad \text{(Eq. 2.2.4.1.6)}$$

with the measured concentrations in the diluted sample (c_{sample}) and the diluted blank sample (c_{blank}), respectively, and m is the soil mass in g.

2.2.4.2 Determination of the effective cation exchange capacity and base saturation level using barium chloride solution

Reagents

Reagents of recognised analytical grade shall be used, including:

- Deionised water (electric conductivity < 0.2 mS m^{-1} at 25 °C)
- Barium chloride ($BaCl_2$) solution; c($BaCl_2$) = 0.1 mol L^{-1}. Preparation: Dissolve 24.43 g of $BaCl_2 \times 2\ H_2O$ in 1000 mL of water (use volumetric flask)
- $BaCl_2$ solution; c($BaCl_2$) = 0.0025 mol L^{-1}. Preparation: Dilute 25 mL of the 0.1 mol L^{-1} solution in 1000 mL of water
- Magnesium sulphate solution; c($MgSO_4$) = 0.020 mol L^{-1}. Preparation: Dissolve 4.930 g magnesium sulphate heptahydrate ($MgSO_4 \times 7\ H_2O$) in 1000 mL of water (use volumetric flask). Prepare fresh solution. Magnesium sulphate can lose crystal water during storage. Protect from that by wrapping the flask in an additional PE bag and storing the chemical in a refrigerator
- Hydrochloric 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 $MgSO_4$ solution D) 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 [$La(NO_3)_3 \times 6\ H_2O$] to a 500 mL volumetric flask, add 42 mL hydrochloric acid, 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 E) and make up to 1000 mL with water
- Sodium and potassium stock solution: c(Na) = 400 mg L^{-1}, c(K) = 1000 mg L^{-1}. 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 j) in 250 mL volumetric flask and fill up with water to the mark. *See Remarks*
- Hydrochloric acid, c(HCl) = 4 mol L^{-1}. Add 83 mL of concentrated HCl (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) = 100 mg L^{-1}. Dissolve 1.0168 g sodium chloride and 0.836 g magnesium chloride hexahydrate ($MgCl_2 \times 6\ H_2O$) in water. Transfer to 1000 mL volumetric flask and fill up to the mark with water. *See Remarks*

Procedure

Leaching:

a. Weigh 2.50 g of air-dried soil, for example, into a polyethylene centrifuge tube of about 50 mL. Close cap tightly. Note the combined mass of tube and soil (m_1)

b. Add 30 mL of of the 0.1 mol L^{-1} $BaCl_2$ solution and shake for 1 h. Subsequently centrifuge the tubes at 3,000 g for 10 min. Note: Balance tubes before centrifugation. Transfer the supernatant

liquid to a 100 mL volumetric flask. Repeat this procedure, i.e. the addition of 30 mL of $BaCl_2$ solution, shaking and centrifugation twice more. Collect all three supernatants in the same volumetric flask. volume of the volumetric flask with the 0.1 mol L^{-1} $BaCl_2$ solution. Mix, filter and store the **extract I** determination of the exchangeable concentration of **Na, K, Ca and Mg**. *See Remarks*

Cleansing:

c. Add 30 mL of the 0.1 mol L^{-1} BaCl2 solution to the soil pellet and shake overnight. (Resulting Ba concentration 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:

d. Weigh the tube with its contents and cap (m_2). Add 30 mL of 0.02 mol L^{-1} $MgSO_4$ solution 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 magnesium (see below)
e. Prepare blank samples without the addition of soil in parallel and follow the above described procedure completely

Determination of CEC:

a. Pipette 0.20 mL from the extracts II of the samples and blank samples into 100 mL volumetric flasks
b. Add 0.3 mL of the 0.1 mol L^{-1} $BaCl_2$ solution and additional 10 mL of acidified lanthanum solution.
c. Fill up with water to the mark and mix
d. For calibration, use dilutions of the magnesium standard solution. Pipette 0 mL, 1 mL, 2 mL, 3 mL, 4 mL, and 5 mL into a series of 100 mL volumetric flasks. Add 10 mL of acidified lanthanum solution 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
e. Analyse magnesium using FAAS at a wavelength of 285.2 nm (or ICP-OES) with instrumentation settings following the manufacturer's instructions for optimum performance of the instrument

Calculation

Correct the concentrations of magnesium in the sample solutions for the volume of the liquid retained by the centrifuged soil after being treated with 0,0025 mol L^{-1} $BaCl_2$ solution:

$$c_2 = [c_1 (30 + m_2 – m_1)] / 30 \quad \text{(Eq. 2.2.4.2.1)}$$

where

$\boldsymbol{c}_2$ is the corrected magnesium concentration in the sample [mmol / L^{-1}],
$\boldsymbol{c}_1$ is the magnesium concentration in the sample [mmol / L^{-1}],
$\boldsymbol{m}_1$ is the mass of the centrifuge tube with air-dried soil [g],
$\boldsymbol{m}_2$ is the mass of the centrifuge tube with wet soil [g].

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

$$CEC = (c_{b1} - c_2)\ 3{,}000 / m \quad \text{(Eq. 2.2.4.2.2)}$$

where

CEC is the cation exchange capacity of the soil [$cmol_c\ kg^{-1}$],

$\boldsymbol{c}_2$ is the corrected magnesium concentration in the sample [$mmol\ L^{-1}$],

$\boldsymbol{c}_{b1}$ is the magnesium concentration in the blank [$mmol\ L^{-1}$],

m is the mass of the air-dried sample [g].

If the CEC exceeds 40 $cmol_c\ kg^{-1}$, the determination should be repeated using less soil, adjusting the calculation accordingly.

Determination of the exchangeable sodium and potassium

a. Determine sodium (Na) and potassium (K) in acidified $BaCl_2$ extract of soil samples using FAAS. To eliminate ionisation interference, a caesium solution is added to the samples

b. *Calibration*: Prepare solutions with 0 mL, 5 mL, 10 mL, 15 mL, 20 mL and 25 mL of the diluted stock solution in 50 mL volumetric flasks. Add 10.0 mL of 0.1 mol L^{-1} $BaCl_2$ 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. The resulting concentrations of K are 0, 10, 20, 30, 40, 50 mg L^{-1}. *See Remarks*

c. *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 Na and K with FAAS at wavelength 589 nm and 766 nm, respectively, with the instrument set according to the manufacturer's instructions for optimum performance

d. *Calculations*: The content of exchangeable Na and K in soil samples is calculated as follows:

$$c(Na, exchangeable) = 2.1749 \times (c_{sample} - c_{blank}) / m \quad [cmol_c\ kg^{-1}] \qquad \text{(Eq. 2.2.4.2.3)}$$

$$c(K, exchangeable) = 1.2788 \times (c_{sample} - c_{blank}) / m \quad [cmol_c\ kg^{-1}] \qquad \text{(Eq. 2.2.4.2.4)}$$

with the measured concentrations in the diluted sample (c_{sample}) and the diluted blank sample (c_{blank}), respectively, and m is the soil mass in g.

Determination of the exchangeable calcium and magnesium

a. Determine calcium (Ca) and magnesium (Mg) in acidified barium chloride/triethanolamine extract of samples using FAAS

b. *Calibration*: Prepare solutions with 0 mL, 2 mL, 4 mL, 6 mL, 8 mL and 10 mL of the sodium and potassium stock solution in 100 mL volumetric flasks. Add 10.0 mL of the 0.1 mol L^{-1} $BaCl_2$ extraction solution and 10.0 mL of 4 mol L^{-1} hydrochloric acid). Fill up to the mark with water. 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}. See Remarks

c. *Analysis*: Fill 1.0 mL of the filtrate I and of the blank sample, respectively, into reaction tubes. Add 1.0 mL of hydrochloric acid I) and 8.0 mL of water and mix (see also above comment). Determine Ca and Mg with FAAS at wavelength 422.7 nm and 285.2 nm, respectively, with the instrument set according to the manufacturer's instructions for optimum performance

d. *Calculations*: The content of exchangeable Ca and Mg in soil samples is calculated as follows:

$$c(Ca, exchangeable) = 8.2288 \times (c_{sample} - c_{blank}) / m \quad [cmol_c\ kg^{-1}] \qquad \text{(Eq. 2.2.4.2.5)}$$

$$c(Mg, exchangeable) = 4.9903 \times (c_{sample} - c_{blank}) / m \quad [cmol_c\ kg^{-1}] \qquad \text{(Eq. 2.2.4.2.6)}$$

with the measured concentrations in the diluted sample (c_{sample}) and the diluted blank sample (c_{blank}), respectively, and m is the soil mass in g.

Examples

Some examples for values of potential and effective cation exchange capacity are given in Table 2.2.4.1. It can be seen that with decreasing soil pH, the base saturation decreases and the difference between potential CEC and ECEC increases. While exchange sites in soils with pH 6.5 and higher are largely dominated by Ca, Al is the dominant cation covering exchange sites in acidic soils

Table 2.2.4.1 Typical values of data on potential CEC, effective CEC, saturation with acidic (Al, Mn, Fe) and basic (Na, K, Ca, Mg) cations and base saturation (Base sat.) in soils of different climates and soil use. Data from Blume et al., (2010) and own data

Soil	Parent rock	pH	OC	pot. CEC	ECEC	Saturation %							Base sat.
		$CaCl_2$	%	$cmol_c\ kg^{-1}$		Al	Mn	Fe	Na	K	Ca	Mg	%
Arable soils (central Europe)													
Luvisol	loess	6.3	1.4	17	14	<LOD[a]	– [b]	–	<1	5	80	15	100
Chernozem	loess	7.2	1.6	18	18	<LOD	–	–	0.4	0.5	90	9	100
Vertisol	mudstone	6.7	2.4	22	17	<LOD	–	–	<LOD	9	83	8	100
Cambisol	terrace mat.	6.6	1.6	13	11	<LOD	0.7	0.02	0.5	3.3	77	20	100
Cambisol	claystone	6.6	1.9	14	11	<LOD	0.7	0.08	0.3	7.2	73	20	100
Forest soils (central Europe)													
Podzol	granite	2.6	12	17	6.8	65	–	–	2.0	5	22	6	35
Stagnosol	loess	3.8	5.7	18	5.4	69	–	–	11	6	13	<2	30
Cambisol	loess	2.9	20	60	12	85	–	–	<LOD	5	5.8	4.2	15
Soils in other climates													
Vertisol		6.8	0.9	45	47	<LOD	–	–	3.6	0.4	71	25	100
Ferralsol		3.5	2.8	13	2.6	89	–	–	1.2	3.1	2.7	3.5	11
Acrisol		3.5	3.3	26	7.2	72	–	–	1.4	2.8	15	8.3	28

[a] <LOD = below limit of detection; [b] – = parameter was not measured

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 NH_4^+ in fertilised agricultural soil and exchangeable Al in acidic and also Fe in very acidic soil
- 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 doing pipette dilutions and using a diluter system, respectively
- Note: The unit cmolc/kg replaces the old unit millli-equivalents/100 g
- There is a contradiction between guidelines ISO 13536 and ISO 11260. In the former an acidified lanthanum solution containing 10 g L^{-1} must be prepared, while here the concentration of La is 10 mg L^{-1}. Typically, a La concentration of 10 g L^{-1} is recommended
- Analysis of sodium and potassium for ECEC is supposed to be done with acidified caesium chloride solution (Section 2.2.4.2), while for potential CEC this is done with hydrochloric acid (Section 2.2.4.1). The user can decide whether to employ acidified caesium chloride or not

References

ad-hoc AG Boden, 2005. Bodenkundliche Kartieranleitung, 5th edition. E. Schweizerbart'sche Verlagsbuchhandlung, Hannover – Stuttgart, 438 p.

Blume, H.-P., Brümmer, G.W., Horn, R., Kandeler, E., Kögel-Knabner, I., Kretzschmar, R., Stahr, K., Wilke, B.-M., 2010. Lehrbuch der Bodenkunde. 16th edition. Scheffer/Schachtschabel, p. 570

ISO, 1995. ISO 13536: Soil quality - Determination of the potential cation exchange capacity and exchangeable cations using barium chloride solution buffered at pH = 8.1. revised 2016

ISO, 2018. ISO 11260: Soil quality - Determination of effective cation exchange capacity and base saturation level using barium chloride solution

Mehlich, A., 1938. Use of triethanolamine acetate-barium buffer for the determination of some base exchange properties and lime requirement of soil. Soil Sci. Soc. Am. Proc., 3, 1–14.

Mehlich, A., 1942. Rapid estimation of base-exchange properties of soil. Soil Sci., 53, 1-14. doi: 10.1097/00010694-194201000-00001

2.2.5 Total nutrients and metals (Ca, Mg, K, Fe, Mn, Cu, Zn, Cd, Pb, Ni, Cr, As, Al, B)

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Importance and applications

All plants require 17 elements to complete their life cycle, and additional four elements have been identified as essential for some plants (Havlin et al., 2005). With the exception of C, H, and O, which plants obtain from air and water, plants derive the remaining 14 elements from the soil or through fertilisers, manures, and amendments (Parikh & James, 2012). The bulk of the soil solid fraction is constituted by soil minerals, which exert significant direct and indirect influences on the supply and availability of most nutrient elements. Soil parent material has a significant direct influence on the nutrient element contents of the soil, and on their concentrations depending on rock type. Therefore, in order to better understand the dynamics of nutrients in soil, it is useful to determine their total concentrations.

In addition, soils may become contaminated by the accumulation of heavy metals and metalloids through anthropogenic activities. The adequate protection and restoration of soil agro-ecosystems contaminated by heavy metals requires a detailed soil characterisation, with total metals concentrations being an essential parameter.

Principle

A representative sample is extracted and/or dissolved in concentrated nitric acid, or alternatively, concentrated nitric acid and concentrated hydrochloric acid using microwave heating with a suitable laboratory microwave unit. The sample and acid(s) are placed in a microwave vessel. The vessel is sealed and heated in the microwave unit for a specified period of time. After cooling, the vessel contents are filtered, centrifuged, or allowed to settle and then diluted to volume and analysed using the appropriate determination method (USEPA, 1997).

Reagents

- Concentrated nitric acid (HNO_3) 65%
- Concentrated hydrochloric acid (HCl) 37%
- Deionised water

Materials and equipment

- Volumetric flask (100 mL)
- Funnels
- Quantitative filter papers of 110 mm diameter (0.45 µm pore size)
- Pipette (10 mL)
- Microwave unit
- Vessels
- Analytical balance
- Flame atomic absorption spectrophotometer (FLAA) or graphite furnace atomic absorption

spectrophotometer (GFAA) or inductively coupled plasma atomic emission spectrometer (ICP-AES) or inductively coupled plasma mass spectrometer (ICP-MS)

Procedure

a. Weigh 0.500 g of well-mixed ground soil sample into a microwave vessel to the nearest 0.001 g with an appropriate analytical balance
b. Add 10 mL concentrated nitric acid or, alternatively, 9 mL concentrated nitric acid and 3 mL concentrated hydrochloric acid to the vessel in a fume hood. The addition of concentrated hydrochloric acid to the nitric acid is appropriate for the stabilisation of high Fe and Al concentrations in solution
c. Seal the vessel according to the manufacturer's instructions. Properly place the vessel in the microwave system according to the manufacturer's recommended specifications and, when applicable, connect appropriate temperature and pressure sensors to vessels according to the manufacturer's specifications
d. Start the microwave program. The temperature of each sample should rise to 175 ± 5ºC in approximately 5.5 ± 0.25 min and remain at 175 ± 5ºC for 4.5 min, and stay for at least 10-min reducing the temperature (Fig. 2.2.5.1)
e. At the end of the microwave program, allow the vessels to cool for a minimum of 30 min before removing them from the microwave system. Cooling of the vessels may be accelerated by internal or external cooling devices
f. Complete the preparation of the sample by venting microwave containers in a fume hood before uncapping, so as to avoid a rush of acid vapour that may still be in the headspace. When cool enough to handle, carefully uncap the vessels
g. Filter the sample solution through quantitative filter paper into a 100 mL volumetric flask, and make up to 100 mL with deionised water
h. The solution is now ready for analysis for elements of interest using appropriate elemental analysis techniques (Flame atomic absorption spectrophotometer (FLAA) or graphite furnace atomic absorption spectrophotometer (GFAA) or inductively coupled plasma atomic emission spectrometer (ICP-AES) or inductively coupled plasma mass spectrometer (ICP-MS)

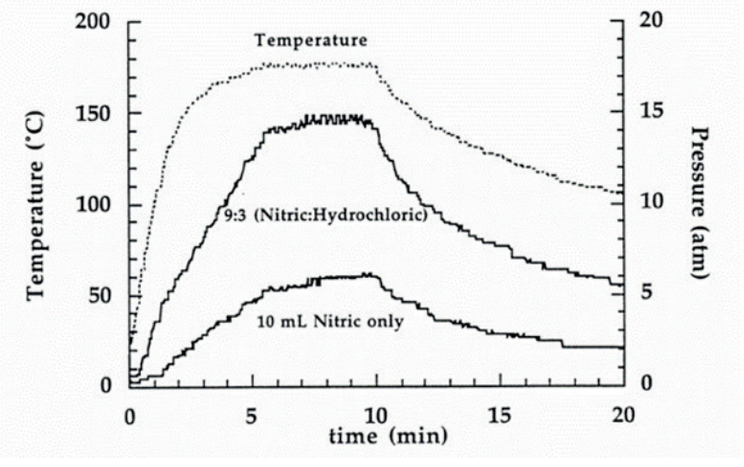

Figure 2.2.5.1 Temperature and pressure profile (Link et al., 1997, 1998)

Calculations

Convert the extract concentration obtained from the instrument in mg/L to mg/kg dry-weight of sample by

$$\textit{Sample concentration (mg kg}^{-1}\textit{)} = \frac{X\ V}{W} DF \qquad \text{(Eq. 2.2.5.1)}$$

where

X is the concentration obtained from the instrument [mg L^{-1}],

V is the final volume of the sample solution [mL] (e.g. volumetric flask of 100 mL),

W is the weight of the sample [g],

DF is the dilution factor (DF = 1.00 with no sample dilution).

Remarks

- All digestion vessels must be carefully acid washed and rinsed with reagent water. When switching between high concentration samples and low concentration samples, all digestion vessels should be cleaned by leaching with hot (1:1) hydrochloric acid (greater than 80ºC, but less than boiling) for a minimum of two hours followed by hot (1:1) nitric acid (greater than 80ºC, but less than boiling) for a minimum of two hours. The vessels should then be rinsed with deionised water and dried in a clean environment
- The addition of hydrochloric acid may limit the quantitation techniques and increase the difficulties of analysis for some quantitation systems

References

Link, D.D., Kingston, H.M., Walter, P.J., 1998. Development and Validation of the New EPA Microwave-assisted Leach Method 3051A. Environ. Sci. Tech., 32. 3628–3632.

Link, D.D., Kingston, H.M., Walter, P.J., 1997. Development and Validation of the EPA Microwave-assisted Methods 3015A and 3051A: Validation Studies for Updated Microwave Leach Methods. Proceedings for the Waste Testing and Quality Assurance Symposium

Havlin, J.L. et al., 2005. Soil Fertility and Fertilizers: An Introduction to Nutrient Management. 7th edition. Prentice Hall, Upper Saddle River, NJ.

Parikh, S.J., James, B.R., 2012. Soil: The foundation of agriculture. Nature Education Knowledge 3, 10.

USEPA, 2007. Method 3051 A Microwave assisted acid digestion of sediments, sludges, soils, and oils.

2.2.6. Bioavailable nutrients and metals (Fe, Mn, Cu, Zn, Cd, Pb, Ni, Cr, As, Al)

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Importance and applications

Metals are associated to different fractions in the soil: (1) in soil solution, as free metal ions and soluble metal complexes, (2) adsorbed in the exchange sites of the soil inorganic constituents, (3) bound to organic matter, (4) precipitated as oxides, hydroxides and carbonates, and (5) in the structures of silicate minerals (Rieuwerts et al., 1998, Lassat, 2001, Reichman, 2002, Basta, 2004). The bioavailability is defined as the heavy metal fraction available for plant uptake. For sustainable farming, the bioavailable nutrients and metals must be monitored to ensure the necessary amount of nutrients for high quality and optimal production, and to be sure that the concentrations of toxic elements (e.g. lead, cadmium, arsenic etc.) remain below the limits of toxicity for crops. In addition, the monitoring of nutrients allows us to optimise the application of the necessary amount of those elements in order to avoid over-application, which could contaminate the soil, subsoil and even groundwater and increase production costs. Finally, the monitoring of potential toxic metals allows to evaluate the risk of transfer to the food chain, and the need to use techniques for reducing their availability which will minimise this risk.

The bioavailability depends on the solubility and adsorption capacity of metals in the colloidal fraction of soil. The interaction between the different processes such as cation exchange, adsorption/desorption, precipitation/dissolution and complex formation affect the distribution of metals between the soil solution and the solid phase, being responsible for their mobility and bioavailability (Rieuwerts et al., 1998). In addition, soil properties and constituents affect metals bioavailability, such as the pH, redox potential, texture, content and type of clays, organic matter, Fe, Mn and Al oxides, and the presence of cations and anions in solution (Rieuwerts et al., 1998, Reichman, 2002, Silveira et al., 2003; Basta, 2004). In order to determine the concentration of bioavailable metals, chelating agents have been widely used, such as is the case of EDTA and DTPA (Kabata-Pendias, 2000, Reichman, 2002).

Principle

A representative sample is extracted by a chelating agent, DTPA or EDTA. The sample and chelating agent are placed in a plastic container and shaken for a specified period of time. After that, the vessel contents are centrifuged, or allowed to settle, and filtered, then analysed by the appropriate determinative method.

Reagents

Soils with pH >6: DTPA 0.05 M at pH 7.30 solution (Lindsay & Norvell, 1978; Crock & Severson, 1980):

- DTPA (diethylene-triamine-pentaacetic acid)
- $CaCl_2$ x $2H_2O$ (0,01 N)

- Triethanolamine (TEA) (0.1 M)
- HCl (37%).

Soils with pH < 6: EDTA 0.005 M at pH 4.65 solution (Lindsay & Norvell, 1978, Borggaard, 1976):

- VEDTA (ethylene-diamine-tetraacetic acid)
- Ammonium acetate ($AcNH_4$)
- HCl (37%)

Materials and equipment

- Beakers (250 and 1000 mL)
- Centrifuge tubes (50 mL)
- Funnels
- Plastic container (50 mL)
- Quantitative filter papers of 110 mm diameter (0.45 µm pore size)
- Analytical balance
- Centrifuge unit
- Orbital shaker
- Flame atomic absorption spectrophotometer (FLAA) or graphite furnace atomic absorption spectrophotometer (GFAA) or inductively coupled plasma atomic emission spectrometer (ICP-AES) or inductively coupled plasma mass spectrometer (ICP-MS)

Procedure

Soil with pH > 6: DTPA 0.05 M at pH 7.30:

a. Preparation of DTPA solution:
   - Weigh the following reagents into a 250 mL beaker:
     - DTPA (diethyene-triamine-pentaacetic acid): 1.9667 g
     - $CaCl_2$ x $2H_2O$ (0,01 N): 0,0735 g
     - Triethanolamine (TEA) (0.1 M): 14 mL (TEA 98 %) or 15.6 mL (TEA 85 %).
   - Shake the solution.
   - Make up to 1 L with deionised water.
   - Measure the initial pH of the solution and adjust it to 7.3 by gradually adding HCl (37%).

b. Weigh 15 g of well-mixed 2 mm-sieved soil sample into a centrifuge tube to the nearest 0.001 g with an appropriate analytical balance.

c. Add 30 mL of DTPA solution (1:2 ratio soil/solution)

d. Shake for 2 h in an orbital shaker unit.

e. Centrifuge the tube at 2100 rpm for 5 min.

f. Filter the sample solution through quantitative filter paper into a 50 mL container.

g. Flame atomic absorption spectrophotometer (FLAA) or graphite furnace atomic absorption spectrophotometer (GFAA) or inductively coupled plasma atomic emission spectrometer (ICP-AES) or inductively coupled plasma mass spectrometer (ICP-MS).

Soil with pH < 6: EDTA 0.005 M at pH 4.65:

a. Preparation of EDTA solution:
   - Weigh the following reagents into a 250 mL beaker:
     - EDTA (ethylene-diamine-tetraacetic acid): 1.8612 g.
     - Ammonium acetate ($AcNH_4$): 77 g.
   - Shake the solution.
   - Make up to 1 L with deionised water.
   - Measure the initial pH of the solution and adjust it to 4.65 by gradually adding HCl (37%).

b. Weigh 8 g of well-mixed 2 mm-sieved soil sample into a centrifuge tube to the nearest 0.001 g with an appropriate analytical balance.

c. Add 40 mL of EDTA solution (1:5 ratio soil/solution)

d. Shake for 1 h in an orbital shaker unit.

e. Centrifuge the tube at 2100 rpm for 5 min.

f. Filter the sample solution through quantitative filter paper into a 50 mL container.

g. Flame atomic absorption spectrophotometer (FLAA) or graphite furnace atomic absorption spectrophotometer (GFAA) or inductively coupled plasma atomic emission spectrometer (ICP-AES) or inductively coupled plasma mass spectrometer (ICP-MS).

Calculations

- Convert the extract concentration obtained from the instrument in mg L^{-1} to mg kg^{-1} dry-weight of sample by:

$$Sample\ concentration\ (mg\ kg^{-1}) = \frac{X\ V}{W}\ DF \qquad \text{(Eq. 2.2.6.1)}$$

where

X is the concentration obtained from the instrument [mg L^{-1}],
V is the final volume of the sample solution [mL],
W is the weight of the sample [g],
DF is the dilution factor (DF = 1.00 with no sample dilution).

Remarks

- In order to choose the correct chelating agent, soil pH must be determined.

References

Basta, N.T., 2004. Heavy metal and trace element chemistry in residual-treated soil: Implications on metal bioavailability and sustainable land application. In: Sustainable land application Conference, January, 4–8, Florida; University of Florida.

Borggarrd, O.K., 1976. The use of EDTA in soil analysis. Acta Agriculturae Scandinavica, 26, 114–150.

Crock, J.G., Severson, R.C., 1980. Four reference soil and rock samples for measuring element availability

in the western energy regions: U.S. Geological Survey Circular 841, 16 p.
Greman, H., Velikonja-Bolta, S., Vodnik, D., Kos, B., Lestan, D., 2001. EDTA enhanced heavy metal phytoextraction: metal accumulation, leaching and toxicity. Plant Soil, 235, 105–114.
Kabata-Pendias, A., 2000. Trace elements in soils and plants. Third Edition. CRC Press, Boca Raton, FL. 432 p.
Lasat, M., 2001. The uses of plants for removal of toxic metal from contaminated soil. US-EPA
Lindsay, W.L., Norvell, W.A., 1978. Development of a DTPA soil test for Zn, Fe, Mn, and Cu. Soil Sci. Soc. Am. J., 42, 421–428.
McBride, M.B., 1995. Toxic metal accumulation from agricultural use of sludge: are USEPA regulations protective? J. Environ. Quality, 24, 5–18.
Reichman, M.S., 2002. The responses of plants to metals toxicity: A review focusing on copper, manganese and zinc. Australian Minerals and Energy Environment Foundation, Melbourne, Australia, 54 p.
Rieuwerts, J.S., Thonton, I., Farago, M.E., Ashmore, M.R., 1998. Factors influencing metals bioavailability in soils: preliminary investigations for the development of a critical loads approach for metals. Chem.Spec. Bioavailab. 10, 2, 61–75.
Silviera, M.L.A., Alleon, L.R.F, Guilherme, L.R.G., 2003. Biosolids and heavy metals in soils. Sci. Agric., 60, 4, 793–806.

2.2.7 Total carbon (organic and inorganic carbon) and nitrogen

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Importance and applications

Organic Carbon (OC) is the main source of energy and nutrients for soil microorganisms, affecting plant growth. It plays a crucial role in aggregate stability and consequently intervenes in the distribution of the porous space, water holding capacity, and soil moisture, amongst other soil properties. Total Organic Carbon (TOC) affects most of the chemical, physical and biological soil properties linked to their quality, sustainability and productive capacity. An increase in Soil Organic Matter (SOM), and therefore total carbon (C), leads to greater biological diversity in the soil, thus increasing biological control of plant diseases and pests. There are management practices that cause a detriment of TOC over time, while there are practices that facilitate its accumulation. The scientific literature points out that conventional agricultural land management, with intensive tillage, promotes the release of C into the atmosphere, while conservation agriculture favours the accumulation of C in organic forms within the soil (Almagro et al., 2016).
Total nitrogen (TN) corresponds to ammonium-N, nitrate-N, nitrite-N and organic N, around 90–95% of TN in soils is in organic form, and therefore is assimilated by plants through mineralisation. The amount of available nitrogen depends on cultivation methods, the environmental conditions, the expected yield, the nutrients available in the soil and their transformations. The amount of nitrogen needed as fertiliser can be estimated by medium-term analysis of the inputs and outputs of nitrogen forms (balance). A TOC/N ratio of 10–12 indicates a correct release of nitrogen, values above or below provide low or excessive release.
Both TOC and TN are indicators of soil quality, and along with P were recently identified as key biological indicators in relation to land use management across Europe. Both relate to two key soil ecosystem services (carbon cycling and storage potential and nutrient cycling) as detailed by Creamer et al., (2016).
This method is used for the determination of TC and TN by an elemental CN analyser, as well as TOC in soil samples, prior to the elimination of carbonates (if present) with HCl.

Principle

The elementary C and N analyser determines the C and N content of a variety of materials and soils. In this method, C is measured as carbon dioxide from the combustion of the sample by means of an infrared detector. The N present is determined by the Dumas method, by complete combustion in the presence of oxygen, reduction of the oxides of nitrogen formed to molecular nitrogen and its detection with a thermal conductivity detector. The quantification of both elements is carried out with certified reference standards of different concentrations of nitrogen and carbon.

Reagents

- 2N Hydrochloric acid

Materials and equipment

- Elemental Analyzer
- Crucible
- Plastic tips for the different micropipettes
- Micro Spatula
- Tin Capsules
- Stainless Steel Plate
- Heating Plate
- Analytical balance with a precision of 0.0001 g
- Micropipettes of 100 µL
- Laminar flow Hood
- Agate mortar and pestle and ball mill
- Desiccator and silica gel

Procedure

Carbon/total nitrogen:

a. Prepare air-dried soil samples
b. Sieve at 2 mm
c. Grind the sample in a ball mill or agate mortar and pestle
d. Weigh 0.05-0.10 ± 0.01 g of the sample in a tin capsule and then close for later insertion into the Elemental Analyzer
e. Weigh soil calibration standards for known carbon/total nitrogen values

Total organic carbon

a. Prepare air-dried soil samples
b. Sieve at 2 mm
c. Grind the sample in a ball mill or agate mortar and pestle
d. Weigh 0.05–0.07 g of sample in a triple tin capsule
e. Place it on a stainless-steel plate in an orderly manner above a heating plate, at a temperature of about 120ºC.
f. Add 100 µL of 2N HCl repeatedly until the carbonates have been destroyed and the effervescence ceased, and allow the samples to dry for 8 h
g. Close them for later introduction in the Elemental Analyzer
h. Verify that the treatment with HCl has been done correctly using two standards
i. Weigh soil calibration standards for known total organic carbon values

Calculations

The final result is displayed as weight percentage, by multiplying it by 10 it can be expressed in mg g^{-1}.

Table 2.2.7.1 Range of values in different agricultural soils

N (mg g^{-1})	Land use	Soil type	Reference
0.8–1.3	Almond RT vs RTG/NT	Mediterranean climate Calcisols (FAO, 2006)	Martinez-Mena et al., (2013)
1.7–6.1	Cropland vs Grassland	Continental climate (Bavarian soils)	Capriel, (2013)
1.0–1.36	Vineyard conventional vs organic	Mediterranean climate Calcareous silty-clay	Coll et al., (2011)
0.5–1.9	Olive conventional vs organic	Mediterranean climate Eutric Cambisols (FAO-ISRICISSS, 2006)	Parras-Alcántara et al., (2015)
TOC (mg g^{-1})	**Land use**	**Soil type**	**Reference**
17.9–26.6	Almond CT vs RT/RTG	Mediterranean climate Calcisols (FAO, 2006)	Almagro et al., (2016)
17–62	Cropland vs Grassland	Continental climate (Bavarian soils)	Capriel, (2013)
10.2–13.5	Vineyard conventional vs organic	Mediterranean climate Calcareous silty-clay	Coll et al., (2011)
4.4–22.5	Olive conventional vs organic	Mediterranean climate Eutric Cambisols (FAO-ISRICISSS, 2006)	Parras-Alcántara et al., (2015)

Remarks

- Sample sizes range from 1 to 10 mg
- A large number of samples can be inserted into the loading head simultaneously
- Total analysis takes less than four minutes
- Carbonates can be inferred through the following equation: $CaCO_3$ = (TC-TOC) x 100/12, if TC and TOC are expressed as percentages, then consequently carbonates also, by multiplying it by 10 it can be expressed in mg g^{-1}.

References

Almagro, M., Vente, J. de, Boix-Fayos, C., García-Franco, N., Aguilar, J. M. de, González, D., Solé-Benet, A., Martínez-Mena, M., 2016. Sustainable land management practices as providers of several ecosystem services under rainfed Mediterranean agroecosystems. Mitig. Adapt. Strateg. Glob. Change, 21, 1029–1043.

Capriel, P., 2013. Trends in organic carbon and nitrogen contents in agricultural soils in Bavaria (south

Germany) between 1986 and 2007. Eur. J. Soil Sci., 64, 445–454.
Coll, P., Le Cadre, E., Blanchart, E., Hinsinger, P., Villenave, C., 2011. Organic viticulture and soil quality: a long-term study in Southern France. Appl. Soil Ecol., 50, 37–44.
Creamer, R.E., Hannula, S.E., Leeuwen, J.P. Van, Stone, D., Rutgers, M., Schmelz, R.M. et al., 2016. Ecological network analysis reveals the inter-connection between soil biodiversity and ecosystem function as affected by land use across Europe. Appl. Soil Ecol., 97, 112–124.
Martinez-Mena, M., Garcia-Franco, N., Almagro, M., Ruiz-Navarro, A., Albaladejo, J., Melgares de Aguilar, J., Gonzalez, D., Querejeta, J.I., 2013. Decreased foliar nitrogen and crop yield in organic rainfed almond trees during transition from reduced tillage to no tillage in dryland farming system. Eur. J. Agron., 49, 149–157.
Parras-Alcántara, L., Díaz-Jaimes. L., Lozano-García. B., 2015. Organic farming affects C and N in soils under olive groves in Mediterranean areas. Land Degrad. Dev., 26, 800–806.

2.2.8 Carbonates

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Importance and applications

The total carbonate content of the soil covers all carbonate CO_3^- minerals. Their formation and importance varies with pedo-climatic regions, but it is their common feature that the soil solution contains carbonic acid. The *primary source* of carbonates is calcite, dolomite, gypsum, marl and calcareous sandstone. Dolomite and vermiculite are also major sources of magnesium. Carbonated lime accumulates in the function of soil water budget during the weathering of $Ca/Mg(CO_3)_2$ present in carbonate rocks. In many cases, soil subtypes are identified by the distribution of carbonates in the profile. Carbonates buffer soil pH and contribute to the formation of soil structure.

Secondary pedogenic carbonates are precipitation forms. The effectiveness of lime heavily depends on its particle size. Lime grains coarser than 250 microns (0.25 mm) have little value in raising soil pH, at least in the short term. Carbonates in the soil profile appear in the following forms:

- uniformLy dispersed, non-visible to the naked eye,
- bound to microchannels, passages, aggregate surfaces, clearly visible;
- in spherical concretions,
- in thick precipitations, horizons, banks.

$CaCO_3$ provides a reactive surface for adsorption and precipitation reactions, for example, of P, trace metals and organic acids (Talibudeen & Arambarri, 1964; Amer et al., 1985). Adequate calcium helps delay leaf senescence and slows down or prevents leaf and fruit fall (abscission). Plants take up calcium in the ionic form (Ca^{2+}).

Carbonate deficit causes ‘blossom-end’ rot in tomatoes. It can be induced by moisture stress, even though the soil may have the adequate calcium levels required for cell elongation and cell division. Deficit is manifested in the chlorosis of young parts, deformation of leaves and browning of leaf veins. In acute cases, root growth stops growth peak gets brown and dies. Wilting may occur even with a good water supply.

Carbonate surplus (higher than 15% carbonate content) leads to phosphorus binding and reduced uptake of microelements (Cu, Zn, Mn, Fe, B).

Principle

When free carbonates are present, the acid will produce effervescence due to the release of CO_2 gas (Loeppert & Suarez, 1996).

The experiments to determine dissolved carbonates in soil samples use Scheibler’s calcimeter, a volumetric method (EN ISO 10693:2014). The carbonates present in the sample are converted into CO_2 by adding hydrochloric acid to the sample:

$$CaCO_3 + 2\,HCl = CO_2 + CaCl_2 + H_2O \qquad \text{(Eq. 2.2.8.1)}$$

As a result of the pressure of the CO_2 released, the water in a burette that is de-aerated rises. The difference in level measured is an indication of the released CO_2, from which the carbonate content can be calculated. At 30 seconds, the pressure is recorded as '$CaCO_3$ pressure'. If the test sample contains any dolomite, there will be a pause, then a slow, second rise in pressure. The reaction is complete when the pressure stops growing (within 30 to 45 minutes). The final pressure value is the total $CaCO_3$ pressure plus the dolomite pressure. The carbonate content is expressed as an equivalent calcium carbonate content. The Scheibler apparatus designed for kinetic dissolution of carbonate is shown in Fig. 2.2.8.1.

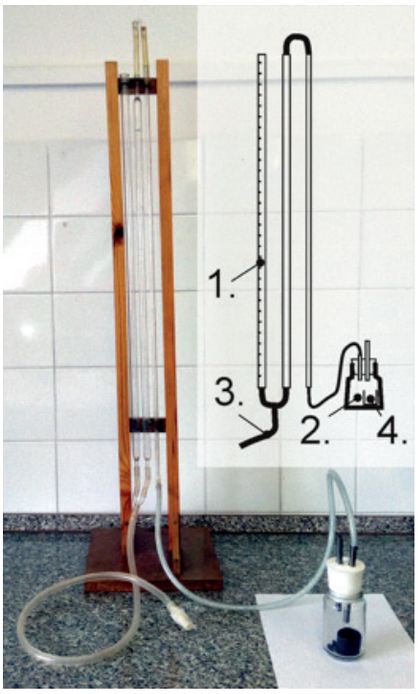

Fig. 2.2.8.1 Scheibler's calcimeter system. 1, U-shaped calibrated manometer with volumetric cm3 degree, 2, lockup glass reaction vessel tube with a cubic capacity of 100-200 cm^3 connected to the manometer, 3, pipe and tap for regulation of water level, 4, small tubes to hold the acid

Reagents

- 10% HCl
- distilled water

Materials and equipment

- Scheibler apparatus
- Thermometer (accuracy: 0.1°C)
- Barometer (accuracy: 1hPa)
- Scale (accuracy: 0.01g)

Procedure

a. Prepare dried soil sample (at 120°C in oven for 24 h)
b. For the preliminary determination, check the carbonate content of the sample through dripping 10% hydrochloric acid on it (Table 2.2.8.1). Weigh the soil sample into the vessel depending on the intensity of effervescence. Fizzle intensity is shown on a four-grade range
c. Fill a reaction vessel with a soil sample
d. Place the test tube with hydrochloric acid in the reaction vessel using a pair of tweezers (prepare one reaction vessel for each burette). Close the reaction vessel.
e. Fill the manometer up to 0 point with distilled water.
f. Enable the hydrochloric acid to flow out of the test tube and react with the soil sample that contains $CaCO_3$, initiating the reaction
g. Swirl the reaction cell and allow sufficient time for the reaction to finish
h. Perform the procedure three times

Table 2.2.8.1 Relative fizzing values and amount of investigated soil sample

Fizzle	Notation	Required soil sample (g)
No fizzle	0	no measurement required
Slight fizzle	x	1.5-2
Medium fizzle	xx	0.5-1.5
Intensive fizzle	xxx	0.2-0.5

Calculations

There are two ways to calculate soil carbonate content (Campbell & Norman, 1998):

1. Using ideal gas law:

$$P\ (theoretical)=(n*R*T)/V\ \ [Pa] \qquad \text{(Eq. 2.2.8.2)}$$

where

P is the value measured,
n is the pure $CaCO_3$ amount which is used in the analysis [mol],
R is the universal gas constant [8.3144 $J \cdot mol^{-1} \cdot K^{-1}$],
T is the temperature [K],
V is the volume of the CO_2 released during the process [mL].

At standard temperature and pressure (STP: 0°C and 101.325 kPa) the molar density (Greek letter: ρ_m) of any gas is 44.615 mol m^{-3} (and 1 mol of any gas occupies 22.4 dm^3). From the Boyle-Charles law, the molar density of CO_2 (and any gas) can be computed:

$$\rho_m = 44.615\ (p\ 101.325^{-1})\ (273.15\ T^{-1}) \qquad \text{(Eq. 2.2.8.3)}$$

where

44.615 is the molar density at STP [mol m^{-3}],

p is the air pressure [kPa],

T is the actual temperature [K].

2. Using auxiliary table (Table 2.2.8.2):

$$w\ (CaCO_3) = (V^*f)\ m\text{-}1\ \ [\%] \qquad \text{(Eq. 2.2.8.4)}$$

where

w is the soil carbonate content [m/m%],

V is the CO_2 volume released during the process [mL],

m is the soil weight [g],

f factor depending on actual air temperature and pressure (from auxiliary table).

Table 2.2.8.2 Auxiliary table. Conversion of the measured CO_2 to carbonate. The numbers express the weight of carbonate in [g x 10^{-6}] (e.g.: 4114 = 0.004114 g) which refer to 1 cm^3 CO_2

Temp. [°C]	Air pressure [Hgmm]									
	749.00	751.00	753.50	756.00	758.00	760.00	762.50	765.00	767.00	769.00
27	4099	4114	4129	4143	4158	4169	4179	4190	4200	4211
26	4114	4139	4144	4158	4172	4183	4193	4204	4214	4225
25	4128	4143	4158	4172	4186	4197	4208	4219	4230	4241
24	4142	4157	4172	4186	4200	4211	4222	4233	4244	4255
23	4156	4171	4186	4200	4214	4226	4237	4248	4259	4270
22	4170	4185	4200	4214	4228	4240	4252	4263	4274	4285
21	4184	4199	4214	4229	4243	4255	4267	4279	4290	4301
20	4199	4214	4229	4243	4257	4269	4281	4292	4303	4214
19	4213	4228	4243	4258	4272	4284	4296	4307	4318	4329
18	4228	4243	4258	4272	4286	4298	4310	4321	4332	4343
17	4242	4257	4272	4296	4300	4312	4324	4335	4346	4357
16	4256	4271	4286	4300	4314	4326	4338	4349	4360	4371
15	4271	4286	4301	4315	4329	4341	4353	4364	4375	4386

To calculate the dolomite pressure, subtract the $CaCO_3$ pressure (30 second reading) from the total pressure (30–45 minute reading).

Table 2.2.8.3 Evaluation of carbonate content

Carbonate (%)	Category
0	Absence
0.1–4.9	Poorly calcareous
5.0–19.9	Moderately calcareous
> 20.0	Strongly calcareous

Remarks

- Equivalent $CaCO_3$ may be overestimated if HCl reacts with non-carbonated substances in the soil.
- Dolomite and magnesite are completely dissolved, but only part of siderite.
- Analysing Ca and Mg in the solution makes it possible to distinguish between $CaCO_3$ and $MgCO_3$.

References

Amer, EA., Mahmoud, A., Sabel, V., 1985. Zeta potential and surface area of calcium carbonate as related to phosphate sorption. Soil Sci. Soc Am. J., 49, 1137–1142.

Campbell, G.S., Norman, J.M., 1998. An introduction to Environmental Biophysics. 2nd edition. Springer, Heidelberg – Berlin. 296 p.

ISO, 2014. EN ISO 10693:2014. Soil quality – Determination of carbonate content – Volumetric method (ISO 10693:1995)

Loeppert, R.H., Suarez, D.L., 1996. Carbonate and Gypsum. U.S. Department of Agriculture: Agricultural Research Service, Lincoln, Nebraska. In: Methods of Soil Analysis. Part 3. Chemical Methods. Soil Science Society of America and American Society of Agronomy, SSSA Book Series 5, 437–474.

Talibudeen, O., Arambarri, P., 1964. The influence of the amount and the origin of calcium carbonates on the isotopically-exchangeable phosphate in calcareous soils. J. Agric. Sci., 62, 93–97.

2.2.9 Soil Organic Carbon. Functional pools

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Importance and applications

Organic matter and organic matter fractions are important attributes of soil quality (Gregorich et al.,1994). Soil organic matter (SOM) consists of various functional pools that are stabilised by specific mechanisms and have certain turnover rates. Particulate organic matter (POM), defined as fresh or decomposing organic material (mainly composed of fine root fragments and other organic debris) between 53 and 250 µm in diameter and serves as a readily decomposable substrate for soil microorganisms (Mrabet et al., 2001). Particulate Organic Carbon (POC) responds in a short time period to the management-induced alterations. It can be used as an early indicator of SOM changes since they are difficult to detect, mostly only in long-term experiments (Chen et al., 2009. Thus, it is a useful index of microbially-important SOM because it consists of recognisable organic matter that can be isolated from mineral soils, and it is sensitive to changes in soil management (Franzluebbers, 2000). In addition, particulate organic C constitutes 8 to 25% of Total Organic Carbon (TOC) (Chan, 2007) and represents a transitional stage in the transformation of plant residue to soil C storage (Mao & Zeng, 2010). Therefore, in the long-term, an increase in POC translates into an increase in TOC (Cambardella & Elliott, 1992).

Principle

The method (Cambardella & Elliot, 1992) is based in the soil physical fractionation, the underlying principle is that the association of soil particles and their spatial arrangement play a key role in SOM dynamics, because bioaccessibility is a prerequisite for decomposition. Physical fractionation of SOM is useful for distinguishing specific C pools responsive to management, identifying the physical control of SOM, and characterising the relationship between SOM and the size distribution of aggregates. The difference between total SOC and POC will give us another C fraction: MOC (mineral associated C) which is the SOM chemically stabilised on silt and clay surfaces. However, this is a more stabilised SOM than the POM, and therefore less sensitive to soil management.

Reagents

- Sodium hexametaphosphate (5 g L^{-1}): dissolve 5 g of sodium hexametaphosphate in distilled water, complete to 1 L and shake well

Materials and equipment

- Reciprocal shaker
- 0.053-mm sieve
- Porcelain crucibles 10-15 cm diameter

- Whatman filter paper 541. Hardened Ashless. CAT No. 1541-125
- Oven
- Agate mortar
- Balance
- Distilled water
- Wash water bottle

Procedure

a. Air-dry soil samples
b. Sieved at 2 mm
c. Weight 20 g of dry mineral soil and dispersed by shaking overnight in a 100 mL solution of sodium hexametaphosphate (5 g L^{-1}).
d. Sieved the mixture through a 0.053-mm sieve and gently washed with deionised water (use 1 L of water approximately) the material retained above the sieved
e. Weight the filters
f. Filter the sample.
g. Introduce the filter + soil in the oven and dry at 60º C for 24 hours.
h. Weight the filter+ soil once dry
i. Remove the stones (in case you have) by hand.
j. Weight the stones (in case you have)
k. Ground the soil using a mortar and stored for carbon analysis. Concentrations of C in the isolated fraction will be determined using a C and N analyser (see section 2.2.7).

Calculations

Soil C in the POC fraction (g C g^{-1} soil) is calculated by multiplying the dry mass of POC (g POC g^{-1} soil) by the respective C concentration (g C g^{-1} POC).

Table 2.2.8.1 Range of values in different agricultural soils

POC (g kg^{-1})	Land use	Soil type	Reference
15–35	Barley/ CT vs NT	Vertisol	Somasundaram et al., (2017)
1.60–4.6	Barley/CC vs NT	Hypercalcic calcisol	Blanco-Moure et al., (2013)
0.58–1.53	Barley/wheat	Calcaric cambisol	Moharana et al., (2017)
2.7	Olive	Hypercalcid calcisol	Martínez-Mena et al., (2008)
0.8	Vegetable cropping system	Gleyc Luvisol	Baiano & Morra (2017)
1.44–4.57	Chestnut orchards	Dystric Cambisols	Borges et al., (2017)

Remarks

- Mineral associated carbon can also be obtained with this method, if the sample passing through the 0.053 mm sieve is collected
- To dry the filters more rapidly, a system can be used to drain the filter before putting it on the oven
- The time in the oven will depend on the quantity of POC to be obtained. 24 hours is the minimum
- As an alternative to removing the stones by hand (step i in the procedure) the dry soil could be sieved with a 1 mm sieve

References

Baiano, S., Morra, L., 2017. Particulate and mineral-associated organic carbon in aggregates as affected by biowaste compost applications in a Mediterranean vegetables cropping system. Commun. Soil Sci. Plant Anal., 48, 2, 2383–2394. doi: 10.1080/00103624.2016.1269798

Blanco-Moure, N., Gracia, R., Bielsa, A.C., López, M.V., 2013. Long-term no-tillage on particulate and mineral-associated soil organic matter under rainfed Mediterranean conditions. Soil Use Manage., 29, 250–259.

Borges, O., Raimundo, F., Coutinho, J., Gonçalves, B., Oliveira, I., Martins, A., Madeira M., 2017. Carbon fractions as indicators of organic matter dynamics in chestnut orchards under different soil management practices. Agrofor. Syst., 92, 2, 301–310. doi: 10.1007/s10457-017-0088-3

Cambardella, C.A., Elliot, E.T., 1992. Particulate soil organic-matter changes across a grassland cultivation sequence. Soil Sci. Soc. Am. J., 56, 777–783.

Chan, C., Kay, B.D., Gregorich, E.G., 2007. Factors influencing mineralizable carbon in a landscape with variable topography. Can. J. Soil Sci., 87, 495–509. doi: 10.4141/CJSS07022

Chen, H., Hou, R., Gong, Y., Li, H., Fan, M., Kuzyakov, Y., 2009. Effects of 11 years of conservation tillage on soil organic matter fractions in wheat monoculture in Loess Plateau of China. Soil Tillage Res., 106, 85–94.

Gregorich, E.G., Carter, M.R., Angers, D.A., Monreal, C.M., Ellert, B.H., 1994. Towards a minimum data set to assess soil organic matter quality in agricultural soils. Can. J. Soil Sci. 74, 367–385.

Franzluebbers, A.J., Stuedemann, J.A., Schomberg, H.H, Wilkinson, S.R., 2000. Soil organic C and N pools under long-term pasture management in the Southern Piedmont, USA. Soil Biol. Biochem., 32 469–478.

Martínez-Mena M., López, J., Almagro M., Boix-Fayos C. Albaladejo J., 2008. Effect of water erosion and cultivation on the soil carbon stock in a semiarid area of South-East Spain. Soil Tillage Res., 99, 119-129.

Mrabet, R., Ibno-Namr, K., Bessam, F., Saber, N., 2001. Soil chemical quality changes and implications for fertilizer management after 11 years of no-tillage wheat production systems in Semiarid Morocco. Land Degrad. Develop., 12, 505–551.

Moharana, P.C., Naitam, R.K., Verma, T.P., Meena, R.L., Kumar, S., Tailor, B., Singh, L., Singh, R.S., Samal, S.K., 2017. Effect of long-term cropping systems on soil organic carbon pools and soil quality in western plain of hot arid India. Arch. Agron. Soil Sci., 63, 1661–1675. doi: 10.1080/03650340.2017.1304637

Somasundaram, J., Reeves, S., Wang, W., Heenan, M., Dalal, R., 2017. Impact of 47 years of no tillage and stubble retention on soil aggregation and carbon distribution in a vertisol. Land Degrad. Develop., 28, 1589–1602. doi: 10.1002/ldr.2689

2.2.10 Soil greenhouse gas emissions

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Importance and applications

The fluxes of nitrous oxide (N_2O), methane (CH_4) and carbon dioxide (CO_2) from soils are indicators of microbial activities in soils. The values measured can be used to estimate the losses of nitrogen and carbon from soils as well as the impact of agricultural production on global warming.

Principle

This method is used to determine gaseous fluxes between the soil and atmosphere. The origin of the flux is microbial metabolism as part of elemental cycles. The principle is to cover an area of soil surface with a measurement chamber and follow the change of gas concentration in the chamber during its enclosure. For this, samples are taken for laboratory analysis or drawn to an analyser in the field to determine the concentration of a gas in the chamber at a defined point in time. The gas flux rate is determined as a function of the concentration in time.

Reagents

- Gases for the gas chromatograph as required by the measurement method
- Reference gases of known concentration

Materials and equipment

- Frames for gas-tight use of chambers
- Vented chambers for gas sample collection. Vent is a 0.5–1 m tube with a diameter of 3–4 mm. Mixing of air during sampling can optionally be achieved by a battery-operated fan or by applying a perforated sampling probe extending from the top to the bottom of the chamber
- Sample vials (glass with rubber septa)
- Syringes with needles
- Stopwatch
- Gas chromatograph or a flow-through instrument for gas analysis

Procedure

N_2O and CH_4 fluxes:

a. Take the chambers to their positions but do not close them yet
b. Close the first chamber and start the stopwatch. Take the first sample from the air inside the chamber
c. Close the second chamber and take the sample 1 or 2 minutes after the first sampling. The time depends on the distance of the chambers and the time available before the next sampling point.

Enough time must be allowed to walk between the chambers. Repeat this procedure as long as the first sample of every chamber has been taken

d. Start the second round of sampling. Proceed the same way as during the first round but mix the air in the chamber by filling and emptying the syringe 3-5 times before taking the sample (if there is no fan in the chambers)
e. Proceed with all 4 rounds as above. Options for timing of sampling rounds include e.g. 0, 15, 30, 45 minutes or 0, 20, 40, 60 minutes
f. Make notes of the air temperature and any deviations of the timing etc. Also remember to take the reading of frame height from the soil surface for determining the total headspace volume
g. Take the samples to the laboratory and proceed with the analysis as defined in your laboratory guidelines

CO_2 flux from soil respiration:

a. Install the chamber on place
b. Take the recordings of CO_2 concentration for 1–3 minutes (or as needed for your instrument) and air temperature during the sampling
c. Repeat the procedure for each chamber

Install the chamber frames at a depth of at least 15 cm to keep the roots out of the chamber area and remove all growing plants the day before the measurement. If it is not possible to install the frames before root growth, take into account that the trenched roots may cause overestimation of soil respiration which may require correction in the calculation phase. Respiration from living roots should be excluded but the dead roots from the previous growing season should be present in the measured plot.

Calculations

N_2O and CH_4 fluxes:

A script for case-wise linear or non-linear method selection is used for calculating the results. It implements a selection algorithm using the minimum detectable flux for selecting between the linear and non-linear calculation (Hüppi et al., submitted).

a. Determine the minimum detectable flux of your measurement system at 95% confidence level as instructed in Appendix 3 of De Klein and Harvey (2015) or using the function in the gas fluxes R-package.
b. Calibrate the detector (concentration vs. peak area). Use a reference gas range similar to that of the samples.
c. Using the calibration curve, first calculate the sample concentration as µmol mol-1 (ppm). Sample concentration (µmol mol^{-1}) is divided by the volume of ideal gas (l mol^{-1}) yielding the chamber air concentration as µmol L^{-1}. Multiply the concentration (µmol L^{-1}1) by the molar weight of the gas (g mol^{-1}) to convert the unit to µg L^{-1}1.

The volume of ideal gas must be temperature-corrected, thus correct for the chamber or air temperature using the ideal gas law. The volume of ideal gas is calculated as V = 0.082056 × (273.15 + T), where

0.082056 is the gas constant (R) and T is the chamber or ambient air temperature (°C).

R=PV/nT; at standard temperature and pressure R= 1 atm × 22.414 litres/1 mol ×273.15 K = 0.082056 L atm mol^{-1} K^{-1}

d. Use the gas fluxes R-package to calculate the fluxes for each chamber from the sample concentration values. For this you will need the headspace volume, frame area and time of sampling from the start of the chamber enclosure. The script will provide both a linear and "robust" non-linear flux estimate

In the winter, the chamber volume needs to be corrected for the volume of snow inside the chamber. For this, sample a known volume of snow and based on this information divide the snow mass in the chamber with the density of ice (0.9168 g cm^{-3}).

The preferable units are mg N_2O m^{-2} h^{-1} or mg CH_4 m^{-2} h^{-1}. This method also provides the estimate of ecosystem respiration if your gas chromatograph measures CO_2.
A minimum of three time points are needed for each flux calculation.

CO_2 flux:
The flux rates will be calculated from the concentration data as above for N_2O and CH_4.
The gaps between measurements will be filled with hourly estimates of soil respiration using empirical modelling for each measurement plot based on the temperature-dependence of soil respiration (Lloyd & Taylor, 1994). Hourly soil temperature measurements (depth of 5 cm) are used for this purpose.

$$R = R_{10}e^{E_0\left(\frac{1}{283.15-T_0}-\frac{1}{T-T_0}\right)} = R_{10}e^{308.56\left(\frac{1}{56.02}-\frac{1}{T-227.13}\right)} \quad \text{(Eq. 2.2.10.1)}$$

where

R is the soil respiration;

$\mathbf{R}_{10}$ is the soil respiration at 10°C;

T is the soil temperature.

Further grouping (e.g. seasonal) of the annual measurement data may be needed in order to increase the reliability of modelling. The hourly modelled values are summed to yield daily and annual values.
The preferable units are mg CO_2 m^{-2} h^{-1}

Remarks

- Every group can use their existing chambers, vials and analysis equipment. Apply one chamber per plot yielding 3–4 replicates. Random chamber placement is recommended but in farmers' fields it is also polite and practical to plan the placement so that e.g. dimensions of spraying equipment are taken into account (so that the farmer can drive between the chamber frames during the growing season).
- As the chambers used for soil respiration measurement are typically smaller than the ones used for N_2O and CH_4 more replicates are needed than for the larger chambers
- For experiments with different subplot areas that are expected to have different emission dynamics (like in vineyards with berms and rows), it is suggested to measure in both conditions on each plot if it

is relevant to the experimental treatment

- The enclosure time varies with the chamber dimensions. General advice is found in Klein and Harvey (2015) but the quality of the results tells us if the enclosure was long enough
- The measurements should be taken between 10.00 and 12.00 to avoid bias by temperature. For the same reason, it is also a good practice to vary the chamber sequence between sampling days
- The intended number of samplings per year is 25 and these can be allocated so that periods of high emissions such as fertilisation, flooding, snowmelt, harvest and other abrupt system changes are well represented. It is very important that a measurement is conducted very soon after such an abrupt system change

References

De Klein, C., Harvey, M., 2015. Nitrous oxide chamber methodology guidelines. Ministry for Primary Industries, Wellington, NZ.
https://globalresearchalliance.org/wp-content/uploads/2015/11/Chamber_Methodology_Guidelines_Final-V1.1-2015.pdf

Hüppi, R., Felber, R., Krauss, M., Six, J., Leifeld, J., Fuss, R., (in press) Restricting HMR's nonlinearity parameter for more reliable soil greenhouse gas flux estimates. Submitted to PLOS.

Lloyd, J., Taylor, J.A., 1994. On the Temperature-Dependence of Soil Respiration. Funct. Ecol., 8, 315−323. doi: 10.2307/2389824

2.2.11 Pesticides

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Importance and applications

492 active substances for pest and weed control are present in more than 2000 pesticides on the European market with 26 active substances pending (Reg. (EC) No 1107/2009; http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.selection&language=EN). Pesticides enter into the soil as a result of plant protection measures (weed control and pest control). Although the persistence of pesticides has strongly decreased in the last decades, a number of studies describe the occurrence of mixtures of (persistent) pesticides in soils as a result of long-term annual applications (e.g., Organochlorines like DDT and its metabolites, forbidden in 1973 in Europe, Ferencz & Balog, 2010; or Glyphosate and its metabolite AMPA, Gui et al., 2014).

Principle

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method seems to be the most consensual option for obtaining a wide spectrum of pesticides residues in soils. Extraction with acetonitrile containing 1% acetic acid (ACN 1% HAc) is used to extract organophosphate, organochlorine, carbamate, thiocarbamate, urea, triazine, and other types of pesticides, according to Mol et al., (2008) (Fig. 2.2.10.1). For Glyphosate and AMPA, column characteristics and instrumentation conditions as followed according to Bento et al., (2016) and Yang et al., (2015, Fig. 2.2.10.1).

Reagents

- See Fig. 2.2.10.1

Materials and equipment

- GC-MS, LC-MS, SRM
- Plastic beakers (200 mL)
- Timer

Procedure

Soils should be collected preferably at 2 depths, 0–10, 10–30 cm, and after being air dried and 2 mm sieved, they must be preserved at -18°C until the determination of pesticides is performed.

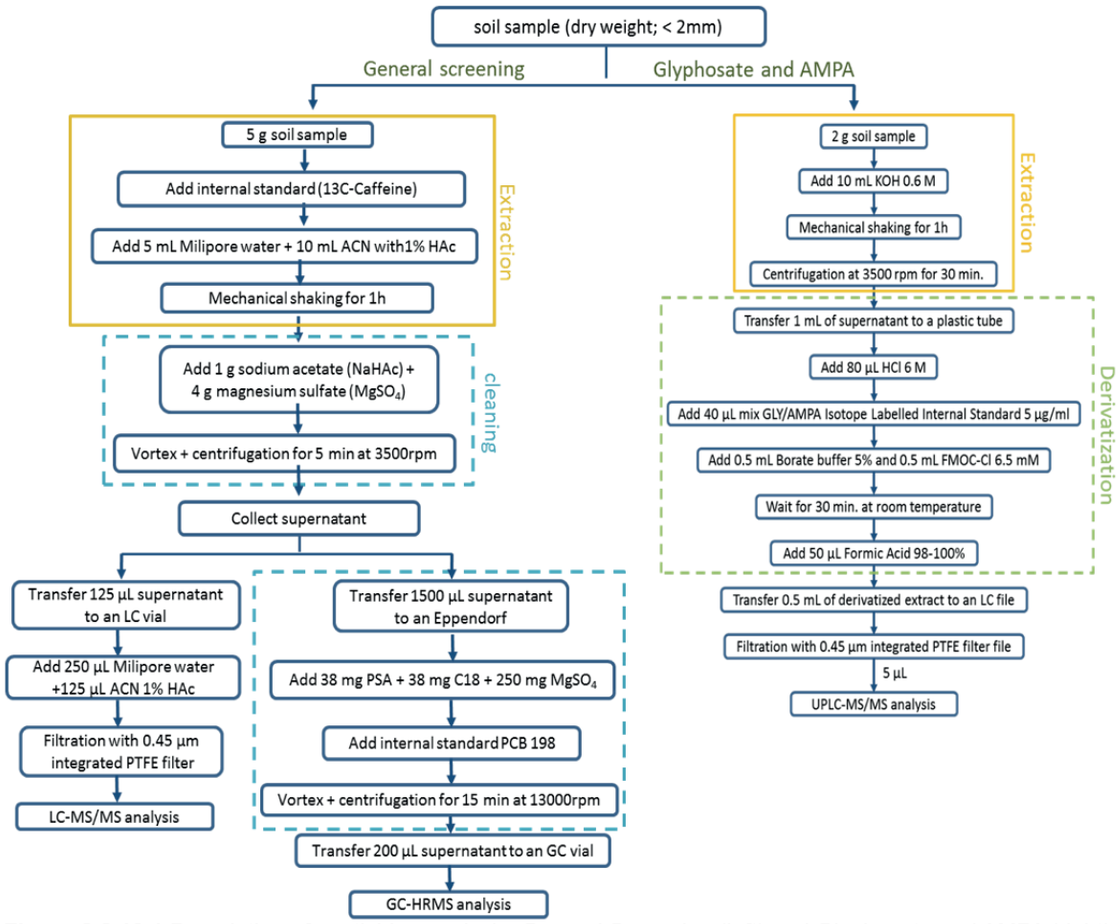

Figure 2.2.10.1 Description of pesticides analysis. General Screening (left) and Glyphosate and AMPA (right). Reagents and procedure

References

Bento, C.P.M., Yang, X., Gort, G., Xue, S., van Dam, R., Zomer, P., Mol, H.G.J., Ritsema, C.J., Geissen, V., 2016. Persistence of glyphosate and aminomethylphosphonic acid in loess soil under different combinations of temperature, soil moisture and light/darkness. Sci. Total Environ., 572, 301–311. doi: 10.1016/j.scitotenv

Ferencz, L., Balog, A., 2010. A Pesticide survey in soil, water and foodstuffs from Central Romania. Carpathian J. Earth Environ. Sci., 5, 1, 111–118.

Gui, Y.-x., Fan, X.-n., Wang, H.-m., Wang, G., 2012. Glyphosate induced cell death through apoptotic and autophagic mechanisms. Neurotoxicol. Teratol., 34, 344–349. doi: 10.1016/j.ntt.2012.03.005

Mol, H.G.J., Plaza-Bolanos, P., Zomer, P., de Rijk, T.C., Stolker, A.A.M., Mulder, P.P.J., 2008. Toward a generic extraction method for simultaneous determination of pesticides, mycotoxins, plant toxins, and veterinary drugs in feed and food matrixes. Anal. Chem., 80, 9450–9459. doi: 10.1021/ac801557f

Yang, X., Wang, F., Bento, C.P.M., Meng, L., van Dam, R., Mol, H., Liu, G., Ritsema, C.J., Geissen, V., 2015. Decay characteristics and erosion-related transport of glyphosate in Chinese loess soil under field conditions. Sci. Total Environ., 530–531, 87–95. doi: 10.1016/j.scitotenv.2015.05.082

PART 3. SOIL BIOLOGICAL ANALYSIS

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

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Importance and applications

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

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

Today, methods in soil biology enable an in-depth analysis of microbiomes and other key species living in the soil. However, it is indispensable to exactly define common methods and protocols in order to come up with comparable and combinable findings between the individual projects in order to achieve an even higher mutual output of the project. Consequently, it is the objective of this handbook to provide detailed information and instructions on parameters and endpoints that will be investigated related soil biology, such as:

- DNA and RNA extraction, storage, shipping and handling.
- Next generation sequencing of DNA for qualitative metagenomics using an Ion Torrent and MiSeq sequencer, respectively.
- Bioinformatics for metagenomics data evaluation.
- qPCR of functional genes, i.e. *amoA, nirK, narG*.
- qPCR of selected pathogens and pests.
- Microbial enzyme activities (fluorogenic or colorimetric determination):
    - dehydrogenase
    - ß-glucosidase
    - leucine-aminopeptidase

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

Soil sampling

Soil samples destined for soil biological testing must be subsamples of the same bulk used to measure all different parameters (i.e. DNA and RNA extraction, enzymatic activities, total carbon, total nitrogen, clays etc.). Mulch layers, litter layers, stone layers and crusts at the soil surface should be excluded from soil biological analysis (unless purposely targeted). Furthermore, rhizosphere and areas with entangled roots (e.g. typical for permanent grassland) should be avoided during sampling, unless it is a specific research question of the project.

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

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

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

Soil preparation

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

the sample.

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

Soil Dry Mass and Water Content (Wilke, 2005)

Principle. Soil samples are dried at 105 ±5°C until mass constancy is reached. The mass difference between moist and dried soil is the measure of the water content. The water content is calculated on a gravimetric basis (g water /g soil), following ISO 11465 (1993). Calculation based on volumes is also possible (cm^3 water/cm^3 soil). Finally, the water content of soil is given as a percentage by weight of oven-dried soil. The optimum water content for microbial processes is in the range of 40–60% of the maximum water-holding capacity (WHC), which corresponds to water suction pressures of −0.01 to −0.031 MPa.

Equipment. Drying oven, thermostatically controlled with forced air ventilation and capable of maintaining a temperature of 105 ±5°C; desiccator with an active drying agent; analytical balance, accuracy 1 mg; glass or porcelain jars with lid (25−100 mL volume).

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

Calculation. Calculate the dry mass content (*w*dm) or water content (wH_2O) on a dry mass basis expressed as percentages by mass with an accuracy of 0.1% (m/m) using the following equations:

$$wdm\ (\%) = (m_2 - m_0) / (m_1 - m_0) \times 100$$

$$wH_2O\ (\%) = (m_1 - m_2) / (m_2 - m_0) \times 100$$

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

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

Soil Water Holding Capacity (WHC)

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

represent useful information for laboratory testing.

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

Equipment. Funnel, glass containers, e.g. measuring cylinders, filter paper (Schleicher-Schuell, 595 ½ or similar), analytical balance, accuracy 10 mg.

Procedure. Prepare a funnel with a filter (e.g. folded filter Schleicher-Schuell, 595 ½). Moisten the filter paper with water (but avoid excess of water that drips off) and record the mass of the filter and funnel with an accuracy of ±10 mg ($\mathbf{m_0}$). Weigh 10 to 20 g of soil into the funnel with filter. Use approximately similar weights for the different samples (for example do not weigh in 10 g of one sample and 20 g of the replicate sample) and record the exact mass with an accuracy of ±10 mg ($\mathbf{m_1}$). If moist soil is used the exact amount of contained water must be known ($\mathbf{m_2}$) (see above the method on "Soil Dry Mass and Water Content"). Place the funnel into a container in order to collect leaching water. If the amount of leaching water is to be determined on a mass basis, the mass of the empty container must be determined beforehand with an accuracy of ±10 mg ($\mathbf{m_3}$). Add an excess of water using a defined volume (e.g. 50 mL = $\mathbf{m_4}$), and wait until no more water drips off (typical waiting time 2 hours but this depends on the soil texture). Place a lid (e.g. a Petri dish) on top of the funnel to avoid losses due to evaporation. Pour the leached water again onto the soil in the funnel. Repeat this step once more (in total three times). Finally, determine the mass of the leached water in the container ($\mathbf{m_5}$) and/or the mass of the funnel with the filter paper and the moist soil ($\mathbf{m_6}$).

Calculation. Calculate WHC (mL/g) using the following equations (a – e). The mass (or volume) of retained water can be either determined from the mass of water saturated soil (a and b) or the mass of leached water (c and d).

For simplicity, the mass and volume of water are considered as 1 g = 1 mL.

a) $m_6 - m_0 = soil_{saturated}$ *[g]*

b) $soil_{saturated} - m_1 + m_2 = water_{retained}$ *[g]*

c) $m_5 - m_3 = water_{leached}$ *[g]*

d) $m_4 + m_2 - water_{leached} = water_{retained}$ *[g]*

e) $water_{retained} / (m_1 - m_2) = WHC$ *[g/g] ~[mL/g]*

3.0.1 Storage of soil samples prior to subsequent biological analyses

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Importance and applications

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

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

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

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

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

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

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

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

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

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Importance and applications

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

Principle

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

Storage of soil samples for DNA extraction. Procedure

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

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

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

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

Collection/storage of soil samples for RNA extraction. Procedure

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

1. Weigh 2.5 g of soil in a 15 mL RNase and DNase free Tube (i.e. a 15 mL screw-cap cryogenic tube) and add 6 mL of LifeGuardTM Soil Preservation Solution (1 g of soil requires 2.5 mL of solution – the solution can be added to the tubes in sterility, before going to the field). If the soil cannot be weighed in the field use a volume of soil as a reference (a 5 mL tube, or equivalent).
2. Vortex or gently mix the soil and the solution by hand to obtain a mixture.
3. Store the soil in the LifeGuardTM Soil Preservation for one month at -20°C (2 weeks at 4°C or 1 week at room temperature.
4. Shipping can be performed at this stage; at 4°C, using boxes with ice packs as in eluted DNA shipping (see section 3.0.3).
5. When you are ready for the total RNA extraction, the samples can be slowly thawed at 4°C, if kept at -20°C, then centrifuged at 2500 x g for 5 min at 4°C to remove the solution and collect the soil to be further processed.

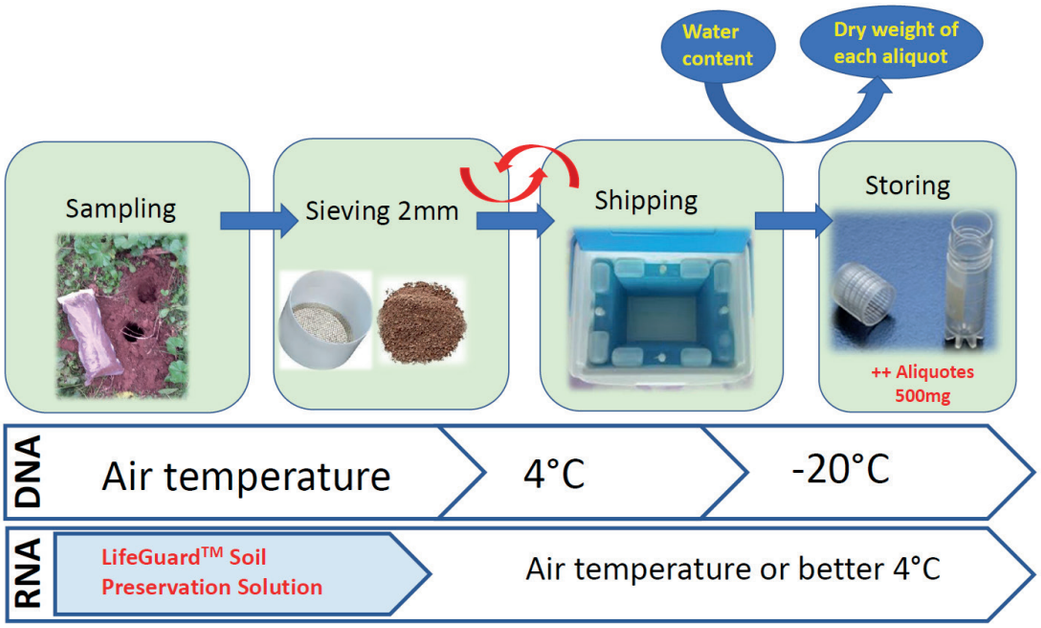

Figure 3.0.1 Diagram of the main steps needed for sampling and handling soil prior to DNA and RNA extraction

Reagents

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

Materials and equipment

- 2 mL screw-cap, sterile, RNase-free, DNase-free cryogenic tubes (i.e. from BRAND, code 114841, or Sigma-Aldrich screw-cap TPP® 2.0 mL cryotubes, code Z760951)
- Cold resistant small labels and/or adhesive tapes
- Permanent-ink pen or labels with cold resistant glue (try before use)
- Sterile 15 mL RNase-free, DNase-free screw-cap tubes (if used for shipping be sure the closure is safe for liquids).
- Box container for 2 mL vials (and for 15 mL vials in case you ship soil for RNA extraction)
- Styrofoam boxes
- wet ice packs
- Parafilm
- -20°C freezer
- Precision scale (4 decimals)
- A field scale (to weigh soil for RNA extraction, 2.5 g/6 mL LifeGuard solution)
- Gloves suitable for RNase and DNase free lab.

3.0.3 Storage and shipping of eluted DNA

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Importance and applications

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

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

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

Procedure

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

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

Remember to always include a shipping information sheet that contains detailed sample information, indicating the person/s receiving the parcel and the sending laboratory. Use glued paper labels to identify your samples. If you write with a marker, it can fade away with ice/freezing.

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

clean and dry. Place all into a thermo-stable shipping box (i.e. Styrofoam, filled with ice packs) and if there is space add other clean packing materials to ensure that the ice packs and the box containing your DNA are not shaken.

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

References

Bainard LD, Klironomos JN, Hart MM (2010) Differential effect of sample preservation methods on plant and arbuscular mycorrhizal fungal DNA, Journal of Microbiological Methods, 82(2)124-130.

Creamer R.E., Hannula S.E., Leeuwen J., Stone D., Rutgers M., Schmelz R.M., Ruiter P., Hendriksen N., Bolger T., Bouffaud M.L., Buee M., Carvalho F., Costa D., Dirilgen T., Francisco R., Griffiths B.S., Griffiths R., Martin F., Silva P.D., Mendes S., Morais P.V., Pereira C., Philippot L., Plassart P., Redecker D., Römbke J., Sousa J.P., Wouterse M., Lemanceau P. (2016) Ecological network analysis reveals the inter-connection between soil biodiversity and ecosystem function as affected by land use across Europe. Applied Soil Ecology, 97, 112-124, doi: 10.1016/j.apsoil.2015.08.006, 2016.

Ivanova NV, Kuzmina ML (2013) Protocols for dry DNA storage and shipment at room temperature. Molecular Ecology Resources. 13(5):890-898. doi:10.1111/1755-0998.12134.

Plassart P, Terrat S, Thomson B, Griffiths R, Dequiedt S, Lelievre M, et al. (2012) Evaluation of the ISO Standard 11063 DNA Extraction Procedure for Assessing Soil Microbial Abundance and Community Structure. PLoS ONE 7(9): e44279.

Roder B, Fruhwirth K, Vogl C, Wagner M and Rossmanith P (2010) Impact of long term storage on stability of standard DNA for nucleic acid based methods Journal of Clinical Microbiology. 48:4260-4262

Rubin BER, Gibbons SM, Kennedy S, Hampton-Marcell J, Owens S, Gilbert JA. (2013) Investigating the Impact of Storage Conditions on Microbial Community Composition in Soil Samples. PLoS ONE.;8(7):e70460.

Schloter M., Nannipieri P., Sørensen, S. J., van Elsas J. D. (2018) Microbial indicators for soil quality. Biology and Fertility of Soils, 54, 1-10, doi: 10.1007/s00374-017-1248-3, 2018.

Terrat S, Plassart P, Bourgeois E, et al. (2015) Meta-barcoded evaluation of the ISO standard 11063 DNA extraction procedure to characterize soil bacterial and fungal community diversity and composition. Microbial Biotechnology. 8(1):131-142.

Wilke B.-M. (2005) Determination of Chemical and Physical Soil Properties. In Margesin, R. Schinner F. (Eds.) Soil Biology -, Vol. 5 Manual for Soil Analysis, pg. 47-49, Springer-Verlag, Berlin Heidelberg 2005

3.1 Deoxyribonucleic acid (DNA) extraction

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Importance and applications

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

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

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

Standardisation in the sense of shared sample handling and analysis protocols, based on strictly defined procedures as defined for example by the International Organization of Standardization (ISO), are needed

when microbial communities from different soils must be compared based on analysis performed by different laboratories. Philippot et al. (2012) developed and validated a protocol for direct extraction of total DNA from soil samples (Philippot et al., 2012), which was formally acknowledged as the ISO-11063 method. This standard has been further modified on the basis of subsequent studies (Plassart et al., 2012; Terrat et al., 2015) aimed at improving its efficiency towards fungal DNA (Terrat et al., 2015).

Principle

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

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

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

One effective kit for isolating total DNA is the DNeasy PowerSoil® DNA Isolation Kit (Qiagen). In principle it is a standard kit that includes three previous steps to disrupt the microbial cells in the soil, precipitate proteins and clean the sample from humic compounds that inhibit further downstream processing such as PCR or ligation. The procedure is very effective for isolating DNA molecules of all types of soil sample with high or low microbial load. This kit uses patented inhibitor removal technology to remove PCR inhibiting compounds, including humic substances associated with soil DNA.

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

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

Soil sampling and pre-processing

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

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

Soil DNA extraction: living and dead biota

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

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

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

1. **Add 0.5 grams of the soil sample to PowerBead Tubes and gently vortex for 30 min.** After the sample has been loaded into the PowerBead Tube, begin dissolution of humic acids, and proteic acid degradation. This is the first critical step to complement the following cell lysis step in which the sample disperses in the PowerBead Solution).
2. Check Solution C1, pre-heated to 70°C for 5 min to dissolve the precipitate. **Add 60 µL of Solution C1 to step 1 and briefly vortex to mix.** Solution C1 contains SDS, thus this is an important step in which the addition of Solution C1 allows the cell lysis to form a white precipitate in the Eppendorf within a few minutes of reaction.
3. **Vortex PowerBead Tubes using a vortex or a flat-bed vortex pad with tape and vortex at maximum speed for 10 min when we use less than 12 preps.** If we use more than 12 preps we extend the vortex up to 20 min.
4. **Centrifuge at room temperature for 2 min at 10 000 x g.** The supernatant may still contain some particles. While centrifuging, **add 250 µL of Solution C2** into each clean tube. **Incubate at 4°C for 15 min** (the incubation can be extended to overnight).
5. **Remove the tube of Step '4' from the centrifuge and carefully transfer the supernatant (between 400 and 500 µL) to step '5'.** Discard the pellet.
6. **While centrifuging, aliquot 200 µL of Solution C3 into each clean tube.**
7. **Remove the tube of Step '6' from the centrifuge and carefully transfer the supernatant to step '6'. Discard the pellet. Incubate at 4°C for 15 min.**
8. **Centrifuge at room temperature for 2 min at 10 000 × g.**
9. **After centrifugation, carefully remove the tube and transfer the entire volume (up to 750 µL) into a clean tube, avoiding the pellet which must be discarded. Add 1.2 mL of Solution C4, and mix**

gently. The pellet at this step contains additional non-DNA organic and non-organic material including humic acid, cell debris, and proteins. The volume at this step is 1850 µL (1200 µL of C4 + 750 µL of sample by step 8).*Take care to shake solution C4 before use; this is a high concentration salt solution allowing binding of DNA to the Spin filters provided by the kit.*

10. **Load 675 µL of step '9' onto a Spin Filter and centrifuge at 10 000 × g for 1 min at room temperature. Load the remaining supernatant onto the spin filter and centrifuge at 10 000 × g for 1 min at room temperature (~ 3 loading).** At this step, DNA is selectively bound to the silica membrane in the spin filter device in high salt solution. Discard the flow through at the end of each centrifugation.
11. **Add 500 µL of Solution C5 to each tube of the spin tube. Apply centrifuge to the tube at room temperature for 5 min at 10 000 × g**
12. **Carefully discard the flow through and repeat centrifugation for 1 min to avoid residual Solution C5.**
13. **Carefully transfer the spin filter to a clean tube and add 100 µL of Solution C6 (preheat to 60ºC) to the centre of the white filter membrane of the spin filter.** (It is advisable to clean the outer part of the filter from any droplet left using clean paper. Solution C6 is a sterile elution buffer. Let C6 sit on the filter for 5' at room temperature before the final centrifugation step.
14. **Centrifuge at room temperature for 3 min at 10 000 x g. Discard the Spin Filter. The DNA is now ready for any downstream analysis.**
15. **Keep DNA frozen** (-20ºC to -80ºC) for medium- to long-term storage. Shipping should take less than one week (see chapter to 3.0.3 for storing and shipping DNA). Split the eluted DNA into three tubes. Tube 1 remains stored in your lab, tube 2 is for analysis of bacteria and tube 3 is for fungi.

DNA yield and quality check

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

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

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

DNA should have a A260/A280 ratio >1.7

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

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

DNA concentration and purification

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

The Amicon Ultra 0.5 mL Centrifugal Filters (EMD Millipore Corporation, Billerica, MA) for DNA Purification and Concentration is the most efficient device used in the extraction process to remove potential PCR inhibitors and concentrate DNA, obtaining optimal downstream analysis results. The Amicon Ultra-0.5 device is supplied with two microcentrifuge tubes. The manufacturer's recommendations for purification and concentration of nucleic acids indicate that the 30K NMWL device is optimal for recovering nucleic acids. After extraction, the total amount of DNA can be added to the microcentrifuge tube provided by the kit. Following the manufacturer's instructions, place the filter device into the centrifuge rotor, and spin for 15' at room temperature, 7.500 g to recover the concentrated solute, invert the filter and concentrate the collection tube. Spin for 2' at 1.000 g to transfer the concentrated and purified sample from the device to the tube.

Dilution of DNA template

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

Amplification and inhibition test

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

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

Materials and equipment

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

Calculations

DNA concentration is estimated automatically both by NanoDrop software and by Qubit 2.0, using the following formula:
Concentration (µg/mL) = (A260 reading – A320 reading) × dilution factor × 50µg/mL.

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

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

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

the centre of the white filter membrane of the spin filter. (It is advisable to clean the outer part of the filter from any droplet left using clean paper. Solution C6 is a sterile elution buffer. Let C6 sit on the filter for 5 min at room temperature before the final centrifugation step.

13. **Centrifuge at room temperature for 3' minutes at 2500 × g. Discard the Spin Filter. The DNA is now ready for any downstream analysis.**
14. **Keep DNA frozen** (-20ºC to -80ºC) for medium- to long-term storage. Shipping should take less than one week (see section 3.0.3 for storing and shipping DNA). Split the eluted DNA into three tubes. Tube 1 remains stored in your lab, tube 2 is for analysis of bacteria and tube 3 is for fungi.

Remarks

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

References

Krsek, M., Wellington, E.M.H., 1999, Comparison of different methods for the isolation and purification of total community DNA from soil, Journal of Microbiological Methods, Volume 39, Issue 1, Pages 1-16, https://doi.org/10.1016/S0167-7012(99)00093-7.

Lombard N, Prestat E, van Elsas JD, Simonet P, 2011, Review: Soil-specific limitations for access and analysis of soil microbial communities by metagenomics. FEMS Microbiol Ecol. 2011 Oct; 78(1):31-49.

Morgan JL, Darling AE, Eisen JA (2010) Metagenomic sequencing of an in vitro-simulated microbial community. PLoS One. 2010 Apr 16; 5(4):e10209.

Philippot L, Ritz K, Pandard P, Hallin S, Martin-Laurent F, 2012, Standardisation of methods in soil microbiology: progress and challenges. FEMS Microbiol Ecol. 2012 Oct; 82(1):1-10.

Picard Ch., C. Ponsonnet, E. Paget, X. Nesme, P. Simonet 1992 Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction, Appl. Environ, Microbiol., 58 (1992), pp. 2717-2722

Plassart P, Terrat S, Thomson B, Griffiths R, Dequiedt S, Lelievre M, Regnier T, Nowak V, Bailey M, Lemanceau P, Bispo A, Chabbi A, Maron PA, Mougel C, Ranjard L. (2012) Evaluation of the ISO standard 11063 DNA extraction procedure for assessing soil microbial abundance and community structure. PLOS ONE. 2012;7:e44279 doi: 10.1371/journal.pone.0044279.

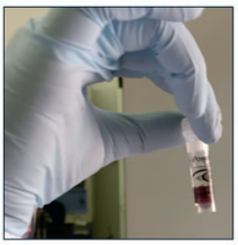
Soliman T., Yang S.-Y., Yamazaki T., and Jenke-Kodama H., 2017 Profiling soil microbial communities with next-generation sequencing: the influence of DNA kit selection and technician technical expertise. PeerJ. 2017; 5: e4178. doi: 10.7717/peerj.4178

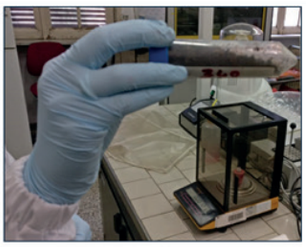
Terrat S, Plassart P, Bourgeois E, Ferreira S, Dequiedt S, Adele-Dit-De-Renseville N, Lemanceau P, Bispo A, Chabbi A, Maron PA, Ranjard L. (2015) Meta-barcoded evaluation of the ISO standard 11063 DNA extraction procedure to characterize soil bacterial and fungal community diversity and composition. Microbial Biotechnology. 2015;8:131–142. doi: 10.1111/1751-7915.12162.

Zielińska S, Radkowski P, Blendowska A, Ludwig-Gałęzowska A, Łoś JM, Łoś M, 2017 The choice of the DNA extraction method may influence the outcome of the soil microbial community structure analysis. robiologyopen. 2017 Aug; 6(4):.

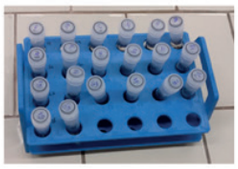
Images on DNA extraction

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

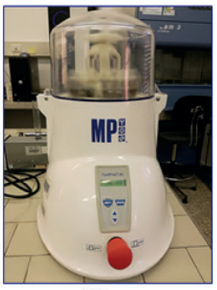




2. Check Solution C1, pre-heated to 70°C for 5' to dissolve precipitate. Add 60 µl of Solution C1 to step 1 and briefly vortex to mix

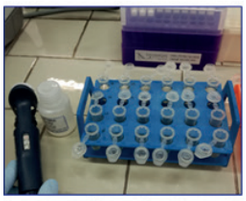
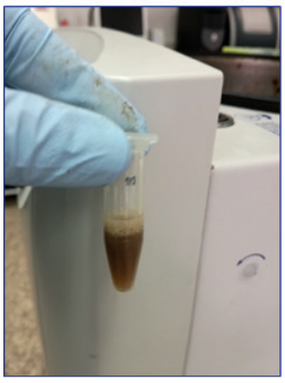
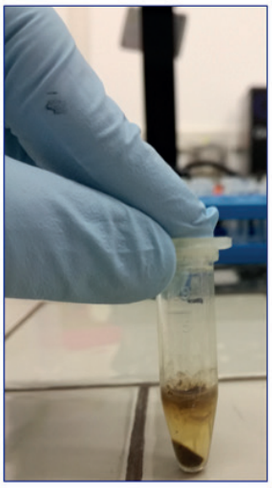
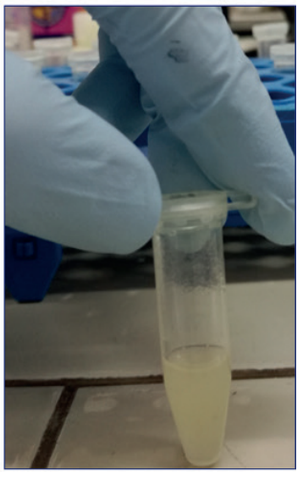



3. Vortex PowerBead Tubes using a vortex or a flat-bed vortex pad with tape and vortex at maximum speed up to 10 min,



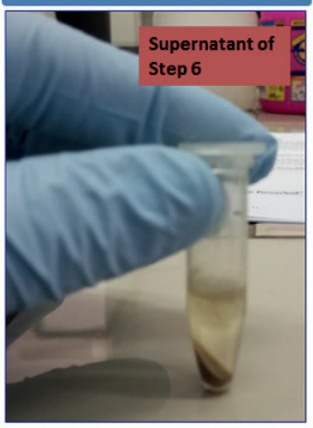
4. Centrifuge at room temperature for 2' minutes at 10000 x g.

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

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

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



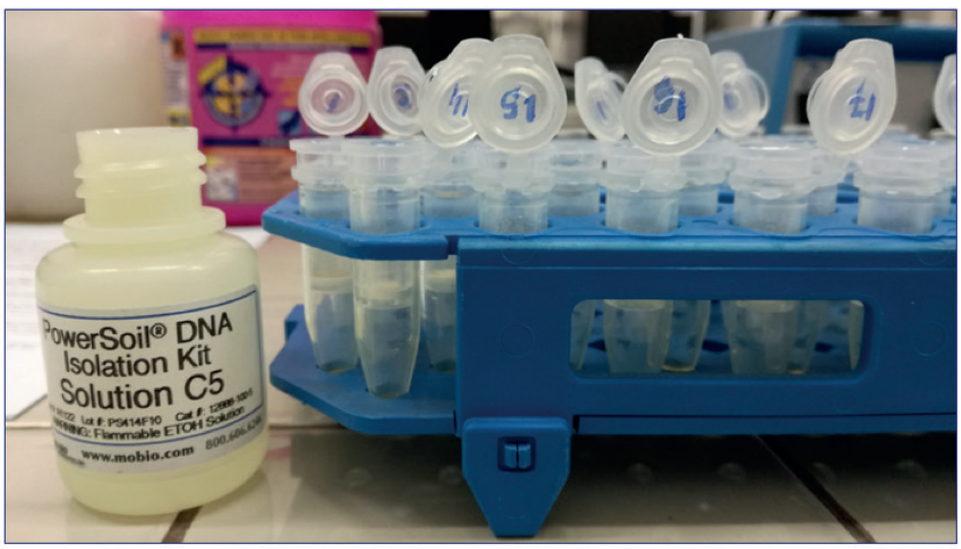
8. Centrifuge at room temperature for 2' minutes at 10000 x g.

Discard the pellet.

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

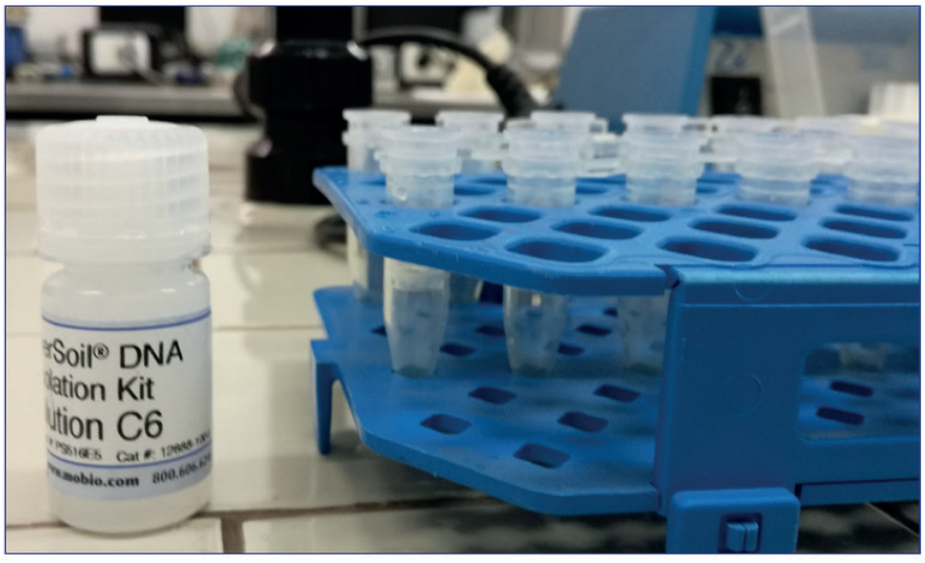
10. Load 675 µl of step '9' onto a Spin Filter and centrifuge at 10000 x g for 1' at room temperature. Load the remaining supernatant onto the spin filter and centrifuge at 10000 x g for 1' at room temperature (~ 3 loading).

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



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

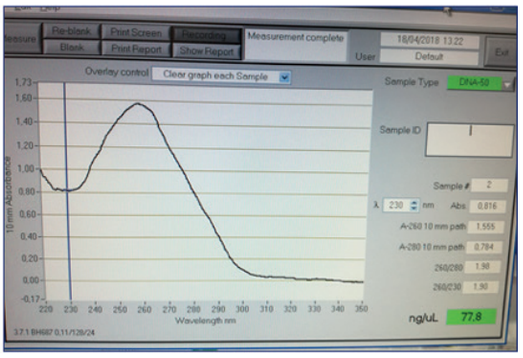
13. Carefully transfer the spin filter to a clean tube and add 100 µl of Solution C6 (preheat to 60ºC) to the centre of white filter membrane of spin filter.



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

Keep DNA

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



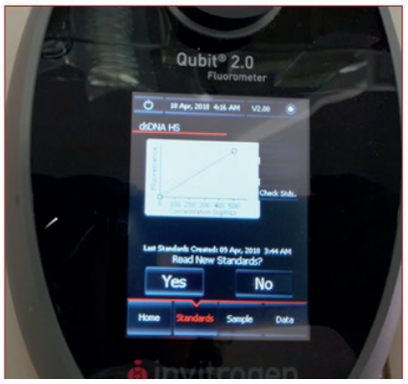


Table 3.1.1 Major requirements for and expected outcome from DNA extraction

			Concen-tration		Initial volume	Final volume	
Subsequent DNA analyses	**Technical replica-tes**	**Extrac-ted DNA samples**	**Ci ng/µL**	**C ng/µL**	**Vi µL**	**Vf µL per reaction**	**A260/ A280**
NGS ILLUMINA - fungi	1	1	minimum 10 ng/µL	10	12.5	12.5	>1.8
NGS Ion Torrent - bacteria	1	1	1.5 ng/µL		18	18	>1.8
qPCR Functional genes	3	3	minimum 10 ng/µL	10	10	90	>1.8
qPCR Pathogens	3	3	minimum 10 ng/µL	10	3	27	>1.8
						Total 147.5	

Table 3.1.2 Expected DNA yield from extraction

	extracted DNA Volume	**yield Range[b]**
250 mg soil[a]	100 µL	2 - 20 ng
10 g soil[a]	5 mL	50 - 400 ng

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

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

3.2 RNA extraction and reverse transcription

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Importance and applications

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

Principle

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

Reagents

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

Materials and equipment

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

Procedure

Soil RNA preparation: sample amount to process

Soil is sampled, pre-processed, stored and/or transported and shipped, respectively, following the common protocol (see chapter 3.0 and subsections). Also take note of the remarks at the end of this chapter. All this and a uniform RNA extraction procedure are important, since soil is a very complex environment, and

the relative abundance of species in soil microbial communities can be biased by the yield and quality of extracted RNA. The **RNeasy PowerSoil Total RNA Kit** is designed to process up to 2.0 g of soil, however it can be scaled up easily to accommodate 2.5 g of soil.

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

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

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

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

*Solution SR1contains SDS and requires heating at 60°C to dissolve the white precipitate.

6. *The following step is an additional step, so it is not included in the manufacturer's protocol.
7. Vortex at maximum speed for 5 min up to 10 min: this step is needed to ensure complete homogenisation and dissociation of nucleoprotein complexes. As an alternative to vortexing, shaking could be introduced using a horizontal shaker set at 25°C for 30 min, attaching the tubes horizontally to the platform.
8. Centrifuge at 2500 x g for 15 min and transfer the suspension obtained to a new tube. Discard the pellet.
9. *The following step is an additional step, so it is not included in the manufacturer's protocol. This step has been introduced to maximise the lysis and optimise the RNA yield. The CTAB reagent improves RNA extraction, giving consistently more RNA yield.
10. Add 2 mL of CTAB 2% (W/V), followed by 3.5 mL of phenol:choloform:isoamyl alcohol. Mix well to enhance the mixture. Remove the tube from the shaker and centrifuge the resulting mixture at 2500 x g for 15 min and transfer the aqueous phase containing RNA into a clean collection tube. Centrifugation separates the mixture into 3 phases: a red organic phase (containing proteins), an interphase (containing

DNA), and a colourless upper aqueous phase (containing RNA). We strongly recommend this step to gain a more complete lysis. Phenol:chloroform:isoamyl alcohol is added to the CTAB to maximise lysing efficiency and yield.

11. Vortex at maximum speed for 15 min. Remove the tube from the vortex and centrifuge at 2500 x g for 10 min at 4°C.

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

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

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

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

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

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

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

20. Add the RNA isolation sample of step 14 to the **RNA Capture column** and allow it to gravity flow through the column.
21. After collecting flow through, wash the column adding 1 mL of solution SR5 and allow it to gravity flow through the column. During this step, the nucleic acids are bound to the column matrix, and the second washing with SR5 ensures the cleaning of unbound contaminants, avoiding the contamination of the eluted RNA.
22. Carefully transfer the RNA Capture Column to a new collection tube. Shake and add 1 mL of Solution SR6: in this step RNA is eluted through the column by gravity flow.
23. Carefully transfer the eluted RNA in a new **2.2 mL collection tube** and add 1 mL of solution SR4. Invert to mix and allow it to stand for 10 min at –20°C.
24. Centrifuge the resulting mixture at 13000 x g for 15 min at 4°C.
25. Discard the supernatant and air dry the pellet decanting the supernatant onto paper (by turning the opened tubes and keeping them upside-down on clean absorbing paper).

26. Resuspend the RNA pellet adding 100 µL of Solution SR7. This product is an RNase/DNase-free water without EDTA.

RNA yield and quality check

The quantification of RNA is essential to guarantee the suitability for downstream molecular biological applications such as PCR amplification, reverse transcription, and sequencing. The quality of RNA can vary depending on the extraction efficiency. RNA integrity and its concentration clearly affect all the downstream processes. There are several approaches used to evaluate RNA yield, purity and integrity. One main quantification approach is based on fluorescence or ultraviolet absorbance of RNA at a specific absorption peak at 260 nm. The intensity of the peak is proportional to the concentration of nucleic acid. The fluorescence method requires a fluorometer and an RNA-binding fluorescent dye that binds specifically to single-strand RNA. The intensity of fluorescence is directly proportional to the amount of binding RNA.

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

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

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

RNA concentration and purification

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

Follow the manufacturer's instructions of RNeasy Mini Kit.

Removal of genomic DNA

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

RNA reverse transcription in cDNA

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

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

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

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

5 × First-Strand Buffer	4 µL
0.1 M DTT	2 µL
RNaseOUT™ (40 units/µL) (optional)[a]	1 µL

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

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

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

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

Calculations

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

$$\textit{Concentration (µg/mL) = (A260 reading – A320 reading) × dilution factor × 40µg/mL.}$$

Remarks

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

To protect from the decay of RNA, it is recommended to use **LifeGuard™ Soil Preservation Solution** for the collection and transportation of soil samples. This product is a specially formulated proprietary fluid developed to protect RNA from degradation. It puts microbial RNA in soil samples into stasis immediately upon contact, preserving gene expression profiles and microbial community structure information. After adding the solution, soil microorganisms are maintained in stasis, and are immediately stabilised. According to the manufacturer's instructions, it is recommended to store nucleic acids for up to 30 days at -20°C, 1 week at 4°C, or 3 days at room temperature.

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

References

Anderson IC, Parkin PI. 2007. Detection of active soil fungi by RT-PCR amplification of precursor rRNA molecules. J. Microbiol. Meth. 68:248–253.

Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ. 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Appl. Environ. Microbiol. 66:5488 –5491.

Wang Y, Nagaoka K, Hayatsu M, Sakai Y, Tago K, Asakawa S, Fujii T. 2012. A novel method for RNA extraction from Andosols using casei and its application to amoA gene expression study in soil. Methods and Protocols. 96:793-802.

Mettel C, Kim Y, Shrestha PM, Liesack W. 2010. Extraction of mRNA from soil. Appl Environ Microbiol 76:5995–6000.

Wang Y, Morimoto S, Ogawa N, Oomori T, Fujii T. 2009. An improved method to extract RNA from soil with efficient removal of humic acids. J Appl Microbiol 107:1168–1177

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

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Importance and applications

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

Sample preparation and storage

Soils are sampled, transported, pre-processed, stored and shipped as described in chapter 3.0 and subsections therein. The DNA extraction is done following the method in chapter 3.1. For convenience, before starting PCR and qPCR dilute template DNA to10 ng/ µL and 1 ng/ µL.

Principle

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

Task 1 qPCR standard preparation and calibration of the qPCR assay

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

Task 2 Preparation of soil DNA template and inhibition test

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

Task 3 qPCR assay

Step 6: qPCR assay

Task 4 Validation and analysis of the qPCR assay

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

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

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

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

Reagents

All required reagents should be freshly prepared.

a. Soil DNA is extracted following the method described in chapter 3.1 of this Handbook.
b. DNA ladder with known lengths and concentrations of fragments.
c. Oligonucleotides used as PCR primers purified with standard desalting (vendor not specified).
d. Competent bacteria: *Escherichia coli* strain, usually used for cloning of PCR product.
e. Plasmid: Cloning vector replicating in *E. coli* containing annealing sites for SP6 and T7primers flanking the cloning site. Commercially-available cloning kits can also be used if they meet the requirements described here.
f. Enzyme: T4 DNA ligase. Not required if a commercial cloning kit is used.
g. Taq polymerase (and a commercial qPCR kit if used).
h. T4 gene T32.
i. Bovine serum albumin (CAS No. 9048-46-8).
j. Ampicilline sodium, $C_{16}H_{18}N_3NaO_4S$ (CAS No. 69-52-3). (Alternative: Kanamycin sulphate, $C_{18}H_{36}N_4O_{11} \times H_2SO_4$ (CAS No. 25389-94-0)).
k. Boric acid, BH_3O_3 (CAS No. 10043-35-3).
l. Deoxynucleotide solution, dNTPs.
m. Ethidium bromide (CAS No. 1239-45-8). **Note: Ethidium bromide is a highly toxic chemical. Although listed in the ISO standard, its use is not recommended, but should be replaced by less harmLess alternatives such as SYBR Green®!**
n. Ethylenediaminetetraacetic acid disodium salt (EDTA), $C_{10}H_{14}N_2O_8Na_2 \cdot \times 2\ H_2O$ (CAS No. 6381-92 6).
o. Glucose, $C_6H_{12}O_6$ (CAS No. 50-99-7).
p. Hydrochloric acid, HCl (CAS No. 7647-01-0).

q. IPTG, isopropyl-beta-D-thiogalactopyranoside (CAS No. 367-93-1).
r. Magnesium chloride, $MgCl_2$ (CAS No. 7786-30-3).
s. Magnesium sulphate, $MgSO_4$ (CAS No. 7487-88-9.
t. Molecular-biology-grade water, H_2O.
u. Potassium chloride, KCl (CAS No. 7447-40-7).
v. Sodium chloride, NaCl (CAS No. 7647-14-5).
w. Tris[hydroxymethyl]aminomethane, $C_4H_{11}NO_3$ (CAS No. 77-86-1).
x. X-Gal, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (CAS No. 7240-90-6).
y. 5.6 Product for bacterial culture medium: 5.6.1 BactoTM3) tryptone, enzymatic digest of casein.
z. 5.6.2 Yeast extract powder (CAS No. 8013-01-2).

aa. Ampicilline solution: 2 g of ampicilline sodium in 4 mL of 0.22 µm filter sterilised H_2O. Adjust to 20 mL with sterilised H_2O, prepare 1 mL aliquots and store at -20°C.
bb. EDTA, 0.5 mol/L, 186.10 g of EDTA in 1 000 mL of H_2O, adjusting with NaOH (10 mol/L) to pH 8.0.
cc. SYBR Green®.
dd. IPTG stock solution: 1 g of IPTG in 8 mL of H_2O. After careful mixing, the solution is adjusted to 10 mL and sterilised under security microbiology post. Prepare 1 mL aliquot of IPTG and store at 20 °C.
ee. Solid LB medium, 10 g of bactoTM tryptone, 5 g of yeast extract, 5 g of sodium chloride, 15 g of agar, in 1000 mL of H_2O. After autoclaving for 20 min at 120°C, 1 mL of ampicilline stock solution at 100 $mg \cdot mL^{-1}$ is added to LB medium and plated in Petri dishes (20 mL) under a security microbiology post.

100 µL of IPTG solution is plated on solid LB-amp medium. When the IPTG solution is entered in LB-ampicilline medium, 20 µL of X-Gal solution is plated on solid LB-amp medium. Solid LB medium is stored at 4°C until its use.

ff. SOC medium, 20 g of bacto™ tryptone, 5 g of yeast extract, 0.58 g of NaCl, 0.95 g of $MgCl_2$, 2.46 g of $MgSO_4$, 3.60 g of glucose in 1 l H_2O. Sterilise by 20 min autoclaving at 120°C. Prepare 950 mL aliquots and store at 20°C.
gg. Tris-HCl, 1 mol/L, 121.14 g of tris in 1 000 mL of H_2O, adjusting with 4 mol/L HCl to pH 8.0.
hh. TBE buffer × 10, pH 8.0, 108 g of tris base, 55 g of boric acid, 40 mL of 0.5 mol/L EDTA (pH 8.0) in 1 000 mL of H_2O.
ii. TBE buffer × 1: 100 mL of TBE buffer × 10 in 900 mL of H_2O.
jj. TE buffer × 10: pH 8.0, 100 mL of 1 mol/L Tris-HCl pH 8.0, 20 mL of 50 mmol/L EDTA pH 8.0 in 880 mL of molecular grade water.
kk. TE buffer × 1, 100 mL of TE buffer × 10 in 900 mL of H_2O.
ll. X-gal solution, 250 mg of X-Gal in 5 mL of dimethylformamide 5 mL. After careful mixing, prepare 0.5 mL aliquot and store at 20°C.

Materials and equipment

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

Procedure

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

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

Primers for qPCR (task1, step 1)

Suitable primer pairs as reported by the literature are listed in Table 3.3.1. General information on the amplicon design and the main parameters to be considered to design oligonucleotide primer pairs is given in the ISO standard 17601 (ISO, 2016).

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

Amplicon	Forward	reverse	Reference
amoA	amoA-1F GGGGTTTCTACTGGTGGT	amoA-2R CCCCTCKGSAAAGCCTTCTTC	Okano et al., (2004)
nirK	nirK876 ATYGGCGGVCAYGGCGA	nirK1040 GCCTCGATCAGRTTRTGGTT	Henry et al., (2004)
narG	narG1960m2F TAYGTSGGGCAGGARAAACTG	narG2050m2R CGTAGAAGAAGCTGGTGCTGTT	Lopez-Gutierrez et al., (2004)
Cloning site	SP6 ATTTAGGTGACACTATAG	T7 TAATACGACTCACTATAGGG	

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

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

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

The procedure used to generate qPCR standards targeting a sequence of the microbial gene of interest (GOI) from different DNA templates (pure bacterial or fungal isolate, environmental DNA or artificial DNA) is described in step 2 of task 1. It also reports the procedure used to insert the qPCR standard in a cloning vector, transform Escherichia coli and purify recombinant plasmids harbouring qPCR standard for further

use for qPCR assays. The quality of the DNA template used for amplifying the qPCR standard by PCR shall be verified by electrophoresis on 1% agarose gel (AGE) in TBE buffer stained with appropriate staining (e.g. SYBR Green®). The concentration of DNA is measured with fluorometer, comparing to known DNA standard in AGE, or spectrophotometer. The DNA template is diluted to10 ng/µL in a final volume of 20 µL and stored at -20°C.

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

amoA
Initial denaturation: 95°C, 10 min
39× 95°C 15 s, 58°C 30 s, 72°C 45 s
1× 95°C 15 s, 58°C 30 s, 72°C 45 s
Final extension: 72°C, 10 min
Expected PCR product length 491 bp

nirK
Initial denaturation: 95°C, 10 min
6× 95°C 15 s, 63 to 58°C 30 s (-1°C by cycle), 72°C 30 s, 80°C 15 s
40× 95°C 15 s, 60°C 30 s, 72°C 30 s, 80°C 15 s
Final extension: 72°C, 10 min
Expected PCR product length 165 bp

narG
Initial denaturation: 95°C, 10 min
6× 95°C 15 s, 65 to 60°C 30 s (-1°C by cycle), 72°C 30 s, 80°C 15 s
40× 95°C 15 s, 60°C 30 s, 72°C 30 s, 80°C 15 s
Final extension: 72°C, 10 min
Expected PCR product length 110 bp

Cloning site
Initial denaturation: 95°C, 10 min
40× 95°C 15 s, 65°C 30 s, 72°C 90 s
Final extension: 72°C, 10 min

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

The expected length of the amplicon is verified by electrophoresis on 2 % agarose gel in TBE buffer stained

with appropriate staining. Amplicons are purified either from the gel using appropriate methods or by using exclusion chromatography columns to remove primers. Purified amplicons are then quantified as described earlier in this chapter.

Cloning and dilution preparation of qPCR standard

Ligation of amplicon of qPCR standard.

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

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

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

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

Transformation of competent Escherichia coli

Thaw competent cells (10^8 cfu/µg of DNA) on ice. Add1 µL of the ligation reaction mixture to cells, mix smoothly (not pipetting up and down) and incubate on ice for 20 min. Heat shock cells by incubating at 42°C for 30 s and immediately place cells on ice and incubate for an additional 2 min. Add 950 µL of SOC medium and incubate at 37°C under agitation at 150 min^{-1} for one hour. Plate 100 µL aliquots of cells suspension onto LB/Amp/IPTG/X-Gal solid medium. Petri dishes are incubated at 37°C overnight in the dark.

Screening for recombinant clone

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

Purification and quantitation of plasmid

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

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

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

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

Prepare an initial solution (100 µL) containing 0.5 × 108 copies / µL in molecular grade water. Prepare tenfold serial dilutions until a concentration of 0.5 × 101 copies / µL is reached. Store dilutions at -80°C until used.

Calibration of the qPCR (task 1, step 3)

SYBR Green® qPCR assay

The qPCR calibration assay is performed on serial dilution of the linearised standard plasmid ranging from 108 to 10^1 copies per reaction. GOI is amplified by using the specific primers pair listed in Table 3.3.1. The amplification is carried out in a final 15 µL volume containing 2 µL of dedicated plasmid standard, 1 µmol/L of each primer, 7.5 µL of 2× Taq master mix or 1.5 µL of 10× Taq master mix containing SYBR Green®, dNTPs, $MgCl_2$ and Taq polymerase. Molecular grade water is added to reach the final reaction volume. qPCR reaction is performed in a real-time thermocycler according to the cycling program described in **qPCR standard preparation** for the amplicon of interest. The final extension in the program is replaced by a dissociation stage (melt analysis). The fluorescence is collected at the end each cycle and in melt analysis where the temperature is gradually raised from 60°C to 95°C. If a commercial qPCR-reaction kit is used, follow the manufacturer's instructions to set up reactions. qPCR calibration is performed in duplicate and two non-template controls (NTC) are also included.

Establishment of the calibration curve and calculation of qPCR efficiency

At the end of the assay, the results are analysed using the automatic option. qPCR is validated with four observations. They are: (1) no amplification in NTC reactions, (2) a single dissociation peak for each dilution of qPCR standard (3) a single qPCR product of correct size in AGE and (4) a linear calibration curve (standard curve) with r^2 equal or superior to 98 %. The qPCR calibration curve gives the number of cycle threshold (Ct) as a function of the amount of the log of the number of copy of standard sequences.

For example, in linear regression y= -3.3386 + 39.574 Ct at copy number 1 represents a Ct value of 39.574 and is the end point forming a line with slope -3.3386 when Ct versus the log standard copy number is plotted. The r^2 is the percentage of the data which matches the hypothesis that the given standards form a standard curve.

Ct can also be represented as an equation Ct=a×q+c where,
q is the copy number of qPCR standard;
a is the slope of the calibration curve;
c is the ordinate at the origin (Ct for 1 copy of qPCR standard);

The efficiency (E) of the qPCR assay is estimated in equation $E= 10^{(-1/\alpha)} - 1$ where,
α is the slope of the calibration curve.
For quick reference a calibration slope -3.32 is equal to 100 % efficiency. A 100 % efficient qPCR reaction in 2-fold- or a 10-fold-dilution of a given DNA template gives a Ct difference of 1 or 3.3, respectively.

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

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

- DNA of a soil mixture (different sub-samples representative of each experimental thesis) is extracted following the method described in chapter 3.1 of this Handbook. The quality of the DNA template used for amplifying the qPCR standard by PCR shall be verified by electrophoresis on 1% agarose gel (AGE) in TBE buffer stained with an appropriate staining (e.g. SYBR Green®; see Note). The concentration of DNA is measured with a fluorometer, comparing to the known DNA standard in AGE, or with a spectrophotometer.
- The extracted DNA is diluted to 10 ng µL-1 in a final volume of 100 µL and stored at -20°C.
- DNA is amplified in duplicate for each target gene, following the SYBR green chemistry protocol. The amplification is carried out in a final volume of 25 µL volume containing 10 µL of starting DNA, 12.5 µL of SYBR Green®, 1.2 µM of each primer, and PCR-grade water up to 15 µL.
- The amplified product is then purified after qPCR reaction, using a commercially-available kit. During this step, qPCR amplicon is validated following that reported here in **'Establishment of the calibration curve and calculation of qPCR efficiency- points 1, 2, 3.** If two PCR products are observed, a first purification by AGE shall be carried out to cut and purify the expected PCR product. A second purification shall be done to obtain a suitable amplicon for qPCR with a good quality.
- The concentration is measured with a fluorometer and the amplicon can be used to calculate the target gene of interest (GOI) copy number, following that described in **'Purification and linearization of plasmid'**.
- Prepare an initial dilution (100 µL) a known amount of copies in molecular grade water (108) and prepare a 10-fold dilution series, covering until 101 dilution.

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

Preparation of soil DNA (task 2, step 4)

As described in **Sample preparation and storage** soil DNA used for qPCR is diluted to 10 ng/ µL and 1

ng/ µL. It is therefore practical in the long run to target the amount of template DNA in qPCR for the lowest practical concentration, such as 10^2 - 10^4 copies of GOI per reaction, as the project continues. In theory, qPCR does not have a detection limit as a single copy can be detected. In practice, 10 copies per reaction can be considered as the lowest reliable detectable concentration.

Inhibition test (task 2, step 5)

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

Spiking of exogenic DNA in soil DNA extract

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

The inhibition test is validated by observing:

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

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

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

Dilution of DNA template

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

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

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

qPCR assay (task 3, step 6)

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

Validation of qPCR assay (task 4, step 7)

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

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

Calculations (task 4, step 8)

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

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

$$\boldsymbol{I} = \frac{GOI\ in\ assay}{volume\ of\ template\ in\ assay\ (\mu L) * concentration\ of\ template\ in\ assay\ \left(\frac{ng}{\mu L}\right)}$$

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

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

References

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nola, M. W. Pfaffl, G. L. Shipley, J. Vandescompele, and C. T. Wittwer. 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry. 55:611-622.

Henry S., Baudoin E., López-Gutiérrez J.C., Martin-Laurent F., Brauman A., Philippot L. 2004. Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. Journal of Microbiological Methods 59, 327-335.

ISO 17061:2016 Soil quality -- Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G. W., Prosser, J. I., Schuster, S. C., and Schleper, C.: Archaea predominate among ammonia-oxidizing prokaryotes in soils, Nature, 442, 806-809, 2006.

Levy-Booth, D. J., Prescott, C. E., and Grayston, S. J.: Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems, Soil Biology and Biochemistry, 75, 11-25, 10.1016/j.soilbio.2014.03.021, 2014.

Okano, Y., Hristova, K. R., Leutenegger, C. M., Jackson, L. E., Denison, R. F., Gebreyesus, B., Lebauer, D., and Scow, K. M.: Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil, Applied and Environmental Microbiology, 70, 1008-1016, 10.1128/aem.70.2.1008-1016.2004, 2004.

Schauss, K., Focks, A., Leininger, S., Kotzerke, A., Heuer, H., Thiele-Bruhn, S., Sharma, S., Wilke, B. M., Matthies, M., Smalla, K., Munch, J. C., Amelung, W., Kaupenjohann, M., Schloter, M., and Schleper, C.: Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils, Environmental Microbiology, 11, 446-456, 2009.

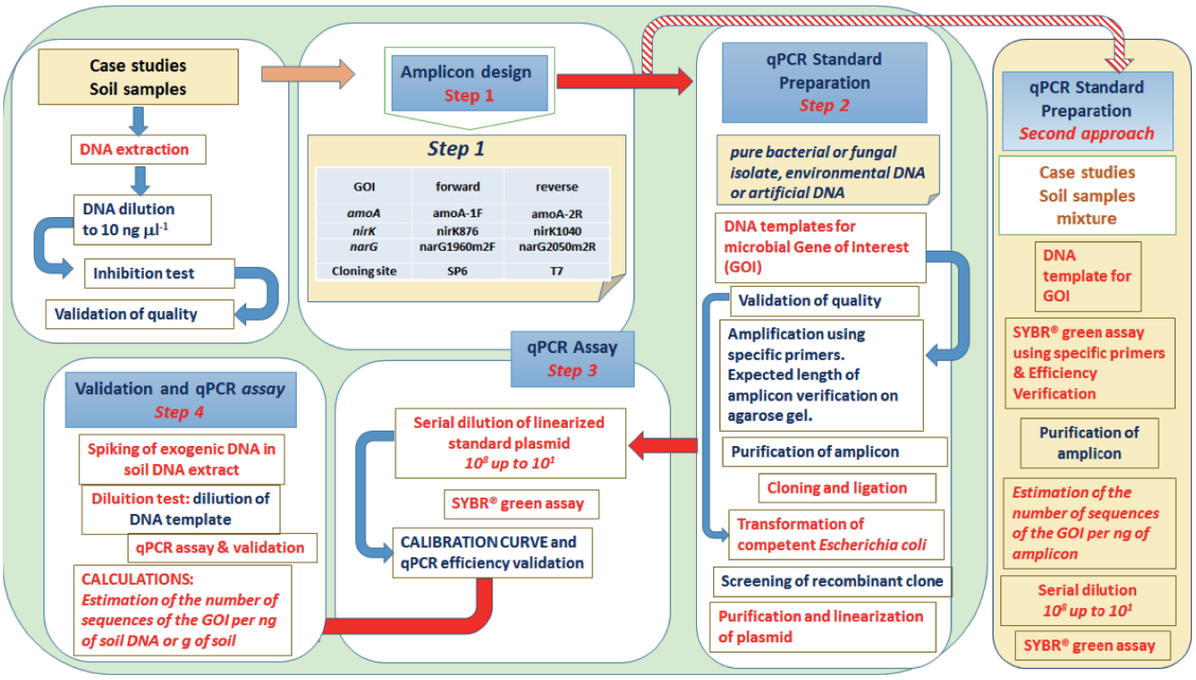

Fig. 3.3.1 General workflow of the procedure for the determination of microbial, functional genes by qPCR from soil extracted DNA

3.4 Detection and quantification of soil borne diseases by qPCR

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Importance and applications

Soil-borne plant pathogens (bacteria, fungi and oomycetes) produce great losses to agricultural crops. One of the most important strategies for controlling plant diseases is an accurate identification and an early detection and monitoring of microorganisms (Lopez-Mondejar et al., 2012). The availability of fast and sensitive methods for the detection of pathogenic species in soil can strongly improve disease control and help decision making. An early detection of the pathogen even before the onset of the symptoms, is of special interest in seeds, nursery plants and plant material to avoid further spreading and introduction of new pathogens into a growing area where it is not yet present (Acero et al., 2011).
The traditional detection methods are time-consuming and require extensive knowledge on classical taxonomy (Capote et al., 2012). Quantitative real-time polymerase chain reaction (qPCR) based technology is a rapid and sensitive method that offers advantages over the traditional diagnosis reducing the time needed for diagnosis. The qPCR technology allows accurate/discriminant detection and/or quantification of pathogens that cannot be extracted or cultivated easily from soil and plant tissue, or are present at low inoculum load in samples. Nowadays a wide range of plant pathogens can be detected and quantified by real-time PCR in numerous hosts or environmental samples, e.g. *Fusarium oxysporum* in muskmelon seedlings (Lopez-Mondejar et al., 2012), *Phytophthora nicotianae* in soil and host tissues (Blaya et al., 2015), *Sclerotinia sclerotiorum* in alfalfa (Parker et al., 2014).

Principle

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

The SYBR Green qPCR assay has been validated by an international ring test, and is widely used starting by an appropriate amplicon design optimising the qPCR assay. Specific and discriminant primer pair design

can be done manually or by using appropriate in silico software or web-based tools and the sequence of microbial gene of interest. Since the specificity and the discriminant character are of crucial importance, several fungal SSR markers nave been reported to be species specific, with a polymorphic character, thus allowing a discriminant and specific qPCR assay.

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

Reagents

- Specific primers and probes according to the target.
- Premix Ex Taq (Probe qPCR) Master Mix + Rox reference dye
- Quanti Fast or Quanti Nova SYBR Green PCR Master mix, (Qiagen)
- Water - molecular biology grade (DNase, RNase, Protease, free)
- Bovine serum albumin (BSA) which enhances PCR amplifications from low purity DNA samples and prevents enzymes from adhering to tubes and tipped surfaces.
- Soil to develop a standard curve
- Pure culture of each pathogen to detect/ quantify
- Internal PCR control (IPC) Phocine Herpesvirus

PhHV-267s:5’- GGGCGAATCACAGATTGAATC-3’
PhHV-337as: 5’-GCGGTTCCAAACGTACCAA-3’
PhHv-305tq: CY5-5’-TTTTTATGTGTCCGCCACCATCTGGAT-3’-BHQ

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

Materials and equipment

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

Procedure

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

1. **Soil DNA extraction.** DNA extraction is a critical pre-step analysis; the quality of the final results can

be significantly affected by the purity and final yield of DNA (for the DNA extraction method, see chapter 3.1).

2. **Selection or design of specific set of primers or primers and probe.** According to the method used to detect and quantify the target microorganisms we have to select or design specific primers and probe.
3. **qPCR assay.** The qPCR assay can be used with any qPCR platform or be anyhow adapted to the specific instrument that each laboratory has available (i.e. 7500 Fast Real-time PCR system (Applied Waltham, MA, USA). The qPCR assay has two steps, qPCR mixture and qPCR conditions.
4. **qPCR analysis and standard curve preparation.** To quantify the amount of pathogen on soil samples a standard curve for each pathogen is made.

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

Target gene selection is a crucial step in real-time PCR assay; sequences of the primer must be unique to identify sequences of the target in the sample of interest with high specificity and efficiency. The ribosomal DNA genes (rDNA) and the internal transcribed spacer (ITS) provide nice targets because they have conserved and variable regions that allow highly sensitive detection. Typically, 16rRNA genes from bacteria and ITS regions of the fungal RNA regions have been used most commonly for PCR-based identification of plant pathogens. Other sequences that are used for identification and monitoring at the species level are for example the β-tubulin gene, the elongation factor 1 alpha (EF1-α), and random amplified polymorphic DNA/ sequence-characterised amplified region (RAPD/SCAR)-based targets (Okubara et al., 2005).

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

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

qPCR assay

For the **non-specific label method**, the amplification reaction is carried out in 25 µL reactions containing

10 µL of template DNA (5-10 ng/µL), 12.5 µL of Quanti Fast SYBR Green PCR Master Mix (Qiagen), 1.2 µM of primer, and up to 15 µL of PCR-grade water. In order to protect soil DNA and microbial DNA extracts from potentially present PCR-inhibitory substances, bovine serum albumin (BSA) should be added to the SYBR Green mix (Quanti Fast or Quanti Nova SYBR Green PCR Master mix, Qiagen). Experiments should be performed in duplicate or, better, in triplicate.

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

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

qPCR analysis and standard curve preparation

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

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

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

   where **a** is the DNA concentration of the plasmid (ng), 6.02×10^{23} is Avogadro's number indicating the number of molecules/mol; **b** is the length of the plasmid containing the insert (bp), 1 × 1012 is used to convert g to pg; and 650 is the average molecular weight of one base pair. The concentration is adjusted to the number of 10^{10} gene copies, and the standard is diluted in 10-fold steps to obtain the standard curve. All qPCR reactions are performed in triplicate (Lopez-Mondejar et al., 2012; Blaya et al., 2015).
   Estimation of the number of sequences of the gene of interest per g of dry soil is developed:
   copy numbers (g dry $soil^{-1}$) = copies μL^{-1} × DNA elution volume µL × g dry $soil^{-1}$
2. The first step of qPCR standard preparation relies on the extraction of DNA templates. This must be done from:
   i. Pre-bacterial of fungal cultures of interest to harbour the gene of interest (thus pathogens) by the DNA extraction method reported in chapter 3.1;
   ii. Soil DNA (a soil used as control in the experimental design) by the DNA extraction method.

A calibration is performed by carrying out microcosm incubation with a known amount of soil DNA (used as

control in the experimental design) added to a known amount of pure bacterial/fungal liquid suspension. DNA is extracted within 48h after the addition of the targeted microbes from the microcosm, amplified for each targeted gene following the SYBR green chemistry protocol (Canfora et al., 2016). Amplicons are purified from the gel using the conventional approach (cut of the bands) to remove primers. The purified amplicons are then quantified and the gene copy number is calculated.

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

gene copy number = (ng _ number _ mol−1)/(base pairs _ ng _ g−1 _ g mol base pairs).

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

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

n $[gene\ of\ interest]_1$ number of sequences of the gene of interest per ng of soil DNA; y is the amount of soil DNA extracted from z gram of soil sample
z is the amount of soil sample from which DNA is extracted (in g of dry mass equivalent).

References

Acero FJ., Carbu M., El-Akhal M.R., Garrido C., Gonzalez-Rodriguez V.E., Cantoral J.M., 2011. Development of proteomics-based fungicides: New strategies for environmentally friendly control of fungal plant diseases. International Journal of Molecular Sciences 12, 795-816.

Blaya J., Lacasa C., Lacasa A., Martinez V., Santisima-Trinidad A.B., Pascual, J.A., Ros M., 2015. Characterization of Phytophthora nicotianae isolates in southeast Spain and their detection and quantification through a real-time Taqman PCR. Journal of the Science of Food and Agriculture 95, 1243-1251.

Canfora L., Malusà E., Tkaczuk C., Tartanus M., Łabanowska B., Pinzari F., 2016. Development of a method for detection and quantification of B. brongniartii and B. bassiana in soil. Scientific Reports, 6:22933; doi: 10.1038/srep22933.

Dreo T., Pirc M., Ravnikar M., 2012. Real-time PCR, a method fit for detection and quantification of Erwinia amylovora. Trees 26, 165-178.

Giulietti A., Overberggh L., Valckx D., Decallone B., Bouillon R., Mathieu C., 2001. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods b25, 386-401.

Lopez-Mondejar R., Beaulieu, R., Ros, M., Pascual, J.A., 2012. SCAR-based real-time TaqMan PCR for early detection of Fusarium oxysporum in melon seedlings under greenhouse nursery conditions. Crop Protection 33, 1-6.

Okubara P.A., Schroeder K.L., Paulitz T.C., 2005. Real-time polymerase chain reaction: applications to studies on soilborne pathogens. Canadian Journal of Plant Pathology 27, 300-313.

Parker M.L., McDonald M.R., Boland G.J., 2014. Evaluation of air sampling and detection methods to quantify airborne ascospores of Sclerotinia sclerotorum. Plant Disease 98, 32-42.

Schena L., Li Destri Nicosia M. G., Sanzani S.M., Faedda R., Ippolito A., Cacciola S.O, 2013. Development of quantitative PCR detection methods for phytopathogenic fungi and oomyctes. Journal of Plant Pathology 95, 7-24.

3.5 Sequencing soil samples for qualitative metagenomics – Ion Torrent Sequencing Protocol

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Importance and applications

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

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

Principle

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

Requirements of DNA quantity for sequencing

The minimum amount to be provided for sequencing is 18 µL with a minimum DNA concentration of

1.5 ng/µL. DNA must also satisfy quality standards with ratios of absorbance A260/A280 = 1.8-1.9, and A260/A230 = 1.9.

Target selection (Amplicon preparation)

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

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

Purification of amplicons

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

Calculation of DNA input for library preparation

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

Quality and quantity requirements of DNA samples to be sequenced

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

Library Preparation

Preparation of the sequencing library is conducted by Ion Plus Fragment Library Kit (Cat. no. 4471252).
An Ion Xpress™ Barcode Adapters 1–16 (Cat. No. 4471250) kit is used to barcode samples so that several samples can be sequenced by pooling them together in the same chip. Barcodes can be further identified by bioinformatics to distinguish between the different samples following the sequencing. There are other Ion Xpress™ Barcode Adapters Kits available as grouped as 16 specific barcodes for each kit up to 96 specific barcodes in total. Other barcoding kits will be used as needed in future (from 16 to 96).
The overview of the library preparation procedure is:

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

Template preparation

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

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

Sequencing the library

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

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

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

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

In our hands, the worst sequencing reaction we obtained had 222 800 reads and the best was 457 362 reads for the 314 chip. **If a sample gives less than 20 000 reads we will check if there is a problem with DNA quality in the Bioanalyzer. If it fails, a new DNA sample must be used.**

Remarks

References

Finley, S. J., Benbow, M. E. and Javan, G. T. (2015) 'Potential applications of soil microbial ecology and next-generation sequencing in criminal investigations', Applied Soil Ecology, pp. 69–78. doi: 10.1016/j.apsoil.2015.01.001.

Kim, R., Eileen, C., Theodora, L., Dorothy, S., Juan, V., Michael, G., Nigel, B., James, G. and S, G. B. (2013) 'Effect of organic, conventional and mixed cultivation practices on soil microbial community structure and nematode abundance in a cultivated onion crop', Journal of the Science of Food and Agriculture. Wiley-Blackwell, 93(15), pp. 3700–3709. doi: 10.1002/jsfa.6206.

Lahens, N. F., Ricciotti, E., Smirnova, O., Toorens, E., Kim, E. J., Baruzzo, G., Hayer, K. E., Ganguly, T., Schug, J. and Grant, G. R. (2017) 'A comparison of Illumina and Ion Torrent sequencing platforms in the context of differential gene expression', BMC Genomics, 18(1), p. 602. doi: 10.1186/s12864-017-4011-0.

Lakshmanan, V., Selvaraj, G. and Bais, H. P. (2014) 'Functional Soil Microbiome: Belowground Solutions to an Aboveground Problem', Plant Physiology, 166(2), pp. 689–700. doi: 10.1104/pp.114.245811.

Life Technologies (2013a) 'De novo assembly using Ion semiconductor sequencing', Ion Torrent: Sequencing for All, (Figure 1), pp. 1–4. Available at: https://tools.thermofisher.com/ content/sfs/brochures/de_novo_assembly_Ion_CO23721_App_Note_V9.pdf.

Life Technologies (2013b) 'The Ion PGM TM System, with 400-base read length chemistry, enables routine high-quality de novo assembly of small genomes', Ion Torrent: Sequencing for All, pp. 1–4. doi: available at https://tools.thermofisher.com/content/sfs/brochures/Small-Genome-Ecoli-De-Novo-App-Note.pdf (accessed 04/07/2017).

Luise, K., Fritz, O. and A., van der H. M. G. (2014) 'Agricultural practices indirectly influence plant productivity and ecosystem services through effects on soil biota', Ecological Applications. Wiley-Blackwell, 24(7), pp. 1842–1853. doi: 10.1890/13-1821.1.

Morozova, O. and Marra, M. A. (2008) 'Applications of next-generation sequencing technologies in functional genomics', Genomics, pp. 255–264. doi: 10.1016/j.ygeno.2008.07.001.

Pace, N. R. (1997) 'A molecular view of microbial diversity and the biosphere', Science, 276(5313), pp. 734–740. doi: 10.1126/science.276.5313.734.

Stoate, C., Báldi, A., Beja, P., Boatman, N. D., Herzon, I., van Doorn, A., de Snoo, G. R., Rakosy, L. and Ramwell, C. (2009) 'Ecological impacts of early 21st century agricultural change in Europe - A review', Journal of Environmental Management, pp. 22–46. doi: 10.1016/j.jenvman.2009.07.005.

Szoboszlay, M., Dohrmann, A. B., Poeplau, C., Don, A. and Tebbe, C. C. (2017) 'Impact of land-use change and soil organic carbon quality on microbial diversity in soils across Europe', FEMS Microbiology Ecology, 93(12), pp. fix146-fix146. Available at: http://dx.doi.org/10. 1093/femsec/fix146.

Vasileiadis, S., Puglisi, E., Arena, M., Cappa, F., Cocconcelli, P. S. and Trevisan, M. (2012) 'Soil Bacterial Diversity Screening Using Single 16S rRNA Gene V Regions Coupled with Multi-Million Read Generating Sequencing Technologies', PLoS ONE. Edited by J. L. Balcazar. San Francisco, USA: Public Library of Science, 7(8), p. e42671. doi: 10.1371/journal.pone.0042671.

Whiteley, A. S., Jenkins, S., Waite, I., Kresoje, N., Payne, H., Mullan, B., Allcock, R. and O'Donnell, A. (2012) 'Microbial 16S rRNA Ion Tag and community metagenome sequencing using the Ion Torrent (PGM) Platform', Journal of Microbiological Methods, 91(1), pp. 80–88. doi: 10.1016/j.mimet.2012.07.008.

Zhou, J., He, Z., Yang, Y., Deng, Y., Tringe, S. G. and Alvarez-Cohen, L. (2015) 'High-Throughput Metagenomic Technologies for Complex Microbial Community Analysis: Open and Closed Formats', mBio. 1752 N St., N.W., Washington, DC: American Society of Microbiology, 6(1), pp. e02288-14. doi: 10.1128/mBio.02288-14.

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

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Overview

Microorganisms play an important role in ecosystem functions by mediating many of the biogeochemical processes that are critical to soil fertility and plant productivity. For this reason, it is important to understand how the agronomic practices impact on microbial biodiversity and the associated function. Advances in sequencing technologies have led to the development of sequencing machines with the ability to generate a large volume of sequence data. These technologies that are generally called "Next Generation Sequencing (NGS)" have profoundly changed the way we approach the studies of the microbial communities, becoming the technology of choice for metagenomics studies. The approaches based on NGS sequencing overcome the limits of the cultivation-based methods and allow to profile the entire microbiome by directly sequencing the DNA taken from environmental samples. PCR amplicon sequencing of specific target regions is a widely used approach to study microbial biodiversity. The target regions commonly used are the ribosomal RNA genes because they are characterised by having highly conserved sequences that enable the design of primers targeting a wide range of taxa and hypervariable regions useful for taxonomic classification (Kim, 2013). The sequencing protocol to study fungal biodiversity using the Illumina (San Diego, CA, USA) MiSeq sequencer is reported below. The protocol is designed to analyse the fungal internal transcribed spacer (ITS1) region.

DNA

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

ITS Illumina amplicon protocol

The protocol described below is based on the method proposed by Smith and Peay (2014) with some modification in the amplification protocol. Sequencing libraries are produced using a single PCR step in which the target region is amplified using locus specific primers (ITS1f-ITS2) tailed with the Illumina adapters. The reverse primers are barcoded to allow multiplexing using the 12-base Golay barcodes (Caporaso et al 2012).

PCR Primers.

Primers are ordered using standard desalting purification. Primers are shipped lyophilized and upon arrival they should be spun down by centrifugation before being resuspended. Resuspension is made in pure water at the concentration of 100 µM (stock solution). Stock solution is diluted to 10 µM (working solution) before use. The primers used for the library preparation are:

Forward Primer 5' **AATGATACGGCGACCACCGAGATCTACACGGCTTGGTCATTTAGAGGAAGTAA**

ReversePrimer5' **CAAGCAGAAGACGGCATACGAGATNNNNNNNNNNNNCGGCTGCGTTCTTCATCGATGC**

In red is highlighted the barcode sequence while in green are highlighted the sequences locus specific. The sequences in black are the Illumina adapter sequences. In orange are highlighted the linker sequences. For more information see the paper of Smith and Peay (2014).

PCR's are performed using the same forward primer and a different barcoded primer for each sample.

Sequencing primers.

Sequencing is performed using custom sequencing primers. These primers are ordered lyophilized and HPLC purified. Resuspension must be done in pure water at the concentration of 100 µM. For more information about these primers see the paper of Smith and Peay (2014).

Read 1 Sequencing Primer	TTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCC
Read2 Sequencing Primer	CGTTCTTCATCGATGCVAGARCCAAGAGATC
Index Sequencing Primer	TCTCGCATCGATGAAGAACGCAGCCG

Amplicon libraries preparation.

The following PCR protocol is used to amplify the ITS1 region producing amplicons tailed with the Illumina adapters. The PCR reactions should include a negative control to assure the lack of contamination.

Prepare the following master mix:

Reagents for one sample	Volume
Template DNA (10ng/mL)	2.0 µL
Forward primer 10 µM	0.7 µL
Reverse primer 10 µM	0.7 µL
Buffer 10X	3.0 µL
$MgSO_4$ (50 nM)	0.9 µL
dNTP (10 nM)	0.6 µL
Platinum taq*	0.12 µL
PCR-grade water	21.98 µL
Total	30 µL

*Platinum Taq DNA polymerase High Fidelity from Invitrogen cat N° 11304-011

Gently mix the reaction and briefly centrifuge the tube

Thermocycling PCR conditions:

Step	Temperature	Time
Denaturation	95°C	3 minutes
35 cycles	95°C	45 seconds
	50°C	1 minute
	72°C	1 minute
Final Extension	72°C	10 minutes
Hold	4°C	

The PCR products are cleaned up from primers and primer dimers using the Agencourt AMPure XP beads (Beckman Coulter) following the manufacturer's instructions. Resuspend your PCR products in 40 µl of 10mM Tris pH 8.5.

PCR products should be checked on a Bioanalyzer DNA 1000 or on an agarose gel to verify the size. The expected size is ~ 340 bp.

Sequencing.

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

Quantify the amplicons with the Qubit using the dsDNA HS Assay kit. To calculate the concentration the amplicon size needs to be determined using the 2100 Bioanalyzer. The DNA concentration is calculated as suggested by the Illumina Technical note number 15044223 using the following equation:

$$concentration\ in\ nM = \frac{concentration\ in\ ng\ /\ \mu l}{(660\ g\ /\ mol\ x\ average\ library\ size)}\ X10^6$$

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

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

To sequence together custom samples and the PhiX control library it is needed to use both the custom sequencing primers and the Illumina primers provided in the reagent cartridge. Primers are mixed together loading 3.4 µl of Read 1 sequencing primers (100 µM) into the reservoir 12, 3.4 µl of Read 2 sequencing primers (100 µM) into the reservoir 14 and 3.4 µl of index sequencing primers (100 µM) into the reservoir 13.

Adding a new assay to the Illumina Experiment Manager (IEM)

Golay barcode indices are not present by default in the Illumina Experiment Manager but needs to be supplied to the IEM. To include these indices to the system, a custom sample prep kit, containing the reverse complement of the index sequences, must be created and added to the following directory:

C:\Program Files(x86)\Illumina\Illumina Experiment Manager\SamplePrepKits.

After this step move to the folder Application

C:\Program Files(x86)\Illumina\Illumina Experiment Manager\Application

and open the file GenerateFASTQ using a text editor such as Notepad. Add the name of the library prep kit file under the [Compatible Sample Prep Kits] section. These two steps make the Golay barcode selectable during the sample sheet generation with the Illumina Experiment Manager software when selecting the GenerateFASTQ application. For more information on how to add a new assay consult the Illumina Experiment Manager software guide.

References

Kim, M., Lee, K. H., Yoon, S. W., Kim, B. S., Chun, J., and Yi, H. (2013). Analytical Tools and Databases for Metagenomics in the Next-Generation Sequencing Era. Genomics Inform 11, 102–113. doi:10.5808/GI.2013.11.3.102.

Claassen, S., du Toit, E., Kaba, M., Moodley, C., Zar, H. J., and Nicol, M. P. (2013). A comparison of the efficiency of five different commercial DNA extraction kits for extraction of DNA from faecal samples. Journal of Microbiological Methods 94, 103–110. doi:10.1016/j.mimet.2013.05.008.

İnceoğlu, Ö., Hoogwout, E. F., Hill, P., and Elsas, J. D. van (2010). Effect of DNA Extraction Method on the Apparent Microbial Diversity of Soil. Appl. Environ. Microbiol. 76, 3378–3382. doi:10.1128/AEM.02715-09.

Smith, D. P., & Peay, K. G. (2014). Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. PLoS ONE, 9(2), e90234–e90234. http://doi.org/10.1371/journal.pone.0090234

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6: 1621–1624

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

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Introduction

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

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

Remark

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

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

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Reads pre-processing

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

Quality filtering

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

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

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

Use Trimmomatic to process the raw reads

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

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

Arguments summary

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

- MINLEN → Eliminate the reads below the specified length.

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

Reads analysis

Assembly paired-end reads

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

Execute the following command to run PEAR with your data:

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

Arguments summary

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

For other options see the software manual.

Proceed with the QIIME pipeline

The fastq files must be converted into a fasta file using the following QIIME script
convert_fastaqual_fastq.py -c fastq_to_fastaqual -f reads_joined.fastq -o output_fasta

Arguments summary

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

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

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

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

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

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

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

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

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

The output is a file called "combined_seqs.fna"

Chimera detection using VSEARCH

Chimeras are sequences artefacts produced during the PCR amplification and derived by joining two or more partial sequences coming from different biological sequences. They are formed when prematurely terminated amplicons generated by incomplete extensions act as primers and anneal to different but similar templates. We use VSEARCH (Rognes, 2016) to detect and remove chimeras. VSEARCH detects chimeras using the UCHIME algorithm. VSEARCH can perform chimera detection using or not a reference database. We suggest running the reference-based chimera detection using the UCHIME reference dataset downloaded from the UNITE database (https://unite.ut.ee/repository.php).

Vsearch -uchime_refpath/combined_seqs.fna -db/path/uchime_sh_refs_dynamic_ original_985_03.07.2014.fasta -nonchimerasno_chimeras.fna -threads 7

Output file = no_chimeras.fna

OTU picking and taxonomy assignment for fungal ITS

In this step, the reads are clustered into OTUs based on a 97% sequence similarity and taxonomy is assigned to OTUs. The sequences similarity threshold can be set by specifying the following option
pick_otus:similarity 0.97
on the QIIME parameters file.

OTU picking is made using an open reference strategy. With this strategy the reads are initially clustered against a reference sequences database. The reads that did not find a match with the reference sequences are clustered de novo.
Pick-open-reference_otus.py by default uses the UCLUST clustering tool.

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

Arguments summary

- -i → The input file
- -o → Output directory
- -p → The parameter file
- -a → Run in parallel
- -0 → Number of job to start in parallel (only with –a)
- --suppress_align_and_tree→ Apply if you are working with ITS amplicons (ITS sequences cannot be aligned).

The output is a file called "otu_table_mc2_w_tax.biom"

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

```
# QIIME parameters file

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

Remove low abundance OTUs

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

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

Now we should normalise the samples by rarefying to the lowest sequence count. We need to know the samples depth to perform this step. To extract this information from the biome table we use the following command
biom summarize-table -i/home/path/otu_table_mc2_w_tax_filtered.biom -o /home/path/table_summary.txt
Arguments summary

- -i → Input file
- -o → Output file

Box 2: Example of summarise-table command output

Counts/sample summary:

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

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

single_rarefaction.py -iotu_table_mc2_w_tax_filtered.biom -o /path/otu_table_mc2_w_tax._rarefied.biom -d 29867

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

Core diversity analysis

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

core_diversity_analyses.py -iotu_table_mc2_w_tax._rarefied.biom -m 'mapping_file.tab' -o /home/RESULTS/Core_diversity--nonphylogenetic_diversity -c condition, treatment

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

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

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Principle

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

Denoising

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

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

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

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

Demultiplex and quality filter reads

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

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

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

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

PCR chimera detection using VSEARCH

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

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

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

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

The script *pick_open_reference_otus.py* executes a number of interesting steps including OTU picking, annealing of representative sequences with a reference GreenGenes alignment, phylogenetic tree construction (possible for the case of rRNA 16S gene) and taxonomy assignments, among others. The different options and parameters for these steps can be easily indicated in the command line and/or in a "parameter file" as in the previous ITS pipeline. For a safe start however, we recommend a threshold of 97% (default option), the combination of methods "sortmeRNA" and "sumaclust", both available in Qiime, and the SILVA database released 128 (Quast et al., 2013), recently updated (2017) in comparison with the Qiime default GreenGenes database (May 2013).
To generate the parameter file for the SILVA database (adjust the number of threads accordingly to the

computer used):

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

Finally, the command to execute the script:

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

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

Remove low-confident OTUs

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

filter_otus_from_otu_table.py -ipath/otu_table.biom -o otu_table_no_singletones.biom--min_count2

Core diversity analysis

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

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

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

References section 3.7.1

Bolger, A. M., Lohse, M., and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. doi:10.1093/bioinformatics/btu170.

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nature Methods 10, 57–59. doi:10.1038/nmeth.2276.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Gonzalez Pena A, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data.Nature Methods 7(5): 335-336.

Illumina Technical note: https://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf

Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F.2016.VSEARCH: a versatile open source tool for metagenomics. PeerJ4:e2584 https://doi.org/10.7717/peerj.2584

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A. 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics. 30:614–20.

References section 3.7.2

Bragg L, Stone G, Imelfort M, Hugenholtz P, Tyson GW. Nat Methods. Fast, accurate error-correction of amplicon pyrosequences using Acacia. 2012 Apr 27;9(5):425-6. doi: 10.1038/nmeth.1990.

Bragg LM, Stone G, Butler MK, Hugenholtz P, Tyson GW (2013) Shining a Light on Dark Sequencing: Characterising Errors in Ion Torrent PGM Data. PLoSComputBiol 9(4): e1003031.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Gonzalez Pena A, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data.Nature Methods 7(5): 335-336.

Fantini E, Gianese G, Giuliano G, Fiore A.Bacterial metabarcoding by 16S rRNA gene ion torrent amplicon sequencing. Methods Mol Biol. 2015;1231:77-90. doi: 10.1007/978-1-4939-1720-4_5.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Opens external link in new windowNucl. Acids Res. 41 (D1): D590-D596.

3.8 Soil enzyme activities

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Importance and applications

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

Sample preparation and storage

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

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

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

Principle

Assays of soil enzyme activity are carried out in the laboratory under manipulated and controlled, and thus largely optimal, conditions. Hence, it has to be stated that the methods described below estimate a potential of soil enzyme activity in soil. In general, a certain amount of soil is mixed with a specific substrate and incubated for some hours. Depending on the enzyme being assayed and the chosen substrate, the reaction product emerging during incubation consists of a quantifiable compound such as MUF (methylumbelliferyl), AMC (aminomethylcoumarin), pNP (para-nitrophenyl) or INTF (iodotetrazolium chloride formazan), which can be extracted and measured against calibration standards either fluorometrically or colorimetrically. Additionally, the method for the determination of the potential nitrification is presented in this chapter.

Table 3.8.1. Commercially-available colorimetric and fluorogenic substrates for soil enzyme assays.

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

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

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

3.8.1 Fluorogenic approach

Reagents

All required reagents should be freshly prepared.

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

Materials and equipment

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

Procedure

a. Prepare a soil suspension by dispersing 1 g of sieved, field moist soil (determine the dry mass beforehand) in 100 mL of sterilised and deionised water. In order to ensure equal dispersion, use an ultrasonic disaggregator at 50 $J*s^{-1}$ for 2 minutes.
b. Combine 50 µL of soil suspension, 50 µL of buffer (Trizma buffer for AMC and MES buffer for MUF) and 100 µL of substrate solution in a microplate-well. Prepare each sample at least in triplicate.
c. Standards are prepared by mixing 50 µL of soil suspension with the corresponding amounts of standard working solution and buffer solution in order to obtain final concentrations of 0, 200, 500, 800, 1200 and 1500 pmol/well in a resulting volume of 200 µL. Standard concentrations may be extended, depending on the activity of assayed enzymes.
d. For the consideration of quenching, add 100 µL of buffer and 100 µL of substrate for each substrate to one well.
e. Multi-well plates are incubated for 4 hours at 30°C.

f. Fluorescence is measured immediately after the addition of the soil suspension to multi-well plates to obtain a start value. In order to evaluate the change of fluorescence i.e. enzyme activity, further readings in constant time intervals (every hour) are obligatory. Measure fluorescence with excitation wavelength set to 355 nm and emission wavelength set to 460 nm.

Calculations

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

With

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

Table 3.8.2. Range of activity values for fluorogenic substrates in agricultural soils.

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

Remarks

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

3.8.2 Colorimetric approach

Reagents

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

Table 3.8.3. Preparation of calibration solutions for INTF.

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

Materials and equipment

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

Procedure

a. Prepare four tubes for each sample and weigh in 2 g of sieved, field moist soil to each of them. (Make sure
the exact dry mass equivalent is known).
b. Add 2 mL of substrate solution to three regular samples.
c. Instead of substrate, the fourth sample (control) receives 2 mL of buffer solution.
d. Use a tube shaker to homogenise samples, close tubes and incubate at 25°C for 4 hours in the dark.
e. Add 8 mL of acetone to all samples and put them on an orbital shaker (250 rpm) for 1 hour in the dark.
f. Centrifuge samples for 5 minutes at 2000 g and transfer supernatants to glass cuvettes.
g. Read absorbance within 1 hour on a spectrophotometer against the calibration curve zero at a wavelength of 485 nm.

Calculations

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

With

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

Table 3.8.4 Range of dehydrogenase activity in agricultural soils.

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

3.8.3 Potential nitrification

Principle

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

Reagents

a. Distilled water (MilliQ).
b. Potassium dihydrogen phosphate, c(KH_2PO_4) = 0.2 mol/l.
c. Dipotassium hydrogen phosphate, c(K_2HPO_4) = 0.2 mol/l.
d. Sodium chlorate, c($NaClO_3$) = 0,5 mol/l.
e. Diammonium sulphate, $(NH_4)_2SO_4$.
f. Sodium hydrogen carbonate, c($NaHCO_3$) = 5 mmol/l
g. Potassium chloride, c(KCl) = 4 mol/l.
h. Stock solution A. Prepare by combining 28 mL of KH_2PO_4 (B), 72 mL of K_2HPO_4 (C), and 100 mL of distilled water (A).
i. Test medium. Prepare by combining 10 mL of stock solution A (H), 10 to 30 mL of $NaClO_3$ (D), and 0.198 g of $(NH_4)_2SO_4$ (E). Dilute to 1000 mL with distilled water (A).

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

Materials and equipment

- Orbital shaking incubator, thermostatically controlled

Procedure

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

Initial incubation. Weigh soil samples into 250 mL flasks and mix with test medium (I) to form slurries. The volume of the test medium plus the water volume contained in the moist soil should give a precise total liquid volume, e.g. 100 mL. Calculate the volume of medium to be added by subtracting the volume of water in the initial soil sample from the desired liquid volume, e.g. 100 mL. Incubate the slurries by placing

the flasks upright on an orbital shaking incubator, thermostatically controlled at 25 ±2°C. Rotation should be sufficient to keep solids suspended (175 rpm). A liquid volume of the slurry >100 mL is required if the water-holding capacity of the soil is >200 % (organic soils).

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

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

The solutions can be stored in a refrigerator (4°C to 8°C) in order to carry out analysis within 24 h.
Calculations
Calculate the rate of ammonium oxidation [ng NO_2-N × (g of dry mass of soil)$^{-1}$ × h^{-1}] from the difference between NO_2-N concentrations at different measuring times.
Typical values may range from less than 10 to more than 500 ng NO_2-N × (g of dry mass of soil)$^{-1}$ × h^{-1}

Remarks on all methods

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

References

Awad, Y.M.; Blagodatskaya, E.; Ok, Y.S.; Kuzyakov, Y. (2012) Effects of polyacrylamide, biopolymer, and biochar on decomposition of soil organic matter and plant residues as determined by ^{14}C and enzyme activities. European Journal of Soil Biology 48, 1–10. DOI: 10.1016/j.ejsobi.2011.09.005.
Benefield, C. B.; Howard, P.J.A.; Howard, Doreen M. (1977) The estimation of dehydrogenase activity in soil. Soil Biology and Biochemistry 9 (1), pp. 67–70. DOI: 10.1016/0038-0717(77)90063-3.

Dick, Richard P. (Ed.) (2011) Methods of soil enzymology. Madison Wisconsin: Soil Science Society of America book series, 9.

Drouillon, M.; Merckx, R. (2005) Performance of para-nitrophenyl phosphate and 4-methylumbelliferyl phosphate as substrate analogues for phosphomonoesterase in soils with different organic matter content. Soil Biology and Biochemistry 37 (8), pp. 1527–1534. DOI: 10.1016/j.soilbio.2005.01.008.

Giacometti, C.; Cavani, L.; Baldoni, G.; Ciavatta, C.; Marzadori, C.; Kandeler, E. (2014) Microplate-scale fluorometric soil enzyme assays as tools to assess soil quality in a long-term agricultural field experiment. Applied Soil Ecology 75, pp. 80–85. DOI: 10.1016/j.apsoil.2013.10.009.

ISO (2005 a) ISO 11732: Water quality -- Determination of ammonium nitrogen -- Method by flow analysis (CFA and FIA) and spectrometric detection.

ISO (2005 b) ISO 14256-2: Soil quality -- Determination of nitrate, nitrite and ammonium in field-moist soils by extraction with potassium chloride solution -- Part 2: Automated method with segmented flow analysis.

ISO (2012) ISO 15685: Soil quality — Determination of potential nitrification and inhibition of nitrification — Rapid test by ammonium oxidation.

Liu, K.; Hammermeister, A. M.; Patriquin, D. G.; Martin, R. C. (2008) Assessing organic potato cropping systems at the end of the first cycle of four-year rotations using principal component analysis. Can. J. Soil. Sci. 88 (4), pp. 543–552. DOI: 10.4141/CJSS07019.

Lopes, Ana R.; Faria, Cátia; Prieto-Fernández, Ángeles; Trasar-Cepeda, Carmen; Manaia, Célia M.; Nunes, Olga C. (2011) Comparative study of the microbial diversity of bulk paddy soil of two rice fields subjected to organic and conventional farming. Soil Biology and Biochemistry 43 (1), pp. 115–125. DOI: 10.1016/j.soilbio.2010.09.021.

Maharjan, M.; Sanaullah, M.; Razavi, B. S.; Kuzyakov, Y. (2017) Effect of land use and management practices on microbial biomass and enzyme activities in subtropical top-and sub-soils. Applied Soil Ecology 113, pp. 22–28. DOI: 10.1016/j.apsoil.2017.01.008.

Marx, M.-C; Wood, M.; Jarvis, S.C (2001: A microplate fluorimetric assay for the study of enzyme diversity in soils. Soil Biology and Biochemistry 33 (12-13), pp. 1633–1640. DOI: 10.1016/S0038-0717(01)00079-7.

Mersi, W. von; Schinner, F. (1991) An improved and accurate method for determining the dehydrogenase activity of soils with iodonitrotetrazolium chloride. Biol Fertil Soils 11 (3), pp. 216–220. DOI: 10.1007/BF00335770.

Nannipieri, P.; Giagnoni, L.; Renella, G.; Puglisi, E.; Ceccanti, B.; Masciandaro, G. et al. (2012) Soil enzymology: classical and molecular approaches. Biol Fertil Soils 48 (7), pp. 743–762. DOI: 10.1007/s00374-012-0723-0.

Paul, Eldor Alvin (Ed.) (2015) Soil microbiology, ecology and biochemistry. Fourth edition: Elsevier; Academic Press.

Shukla, Girish (Ed.) (2011: Soil Enzymology. Berlin, Heidelberg: Springer Berlin Heidelberg (Soil Biology).

3.9 Soil microbial biomass

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General remark

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

3.9.1 Soil microbial biomass carbon and nitrogen – fumigation extraction method (based on IS0 14240-2)

Importance and applications

The soil microflora governs major soil functions and ecosystem services such as organic matter turnover and nutrient cycling, and thus, soil fertility and overall quality. This functioning very much depends – among other factors – on the number of microorganisms present in soil. The fumigation extraction method is meant to determine the biomass of the living soil microbial community (mass of intact microbial cells in a given soil) that is assessed from its carbon (C) content. Additionally, the nitrogen (N) content can also be measured. Results on soil microbial biomass carbon (MBC) and nitrogen (MBN) can also be used to better evaluate results from test methods on microbial functions (see chapters 3.3 and 3.7). This is done by relating microbial enzyme activities (especially of endoenzymes such as dehydrogenase) to the MBC. Furthermore, the ratio of MBC to MBN is a measure of the dominance of bacteria and fungi, respectively. A typical MBC/MBN ratio of soil microbial communities has a value of 6. Lower values indicate the dominance of bacteria, higher values the dominance of fungi (Ottow, 2011).

Principle

The method described here is largely based on the ISO standard 14240-2 (ISO 1997). Basically, intact microbial cells are lysed upon fumigation of the soil sample. For fumigation, the sample is exposed to a chloroform saturated atmosphere for 24 h. Subsequently, the microbial organic matter can be easily released by extraction using 0.5 M potassium sulphate (K_2SO_4). Alternatively, the use of 0.01 M calcium chloride ($CaCl_2$) is recommended (Joergensen, 1995). The carbon content in the extract is determined in fumigated and non-fumigated samples using a TOC analyser, and the difference in extracted organic carbon is calculated. To calculate the microbial biomass carbon (MBC), the difference is divided by the correction factor kEC according to Joergensen (1996) and Joergensen and Mueller (1996). Microbial biomass nitrogen (MBN) is determined in a similar way, extracting nitrogen from fumigated and non-fumigated samples, calculating the difference in contents and dividing it by the correction factor kEN.

The carbon content of microorganisms in a soil sample is determined analytically and can be used to make

comparisons between different soil samples. If a value for actual microbial biomass is required, then such analyses are multiplied by a conversion factor derived from experiments correlating a known cell mass to carbon analysis after fumigation-extraction. All conversion factors used are related to this initial factor.

Soils

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

Reagents

Reagents of recognised analytical grade shall be used, including:

- Silicone grease (medium viscosity).
- Ethanol-free chloroform (Trichloromethane; e.g., Merck Art.1.02444, contains stabilisers: 2-methyl-2-butene and methanol) - WARNING - chloroform is a hazardous and highly volatile; narcotic chemical. All work must be done under a fume hood. Waste must be properly disposed of. In the presence of light, ethanol-free chloroform degrades rapidly to form phosgene gas (COCl,) which is odourless and highly toxic. See the respective safety instructions.
- Distilled H_2O (MilliQ)
- Calcium chloride $CaCl_2$* 2 H_2O (e.g., Merck Art.1.02382), c($CaCl_2$) = 0.01 mol/l; 1.47 g $CaCl_2$*2 H_2O in 1 L volumetric flask; dissolve in distilled H_2O. Fill up to the mark after complete dissolution. The use of calcium chloride is recommended as an alternative to potassium sulphate solution, c(K_2SO_4) = 0.5 mol/l (p = 87.135 gA) which is described in ISO 14240-2.
- Soda lime (e.g., Merck Art.6839)
- Small boiling stones (‘Antibumping granules’; e.g., Merck Art.7913).

Materials and equipment

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

Procedure

Fumigation

Weigh from each soil sample at least two moist samples (mass equivalent to 25.00 g of oven-dry soil)*,

one – for fumigation – in a 50 mL glass beaker and one – the non-fumigated control sample – in a 250 mL PE flask. Immediately extract the control sample (see below).

* NOTE: In case it is necessary to use less soil, the soil mass can be reduced to 10 g. However, increasing uncertainties due to sample inhomogeneity must be expected. It is not recommended to reduce the soil mass to less than 10 g. For the following extraction, the ratio of soil mass-to-volume of extractant must be kept the same (1:4). In soils containing more than 20 % organic material (as determined according to IS0 10694), reduce the ratio of soil-to-extract to 1:4 and less (to a minimum of 1:30 for soils containing 95% organic matter, e.g. organic layers) in order to obtain sufficient extracted matter. Record the mass of soil used.

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

After fumigation is completed, remove the beaker containing the chloroform and the filter paper from the desiccators. Remove the chloroform vapour from the soil by repeated evacuation (6 times for 2 min each) in the desiccator. Chloroform removal must be done with care, especially in clay-rich soils. The remaining chloroform will be subsequently extracted and bias the carbon determination (Alessi et al., 2011).

Extraction

Transfer the soil quantitatively to PE flasks. Extract fumigated and non-fumigated control samples in the same way. Add 100 mL 0.01 M $CaCl_2$ (or 0.5 M K_2SO_4) and shake immediately for ½ hour on a horizontal shaker. Subsequently, filtrate through a folded filter and a funnel into a 100 mL PE flask.
(ISO method: Add 200 mL of potassium sulphate, shake bottles on a horizontal shaker at 200 r/min for 30 min or an overhead shaker at 60 r/min for 45 min and filter the extracts through a folded filter paper.)
Store extracts in a refrigerator (not more than 24 h) until further analysis. If not analysed at once, store the extracts of fumigated and non-fumigated soil samples in the freezer at between -15°C and -20°C. Homogenise frozen extracts before use, after thawing at room temperature.

NOTES: A white precipitate occurs during the storage of K_2SO_4 soil extracts (especially if the samples are frozen) because they are usually supersaturated with calcium sulphate ($CaSO_4$). It is unnecessary to dissolve this excess $CaSO_4$ because it does not interfere with any of the analytical procedures described in this method.
Cell membranes of young, living roots are also affected by chloroform fumigation. In soils containing large amounts of living roots, the pre-extraction procedure given in annex B of ISO 12420-2 should be used.

Determination of carbon in the extracts

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

Calculations

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

$$E_C\ (\mu g\ /\ g\ dry\ Soil) = [(V \times D_V) - (B \times D_B)] \times (P_k\ /\ D_W + S_W)$$

where
E_C = (organic C extracted from fumigated samples) - (organic C extracted from non-fumigated samples);
V is the C concentration (µg / mL) of the sample;
B is the C concentration (µg / mL) of the blank;
D_V is the dilution of the sample, in mL;
D_B is the dilution of the blank, in mL;
P_k, see equation (2);
D_W, see equation (2);
S_W, see equation (2).

Calculate the microbial biomass carbon MBC using the following equation:

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

where

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

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

Table 3.9.1. Range of values in different agricultural soils.

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

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

Remarks

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

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

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Importance and applications

The MicroResp™ is a respirometry method that measures the CO_2 evolved from soil samples over short periods of time (4 to 6 h at 25°C), using a colour-forming reaction. The method can be used to estimate active microorganisms in soil and their growth rates at the presence of various substrates (Campbell et al., 2003). The original MicroResp™ protocol was developed to be used with 96-well microtiter plates, which can be read with conventional automated plate readers (Campbell et al., 2003). The protocol was later and more recently modified to be used both in other contexts with respect to soil (Drage et al., 2012), and with larger sample quantities (Mathew et al., 2015).

The incubation of soil samples in the MicroResp™ system with a selection of carbon sources such as sugars, carboxylic acids, amino acids, polymers, amines and amides (Anderson & Domsch, 1978) allows the measurement of substrate-induced respiration (SIR) of soil microbial biomass. The magnitude of the SIR response over 0-6 h characterises the initial microbial community in soil before the growth/selection of organisms occurs by the added substrates (Degens & Harris, 1997; Anderson & Domsch, 1978). The evaluation of SIR differences after the addition of different C-sources can be used to assess the structure and functional diversity of microbial communities in soils (Degens & Harris, 1997; Campbell et al., 2003, 2008). Soil respiration induced by the addition of glucose (Glucose-SIR) has been widely used as an estimate of microbial biomass (Anderson & Domsch, 1975, I978; West et al., 1986). It identifies a metabolically active component of the microbial community (namely a glucose inducible, or potentially active microbial biomass) and when used with selective inhibitors allows for the separation of fungi and bacteria (or other groups) contributions to the total respiratory response (Sassi et al., 2012).

The MicroResp™ method has been developed and applied mainly on soils with pH < 7, but has been occasionally used on soils with higher pH and calcite. In this regard, the original method has been modified by considering the CO_2 of the soil solution, the effects of substrates and of CO_2 itself on pH and calcite dissolution (Renault et al., 2013 and references therein).

Principle

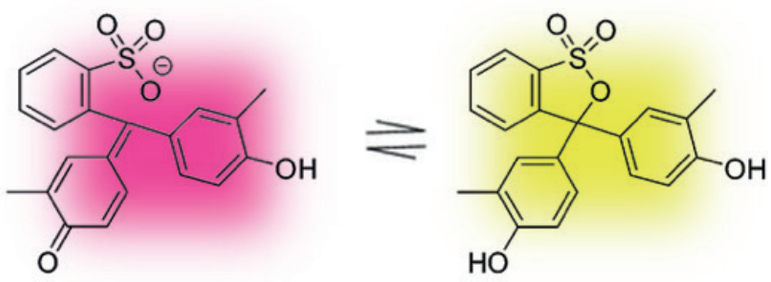
The MicroResp™ respiration system is based on the trapping of CO_2 from soil in a gel-based bicarbonate buffer and its quantification with an indicator dye (cresol red) that responds to the pH change within the same gel (Fig. 3.9.1). The colour change is read on a standard laboratory microplate reader, after a defined incubation time. The trapping system consists of two microtiter plates placed face-to-face: a deep-well plate that holds the soil samples and a plate that contains the detection gel. The deep-well plate has a capacity of 1.2 mL well^{-1} and holds about 0.45 g well^{-1} of soil, with or without substrate. The detection plate has a capacity of 300 µL well^{-1}, holds 150 µL well^{-1} of gel with indicator. The two plates are sealed together with a silicone rubber gasket with interconnecting holes to allow free gas exchange between the deep well

containing soil and the detection well containing the detection system (Cameron, 2007). It is possible to adapt the method to microplates with different number and size of wells (Renault et al., 2013; Swallow et al., 2015). The indicator plate is read with an absorbance microplate reader (Absorbance at 570nm = A570) just before and after 6 h of incubation. The incubation time is defined as the best compromise between the need for short incubations to prevent microbial growth/selection and the need for long incubations to reduce the CO_2 gradient between the gel and the well headspace. Since CO_2 equilibration is a slow process, an incubation time of less than 4 h may underestimate microbial respiration.
According to the work of Rowell (1995), the CO_2 evolved from soil reacts with bicarbonate:

$$CO_2 \text{ (gas)} + H_2O + HCO_3^{2-} \leftrightarrow 2CO_3^{2-} + 3H+$$

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

Figure 3.9.1 Reaction of cresol red (indicator dye): the H+ produced when CO_2 reacts with bicarbonate turns the



indicator from purple to yellow (protonation).

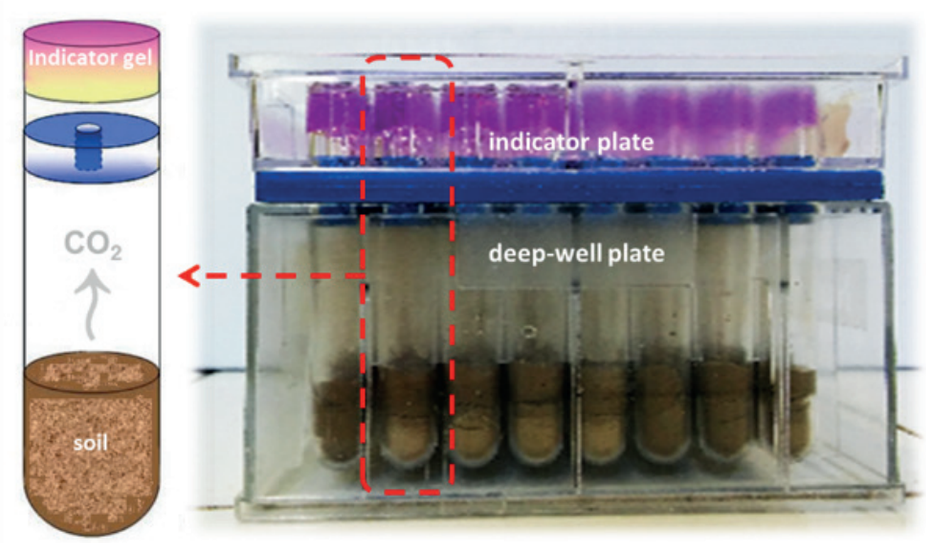


Figure 3.9.2 MicroResp™ sealed microplates and schematic diagram of the dye detection system: a deep well containing the soil sample is connected to a detection well with agar gel carrying cresol red as indicator dye. After incubation (4-6 h), the cresol red changes from pink to yellow as the pH decreases.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

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

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

$$\% \ CO_2 = A + B \times e^{-kx}$$

where A = 0.222, B = 0.384, k = ln(R), x = A570, R = 0.106 (P = 0.001; r2 = 0.79) (Fig. 3.9.2).

However, Cameron (2007) slightly modified the protocol by using the 570 nm optical density as the closest optimum wavelength and changing the formula used to convert the absorbance reading to $\%CO_2$. The author found a better fit for a linear-to-linear (rectangular hyperbola) curve, compared to the original formula of Campbell (2003):

$$\% \ CO_2 = \frac{A+B}{1+D \times A_i}$$

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

Reagents

- Cresol red indicator grade CAS 1733-12-6
- Potassium chloride KCl CAS 7447-40-7
- Sodium bicarbonate $NaHCO_3$ CAS 144-55-8
- Purified agar CAS 9002-18-0
- Indicator solution (1 L) (agar gel 10 g L^{-1}, KCl 0.15 mol L^{-1}, NaHCO3 2.5 mmol L^{-1} and cresol red dye 32.7 µmol L^{-1}): dissolve 18.75 mg cresol red, 16.77 g KCl, 0.315 g $NaHCO_3$ in 0.5 L of water in a 1 L volumetric flask over a low heat (< 50°C), bring to volume. Store at 4°C for a maximum of 2 weeks. Do not autoclave the indicator solution.
- Substrates of interest, i.e. glucose.

Materials and equipment

- 96 deep-well 1.2mL microplate (Thermo LifeSciences, Basingstoke, United Kingdom; MicroRespTM).
- 96 well microplate (detection plate) (Thermo LifeSciences, Basingstoke, United Kingdom; MicroRespTM).
- MicroRespTM seal
- Filling device

- Metal clamp
- Multi-channel pipettes
- Spectrophotometer Microplate Reader at 570 nm Optical Density (Absorbance = A570)
- Conditioning unit (thermostated dark room) for soil incubation
- Dessicator (air-tight container)
- Hot-plate stirrer for detection gel preparation
- Common laboratory equipment.

Procedure

g. Preparation of soil samples: grind gently to disaggregate the soil and sieve through 2 mm stainless-steel sieve. Determine the soil moisture content and adjust to 40 % of the water-holding capacity (WHC) by adding deionised water. Usually, about 35-50 g of soil fresh weight is sufficient for a 96-well plate. Incubate the soil samples in a conditioning unit together with a beaker of water and a beaker of soda lime, in the dark at 25°C for 48 h (for 5 days if soil moisture has been adjusted).

h. Preparation of detection plates: prepare the gel (3% agar) by dissolving 3g of purified agar in 100 mL of deionised water, autoclave at 121°C and allow to cool at 65°C in a water bath. Transfer 200 mL of the indicator solution to a 0.5 L beaker and warm at 65°C on a hot plate stirring constantly. When both the indicator solution and the agar gel reach about 65°C, add the purified agar (100 mL) in the indicator solution (200 mL) and mix thoroughly (agar:indicator = 1:2). Using a multi-channel pipette, dispense 150 µL aliquots per well of the indicator plate. When dispensing the agar gel into the wells of the indicator plate, take care to pipette the agar slowly into the centre of the well, omitting inclined surfaces and trapping of bubbles.

   Discard the first and the last dispenses and keep pipette tips warm to aid in dispensing. Store the indicator plates in the dark in a small desiccator or plastic box containing soda lime and a beaker of water (CO_2-free, moistened atmosphere). For longer storage cover the indicator plates with Parafilm.

i. Preparation of substrates: 30 mg of glucose is used as C-source per gram of soil water (water contained in the soil sample). Using deionised water, prepare a stock solution of each carbon source. Deliver the stock solution in 25 µL aliquots per well of the deep-well plates, performing this rapidly for each plate because adding carbon sources increases respiration within minutes. The concentration of the stock solution must be designed to deliver 30 mg C * g $H_20_{soil}^{-1}$. Calculate the amount of substrate per well (in mg) by multiplying 30 * g soil well-1 * g $H_20_{gsoil-1}$. To calculate the weight of soil in each well (g soil $well^{-1}$), divide the weight of the soil in one plate by the number of wells filled. Remember to record the position of each C-source on a template (the detection plate configuration will be the reverse of the deep-well plate configuration). According to the soil characteristic, the C-sources can be dispensed before or after the addition of soil to the deep-well plate, ensuring that the soil contacts the substrates at the same time for all wells.

j. Calibration: determine the calibration curve for absorbance A_{570} versus headspace equilibrium CO_2 concentration by parallel measurement of soil respiration. Equilibrate the dye solutions at different CO_2 concentrations prepared with standard gas mixtures (Rowell, 1995). Alternatively,

incubate four different soils in jars for 6 h, with or without glucose (30 mg C × (g H_2O_{soil})$_{-1}$), and measure the headspace CO_2 concentration every 2 h. Place 4 microtiter detection wells (breakable CombiStrips of the Thermo LifeSciences) in each jar, prepared as prescribed by the MicroRespTM protocol, to be reassembled and read with the plate reader when required.

k. Setting-up: place the soil into the deep-well plate by using the MicroRespTM filling device, which is positioned over the deep-well plate through a false bottom[1]. Place 300 µL of soil into the filling device, tapping gently to ensure consistent packing[2]. The soil moisture content must be within 30 and 60% of its maximum WHC to not affect the microbial activity and to easily manipulate the soil, it is therefore possible to adjust the aliquot of substrate stock solution delivered to the soil samples according to the soil characteristics. Record the weight of the placed soil, and then remove the false bottom, allowing the soil o fall into the deep wells and make contact with the C-source solutions, if already dispensed. The weight of the sample per well is approximate, since the method is on a volumetric basis. Add the C-sources (if not already dispensed), and immediately seal the deep-well plate with the gasket and proceed with the measurement. When using more than one soil, use tape to isolate columns of the filling device and of the plates before filling.

l. Measurement: switch on the spectrophotometer and read the indicator plate with a microtiter plate reader at 570 nm (time 0, store electronically), then place it firmLy on the MicroResp gasket and seal the system closing the metal clamps (be aware that the soil sample in slot A1 is measured by slot A12 of the indicator plate). Incubate for 6 hours at 25°C in the dark, then disassemble, peel off the gasket and repeat the absorbance measurement at 570 nm (time 6). A new indicator plate may be attached to continue respiration measurements over time (time ith). It is advisable to estimate the initial CO2 partial pressure (usually 0.04 - 0.1% in lab air), by reading the absorbance of empty wells.

Calculations

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

$$\%CO_2 = \frac{A+B}{1+D\times A_i}$$

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

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

[1]It is recommended that samples are run at least in triplicate to ensure the best estimate of a mean absorbance per carbon source.
[2]The deep-well plates can be prepared the day before the MicroRespTM set-up, in this case cover the plates with Parafilm and store overnight at 4°C. Allow to warm at room temperature before use.

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

The CO_2 respiration rate per gram of dry soil per hour RESP (µg CO_2-C g^{-1} h^{-1}) is calculated as follows:

$$RESP = \frac{\left(\%\ \frac{CO_2}{100}\right) x\ V\ x\ \left(\frac{44}{22.4}\right) x \left(\frac{12}{44}\right) x \left(\frac{273}{273 + T}\right)}{Wf\ x\ \left(\frac{Wd}{100}\right)} \Big/ h$$

T = the incubation temperature (°C);
V = the well headspace volume (µL), normally 945 µL for the standard method set-up;
Wf = the soil fresh weight per well (g);
Wd = the soil sample dry weight (%);
h = the incubation time (h);

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

$$MBC = 40.4 \times basal\ respiration + 0.37$$

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

$$qCO_2 = \frac{basal\ respiration\ x\ 1000}{MBC}$$

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

Remarks

- Suitable substrates: amino acids (L-arginine, γ-amino butyric acid, L-alanine, L-cysteine-HCl, L-lysine-HCl, and N-acetyl-glucosamine), aromatic carboxylic acid (3,4-dihydroxybenzoic acid), carbohydrates (D-Fructose, D-galactose, D-glucose, L -arabinose, and D-trehalose), carboxylic acids (citric acid, L -malic acid, and oxalic acid) etc.
- Indicator plates can be regenerated in a plastic box containing soda lime and a wet tissue paper, re-equilibration takes between 24 and 36 hours after which the indicator gel returns from yellow/orange to dark red/purple. However, storage affects the properties of the gel: soda lime partly extracts CO2 supplied as NaHCO3, and dries out the gel, which can skew the calibration (Renault et al., 2013). Therefore, discard all

plates that at time 0 display a coefficient of variation > 5% (% CoV).

- Deep-well plates can be cleaned from soil and be reused.
- For some soils, the CO_2 evolution can be overestimated due to calcite dissolution associated with CO_2-induced change in soil solution pH (Oren & Steinberger, 2008).
- Keep firm control over temperature, which can affect all the thermodynamic constants as well as microbial activity.

References section 3.9.1

Alessi, D.S., Walsh, D.M., Fein, J.B., 2011. Uncertainties in determining microbial biomass C using the chloroform fumigation–extraction method. Chem. Geol. 280, 58-64, 10.1016/j.chemgeo.2010.10.014.

Furtak, K., Gawryjołek, K., Gajda, A.M., Gałązka, A., 2017. Effects of maize and winter wheat grown under different cultivation techniques on biological activity of soil. Plant Soil Environ. 63, 449-454.

ISO, 1997. ISO 14240-2: Soil quality -- Determination of soil microbial biomass -- Part 2: Fumigation-extraction method.

ISO, 2009. ISO 10381-6: Soil quality -- Sampling -- Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory.

Joergensen, R.G., 1995. The fumigation-extraction method to estimate soil microbial biomass: extraction with 0.01 M $CaCl_2$. Agrobiol. Res. 48, 319–324.

Joergensen, R.G., 1996. The fumigation-extraction method to estimate soil microbial biomass: Calibration of the k_{EC} value. Soil Biol. Biochem., 28, 25-31, 10.1016/0038-0717(95)00102-6.

Joergensen, R.G., and Mueller, T., 1996. The fumigation-extraction method to estimate soil microbial biomass: Calibration of the k_{EN} value. Soil Biol. Biochem. 28, 33-37, 10.1016/0038-0717(95)00101-8.

Ottow, J.C.G. 2011. Mikrobiologie von Böden - Biodiversität, Ökophysiologie und Metagenomik, Springer-Verlag, Berlin, Heidelberg, 485 pp.

Vohland M., Harbich M., Ludwig M., Emmerling C., Thiele-Bruhn S., 2016. Quantification of soil variables in a heterogeneous soil region with VIS-NIR-SWIR data using different statistical sampling and modelling strategies. IEEE J. Select. Topics Appl. Earth Observ. Remote Sens., 1-11 doi:10.1109/JSTARS.2016.2572879.

References section 3.9.2

Anderson J. P. E. and Domsch K. H. (1975) Measurement of bacterial and fungal contributions to respiration of selected agricultural and forest soils. Canadian Journal of microbiology 21, 314-322.

Anderson, J. P. E., and Domsch K. H.. 1978. A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biol. Biochem. 10:215–221.

Anderson, T.H., 2003. Microbial eco-physiological indicators to asses soil quality. Agriculture, Ecosystems & Environment, 98(1-3), pp.285-293.

Bérard, A., Mazzia, C., Sappin-Didier, V., Capowiez, L. and Capowiez, Y., 2014. Use of the MicroResp™ method to assess pollution-induced community tolerance in the context of metal soil contamination.

Ecological indicators, 40, pp.27-33.
Cameron, C. 2007. MicroResp™ technical manual. Macaulay Institute, Aberdeen, Scotland, UK.
Campbell, C.D., Chapman, S.J., Cameron, C.M., Davidson, M.S., Potts, J.M., 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Appl. Environ. Microbiol. 69, 3593-3599.
Campbell, C.D., Cameron, C.M., Bastias, B.A., Chen, C., Cairney, J.W.G., 2008. Long term repeated burning in a wet sclerophyll forest reduces fungal and bacterial biomass and responses to carbon substrates. Soil Biol. Biochem. 40, 2246-2252
Degens, B. P., and J. A. Harris. 1997. Development of a physiological approach to measuring the catabolic diversity of soil microbial communities. Soil Biol. Biochem. 29:1309–1320.
Drage et al. Journal of Microbiological Methods 88 (2012) 399–412
Oren, A., Steinberger, Y., 2008. Coping with artifacts induced by CaCO3-CO2-H2O equilibria in substrate utilization profiling of calcareous soils. Soil Biol. Biochem. 40, 2569-2577.
Renault, P., Ben-Sassi, M. and Berard, A., 2013. Improving the MicroResp™ substrate-induced respiration method by a more complete description of CO2 behavior in closed incubation wells. Geoderma, 207, pp.82-91.
Rowell, M.J. 1995. Colorimetric Method for CO2 Measurement in Soils. Soil Biol. Biochem. 27(3), 373-375.
Sassi, M.B., Dollinger, J., Renault, P., Tlili, A. and Bérard, A., 2012. The FungiResp method: an application of the MicroResp™ method to assess fungi in microbial communities as soil biological indicators. Ecological Indicators, 23, pp.482-490.
Swallow, M.J. and Quideau, S.A., 2015. A method for determining community level physiological profiles of organic soil horizons. Soil Sci. Soc. Am. J. 79(2), pp.536-542.
West A. W.. Sparling G. P. and Grant W. D. (1986) Correlation between four methods to estimate total biomass in stored. air-dried and glucose amended soils. Soil Biol. Biochem. 18, 569-576.

3.10 Earthworm sampling

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Importance and applications

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

Principle

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

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

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

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

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

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

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

Reagents

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

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

Materials and equipment

In the field:

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

- large water container, 20 litres or more (for preparing the mustard oil solution)
- sprinkling cans (for pouring the mustard oil solution)
- watch/timer (to control the length of the chemical extraction)
- sample vials with watertight tops for each individual sample (e.g. 250 mL)
- fine-meshed sieve (to ease the transfer of earthworms from water to sample vials)
- water resistant marker/pencil and paper labels to put into sample vials (vials can be additionally marked outside)
- 4% formalin preservative (1:9 dilution of 40% formaldehyde in water)
- notebook for keeping diary

Additional notes for the field:

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

In the laboratory:

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

Procedure

Field work:

m. Preparation of mustard oil solution. (*Carefully follow safety instructions.*) In the laboratory, 2 mL of allyl- isothiocyanate is mixed into 40 mL of isopropanol in small bottles. One bottle (42 mL) of the concentrate is enough to make 20 litres of mustard oil solution used in extraction. Several bottles can be made and stored in a refrigerator. Bottles are transported to the field in a cool box.

n. Sampling point location. (*One should take into consideration the need to adjust the points spatially with other soil sampling points.*) While the random positioning of samples could be preferable, systematic placing is practically convenient, e.g. for later sampling at the site, when sampling at the exact same points needs to be avoided. Replicate samples can be positioned along a transect at the central area of the treatment. The number of samples and their intervals depends on the area under investigation. In an experimental plot scale 4-6 samples at a few metre distances is suitable. When

treatments are compared at field scale, a larger number of samples with wider distances are needed (for instance 10 samples separated so that the whole field is covered). In selecting the exact sampling spot, it is good to avoid clearly aberrant spots such as deep wheel tracks. Margin areas with high field traffic should in general be avoided. Meter readings (or coordinates) of sampling point positions are taken for later reference. When the sampling is repeated, the sampling should follow the same transect pattern, with the new samples taken at least 2-3 m away from the earlier sampling spots.

o. Soil block hand-sorting. Vegetation is cut and removed from the sampling point. The frame or board (25 cm x 25 cm) is placed on the ground to mark the sample area and a soil block is taken with a spade to a depth of 20 cm. Digging is done as much as possible along the sample margins, as cutting inside the block increases the proportion of injured worms. The sample is placed on the sheet or tray and earthworms are hand-sorted from the soil aiming at finding even the smallest specimens (newborn individuals can be only 10 mm in length). The bottom of the pit can be levelled with a trowel or knife to ease the subsequent chemical extraction. When the soil is not easily friable and needs to be actively broken during sorting (as in the case of plastic clay) one must decide roughly to how small pieces breaking is done (“tip of a thumb” -size, for instance). Dense roots can be hard to handle but they need to be closely sorted. Earthworms and pieces of them are placed in a deep vessel which has cool water and is kept in shadow (e.g. in a cool box). It is necessary to keep an eye on this to ensure that earthworms do not escape from the vessel.

p. Chemical extraction. (*Done when the presence of anecic earthworm species can be expected*). Mustard oil solution is prepared in the field just before the sampling starts. The concentrate prepared mL volume of AITC + isopropanol) is added in 20 litres of water and mixed thoroughly. Despite the dilution, the solution is irritating and the safety precautions apply. The amount of solution needed for the extraction depends on the infiltration rate which varies greatly depending on soil conditions. It is therefore not necessary to aim for the same application rate for all pits. A maximum of 5 litres of solution per pit should be enough and the extraction time is set to 20-30 minutes (the same for all pits) depending on the pace of earthworm emergence. One can start by pouring 1-2 litres of the solution from the sprinkling can at the bottom of the pit and follow the infiltration. When the infiltration rate is high, application is continued with fair additions over the whole extraction time. In case of extremely low infiltration, one can keep the bottom of the pit slightly covered with solution. Emerging earthworms are picked up with forceps or by hand (using rubber gloves) in a vessel with water (as above). One should pick up a worm only when it has fully emerged from its burrow, preferably touching the head end of the worm (to avoid autotomy).

q. Preservation of the specimens. Water vessels with the collected earthworms are poured on a fine sieve and the worms are picked in sample vial with 4% formalin. Samples are coded on paper labels which are placed in the vials. It is helpful to also mark the code outside the bottle.

r. Filling of the pit. After the sampling, the pit is filled with the hand-sorted soil.

Laboratory work:

a. Change of preservation liquid. The samples should be kept in formalin for at least four days, but

preferably for one to two weeks. After that the samples are changed to 70% ethanol where they can unlimited time waiting for identification and measurements. Rinsing the sample in fresh water before transfer into ethanol is recommendable.

b. Determination of mass and species identification. Before weighing and identification, earthworms are rinsed in water, quickly dried on paper towel and subsequently have their mass determined using a suitable balance. The specimens are then identified as closely as possible (to species or stereo microscope and using standard keys. If no taxonomical identification is possible, a note on the pigmentation is useful ("pigmented" (epigeic and anecic species) or "non-pigmented" (endogeic species)). Developmental stage is finally recorded: adult (fully developed clitellum ("saddle")), sub-adult (tubercula pubertatis visible, no fully developed clitellum), juvenile.

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

Calculations

For each sample point total, species-wise and ecological-group-wise density and mass are calculated by first summing the figures in the sample appropriately and then multiplying by 16 to obtain per square metre values which are commonly used in earthworm ecology (sample area is 1/16 m^2). In case of earthworm pieces, all parts are used for total mass, only head pieces for total density. For the description of community composition percentage values of species, genera or ecological groups will be calculated. In the statistical comparisons of the treatments, plot (or field) means can be used.

References

Bertrand, M., Barot, S., Blouin, M., Whalen, J., de Oliveira, T., Roger-Estrade, J., 2015. Earthworm services for cropping systems. A review. Agronomy for Sustainable Development 35, 553-567.

Epie, K.E., Cass, S., Stoddard, F.L. 2015. Earthworm communities under boreal grass and legume bioenergy crops in pure stands and mixtures. Pedobiologia 58, 49-54.

Griffiths, B.S. et al. 2016. Selecting cost effective and policy relevant biological indicators for European monitoring of soil biodiversity and ecosystem function. Ecological Indicators 69, 213-223.

ISO 23611-1:2006. Soil quality. Sampling of soil invertebrates. Part 1: Hand sorting and formalin extraction of earthworms. International Organisation for Standardisation, Geneva, Switzerland.

van Groenigen, J.W., Lubbers, I.M., Vos, H.M.J., Brown, G,G., De Deys, G.B., van Groenigen, K.J. 2014. Earthworms increase plant production: a meta-analysis. Scientific Reports 4, 6365.

3.11 Vegetation diversity, cover and structure

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Importance and applications

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

Principle

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

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

Reagents

- None

Materials and equipment

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

Procedure

s. Identify, seasonally, all plant species present in your plots, including borders with native vegetation to increase biodiversity and attract beneficiary fauna. Distinguish crop species from other native or spontaneous species. Use a guide for plant species identification if needed. Guides with regional information are more effective for field identification than generalist guides. It is important that all seasons are well characterised. Record the name of all different species per season. The number of different species identified is the Richness.

t. Place the quadrant (select the size depending on your crop type) randomLy on the plot. Record the vegetation cover as the percentage of land covered by plants in relation with the entire quadrant surface, considering the entire area covered by the quadrant as 100%. Repeat this process at least four times in each plot, making sure that the selection of the quadrant location is random. Take the average of the vegetation cover for all repetitions. This procedure should be seasonally performed to assess the effect of crop cycles of the different cropping systems.

Calculations

a. Richness: number of different plant species present in each plot, per season and per year.

b. Vegetation cover: percentage of the total land surface covered by vegetation in each plot estimated by the quadrant method. Provide the average value of at least four different measures.

c. Similarity index (SI). The similarity index is calculated as follows:

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

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

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

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

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

References

Gurr, G. M., et al., 2012. Biodiversity and insect pests: key issues for sustainable management. John Wiley & Sons.

Lavelle, P., Moreira, F., Spain, A., 2014. Biodiversity: Conserving Biodiversity in Agroecosystems, in: Van Alfen, N.K. (Ed), Encyclopedia of Agriculture and Food Systems. Elsevier, Amsterdam, pp. 41-60.

Lopes T., et al., 2012. Pest regulation and support of natural enemies in agriculture: Experimental evidence of within field wildflower strips. Ecol. Eng. 98, 240-245.

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