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Environment (including Climate Change)

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Publication of drafts of ISO standards and OECD guidelines of the selected recommended indicators

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Overview

One of the strategic goals of EcoFINDERS is to provide the EC with tools to design and implement soil strategies aimed at ensuring sustainable use of soils. In particular, policy-relevant and cost-effective indicators for monitoring soil biodiversity have to be identified. From the very beginning of the project it was clear that especially methods for the description, measurement and assessment of microbial and faunal diversity are needed. At the same time, these bioindicators have to be accepted by the various stakeholders involved in soil biodiversity assessment. Besides practicability and sensitivity, the comparability and robustness of any data to be gained has to be secured – and the most efficient way of getting acceptance of methods is to use standardized procedures. Due to the personal experience of several partners of EcoFINDERS and the work previously done in the area of soil biology, the International Organization of Standardization (ISO) was quickly identified as the most suitable partner in this respect. The second large international standardization body, the Organization for Economic Cooperation and Development (OECD) is very focused on (mainly laboratory) test methods; thus, it does not play an important role here.

Fortunately, in soil ecology many methods for using the structure and functions of soil organisms as indicators for the biological quality of the soil have been described in the past. However, while standards for sampling soil organisms or measuring basic functions such as respiration are available, both new (mainly genetic) methods usable for describing microbial or invertebrate communities as well as more complex assessment approaches are not standardized so far. These methods are urgently needed for addressing the general hypotheses of EcoFINDERS: changes in soil biodiversity indicate the direction and rate of changes in soil functions and associated ecosystem services. Application of cost-effective bioindicators brings an economic added value to sustainable soil management.

In the following, information gathered from the Work Packages 1 – 4 has been compiled in four main chapters, focusing on the following issues:

1. Compilation of a list of existing guidelines in the area of soil biodiversity and soil functions;
2. Identification of those ISO guidelines which – in the light of literature data and practical experience within EcoFINDERS - have to be improved;
3. Standardization of new methods, which have been developed or modified within EcoFINDERS, in a way that they can be taken over by (mainly) ISO;
4. Publication of draft versions of these new or modified methods.

This work has been (and still is) performed in close co-operation with colleagues in the ISO Technical Committee 190 “Soil Quality”. The whole process of standardizing a new method lasts at least for about 2 – 4 years. Hence, is not achievable within the lifetime of this project. However, EcoFINDERS is contributing to the on-going process.

Introduction

Background and justification

As described in detail in other reports of this project, there is a strong and increasing policy requirement for the monitoring of soils using effective indicators at local, regional and national scales (Ritz et al., 2009). In Work Package 4 of EcoFINDERS, potential indicators of soil diversity and soil function were identified by means of data obtained from the literature, especially from national monitoring programs (e.g. The Netherlands, France, Great Britain and Germany). In addition, data were generated within EcoFINDERS, both at six long-term observatory sites (LTOs) as well as on more than 90 so-called transect sites distributed all over Europe. Both groups of sites were selected in order to represent different ecological and climatic regions of Europe, differing for example in soil properties (e.g. texture, pH, and organic matter content). The information from these various sources was used to perform an expert evaluation of potential indicators, which was based on the logical sieve approach (Ritz et al., 2009), in which a structured and weighted scoring system ranks potential indicators, in combination with a meta-analysis of current soil indicators.

According to the latest stage of the discussions within WP 4, no final decision has been made which will be the top five indicators to be recommended for the monitoring of soil biodiversity and soil functions. Right now, it is assumed that faunal diversity will, among others, be addressed by using earthworm and enchytraeid species composition, abundance and biomass. Several microbial methods were investigated, and probably DNA extraction, e.g. a modified ISO method as described by Plassart et al. (2012) and PLFA will be recommended. Among the functional methods microbial tests (in particular those related to the N-cycle) such as nitrification assays, already standardized by ISO, scored highly. In addition, the bait-lamina test seems to be on the list (for details see Deliverable D 4.1). While there is a general agreement that the indicators to be recommended should be standardized in the foreseeable future also other structural or functional indicators among the top 5 should be standardized – as long as there is a need for them (e.g. in the area of the environmental risk assessment of contaminated or remediated soils).

However, in order to be meet the policy requirements mentioned above, the selected indicators have not only to be practical, cost-efficient and sensitive. In order to be useful for regulatory decisions comparable and robust data have to produced, meaning that the indicator methods have to be standardized. The usage of standardized methods is not only necessary for scientific reasons (including data quality), it is also a pre-condition for the acceptance of information on soil biodiversity and soil functions by various stakeholders: Data that have been prepared in a standard and transparent way can act as a “neutral

ground" in the discussions between stakeholders with often varying interests, background knowledge and language. Due to the use of standards in various areas of "daily life" such information has a much higher value in stakeholder discussions than just a scientific report or paper.

Organizations and processes: Background information

It is an important goal of EcoFINDERS to specify, validate and standardize indicators of soil biodiversity and soil functions in a way that these indicator methods can formally be taken over by an international standardization organization. In fact, as mentioned already in the title of this deliverable, in theory two organizations are potentially relevant for this purpose: the Organization for Economic Cooperation and Development (OECD) and the International Organization for Standardization (ISO). However, in discussions with representatives from both OECD and ISO it became clear that the latter is more relevant for the work done by EcoFINDERS, for the following reason: OECD is focusing on, mainly, laboratory methods suitable for the assessment of individual stressors, mainly chemicals. The relevant ISO Technical Committee (TC) 190 "Soil quality", however, focuses more and more on field methods addressing directly the biological quality of soils. Therefore, this report will mainly describe focus on the work with ISO TC 190, which is also facilitated by the fact that several of the partners and stakeholders of EcoFINDERS are already – partly for many years – involved in the standardization work of ISO and in particular of its ISO TC 190. To a lesser extent, and in a more indirect way, OECD will also benefit from the work described in this report.

Before addressing ISO standards in detail, the standardization process as such has briefly to be summarized (a slightly outdated description is also given by Philippot et al. 2012):

There are different deliverables that can be developed by an ISO committee:

- ISO Standards:
  A normative document, developed according to consensus procedures, which has been approved by the ISO membership and the members of the responsible committee. Typical the description of practical work.
- ISO/TS Technical Specifications:
  A normative document representing the technical consensus within an ISO committee, but with insufficient support by member countries for publication as a Standard (e.g. when sufficient validation is missing).
- ISO/TR Technical Reports:
  An informative document containing information of a different kind from that normally published in a normative document (e.g. background data or proposals for data evaluation).

Within EcoFINDERS, the aim is to prepare or to modify ISO standards. Whatever document is going to be prepared, in most cases it will go through several stages:

1. WD: Working draft
2. CD: Committee draft
3. DIS Draft international standard
4. FDIS: Final draft international standard

Between each stage the respective document will be commented by committee members and/or national mirror committees. These questions and comments are collected by the National Standardisation Bodies (NSB, e.g. DIN, NEN, BSI or AFNOR). Afterwards, in most cases the secretariat of the international committee compiles the comments in one document which is then discussed by the expert round (e.g. Working Group) during the Annual Conference of ISO/TC 190 or within intermediate meetings. The revised version will, if appropriate, move to the next stage. The whole process lasts at least for about 2 – 4 years and hence is not achievable within the lifetime of this project. However, EcoFINDERS is contributing to the on-going process.

The final standard will be published by the ISO Central Secretariat. Every 5 years the ISO Standard will be reviewed (TS every 3 years). Depending on the number and nature of the comments the standard is revised, confirmed or, rarely, completely removed.

EcoFINDERS as such or, more likely, individual partners will submit comments (e.g. recommendations for modifications) for existing standards to be considered during the next regular review, via their national standardisation organisations. In principle, the same approach will be used for proposals of new standards as well, but in this case a whole Working Draft (WD) will be provided. In fact, the WD for the bait-lamina test which has been prepared in WP 3 was submitted via the German National Standardization Organisation (DIN).

It should be remembered, that all documents submitted to ISO are covered by Copyright once they have entered the ISO standardization process. Therefore, ISO does not make them freely available. Due to this reason, in the content of a WD as well as the process leading to a standard can be presented in this report, but not the formal ISO version.

In addition, it is not always the case that a WD submitted to ISO will reach the level of an International Standard. First of all it has to be proven that there is a need for the modified or new method, i.e. as indicated by national or international legal requirements. In fact, at least five countries must express their interest in a new or modified standard in order to avoid the preparation of (maybe scientifically interesting) standards which no stakeholder will ever use. In fact, ISO member states do not only have to state their interest but also to prove it by

naming a national expert who will actively support the preparation of a new or modified standard. Besides these formal criteria it is very helpful when peer-reviewed data gained by this method as well as practical experiences can be provided. Ideally, an international ring test will be prepared.

Objectives for Deliverable 6.4

- Compile a list of existing guidelines in the area of soil biodiversity and soil functions;
- Identify ISO guidelines which – in the light of literature data and practical experience within EcoFINDERS - have to be improved;
- Standardize new methods, which have been developed or modified within EcoFINDERS (WP 1, WP 3), in a way that they can be taken over by (mainly) ISO;
- Publish draft versions of these new or modified methods.

In order to support the implementation, an ad-hoc group of partners and stakeholders of EcoFINDERS was set up in early 2012. Partners from various institutions and representing different institutions within EcoFINDERS regularly exchanged ideas and met during the Annual Conferences. In this context it should be noted that several colleagues (e.g. Laurent Philippot, Jörg Römbke, Paulo Sousa) were already active within their national standardization organizations before joining EcoFINDERS. In addition, some of the stakeholders (especially Antonio Bispo, ADEME, France) were joining these discussions.

1. Compilation of a list of existing guidelines in the area of soil biodiversity and soil functions

As already explained, this report will mainly focus on ISO methods. Those being of interest have been prepared by the ISO Technical Committee (TC) 190 “Soil quality”. These methods can be divided into three groups which are listed and discussed separately in the following subchapters.

1.1. Physico-chemical properties of the soil

Any assessment of soil biodiversity or soil functions has to accept that soil and site properties have a huge influence on the composition and activity of soil organism communities. Therefore, any study or monitoring of these organisms must describe in detail these properties. As a minimum the following parameters are recommended (Turbé et al. 2010, ISO 2004a): pH-value (CaCl2, KCl), soil organic matter (SOM) content, cation exchange capacity, soil dry mass, texture, and soil density. With respect to the biological monitoring focus, nitrogen content, C/N-ratio, water holding capacity and humus form (especially for forest sites) should also be recorded. Fortunately, for most of the soil properties ISO standards are available (Table 1). They have been and still are regularly applied in a wide range of soil investigations or monitoring programs (partly for decades), including the practical work of EcoFINDERS. Early in this project the usage of these methods as well as any comments or problems were reviewed by directly contacting all partners. According to this inquiry, most of the guidelines are used without problems. However, some hints were given for further improvements (e.g. it was recommended to standardize the “Hot Water extractable Carbon” method which is useful for the differentiation of the organic carbon pool, especially in forest soils (Gaublomme et al. 2006). However, only limited experience is available in this area within EcoFINDERS, since forest sites were not the main focus of this project.

Table 1: List of ISO guidelines in the area of soil characterization

ISO No.	Short title (details given in the reference list)	Reference	Comments
10381-1	Soil sampling - design	ISO 2002b	Also useful for microbial and faunal samplings: currently under revision.
10390	Soil pH	ISO 2005a	None
10694	Carbon content	ISO 1995a	None
11260	CEC using BaCl	ISO 1994a	None
11261	Total N - Kjeldahl	ISO 1995b	None
11464	Soil pretreatment	ISO 1994b	None
11465	Moisture content	ISO 1993a	None
13878	Total nitrogen	ISO 1998a	None
14235	Organic Carbon	ISO 1998b	More differentiation of the pool of organic carbon would be useful.
14256-1	Nitrogen – Manual	ISO 2003a	None
14256-2	Nitrogen - Automatic	ISO 2005b	None

Please note that anthropogenic impact, especially chemical (concentrations of common contaminants, e.g., heavy metals, PAH, etc.) or physical stress (management practice, compaction, fertilization, erosion, etc.) is not listed. The measurement of most chemical stressors is covered by ISO standards as well, but since they are not the main focus of this report they were not listed in the Annex. Physical stress is less covered, but ISO started to address these issues (e.g. erosion) recently at the ISO annual conference in 2012.

1.2. Biological Methods: Soil Microorganisms

Most of the biological methods standardized by ISO so far focus on microbial activities (Tab. 2). In contrast to the soil fauna methods listed in the following subchapter the methods of microbial activities usually describe functions of the whole microbial community living in a soil sample, i.e. no distinction is made between individual taxa. After the development of genetic methods this approach is less dominant despite the fact that the number of standards does not (yet) reflects this change. According to the present compilation of experience, four actions have been launched in the context of soil microbiology, which are described in Chapter 2.1:

- Improvement of available guidance (ISO 10381-6) on the sampling of soil micro-organisms (currently at the stage of standard operation procedures (SOP));
- Modification of the already published standard of extraction of DNA from soil (ISO-11063) in order to widen the range of organisms which are covered (recommendations given by Plassart et al. 2012);
- Modification of the existing PLFA standard (ISO 29843-2), based on experiences made in a still running interlaboratory comparison study (currently at the SOP stage);

- Clarification of methodological details of ISO 16072: recommendations presented and discussed in Creamer et al. (2014).

Two microbial methods, PMN (Potentially Mineralizable Nitrogen) and HWC (Hot Water extractable Carbon) are used in EcoFINDERS, but are not intended to be submitted to ISO.

Table 2: List of ISO guidelines in the area of soil microbiology, plus EcoFINDERS comments

ISO No.	Short title (details given in the reference list)	Reference	Comments
10381-6	Sampling – Microbes	ISO 1993b	Identified as not sufficient
11063	DNA soil extraction	ISO 2011b	First example of „modern“ methods
14238	N mineralization	ISO 1997a	Not used within EcoFINDERS
14240-1	Substrate-induced respiration	ISO 1997b	None
14240-2	Fumigation-Extraction method	ISO 1997c	None
15685	Potential Nitrification	ISO 2004b	None
16072	Microbial soil respiration	ISO 2002a	Clarification on temperature needed
17155	Respiration curves	ISO 2011a	None
17601	Microbial gene estimation	ISO 2012	Just at the CD stage
22939	Extracellular Enzyme Acitivity Assay	ISO 2010c	Checked and a modification used within EcoFINDERS
23753-1	Dehydrogenase TTC	ISO 2005c	None
23753-2	Dehydrogenase INT	ISO 2005d	Not used within EcoFINDERS
29843-1	PLFA and PLEL analysis	ISO 2010d	Not used within EcoFINDERS
29843-2	PLFA and simple PLFA	ISO 2011d	Checked intensively within EcoFINDERS

Actually, the standardization of methods in soil microbiology was recently addressed in a review mainly written by partners from EcoFINDERS (Philippot et al. 2012). In this paper the existing methods listed in Table 2 are briefly described and their Pro`s and Con`s are intensively discussed. In addition, this paper also provides a fine overview on further developments in the area of the development and use of standardized methods with soil microorganisms. Since that paper covers the whole topic very adequately the summary prepared by Philippot et al. (2012) is given here (with authorization by the lead author).

“The standardization effort in soil microbiology is uneven between methods addressing the abundance, the diversity and the activity of the soil microbial community. Indeed, while there are already three ISO standards for quantifying soil microbial biomass (substrate induced respiration method (ISO 14240-1), fumigation-extraction method (ISO 14240-2) or by analysing respiration curves (ISO 17155)) deriving from methods described in the 1980’s, a new work item was recently adopted by ISO TC 190 and discussed during its annual meeting (2011). Its objective was to propose a standard to estimate the abundance of soil bacterial community by 16S rRNA gene targeted quantitative PCR (qPCR) using extracted

soil DNA as template. The recent developments of qPCR analyses also allow the quantification of the abundances of specific functional or taxonomical microbial groups, which may represent useful bioindicators (Wessen et al. 2011). With the use of appropriate blanks, internal and surrogate standards, qPCR is a reliable method having the advantage to offer high throughput and cost-effective analyses. For a better understanding of soil microbial activity, or more generally of soil functioning, several methods for quantifying potential enzyme activity have been developed. Even though these methods providing an insight of the size of the enzyme pool have some limits (Wallenstein & Weintraub 2008), they are commonly used as microbiological indicators of soil quality and should therefore be standardized for comparison of microbial activities both between soils and laboratories. For example, due to their environmental and agronomical importance, microorganisms involved in N-cycling are popular models in soil microbial ecology for relating microbial diversity and soil functioning. However, only measurement of potential nitrification has been internationally standardized up to now while methods for monitoring other N-processes such as nitrogen fixation and denitrification also necessitate standardization. For example, the original protocol for estimating potential denitrification (Smith & Tiedje1979) has been modified in many ways. In this assay, to measure the activity of the pool of denitrification enzymes in the soil at the time of sampling, soil slurries are incubated in the laboratory in non-limiting denitrification conditions (without oxygen, addition of nitrate, and carbon, and of chloramphenicol to avoid de novosynthesis) so that only the amount of enzyme is rate-limiting. Changes in the original protocol include excluding the chloramphenicol, which can also decrease the activity of synthesized enzymes, addition of different carbon types and amount (glucose, acetate, glumatic acid, etc) and incubation of the soil slurries in various conditions. Similarly, determination of the nitrogenase activity using the acetylene reduction technique (Hardy et al., 1968) is subjected to various modifications of the protocol resulting, for example, in variants of the acetylene concentration (0.03 to 0.1 v/v). In contrast to other methods, most modifications of these methods are not soil-specific and both potential denitrification and nitrogen-fixation assays could readily be standardized in the future. Finally, regarding methods to monitor the diversity and the structure of the soil microbial community, the adoption of the ISO 29843-1 in 2010 (ISO 2010d) describing phospholipid fatty acid (PLFA) and phospholipid ether lipids (PLEL) analyses opens the path for other standards. While it is too early to propose any standardisation of the new high-throughput sequencing technologies (e.g. 454 pyrosequencing, etc.), other powerful approaches such as those based on taxonomic and functional microarrays meet the criteria to become standards. Of course these perspectives for the development of future standards in soil microbiology are not exhaustive, and we encourage soil microbiologists to expand it by proposing other popular methods for standardization."

1.3. Biological Methods: Soil Fauna

The sampling methods for soil invertebrates have only quite recently been standardized by ISO. Efforts started in 2001 when the German Ministry of the Environment organized an official hearing in Bonn, in which the function of the soil as a habitat for organisms was discussed (for an overview on these ideas see Beck et al. 2005). While the attendants could not agree how this function could be assessed most efficiently it became clear that any approach should be based on robust and reliable data. Since the biological characterization of a soil could lead to site-specific regulations (theoretically even a remediation of that site), any monitoring method must be standardized in order to be legally valid. Therefore, the German Federal Environmental Agency in co-operation with some institutions being active in soil ecology contacted the ISO Technical Committee “Soil Quality” in order to standardize sampling methods for the most important groups of soil invertebrates (Römbke et al. 2002). A working group of ISO TC 190 Subcommittee “soil biology” which was mainly working on ecotoxicological tests with soil invertebrates, reviewed appropriate candidates and proposed four methods for inclusion into the ISO working program: the sampling of earthworms, micro-arthropods, enchytraeids and nematodes was covered in four standards of the series 23611. Later on, the sampling of soil macrofauna (e.g. isopods or diplopods) and the design of such monitoring studies were assessed (Table 3).

Especially the four original methods are regularly used in the Indicator and Transect samplings of EcoFINDERS. Existing ISO guidelines were used as a source for SOPs. During this work the need for several improvements became obvious (especially the determination using genetic methods has to be added). At the same time two gaps in the ISO list of standard methods for soil fauna were identified: no standard sampling method for protists (an organism group intensively studied in EcoFINDERS) is available, and, even more importantly, no functional method regarding the activities of soil fauna has been standardized. According to this compilation of experience, three actions have been launched in the context of soil fauna, which are described in detail in Chapter 2.2:

- Modification of existing sampling methods for invertebrates (ISO 23611-1-3), mainly regarding technical details and the implementation of genetic methods (barcoding). These issues are compiled as notes to be submitted at the next regular review.
- Proposal for a new standard describing the sampling of soil protists in the field (currently in the SOP stage);
- Proposal for the standardization of a functional method, which is measuring the feeding activity of the soil organism community. During the first years of the EcoFINDERS project, a SOP was prepared and, based on practical experiences, an ISO Working Draft was submitted. This WD moved already to the ISO CD stage.

Table 3: List of ISO guidelines in the area of soil fauna

ISO No.	Short title (details given in the reference list)	Reference	Comments
23611-1	Earthworm sampling	ISO 2006a	Several modifications needed
23611-2	Microarthropod sampling	ISO 2006b	Modifications needed
23611-3	Enchytraeid sampling	ISO 2007a	Modifications needed
23611-4	Nematod sampling	ISO 2007b	Modifications needed
23611-5	Macrofauna sampling	ISO 2010a	Modifications needed
23611-6	Design monitoring prog.	ISO 2010b	None
?	Bait lamina	None so far	Not appropriate

2. Modification of existing guidelines and standardization of new guidelines (based on literature data and practical experience within EcoFINDERS)

2.1. Physico-chemical properties of the soil

Actually, no improvements have been made for individual abiotic methods since this was not the main aim of EcoFINDERS. However, experiences made when using them (e.g. when characterizing the soils of transect and indicator sites; see e.g. Deliverable 4.1) will be transferred to the appropriate ISO committees. In this context, it is important to know that the whole approach of standardizing soil sampling with focus on soil characteristics is under review at ISO at the moment.

Finally, the water infiltration rate into soil was measured at several indicator sites of EcoFINDERS, using a German national standard (DIN 19882-7 (2007). First experiences indicate that this method is very labor-intensive. The results gained are difficult to interpret, meaning that further work has to be performed before a decision on its suitability is possible.

2.2. Biological Methods: Soil Microorganisms

2.2.1. Sampling of soil microorganisms

Based on the experiences made during the first sampling campaign (2011) in EcoFINDERS it became obvious that the currently available guidelines (ISO 10381-6; ISO 23611-6) are not sufficient for the sampling of soil microorganisms. Therefore, for the following sampling campaigns (2012 / 2013) a SOP was prepared (see Annex III). This SOP will be extended so that it can be fitted into the existing ISO standards. Since soil sampling in general is under review within ISO there is a high probability that sampling of microorganisms will be improved in the future.

2.2.2. Modification of DNA extraction from soil (ISO 11063, ISO 17601)

The already published standard ISO 11063 was modified in order to widen the range of organisms which could be covered: so far it is focusing on bacteria, but not other microorganisms such as fungi (recommendations published by Plassart et al. 2012). In the following, only the abstract of this publication is given. The experiences made will be submitted to ISO right in time for the next regular revision of this guideline (actually, discussions on this topic have already started in TC 190).

“Soil DNA extraction has become a critical step in describing microbial biodiversity. Historically, ascertaining overarching microbial ecological theories has been hindered as independent studies have used numerous custom and commercial DNA extraction

procedures. For that reason, a standardized soil DNA extraction method (ISO-11063) was previously published. However, although this ISO method is suited for molecular tools such as quantitative PCR and community fingerprinting techniques, it has only been optimized for examining soil bacteria. Therefore, the aim of this study was to assess an appropriate soil DNA extraction procedure for examining bacterial, archaeal and fungal diversity in soils of contrasting land-use and physico-chemical properties. Three different procedures were tested: the ISO-11063 standard; a custom procedure (GnS-GII); and a modified ISO procedure (ISOm) which includes a different mechanical lysis step (a FastPrep H-24 lysis step instead of the recommended bead-beating). The efficacy of each method was first assessed by estimating microbial biomass through total DNA quantification. Then, the abundances and community structure of bacteria, archaea and fungi were determined using real-time PCR and terminal restriction fragment length polymorphism approaches. Results showed that DNA yield was improved with the GnS-GII and ISOm procedures, and fungal community patterns were found to be strongly dependent on the extraction method. The main methodological factor responsible for differences between extraction procedure efficiencies was found to be the soil homogenization step. For integrative studies which aim to examine bacteria, archaea and fungi simultaneously, the ISOm procedure results in higher DNA recovery and better represents microbial communities."

Currently, a second method aiming to estimate the abundance of selected microbial gene sequences by quantitative real time PCR from DNA directly extracted from soil is discussed within ISO (the standard 17601 is now at the CD stage). In order to clarify the potential of this method an international ring test has been launched by partners from EcoFINDERS (but participation is of course open to other scientists too). The purpose of this ring test is to evaluate the efficiency of different SyberGreen and TaqMan qPCR assays, which were chosen at the kick-off meeting, in different laboratories by considering two different parameters: (i) qPCR machine, (ii) qPCR kit. In addition, the ring test will also aim at estimating the variability of the quantification of the abundances of selected microbial gene sequences from different soil samples. An intermediate report, summarizing first results of the work of 10 partners, has been published by F. Martin-Laurent in December 2013. In March 2014, the next steps of the standardization process will be discussed at an intermediary ISO meeting in Paris, France.

2.2.3. Modification of the PLFA standard

Within WP3 a SOP for the quantification of phospholipid fatty acids (PLFA) in soils was elaborated based on the standard ISO 29843-2 (see Annex III); see also Deliverable Report 3.1. Several modifications and improvements were made to the ISO standard aiming to

shorten the operational time and to standardize the water content of each soil sample to be analyzed, making the PLFA identification more reliable without modifying their relative balance. All modifications contributed to reduce the protocol operational time (at least one overnight incubation less and a reduction to a maximum of 4 hours of the MIDI Derivatisation – Transmethylation – Clean-up protocol). None of the modifications introduced changes to the balance of PLFA in comparison to the method described in the ISO 29843-2. However, the modifications increased the total PLFA mass extracted. The major modifications introduced in this SOP are:

1. Freeze- drying (lyophilization) of soil samples in order to standardize the water content of the samples. The lyophilized sample allows preservation for microbiological analysis when sealed and kept at 4ºC.
2. The major steps that were removed from the ISO standard are: soil water content determination is not necessary after lyophilization; the first 24h incubation step during the lipid extraction at 4ºC was removed; for the separation of lipids in SI-columns, activated silica gel was used, therefore removing the need for an activation step (this shortens the methodology in at least 1 hour).
3. In order to make the identification of the PLFA more reliable, the Sherlock MIS (MIDI) software was used. To comply with the identification standards, the PLFA analysis (GC conditions and the ISTD solvent where samples are dissolved before GC injection) was adapted in conformity.
4. The ISO protocol for FAME extraction and methylation was compared with the MIDI methodology. The results obtained indicate that the MIDI protocol for FAME analysis is reproducible and the results are comparable to the ones obtained with the ISO standard.

Samples used to develop this SOP resulted from the 2011 sampling campaign on the LTOs. Results from control sites only showed a clear separation of the different sites, with the methods being able to discriminate the different PLFA profiles of the different LTOs. Currently, the power of the method in discriminating the different land-use intensity treatments within each LTO is being tested with samples collected in 2012 and 2013.

This on-going work is an interlaboratory comparison test, running between two partners of EcoFINDERS (the University of Coimbra, Portugal and the University of Lund, Sweden) in order to determine comparability of the basic and modified version as well as issues such as practicability. The results of this exercise are planned to be send to ISO to be considered in the next round of reviewing of the standard ISO 29843-2.

2.2.4. Clarification of methodological details of ISO 16072

In the following, the abstract of the recent publication of Creamer et al. (2014) is presented:

"This study evaluated the measurement of the initial rate of soil basal respiration (BR) as a potential biological indicator of ecosystem service provision. The purpose of this study was to test ISO 16072:2002 (Soil Quality: Laboratory methods for the determining of microbial soil respiration). In the literature a range of pre-incubation temperatures (pre-inc) and experimental incubation temperatures (exp-inc) have been applied when using the ISO method for the establishment of basal respiration. This study evaluated whether the range of temperatures applied during pre- and exp-incubation had a significant effect on the rate of respiration determined when following the protocol established in ISO 16072:2002. The evaluation was carried out on a pedo-climatic gradient spanning ten countries across Europe and covering four biogeographical regions. Three sites were sampled in each country providing a range of soil and land-use parameters. Our results suggest that experimental incubation temperatures of 20◦C or above should be used in the application of the methodology ISO 16072:2002 (incubation at 15◦ C resulted in erratic variation between replicates). However, pre-incubation temperature did not affect the soil basal respiration rate, when following the standard recommendations. The time interval with the best prediction of the initial rate of basal respiration was 6 h." It is expected that these experiences will be discussed at the next meeting of the ISO TC 190, Subcommittee "Microbiology".

In this context it should be mentioned that in the Deliverable 3.1 an improved, i.e. miniaturized, method for the measurement of soil microbial respiration has been described, based on a proposal by Black et al. (2011). A short overview on the content of this SOP is given in the following: This SOP was tested against 15 soils from 5 LTO's in EcoFINDERS sampled during May – July 2011. The SOP worked well and the MicroResp analysis was able to distinguish between the different soils with a high level of significance, indicating that MicroResp is a highly sensitive method for measuring catabolic activity and functional diversity of microbial communities in soil. Standing issues of the soil pre-treatment, number and identity of carbon substrates used, incubation time and the level of reproducibility are discussed. We recommend performing an inter-laboratory ring test using the same soils and assay setup, necessary for developing the SOP into an ISO standard.

This step has not been undertaken so far. However, a review on functional microbial tests is scheduled by ISO TC 190 for March 2014.

2.3. Biological Methods: Soil Fauna

2.3.1. Modification of sampling soil invertebrates

Since 2006, methods being suitable for the assessment of the habitat function of soil (i.e. the ability of soil to act as an environment for organisms) were increasingly looked for. In particular, this demand became obvious when concepts for the biological classification and assessment of soils were formulated in Germany and The Netherlands. Since the biological assessment of a soil at a specific site could lead to actions (theoretically even a remediation of that site), only standardized methods can be used for producing legally valid results.

In the meantime six monitoring standards focusing on soil invertebrates were prepared, all under one number (ISO 23611). With the exception of ISO 23611-5 (macrofauna), all standards have been used in EcoFINDERS, usually for producing specific SOPs. One general comment to be relevant for all organism-specific standards (i.e. all of 23611 except 23611-6) is the recommendation to add barcoding as possibility when describing the preparation of species determination (e.g. warning that some chemicals used during the fixation process may destroy DNA or by adding relevant references). Some detailed experiences made by partners are listed in the following. All of them will be used in the upcoming regular ISO reviews.

23611-1 (earthworms):

Currently, deep-burrowing (= anecic) earthworms are extracted by the chemical formalin, which is under pressure due to its toxicological properties. Probably AITC (Allyl-isothiocyanate) is a suitable alternative (Zaborski 2003). Efficiency of the method might be increased by time-limited sorting as already proposed by Schmidt (2001). Further guidance is also needed for site-specific conditions, e.g. sampling on slopes.

23611-2 (micro-arthropods):

Further guidance regarding extraction efficiency is needed. However, further work is needed to give specific advice. In addition, it could be added that fixation of the animals is improved by adding benzoic acid (plus a few drops of a detergent).

23611-6 (study design):

Right now, this standard focuses almost completely on soil invertebrates. However, since in many monitoring schemes both microbes and animals are studied it would make sense to add some explanatory sentences regarding the sampling of microorganisms. Again, referencing new standards as already discussed above may be sufficient. As an additional case-study, the Transect Sampling performed within EcoFINDERS could be used as a good example of efficient biological soil monitoring.

2.3.2. Proposal for a new guideline on the sampling of soil protists

When working on the genetic characterization of protists within EcoFINDERS it became obvious that these organisms are not yet covered in the ISO series of standards on soil organism sampling methods (ISO 23611). Therefore, a first protocol was prepared by the specialist working with protists which could be transformed into an ISO Working Document (see Annex III). Currently, the need for such a method in various ISO member states is discussed. A first draft of the "Modified Liquid Aliquot Method (LAM)" will be presented at the next ISO Annual Conference to be held in Berlin (Germany) in September 2014.

2.3.3. Proposal for a new functional standard method: the bait-lamina test

The bait-lamina test, already proposed more than twenty years ago (Von Törne 1990a, b), has been further developed in the WP 3 of EcoFINDERS. Later on it became the only functional method directly addressing the activity of soil fauna of the INDICATOR program of WP4. The results of the work performed in WP 3 have already been presented in the Deliverable Report D 3.3. Results of the sampling at the six INDICATOR sites support the proposal to standardize this method (see Deliverable 4.1, which has been prepared in parallel to this report). In D 4.1 not only the results of the individual tests but also a general assessment of the test, using the same criteria as during the "Indicator Selection Workshop" during the first EcoFINDERS Conference (Coimbra, 2011).

In both cases the SOP originally developed for EcoFINDERS has proven its suitability for the assessment of the activity of soil organisms (see Annex III). Therefore, this method has been submitted to ISO TC 190 "soil quality". In the following, an overview on the activities in the context of the ISO standardization is given which also provides an overview of the process of suggesting an ISO standard. Furthermore, the very detailed SOP is attached to this report.

The standardization process within ISO TC 190 can be summarized as follows:

2011, July:

Working Draft prepared by J. Römbke and submitted to ISO

2011, September:

After discussion within the Subcommittee Soil fauna the proposal was adopted at the annual conference of TC 190 (Adelaide, Australia).

2012, April:

The WD document was transferred to ISO format by the DIN secretariat. Afterwards it was distributed to all member countries in order get comments from their experts.

2012, August:

The document got an official number: ISO 18311

2012, September:

The formal version and the collected comments (22) from member countries were discussed at the annual conference of TC 190 (Helsinki, Finland). The group agreed to proceed with this test to the next step (i.e. it became a Committee Draft (CD)). Later on, the CD version was distributed to all member countries in order get comments from their experts.

2013, May:

In parallel to the international standardization process the latest version of the ISO document was translated in order to become a German DIN standard (similar steps have been (or will be) made in other member states such as France).

2013, September:

The CD version and the collected comments (40) from member countries were discussed at the annual conference of TC 190 (Fukuoka, Japan). The group agreed to proceed with this test to the next step (i.e. it became a Draft International Standard (DIS)).

Outlook:

The standard will probably be finalized at the next annual ISO conference in Berlin in 2014.

3. Other standardization efforts within EcoFINDERS

3.1. Standardization of site-specific properties

There is a general agreement that, when studying or monitoring soil biodiversity or soil functions, the following site properties should be recorded: site history (land use, prior samplings), exact geographical location (coordinates), current land-use type, climate data (at least: mean annual and monthly air temperature and precipitation; annual course of surface soil temperature), and ground-water level. However, there are few international guidelines available how to collect this kind of information. Some kind of general requirements can be found in the ISO standard 16133 (2004a), but no details are provided. Interestingly, an ISO standard which exactly described data requirements concerning land use, climate, etc. was published about ten years ago (ISO 2005e), but was not confirmed during its first review for reasons unknown. The preparation of such a guideline addressing the determination of site-specific properties was outside the focus of EcoFINDERS, but it should be kept in mind that in the context of the usage of indicators, e.g. in monitoring programs, such guidance would be very helpful.

3.2. Use of Soil Organisms for the Assessment of Contaminated Sites: the TRIAD Approach

Since 2010, ISO TC 190 has discussed the TRIAD approach, a test and evaluation strategy mainly aiming at the assessment of contaminated land, but in fact useful for various types of site assessment (Jensen & Mesman 2006). Originally, it has been proposed to ISO by scientists from the Netherlands (among them partners from EcoFINDERS, e.g. Michiel Rutgers). Currently, it has reached CD status (ISO 19204). One part of that standard is the use of monitoring methods (no detailed description, just a list of potentially useful methods). So far, results from monitoring are difficult to assess, but the NOR (Normal Operating Range) of soil organisms as going to be defined within EcoFINDERS will be a very useful tool to perform an environmental risk assessment of potentially impacted soils.

3.3. Genetic Characterization of Test Organisms in Ecotoxicological Tests

Another “side-effect” of the work of EcoFINDERS is the “Eisenia barcoding Initiative” where bar codes for the earthworm Eisenia are tested. Mainly based on experiences with earthworm barcoding in this project, an international ring test was launched, organized by, among others, ECT GmbH. It has the aim to assess the practicability and robustness of genetic characterization methods of mainly earthworms for an improved quality assurance of ecotoxicological tests. In particular, it is checked whether the species Eisenia fetida and Eisenia andrei, both regularly used in legal testing, can easily be distinguished by barcoding. These two species are difficult to separate morphologically. The results of this ring test will be used by ISO TC 190 in order to improve ecotoxicological routine testing. Furthermore, these results will also be useful for the standardization efforts undertaken by OECD, as this organization encounter the same problem as ISO when it comes to distinct identification of test species.

4. Publication of draft versions of new or modified guidelines and SOPs

In the following, two lists are provided:

- ISO documents which have been modified or proposed by members of the EcoFINDERS project. It should be noted that they are not publicly available until finalization of the modified or new guideline;
- Publications in which the modification of an existing or the preparation of a new guideline are proposed or discussed.

4.1. Modified or new ISO guidelines (with EcoFINDERS impacts)

ISO (International Organization for Standardization) (1993b): Soil quality — Sampling - Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory. ISO 10381-6. Geneva, Switzerland.

ISO (International Organization for Standardization) (2002b): Soil quality — Sampling - Part 1: Guidance on the design of sampling programs. ISO 10381-1. Geneva, Switzerland.

ISO (International Organization for Standardization) (2006a): Soil quality - Sampling of soil invertebrates Part 1: Hand-sorting and formalin extraction of earthworms. ISO 23611-1. Geneva, Switzerland.

ISO (International Organization for Standardization) (2010b): Soil quality - Sampling of soil invertebrates Part 6: Guidance for the design of sampling programmes with soil invertebrates. ISO 23611-6. Geneva, Switzerland.

ISO (International Organization for Standardization) (2011a): Soil quality - Determination of abundance and activity of the soil microflora using respiration curves. ISO 17155. Geneva, Switzerland.

ISO (International Organization for Standardization) (2011b): Soil quality - Method to directly extract DNA from soil samples. ISO 11063. Geneva, Switzerland.

ISO (International Organization for Standardization) (2011c): Soil quality - Procedure for site-specific ecological risk assessment of soil contamination (TRIAD approach). Draft. Geneva, Switzerland.

ISO (International Organization for Standardization) (2011d): Soil quality - Determination of soil microbial diversity - Part 2: Method by phospholipid fatty acid analysis (PLFA) using the "simple PLFA extraction method". ISO 29843-2. Geneva, Switzerland.

ISO (International Organization for Standardization) (2013): Soil quality — Method for testing effects of soil contaminants on the feeding activity of soil dwelling organisms — Bait-lamina test. ISO/CD 18311. Geneva, Switzerland.

ISO (International Organization for Standardization) (2012): Soil quality – Estimation of abundance of selected microbial gene sequences by quantitative realtime PCR from DNA directly extracted from soil. ISO 17601. Geneva, Switzerland.

4.2. Publication in which modified or new ISO guidelines are discussed (with EcoFINDERS impacts)

Creamer, R.E., Schulte, R.P.O., Stone, D., Galb, A., Krogh, P.H., Lo Papa, G., Murraye, P.J., Pérès, G., Foerster, B., Rutgers, M., Sousa, J.P. & Winding, A. (2014): Measuring basal soil respiration across Europe: Do incubation temperature and incubation period matter? Ecological Indicators 36: 409–418.

Philippot, L., Ritz, K., Pandard, P., Hallin, S. & Martin-Laurent, F. (2012): Standardization of methods in soil microbiology: progress and challenges. FEMS Microbial Ecol. 82: 1-10.

Plassart, P., Terrat, S., Thomson, B., Griffiths, R., Dequiedt, S., et al. (2012): Evaluation of the ISO Standard 11063 DNA Extraction Procedure for Assessing Soil. Microbial Abundance and Community Structure. PLoS ONE 7: e44279. doi:10.1371/journal.pone.0044279.

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Annex I: List of current ISO Standards

ISO (International Organization for Standardization) (1993a): Soil quality - Determination of dry matter and water content on a mass basis - Gravimetric method. ISO 11465. Geneva, Switzerland.

ISO (International Organization for Standardization) (1993b): Soil quality — Sampling - Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory. ISO 10381-6. Geneva, Switzerland.

ISO (International Organization for Standardization) (1994a): Soil quality - Determination of effective cation exchange capacity and base saturation level using barium chloride solution. ISO 11260. Geneva, Switzerland.

ISO (International Organization for Standardization) (1994b): Soil quality - Pretreatment of samples for physico-chemical analysis. ISO 11464. Geneva, Switzerland.

ISO (International Organization for Standardization) (1995a): Soil quality - Determination of organic and total carbon after dry combustion (elementary analysis). ISO 10694. Geneva, Switzerland.

ISO (International Organization for Standardization) (1995b): Soil quality - Determination of total nitrogen - Modified Kjeldahl method. ISO 11261. Geneva, Switzerland.

ISO (International Organization for Standardization) (1995c): Soil quality — Sampling . ISO 10381-5. Geneva, Switzerland.

ISO (International Organization for Standardization) (1997a): Soil Quality - Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes. ISO 14238, Geneva, Switzerland.

ISO (International Organization for Standardization) (1997b): Soil Quality - Determination of soil microbial biomass. Part 1: Substrate induced respiration method. ISO 14240-1, Geneva, Switzerland.

ISO (International Organization for Standardization) (1997c): Soil Quality - Determination of soil microbial biomass. Part 2: Fumigation-extraction method. ISO 14240-2, Geneva, Switzerland.

ISO (International Organization for Standardization) (1998a): Soil quality - Determination of total nitrogen content by dry combustion. ISO 13878. Geneva, Switzerland.

ISO (International Organization for Standardization) (1998b): Soil quality - Determination of organic carbon by sulfochromic oxidation. ISO 14235. Geneva, Switzerland.

ISO (International Organization for Standardization) (2001): Soil quality — Sampling - Part 3: Guidance on safety. ISO 10381-3. Geneva, Switzerland.

ISO (International Organization for Standardization) (2002a): Soil quality — Laboratory methods for determination of microbial soil respiration. ISO 16072. Geneva, Switzerland.

ISO (International Organization for Standardization) (2002b): Soil quality — Sampling - Part 1: Guidance on the design of sampling programs. ISO 10381-1. Geneva, Switzerland.

ISO (International Organization for Standardization) (2002c): Soil quality — Sampling - Part 2: Guidance on sampling techniques. ISO 10381-2. Geneva, Switzerland.

ISO (International Organization for Standardization) (2002d): Soil quality — Format for recording soil and site information. ISO 15903. Geneva, Switzerland.

ISO (International Organization for Standardization) (2003a): Soil quality — Determination of nitrate, nitrite and ammonium in field-moist soils by extraction with potassium chloride solution. Part 1: Manual method. ISO 14256-1. Geneva, Switzerland.

ISO (International Organization for Standardization) (2003b): Soil quality — Sampling - Part 4: Guidance on the procedure for investigation of natural, near-natural and cultivated sites. ISO 10381-4. Geneva, Switzerland.

ISO (International Organization for Standardization) (2004a): Soil quality — Guidance on the establishment and maintenance of monitoring programmes. ISO 16133. Geneva, Switzerland.

ISO (International Organization for Standardization) (2004b): Soil quality — Determination of potential nitrification and inhibition of nitrification - Rapid test by ammonium oxidation. ISO 15685. Geneva, Switzerland.

ISO (International Organization for Standardization) (2005a): Soil quality - Determination of pH. ISO 10390. Geneva, Switzerland.

ISO (International Organization for Standardization) (2005b): 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 14256-2. Geneva, Switzerland.

ISO (International Organization for Standardization) (2005c): Soil quality — Determination of dehydrogenase activity in soils - Part 1: Method using triphenyltetrazolium chloride (TTC). ISO 23753-1. Geneva, Switzerland.

ISO (International Organization for Standardization) (2005d): Soil quality — Determination of dehydrogenase activity in soils - Part 2: Method using iodotetrazolium chloride (INT). ISO 23753-2. Geneva, Switzerland.

ISO (International Organization for Standardization) (2005e): Soil quality — Simplified soil description. ISO 11259. Geneva, Switzerland.

ISO (International Organization for Standardization) (2006a): Soil quality - Sampling of soil invertebrates Part 1: Hand-sorting and formalin extraction of earthworms. ISO 23611-1. Geneva, Switzerland.

ISO (International Organization for Standardization) (2006b): Soil quality - Sampling of soil invertebrates Part 2: Sampling and extraction of microarthropods (Collembola and Acarina). ISO 23611-2. Geneva, Switzerland.

ISO (International Organization for Standardization) (2006c): Soil quality - Guidance on long and short-term storage of soil samples. ISO 18512. Geneva, Switzerland.

ISO (International Organization for Standardization) (2007a): Soil quality - Sampling of soil invertebrates Part 3: Sampling and soil extraction of enchytraeids. ISO 23611-3. Geneva, Switzerland.

ISO (International Organization for Standardization) (2007b): Soil quality - Sampling of soil invertebrates Part 4: Sampling, extraction and identification of free-living stages of nematodes. ISO 23611-4. Geneva, Switzerland.

ISO (International Organization for Standardization) (2010a): Soil quality - Sampling of soil invertebrates Part 5: Sampling and extraction of soil macro-invertebrates. ISO 23611-5. Geneva, Switzerland.

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ISO (International Organization for Standardization) (2010c): Soil quality -- Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates. ISO/TS 22939.

ISO (International Organization for Standardization) (2010d): Soil quality - Determination of soil microbial diversity - Part 1: Method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis. ISO 29843-1. Geneva, Switzerland.

ISO (International Organization for Standardization) (2011a): Soil quality - Determination of abundance and activity of the soil microflora using respiration curves. ISO 17155. Geneva, Switzerland.

ISO (International Organization for Standardization) (2011b): Soil quality - Method to directly extract DNA from soil samples. ISO 11063. Geneva, Switzerland.

ISO (International Organization for Standardization) (2011c): Soil quality - Procedure for site-specific ecological risk assessment of soil contamination (TRIAD approach). Draft. Geneva, Switzerland.

ISO (International Organization for Standardization) (2011d): Soil quality - Determination of soil microbial diversity - Part 2: Method by phospholipid fatty acid analysis (PLFA) using the "simple PLFA extraction method". ISO 29843-2. Geneva, Switzerland.

ISO (International Organization for Standardization) (2012): Soil quality – Estimation of abundance of selected microbial gene sequences by quantitative realtime PCR from DNA irectly extracted from soil. ISO 17601. Geneva, Switzerland.

ISO (International Organization for Standardization) (2013): Soil quality — Method for testing effects of soil contaminants on the feeding activity of soil dwelling organisms — Bait-lamina test. ISO/CD 18311. Geneva, Switzerland.

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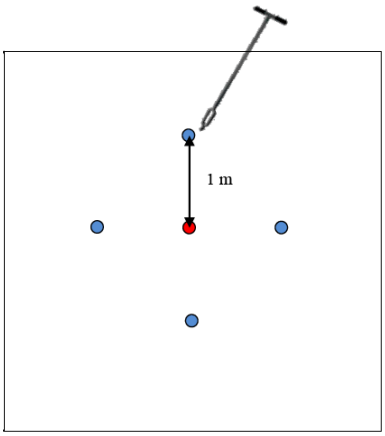
Annex III: SOPs

SOP for Chapter 2.2.1: Sampling of soil microorganisms

1. Materials needed

- Sealable plastic bags with sample names / code
- Spare bags and permanent markers
- Soil corers
- Plastic trays to carry or homogenize samples
- Brush to clean trays
- Paper wipes (there is always something that needs to be cleaned)
- Protective clothing, including gardening gloves
- Lab gloves
- Pickets to mark sampling points
- GPS
- Cool boxes and cool blocks
- Sterile, labeled bags
- Spare pillboxes and labels
- Balance
- Sample list to record weights
- Stainless steel 4mm sieve



2. Soil sampling

Sampling will be done down to 15-20cm depth with a soil corer / auger. Every sample should be a composite of at least five soil cores: one central core and the other ones taken 1m around. These cores should be stored in a sealable plastic bag to roughly homogenize them until sieving.

3. Processing soil samples

This part aims to describing how to prepare a soil sample representative of the sampling point.

3.1 Conditions

Samples preparation should be done in optimized conditions to avoid inter-sample contamination. This means:

- Clean trays should be used
- Trays should be cleaned between samples
- Gloves should be worn and changed between samples

3.2 Sample preparation

Empty the whole sample bag in a tray

Break the macro-aggregates by hand (with gloves on)

Mix the soil to obtain an homogeneous sample

Sieve (4mm) the sample, need at least 1.62kg sieved soil per plot

Weigh the fresh sample and store in a cool box. Post to recipients as soon as possible and inform recipients that soil has been sent.

SOP for Chapter 2.2.2: Modification of DNA extraction from soil

No SOP provided since this information was already published by Plassart et al. (2012).

SOP for Chapter 2.2.3: Modification of the existing PLFA-Standard (ISO 29843-2) (see also Deliverable D 3.1)

Aim of the Standard Operating Procedure:

To extract and identify PLFA from soil samples

Justification of the Method:

Different methodologies for determination of soil fatty acids are available. These methodologies present different levels of complexity when applied and provide different levels of resolution in the description of soil microbial communities.

The method presented in this SOP is accessible for most research and analytical laboratories involved in soil sciences. The method simplifies the one described in the standard ISO 29843-2 [4], and can be used with a wide range of soils with different soil properties. It provides a broad diversity measurement of a soil microbial community at the phenotypic level. It can be applied to biomass estimation and can be used to differentiate microbial communities among different soil samples. It can also be used for a rough description of microbial groups present in soil samples (e.g. gram-positive bacteria, actinomycetes, fungi, etc.). The method described in the standard ISO 29843-2 [4] was derived from the one first proposed by Bligh and Dyer [1] and later modified by White et al. [8]. The method proposed in this SOP results from a modification of the ISO guideline, including a second method for fatty acids identification.

Summary:

Soils are lyophilized and lipids are extracted using the Bligh and Dyer [1] extraction procedure. Lipid extracts are separated by liquid chromatography using an SI-column. Phospholipids are transformed into fatty acid methyl esters (FAME) by mild alkaline hydrolysis followed by methylation. The different FAMEs are measured by Gas Chromatography (GC), identified and quantified using standards or Sherlock MIS data base. Alternative methods are included for derivatisation, transmethylation and clean-up of the fatty acids.

1. Test materials: Soil

Collect soil samples and keep them at -80ºC. Deep-freezing should be done as soon as possible after soil sampling. NOTE: Between collection and deep-freezing samples should be kept at 4ºC but not be frozen at -20ºC.

2. Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade or HPLC grade when specified.

Organic Solvents: Acetone, C3H6O (HPLC grade); Chloroform, CHCl3 (HPLC grade); Hexane, C6H14, Methanol, CH4O (HPLC grade); Toluene, C7H8, Methyl tert-butyl ether;

Chemicals: 2.2.1 2,6-Di-tert-butyl-4-methylphenol (BHT), C15H24O; Citric acid, C6H8O7, H2O; Tri-sodium citrate, C6H5Na3O7,2H2O; Silica Gel 230-400 (40-63 µ) Average pore diameter: 60 Å; Anhydrous sodium sulphate, Na2O4S; Potassium hydroxide, KOH; Acetic acid, C2H4O2; Sodium hydroxide, NaOH; Nonadecanoic acid methyl ester, C20H40O2; Nitrogen gas (N2) HCl.

Solutions and standards: CM (Chloroform : Methanol) solution; Chloroform : Methanol (1 :2) + 0.005 % 2,6-Di-tert-butyl-4-methylphenol (BHT); CB (citrate buffer) solution: – 0.15 mol l-1 citric acid monohydrate: dissolve 15.76 g C6H8O7, H2O in 500 ml H2O; 0.15 mol l-1 tri-sodium citrate: dissolve 22.06 g C6H5Na3O7,2H2O in 500 ml H2O; For pH = 4 , add 59 ml of CB solution to 41 ml of tri-sodium citrate solution.
BD : Bligh and Dyer solvent: Chloroform :Methanol :citrate buffer (ratio 1 :2 :0.8) + 0,005 % 2,6-Di-tert-butyl-4-methylphenol (BHT). Example (100 ml Chloroform: 200 ml Methanol : 80 ml CB) + BHT; Methanolic KOH: 0.2 mol l-1 KOH: dissolve 0.56 g KOH in 50 ml dry methanol (anhydrous sodium sulfate); SE Solvent for extraction: Hexane : chloroform (4 :1 vol/vol); Acetic acid 1 mol l-1 : 58 ml l-1; Sodium Hydroxide 0.3 mol l-1 : 12 g l-1; Standard ISTD (C19:0 FAME): 10 mg nonadecanoic acid methyl ester in 1 ml hexane stock solution => dilution 1 : 100 with hexane.
MIDI solutions and standards: Reagent 1: 45g sodium hydroxide, 150ml methanol, and 150ml distilled water; Reagent 2: 325ml certified 6.0N hydrochloric acid and 275ml methyl alcohol; Reagent 3: 200ml hexane and 200ml methyl tert-butyl ether; Reagent 4: 10.8g sodium hydroxide dissolved in 900ml distilled water.

3. Consumables

Polypropylene 20 ml tubes; Pasteur pipettes ; Glass tubes with teflon lined caps, for centrifuging (20 ml); Glass flasks with teflon line caps (10 ml); For homemade columns: Polypropylene pipette tips (1, 5 or 10 ml); Ashless Flocks; MIDI extraction tubes (13x100 culture tube sealed with teflon lined caps). You can also use 12ml vials with Teflon lined caps.

4. Apparatus

Usual laboratory equipment: Fume cupboard; Ultrasonic bath; Centrifuge; Fridge, freezer; Oven; Vortex shaker; Water bath; Lyophilizer with dissecator jar; Gas chromatograph; GC; Flame ionisation detector; Fused silica capillary column (30 m x 0,,25 mm x 0,,25 µm film thickness; Helium as carrier gas.

5. Procedures

5.1 Soil lyophilisation

6 g of fresh soil are placed in a Petri dish and frozen at -80ºC for 3h. Frozen soil is subjected to vacuum under 280 mT overnight.

NOTE: In case you are using soil kept at -80ºC you perform lyophilisation directly.

5.2 Lipid extraction (Bligh-Dyer-extraction) (chloroform/methanol extraction)

2 g of lyophilized soil are placed in a 20 ml centrifuge tube. Add 11.9 ml of CM (chloroform/methanol) solution to the tube and 3.16 of CB (citrate buffer) solution. The sample is then placed on a vortex shaker and put into the ultrasonic bath for 30 min. Leave this sample at 4ºC overnight (leaving the solution stand overnight may increase the yield).

Next day, the sample is shaken and centrifuged for 10 min at 1 200 rpm. The supernatant is transferred into a clean, labelled 40 ml flask. Add 5 ml of the BD (chlormeth : citrate) solvent to the tube containing the soil slurry. The obtained mixture is shaken again and centrifuged for further 10 min at 1 200 rpm. The supernatant is then transferred to the 40 ml flask and another 5 ml of the BD solvent are added to the remnant tube with the soil slurry. The mixture is shaken a third time and centrifuged for 10 min at 1 200 rpm. The supernatant of this third centrifugation is added to the previous two in the 40ml flask. 4 ml of chloroform and 4 ml of CB are then added to the supernatants flask to split the phase. The flask is then centrifuged at 1 200 rpm for 10 min, for phase separation. The upper layer of the sample is removed and discarded. The lower layer is dried under N2 at 50°C. Samples can then be stored either in a fridge (at 4ºC) or deep-freezer (at -80ºC).

NOTE: If maintained at 4ºC one should proceed as soon as possible

5.3 Separation of lipids by SI-column

Use an activated silica cartridge. Introduce the activated silica in the tip and add 3 ml chloroform. Allow the chloroform to dry. The cartridge is first conditioned with 2 ml of methanol, then 2 ml of acetone, followed by 2 ml of chloroform. Finally, the cartridge is then conditioned with 2 ml of chloroform.

NOTE: from now on, do not allow the sorbent (silica) to dry out between solvents.

The lipid extract is reconstituted in 300 µL of chloroform and added to the top of the cartridge through a filter. The filter may be made from a 5 ml pipette tip containing ashless flock and 2.5 cm of anhydrous sodium sulphate. First add 5 ml of chloroform, then 12 ml of acetone followed by 8 ml of methanol to the lipid extract. The elutant obtained from the methanol addition is collected in a clean, labelled 20 ml tube. Evaporate this elutant to dryness under N2 at 40°C and deep-freeze (-80ºC).

5.4 Derivatisation – Transmethylation – Clean-up

5.4.1 Simple PLFA extraction method

The fractionated sample (resulting from previous step) is dissolved in 0.5 ml of dry methanol and 0.5 ml of dry toluene and then 1 ml of methanolic KOH is added. Place the sample on the shaker and then incubate it at 37°C for a minimum of 30 min. The reaction is stopped by the addition of 0.3 ml acetic acid (1M), 5 ml of the SE solvent and 3 ml of water. The sample is shaken and cleaned in the ultrasonic bath for 30 min. The sample is then centrifuged at 1 200 rpm for 5 min. The aqueous phase (bottom) is then removed and discarded.

For the final cleaning, add 3 ml of base wash reagent (NaOH 12 g l-1). This time the sample is shaken for 30 sec and then centrifuged for 15 min at 1 200 rpm. The supernatant is transferred into a clean, labelled 12ml vial with a teflon lined cap (or a MIDI extraction tube) through a filter (eg. pipette tip with ashless flock and anhydrous sodium sulphate). The aqueous layer is washed again with 3 ml of SE solvent. Centrifuge this for 5 min at 1 200 rpm. Transfer the supernatant into the same vial using the filter. Repeat these last two steps (ie. SE addition and centrifuge) and collect all three supernatants in one vial and discard the aqueous layer. Evaporate these supernatants to dryness under N2 at 40°C. Freeze samples (-20°C) until required for GC analysis.

5.4.2 MIDI Protocol

Saponification - 1.0ml of Reagent 1 is added to each tube containing the evaporated lipid extract. The tubes are securely sealed with teflon lined caps, vortexed briefly and heated in a boiling water bath for ca. 5 minutes, at which time the tubes are vigorously vortexed for 5-10 seconds and returned to the water bath to complete the 30 minute heating.

Methylation - The cooled tubes are uncapped, 2ml of Reagent 2 are added. The tubes are capped and briefly vortexed. After vortexing, the tubes are heated for 10 ± 1 minutes at 80° ± 1°C. (This step is critical in time and temperature.)

Extraction - Addition of 1.25ml of Reagent 3 to the cooled tubes is followed by recapping and gentle tumbling on a clinical rotator for about 10 minutes. The tubes are uncapped and the aqueous (lower) phase is pipetted out and discarded.

Base Wash - 3ml of Reagent 4 is added to the organic phase remaining in the tubes, the tubes are recapped, and tumbled for 5 minutes. Following uncapping, about 2/3 of the organic phase is pipetted into a GC vial. Samples are then dried under N2 at 40°C, capped and stored at -20°C until required for GC analysis.

5.5 PLFA analysis

Dissolve the sample in 1.35 ml of MIDI solvent 3 (smaller volumes may not allow complete lipid recovering) and add it to a 200 µl tube adapter for GC vials, with an Hamilton syringe. The sample was concentrated under N2 flux, to dryness. 50 µl of MIDI reagent 3 is added to the dry sample for reconstitution. 50 µl standard ISTD, including 19:0 FAME reference, are added in order to have 0.5 ng in a volume of 100 µl. 10 µl of the sample are injected into the capillary column (injector temperature 170 °C) of the GC. The temperature program ramps from 170°C to 270°C at 5°C per minute. Hydrogen is the carrier gas, nitrogen is the "makeup" gas, and air is used to support the flame. Following the analysis, a ballistic increase to 300°C allows cleaning of the column during a hold of 2 minutes. Identification of fatty acids is performed by comparing the results with spectra that were obtained from standards.

6. Measurements and Records:

Calculation of PLFA biomass: [(PLFA Peak Area/Reference Peak Area)*injected reference mass]/soil sample weight. The injected reference mass (19:0 FAME) was of 0.5 ng.

Calculation of %PLFA in samples: [PLFA area peak/(total peak area-Reference peak Area)]*100.

Comments:

All modifications contributed to reduce the protocol operational time (at least one overnight incubation less, and the MIDI Derivatisation – Transmethylation – Clean-up protocol takes only 4 hours). None of the modifications introduced changes the balance of PLFA in comparison to the method described in the ISO 29843-2 [4]. In addition, the modifications increased the total PLFA mass. The major modifications introduced in this SOP are:

1- Freeze- drying (lyophilization) of soil samples in order to standardize the water content of the samples. The lyophilized sample allows preservation for microbiological analysis when sealed and kept at 4ºC.

2- The major steps were removed from ISO are: soil water content determination is not necessary after lyophilization; the first 24h incubation step during the lipid extraction at 4ºC was removed; for the separation of lipids in SI-columns, activated silica gel was used,

therefore removing the need for an activation step (this shortens the methodology in at least 1 hour).

3- In order to make the identification of the PLFA more reliable, the Sherlock MIS (MIDI) software was used. To comply with the identification standards, the PLFA analysis (GC conditions and the ISTD solvent where samples are dissolved before GC injection) was adapted in conformity.

4- The ISO protocol for FAME extraction and methylation was compared with the MIDI methodology. The results obtained indicate that the MIDI protocol for FAME analysis is reproducible and the results are comparable to the ones obtained with the ISO.

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SOP for Chapter 2.3.1: Modification of sampling soil invertebrates

No SOP has been prepared for this activity. The items to be modified in or added to the existing ISO guidelines are already listed in Chapter 2.3.1.

SOP for Chapter 2.3.2: Proposal for a new guideline on the sampling of soil protists

The liquid aliquot method (LAM) is an enrichment cultivation technique for enumeration and determination of soil protists, which was developed by Butler and Rogerson (1995) with slight modifications to simplify the procedure. We modified the LAM to reduce the workload and increase the sample-throughput. The procedure is outlined below.

- Homogenize 10-20 g fresh weight (fw) soil in a plastic bag by vigorous shaking for ten seconds.
- Take a subsample of ~ 2g and oven dry at 80 °C for 2 days to determine the soil water content
- Suspend a 1 g subsample in 200 ml Neff's Modified Amoeba Saline (NMAS (Page 1976)) and vigorously shake on an orbital shaker/overhead shaker at 100 rpm for 10 min
- Inverse the suspension by hand and shake vigorously for 10 sec to detach protozoa from soil particles.
- Let homogenized samples settle for 5 min
- Pre-fill 144 wells of flat bottom, 96-well microtitre plates with 195 µl Prescott-James (PJ) medium (Page 1991) enriched with 0.15 % wheat grass (WG) (Weizengras, Sanatur GmbH, Germany)
- Inoculate 5 µl aliquots from the centre of the soil suspension into each of the 144 pre-filled wells
- Seal plates with Parafilm and incubate at 15°C in the dark

To enumerate and identify protists, examine the wells with an inverted microscope at 100x - 400x magnification and record identified protists twice after 14 and 28 days. Calculate protist abundances determined in fresh weight soil to dry weight soil.

Advantages of this method

- Detailed identification of protists in a transparent medium possible, as soil particles are largely removed and particulate nutrient sources are not needed in the medium used
- Little space needed (1.5 x 96well plates per sample)

- Standardized, easy-accessible and easy-to-prepare media used
- Very fast and easy sample preparation/setup
- Usually a maximum of one species per well
- Avoids problems of differentiating morphologically similar species in mixed samples
- Allows follow-up molecular studies as distinct taxa are separated

Remaining problems

- Time consuming identification necessitating expert knowledge for reliable identifications
- Cultivation bias towards cultivable and bacterivorous taxa
- Also inactive, dormant stages (cysts) are being cultivated (problem of all enrichment cultivation methods- no alternatives for flagellates and amoebae except molecular methods).

Butler, H. and A. Rogerson. 1995. Temporal and Spatial Abundance of Naked Amoebae (Gymnamoebae) in Marine Benthic Sediments of the Clyde Sea Area, Scotland. Journal of Eukaryotic Microbiology 42:724-730.

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SOP for Chapter 2.3.3: Proposal for a new functional standard method: the bait-lamina test (see also Deliverable 3.3)

Aim of the Standard Operating Procedure

In this SOP test set-up, sampling and optical evaluation of the feeding activity of soil organisms on small portions of a thin-layered bait substrate exposed in the field is described in detail.

Summary

This standard describes a technique for determining the effects of substances and the prevailing environmental conditions on the feeding rate of soil organisms in the field. The information gained can be used as a prerequisite for using this functional endpoint as an indicator for the assessment of the quality of a soil as a habitat for organisms.

Background

The bait-lamina test was developed for measuring the biological activity of soils (Von Törne 1990). In order to evaluate this biological activity in field soils, it is necessary to standardise the assessment of the feeding activity of soil invertebrates (e.g. earthworms, Collembola, Diplopoda, Enchytraeidae) and, to a lesser extent, microorganisms (Helling et al. 1998). The bait-lamina test consists of small plastic sticks with holes, in which bait material is filled-in (Kratz 1998). In principle, the loss of the bait material is assessed by counting the empty apertures after a certain exposure time. The number of empty apertures (i.e. areas from which the bait material has been removed) as well as their vertical distribution are used as assessment endpoints. As environmental conditions such as climate or soil moisture may influence the results (Kratz & Pieper 1999), the test should preferably be applied for comparing the biological activity between closely situated plots (e.g. contaminated versus reference sites) (André et al. 2009, Geissen et al. 2007, Geissen et al. 2008, Hamel et al. 2007).

Necessary Material and Equipment

Bait-lamina sticks (see Fig. 1)

Plastic sticks of 120 mm x 6 mm x 1 mm, which have a pointed tip at the lower end. In the lower part (85 mm) of each stick 16 bi-conical apertures of 1.5 mm diameter are drilled, which are 5 mm apart from each other.

Bait material

Organic material used as bait for soil organisms (a mixture of Cellulose (70 %), finely ground wheat bran (25 %) and activated charcoal (5 %))

NOTE: In this case, the bait material will be already filled in the bait-lamina sticks, i.e. they can directly be used in the field.

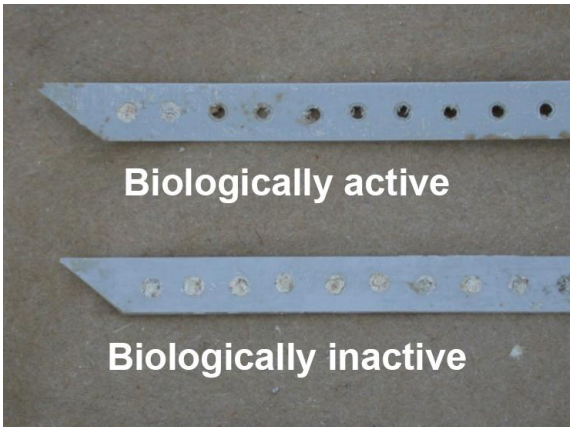

Fig. 1: Bait-lamina stick (lower end) exposed in the field: up: bait-material is gone (= fed); below: still filled with bait-material

Sharp tool

Tool formed like a long knife or a screw-driver which has to be used to drill a small slit into the soil in which the individual bait-lamina sticks is inserted when the soil is too dry or compact for direct manual insertion.

Tissue paper

Laboratory paper used to clean dishes or tables; here needed for the wrapping of the bait-lamina sticks as a protection while transported.

Small plastic sacks (e.g. 20 x 10 cm)

Needed for the transport of the wrapped-in bait-lamina sticks

Tags or labels

Used for the identification of the small plastic sacks; in case no labels etc. are provided centrally, a water-proof marker has to be used.

Marker sticks

Wooden or plastic sticks of 30 – 50 cm length (preferably coloured) to be used in the field to improve the retrieval of the bait-lamina sticks (Fig. 2).

Fig. 2: Bait-lamina sticks at a forest site, together with a coloured marker.

Performance of the test: start

At the beginning of the test, the sticks are inserted vertically by hand in the soil such that the uppermost aperture is just beneath the soil surface. The depth of the insertion of the sticks has to be determined carefully, since it is often not really clear where the soil surface actually starts (e.g. due to a litter layer or extended roots (turf)). The main criterion is that soil organisms have access to the bait and that the way of exposure of all sticks is similar. In

dry soils as well as in those with high clay content, the insertion by hand is often not possible. In such cases a metallic (or wooden) tool has to be used (Fig. 3, 4). However, it must be secured that the holes made with this tool are not larger than the diameter of the plastic stick itself. The sticks should always be in close contact with the surrounding soil.

Fig. 3: Bait-lamina sticks at a grassland site

Fig. 4: Bait-lamina sticks at a crop site

Finally, the location of the sticks has to be marked since due to their (usually) grey colour and small size individual sticks are often difficult to find, especially after long exposure periods (see Figure 2).

Performance of the test: exposure period and test end

The prepared bait-lamina sticks are exposed in the soil for different periods of time, depending mainly on the climate, the soil properties and the composition and abundance of the soil organism community at the test site. On average, the exposure time lasts 10 - 20 d in temperate zones (Förster et al. 2004) in order to reach a feeding rate of at least 50 % (i.e., 50% of empty holes in the control treatment). However, these numbers can differ considerably: e.g., at a coniferous forest site in Southern Germany, with an acid soil and with few macrofauna, the best exposure time was 100 days (Römbke, pers. comm.).

Because of these differences it is recommended to check visually (already in the field) the feeding rate at individual sticks (in the case of the sampling design adopted for Ecofinders LTO sampling, at least five per plot (= 5 out of 40); i.e. 15 in total at the three control or treatment plots) after two weeks in order to identify if at least 50 % of all bait has been consumed (holes are empty). This has to be done as follows: the individual stick is drawn out of the soil and cleaned from large soil or organic particles using a tissue paper. By holding it against the light (see Figure 5), it can easily be decided whether the bait of a hole has been fed or not: in case light is crossing through the hole the respective bait is classified as “empty” (= fed).

Fig. 5 Bait-lamina sticks hold against the light after exposure in the field. (Foto: Dr. H. Hoefer)

After this check, two options are then possible:

Option 1: In case the feeding rate is still lower than 50 %, the sticks are put back into their holes in the soil and the next assessment of the bait-lamina sticks is postponed for at least another week, depending on the amount of empty holes (for the distinction between fed and non-fed holes see also Figure 1). This procedure has to be repeated until the feeding rate is higher than 50% of the total number of baits.

Option 2: In case the feeding rate is > 50% in the selected sticks, all bait-lamina sticks of all plots are sampled.

Then a visual evaluation has to be done in the field as described in the previous paragraph. The number and the location of the empty apertures are counted and the results in the attached form (see the end of this document). For each sample an own form has to be used. Afterwards (still in the field), all bait-lamina sticks belonging to the same sample are wrapped together in tissue paper (or comparable material). The bundle is put into a plastic sack which has to be labelled (both at inside and outside). The bundles have to be kept under dry conditions, preferably at temperatures below 25°C.

In the laboratory, each bait-lamina is then checked under a stereo microscope for empty holes and the data recorded in the corresponding evaluation form. The percentage of feeding activity (number of empty holes in relation to the total number of holes) can be calculated for each sample either considering the total depth (12 cm) or at each individual depth (down to a 0.5cm interval), according to the goal of the study.

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SOP - Feeding activity of soil organism using the bait-lamina test method – Evaluation form

This evaluation form is refers to the sampling design adopted at the Ecofinders fall sampling campaign (each sample is composed of 8 bait strips). However it can be adapted according to the number of bait strips used.

Site Name: _____

Treatment: _____

Plot No.:_____

Sample No.:_____

Date of insertion: _____

Date of retrieval: _____

Hole No.	Bait-Lamina Stick No.: 1	2	3	4	5	6	7	8
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								

X = Hole empty

— = Hole not empty