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Soil Biodiversity Pilot second year draft report

Methodological challenges and lessons learnt

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What is Biodiversa+

The European Biodiversity Partnership, Biodiversa+, supports excellent research on biodiversity with an impact for policy and society. Connecting science, policy and practice for transformative change, Biodiversa+ is part of the European Biodiversity Strategy for 2030 that aims to put Europe's biodiversity on a path to recovery by 2030. Co-funded by the European Commission, Biodiversa+ gathers partners from research funding, programming and environmental policy actors in European and associated countries to work on 5 main objectives:

1. Plan and support research and innovation on biodiversity through a shared strategy, annual joint calls for research projects and capacity building activities
2. Set up a network of harmonised schemes to improve monitoring of biodiversity and ecosystem services across Europe
3. Contribute to high-end knowledge for deploying Nature-based Solutions and valuation of biodiversity in the private sector
4. Ensure efficient science-based support for policy-making and implementation in Europe
5. Strengthen the relevance and impact of pan-European research on biodiversity in a global context.

More information at: https://www.biodiversa.eu/

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List of acronyms

ASV	Amplicon Sequence Variant
DNA	Deoxyribonucleic Acid
eDNA	Environmental Deoxyribonucleic Acid
EU	European Union
EBV	Essential Biodiversity Variable
FAO	Food and Agricultural Organisation of the United Nations
GLOSOLAN	Global Soil Laboratory Network
GPS	Global Positioning System
HTS	High-throughput Sequencing
LUCAS	Land Use/Cover Area frame Survey
MBAG	Monitoring Network for Biodiversity in the Agricultural area of Flanders
NETSOB	International Network on Soil Biodiversity
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
RMQS	Le Réseau de Mesures de la Qualité des Sols
SDG	Sustainable Development Goals
SISEBIO	Catalan Plot System for Terrestrial Biodiversity Monitoring
SoilBON	Soil Biodiversity Observation Network

Table of abbreviations of forest types

Code	Category
FDB	Beech forests of the nemoral and Alpine region
FDC	Broadleaved forests of the Black Sea region
FDE	Oak forests of Continental and Atlantic Europe
FDS	Thermophilous deciduous forest (supramediterranean)
FEH	Broadleaved evergreen forest of the Mediterranean region
FEL	Broadleaved evergreen forest of Macaronesian region
FCM	Coniferous forests of the Mediterranean, Anatolian and Macaronesian region
FCP	Boreal and Alpine spruce forests
FCL	Alpine Swiss pine and larch forests
FCY	Boreal and hemi-boreal Scots pine forests

Executive Summary

Biodiversa+ has established a series of pilots as a proof of concept for its tasks leading to the establishment of a transnational network of biodiversity monitoring systems. In line with one of the priorities of Biodiversa+, the pilot on "Soil biodiversity in protected semi-natural forests" was launched in January 2023.

The aims of the pilot are:

- To develop a feasible experimental design and to define common protocols for field and laboratory work;
- To test the applicability and requirements of eDNA methods in such a scheme to obtain high resolution taxonomic data;
- To test the applicability of EBVs related to soil biodiversity to such monitoring schemes;
- To link the pilot to international and EU policies;
- To evaluate the coordination, cooperation and governance of a transnational soil biodiversity monitoring.

This report focuses on the methodological challenges of transnational monitoring and describes the advantages and limitations of suitable methods (established traditional methods and molecular methods). It also compares the results of the pilot for both methods. The applicability of molecular (eDNA) methods for soil invertebrate monitoring is still limited and problems and possible solutions are discussed.

The pilot was coordinated by the Autonomous Province of Bolzano (Italy) through Eurac Research as third party, and was conducted with ten active partners: Autonomous Region of the Azores (Portugal - FRCT), Belgium (VL O), Denmark (MoE_DK), France (OFB), Germany (BMUV), Israel (MoEP), Province of Bolzano (Italy - BOZEN), Slovakia (SAS), Sweden (SEPA), and Turkey (TAGEM).

1. Introduction

Biodiversa+ aims at promoting and supporting transnational biodiversity monitoring by building a transnational network of harmonised biodiversity monitoring schemes on specific priority topics[1]. One of these Biodiversa+ priorities focuses on soil biodiversity.

To advance such a transnational soil biodiversity monitoring scheme, the aims of the pilot are:

- To develop a feasible experimental design and to define common protocols for field and laboratory work;
- To test the applicability and requirements of eDNA methods in such a scheme to obtain high resolution taxonomic data and compare the results with results obtained by traditional methods;
- To test the applicability of EBVs related to soil biodiversity to such monitoring schemes;
- To link the pilot to international and EU policies;
- To evaluate the coordination, cooperation and governance of a transnational soil biodiversity monitoring.

The results of the pilot should not only contribute to a better understanding of soil biodiversity, but also identify ways to take action in order to conserve or restore it. In this way, the pilot supports actions for the forthcoming European Commission's Biodiversity Strategy for 2030 and the Soil Monitoring Law. On an international level, the pilot is relevant for the SDG framework, the recently adopted Kunming-Montreal Global Biodiversity Framework (United Nations, 2015; United Nations Convention on Biological Diversity, 2022), the International Network on Soil Biodiversity (NETSOB), and the Global Soil Laboratory Network (GLOSOLAN), all of which aim at providing the biological and ecosystem information needed to implement sustainable management and conservation of soils. While the SDG framework and the Global Biodiversity Framework operate on a broader scale (not focused on soil), NETSOB and GLOSOLAN, both initiated by FAO, aim to provide the means to globally promote the sustainable use and conservation of soil biodiversity, and to establish a global network of laboratories to analyse soil properties in a harmonised manner, respectively. The pilot project can contribute to these initiatives by providing the data and indicators needed to monitor and evaluate local soil biodiversity and by testing harmonised protocols.

According to a recent study, soils are home to the vast majority (59 % (± 15)) of life on earth (Anthony et al., 2023), which is twice as much as the previous estimate (25 % by Decaens et al., 2006), and many more, particularly from the microbial species pool, are still unknown. The importance of soil organisms for ecosystem functioning is undisputed, but how soil organisms are affected by human intervention and global change, and how changes in community composition affect ecosystem processes, remains largely unexplored. To obtain a holistic view of local soil biodiversity, many different methods are needed to cover this enormous diversity of soil organisms (microbes, micro-, meso- and macrofauna, Potapov et al. 2022). Furthermore, until the advent of high-throughput sequencing (HTS), species identification has been difficult due to the wide range of taxa that make up soil communities, and taxa have mostly only been identified at the order, family (for invertebrates) or operational taxonomic unit (OTU, for microorganisms)

[1] Biodiversa+ priorities for biodiversity monitoring: https://www.biodiversa.eu/biodiversity-monitoring/priorities/

level. The selection of appropriate methods will therefore be of paramount importance for a future transnational monitoring scheme.

To support and prepare for a transnational soil biodiversity monitoring scheme, the Biodiversa+ partners agreed to launch a pilot in January 2023. During its second year, the pilot involved ten countries: Italy - Autonomous Province of Bolzano, Belgium - Flanders, Denmark, France, Germany, Israel, Slovakia, Sweden, Portugal - Autonomous Region of the Azores, and Turkey. Additionally, Catalonia, financed by the Departament d'Acció Climàtica, Alimentació i Agenda Rural (DACC) of the Catalonia Government (a key partner in the Biodiversa+ project), contributes with a single site.

This year 2 report focuses on methodological challenges of the soil biodiversity monitoring pilot, which need to be considered when planning a transnational monitoring programme, and describes suggestions for common protocols for field and laboratory work. By this, it builds up on the year 1 report published in February 2024[2].

2. Experimental design

2.1. Site selection

The coordinators chose to sample forest types from the different biogeographical regions of Europe to complement existing initiatives such as LUCAS (focus on agricultural sites) and SoilBON (paired approach with only few sites in Europe) as well as national initiatives such as RMQS Biodiversity (France), SISEBIO (Catalonia, Spain), and MBAG (Flanders, Belgium), which monitor various habitat types. The categorisation of the forest types followed the classification provided by the European Environment Agency[3]. The sites for each country were selected in bilateral online meetings between coordinators and each partner to ensure a good representation of the main European forest types. However, some forest types were under-represented due to lack of coverage by the participating countries. Additional criteria for selecting a site included a protection status (as far as possible) and that it had a high degree of naturalness.

2.2. Field and lab work

The SoilBON protocol was used as the starting point for the sampling in the field and the sample preparation in the laboratory. For DNA extraction and sequencing, this protocol also corresponds to the LUCAS protocol (see below). For the vegetation survey, a minimal protocol to create a list of plant species present in the plots was developed by the coordinators. All work steps were summarised in a step-by-step protocol and shared with the participants along with a list of materials. For the second sampling year, the step-by-step protocol was updated, based on discussions during the first workshop in Bolzano in November 2023. Please refer to the first mid-term report for more detailed information on protocols and field work[2].

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2 First report of the Biodiversa+ soil pilot: https://www.biodiversa.eu/2024/02/01/pilot-on-soil-biodiversity-monitoring-first-year-report/

3 EEA. European Forest types. EEA technical report. N°9/2006: https://www.eea.europa.eu/publications/technical_report_2006_9

Soil properties are analysed by the coordinator, who also pre-sorts pitfall traps and soil core samples. Two external companies were contracted to carry out morphological species identification (ÖKOTEAM, Austria) and eDNA metabarcoding analysis (biome-ID, Germany), the latter in accordance with the specifications published in the LUCAS protocol[4]. In brief, DNA from three 0.2 g aliquots of each sample is extracted using the Qiagen DNeasy PowerSoil HTP 96 Kit and amplified by PCR. Primers used to amplify DNA from archaea, bacteria and eukaryotes (fungi and other eukaryotes) are shown in Table 1. All amplicon libraries were sequenced on Illumina or PacBio platforms. The raw sequencing data (FASTQ files) was further bioinformatically processed by the contracted company. Briefly, all reads were subjected to preprocessing and quality control, and sequences that did not meet basic quality criteria (e.g. minimum length, maximum expected errors, chimeric sequences) were discarded. The resulting data was then clustered into Amplicon Sequence Variants (ASVs) or Operational Taxonomic Units (OTUs) depending on the primers used, which represent species-level proxies. OTU- or ASV-representative sequences were then compared to public reference databases (e.g. PR2 for 18S, UNITE for ITS2) for taxonomic classification.

Table 1: Primers used in the pilot

Taxon	Primers
Archaea 16S (SSU)	SSU1ArF (TCCGGTTGATCCYGCBRG) and SSU1000ArR (GGCCATGCAMYWCCTCTC)
Bacteria 16S (SSU)	515F (GTGYCAGCMGCCGCGGTAA) and 926R (GGCCGYCAATTYMTTTRAGTTT)
Eukaryote ITS2	ITS9mun (GTACACACCGCCCGTCG) and ITS4ngsUni (CGCCTSCSCTTANTDATATGC)
Eukaryote 18S (SSU)	Euk575F (ASCYGYGGTAAYWCCAGC) and Euk895R (TCHNHGNATTTCACCNCT)

Additionally, biomass of carabid beetles will be measured by the University of Aarhus using an automatic image-based technology (BIODISCOVER[5]).

3. Methodological challenges

General remark:

The implementation of a harmonised protocol raises a number of methodological questions. This is particularly true for a large-scale study such as the soil pilot and for a future transnational monitoring programme. Despite a detailed step-by-step protocol, each partner sets up the protocol depending on their own experience and equipment at hand. Minor variations in carrying-out the work must be accepted.

[4] Orgiazzi et al., (2022) LUCAS Soil Biodiversity and LUCAS Soil Pesticides, new tools for research and policy development. *European Journal of Soil Science*. https://doi.org/10.1111/ejss.13299

[5] Ärje et al., (2020) Automatic image-based identification and biomass estimation of invertebrates. *Methods in Ecology and Evolution*. https://doi.org/10.1111/2041-210X.13428

One aim of the Biodiversa+ soil pilot is to compare the results of traditional and molecular methods, with a view to making recommendations on the methodology of large-scale monitoring in the final report. Here we discuss advantages and limitations of each of the methods employed in the pilot.

3.1. Traditional methods (pitfall traps and hand-sorting of soil cores)

The use of pitfall traps to collect soil-dwelling invertebrates and the manual sorting of soil cores to collect soil-dwelling invertebrates are long-established and standard methods in soil ecology (Potapov et al., 2022). Both methods are used to collect macrofauna only (i.e. all invertebrates larger than 2 mm). The advantages and disadvantages of these methods are generally well known but need to be reviewed with a view to large-scale monitoring, which will include many partners and a wide variety of habitats with different site and soil conditions.

3.1.1. Pitfall traps

Fig. 1: Pitfall trap at Montiggl (Province of Bolzano, Italy) and Kastamonu Yenice (Turkey)

General remarks

Pitfall traps provide a measure of activity density rather than abundance per surface area. This aspect has to be taken into account when analysing and interpreting the data. Target taxa of pitfall traps are mainly highly mobile organisms (e.g. spiders, ground and rove beetles, millipedes), therefore the data are not representative for the entire macrofauna.

Advantages:

The traps are cheap and easy to install and there is flexibility in the use of collection fluids (in the pilot salt water is used, but also ethylene glycol, propylene glycol, or ethanol mixtures are common). Covered by a roof, they can be installed regardless of the weather and in almost any type of habitat, as long as a deep enough hole can be dug. Also, there is no observer bias, which is an important advantage for large-scale studies. Pitfall traps can be used throughout the season and, emptied regularly, can provide important data on seasonality of invertebrates. In heterogeneous habitats, a higher number of replicates can easily be installed to account for a high variability in the occurrence of invertebrates.

Disadvantages:

Traps must be collected after a fixed number of days (in the pilot 14 days), which might result in an additional effort, in particular for remote or far away sites. The traps are susceptible to damage by wild animals or even removal by humans, therefore a minimum number of traps needs to be installed to avoid losing data. Snails and slugs can also contaminate samples with their mucus, making sample processing difficult. In addition, small vertebrates such as shrews and lizards can be trapped and have no means of escape.

Results from the pilot

Taxon diversity of ground-dwelling invertebrates on family level (Fig. 2), based on two sampling dates in 2023, is significantly different between forest types ($F_{9,237} = 10.54$, $p < 0.001$). Interestingly, the highest taxon diversity was found in alpine larch forests, but due to a low replicate number, this result has to be taken with caution (FCL, only sampled in the Province of Bolzano, n = 2 forests). High taxon diversity of ground-dwelling invertebrates was also found in deciduous oak forests (FDE, n = 11 forests). The lowest diversity was found in evergreen forests of the Macaronesian region (FEL, only sampled on the Azores, n = 4 forests). However, these results for the Azores are inconsistent with data from Borges et al. (2005) and Cardoso et al. (2007), who used identical methodology (pitfalls) and found a much higher diversity. This discrepancy is likely due to specific abiotic factors in the selected sampling areas (steep slopes on volcanic cones), as well as unusually high rainfall during the 2023 campaign. These factors were considered in the methodological adjustments for the 2024 campaign, which resulted in a significantly higher number of specimens captured by the same traps. Patterns are already well established at this taxonomic resolution, but will be compared to patterns at species level for selected taxa (spiders, ground and rove beetles) as soon as the data are available for the full year 2023.

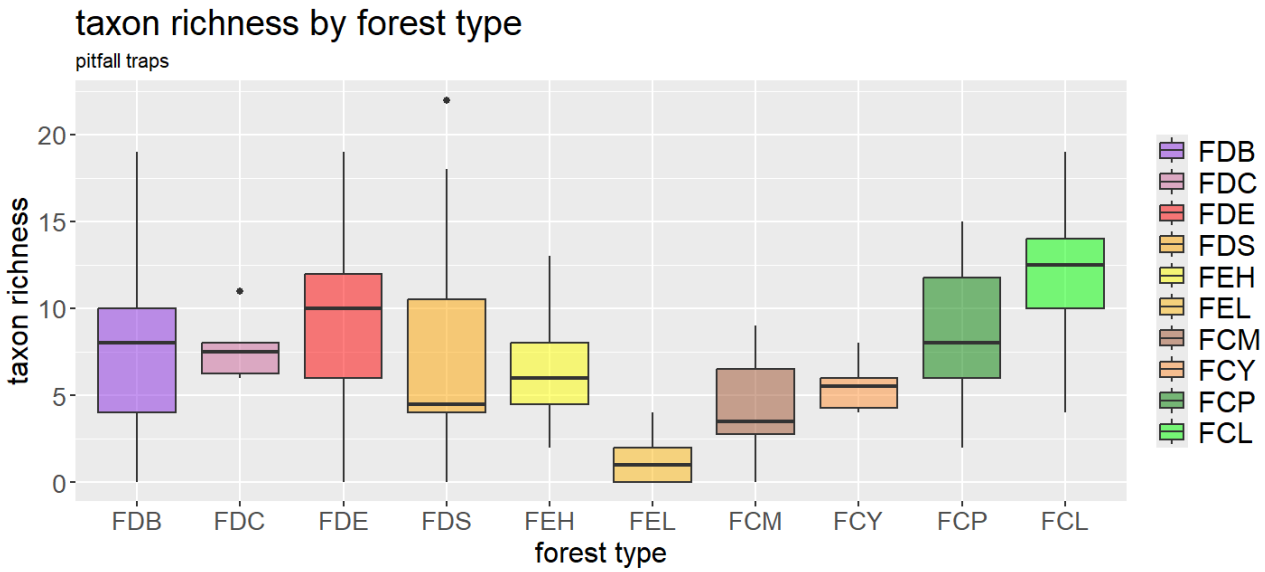

Fig. 2: Taxon diversity (family level) of ground-dwelling invertebrates in different forest types, collected by pitfall traps during two sampling campaigns (spring and autumn 2023). For abbreviations of forest types please see Table of abbreviations.

3.1.2. Hand-sorting of soil cores

General remarks

Hand-sorting of soil cores is the standard method to collect soil-dwelling invertebrates, especially since the alternative method, heat-extraction, needs lab equipment (Kempson or Berlese extractor), which is not readily available. It is also the only standardised method to collect earthworms. It provides abundance data (individuals per unit of area). Hand-sorting is usually carried out for a fixed period of time (e.g. 30 minutes for one person) and can be reduced if several people work on the same sample.

Fig. 3: Hand-sorting of soil cores at sites in the Province of Bolzano, in Israel and in France (clockwise from left to right)

Advantages:

Cheap and easy method, which needs almost no equipment and does not require soil to be carried to a laboratory (especially from remote sites). It also leaves the soil on site, reducing the general destructiveness of soil sampling.

Disadvantages:

The two main limitations of hand-sorting are that it is time-consuming (30 minutes for one replicate, less, if more people work on one sample) and the observer bias is very high. It should also be noted that, depending on the type of soil, extraction of the soil block can be quite demanding. If the soil is stony, it is more difficult to get the block out in one go, and the vibrations cause certain organisms such as earthworms to flee. It might also be very difficult, especially in grasslands, to tear up the turf, find the invertebrates living between the roots and extract them without damaging them. Due to the high spatial variability in invertebrate distribution within a site (e.g. due to aggregated occurrence of dipteran larvae), the number of replicates must be well chosen. Statistically, at least 5 samples should be taken, but this is again very time-consuming (therefore, only two replicates were taken in the pilot).

Results from the pilot

Also, taxon diversity of soil-dwelling invertebrates on family level (Fig. 4), based on two sampling dates in 2023, is significantly different between forest types ($F_{8,178} = 10.97$, $p < 0.001$). The highest taxon diversity was found in deciduous oak forests (FDE, n = 7 forests), followed by coniferous pine (FCP, n = 4 forests) and larch forests (FCL, n = 2 forests). The lowest diversity was again found in evergreen forests of the Macaronesian region (FEL, only sampled on the Azores, n = 4 forests).

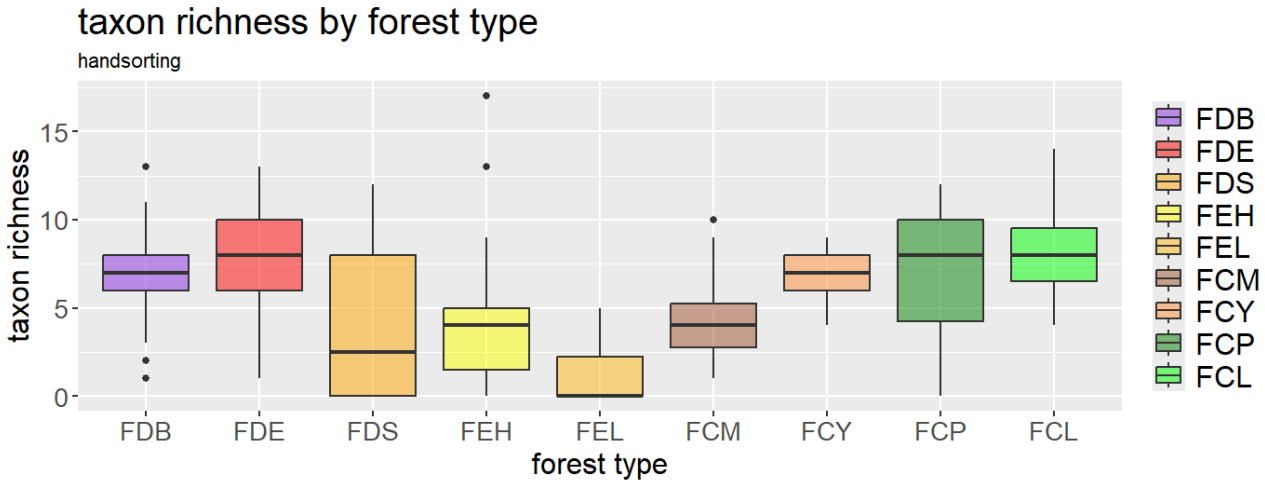

Fig. 4: Taxon diversity (family level) of soil-dwelling invertebrates in different forest types obtained by hand-sorting during two sampling campaigns (spring and autumn 2023). For abbreviations of forest types please see Table of abbreviations.

3.1.3. Taxonomic resolution

A general remark on taxonomic resolution: The pilot will identify spiders and harvestmen (Araneae and Opiliones), ground beetles (Carabidae) and rove beetles (Staphylinidae) to species level. The selection is based on relevance to the soil macrofauna and on the taxonomic expertise of the contracted external

company. In general, species identification of soil organisms is hampered by the lack of specialists and by costs. For transnational monitoring it will be important to decide on the taxonomic resolution required (description of local biodiversity vs. patterns across the European Union).

3.2. Molecular methods (eDNA)

General remarks

eDNA metabarcoding has been demonstrated as a powerful technique to survey aquatic biodiversity (Deiner et al., 2017; Keck et al., 2022; Ruppert et al., 2019; Serrana et al., 2019), soil microbial biodiversity (Heyde et al., 2022; Romero et al., 2024), and soil invertebrate biodiversity (Arribas et al., 2021; Calderón-Sanou et al., 2022; Hermans et al., 2022; Leclerc et al., 2023; Lilja et al., 2023; Llanos et al., 2023; Porter et al., 2019; Remmel et al., 2024). The quality of the data as a measure of soil biodiversity highly depends on primer choice, the depth of the soil layer sampled, the amount of starting material, the quality of the reference databases used, and to a lesser extent, other factors such as the DNA-extraction method, and the processing of the eDNA sequence data (Blackman et al., 2024; Dopheide et al., 2019; Jurburg et al., 2021; Kirse et al., 2021; Taberlet et al., 2012).

Advantages:

The main advantage of the eDNA method is that the entire soil community, from microorganisms to micro-, meso- and macrofauna, can be sequenced from a single bulk soil sample. The sampling of the soil is fast, easy and less invasive than any traditional method. DNA extraction and sequencing procedures can be harmonized and centralised in a single laboratory, limiting handling bias. Also, it can detect a wider range of taxa, including cryptic or rare ones, juveniles, etc, that are often missed by traditional methods.

Disadvantages:

The method requires measures to avoid contamination during sampling, processing and storing, and a minimum of laboratory equipment is necessary. For soil invertebrates, the method is less developed compared to applications in aquatic biodiversity monitoring or soil microbial biodiversity monitoring. The primers used by LUCAS and SoilBON to target Eukaryotes are very general, but have limited taxonomic resolution, and do not recover all taxa equally, thereby creating amplification bias (Fig. 5, see 3.3 for more details). Reference sequences are not available for many species, leaving the taxonomic identifications of many Operational Taxonomic Units (OTUs) unclassified. In addition, the current protocol uses a very small amount of soil for DNA extraction and is therefore likely to miss DNA fragments from larger animals.

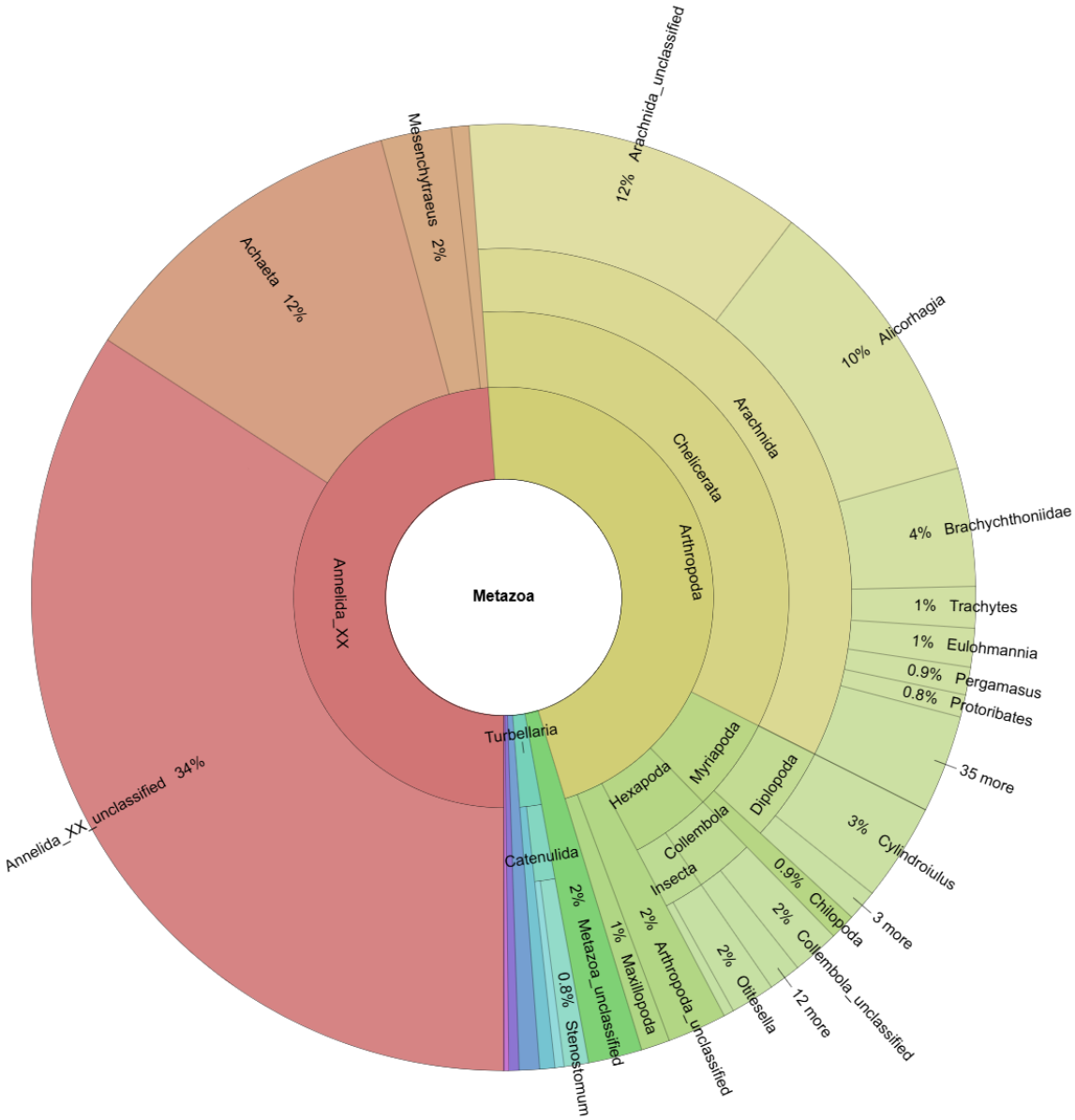

Fig. 5: Overview of invertebrate taxa identified with molecular methods.

3.3. Methodological issues tested in the pilot

Here we focus on comparing the results for soil macrofauna, in particular for representatives of the phylum Arthropoda, considering them to be the most representative taxa in soil samples. Arthropods from hand-sorted soil samples were identified to family level, while sequences were translated using the PR2 reference database.

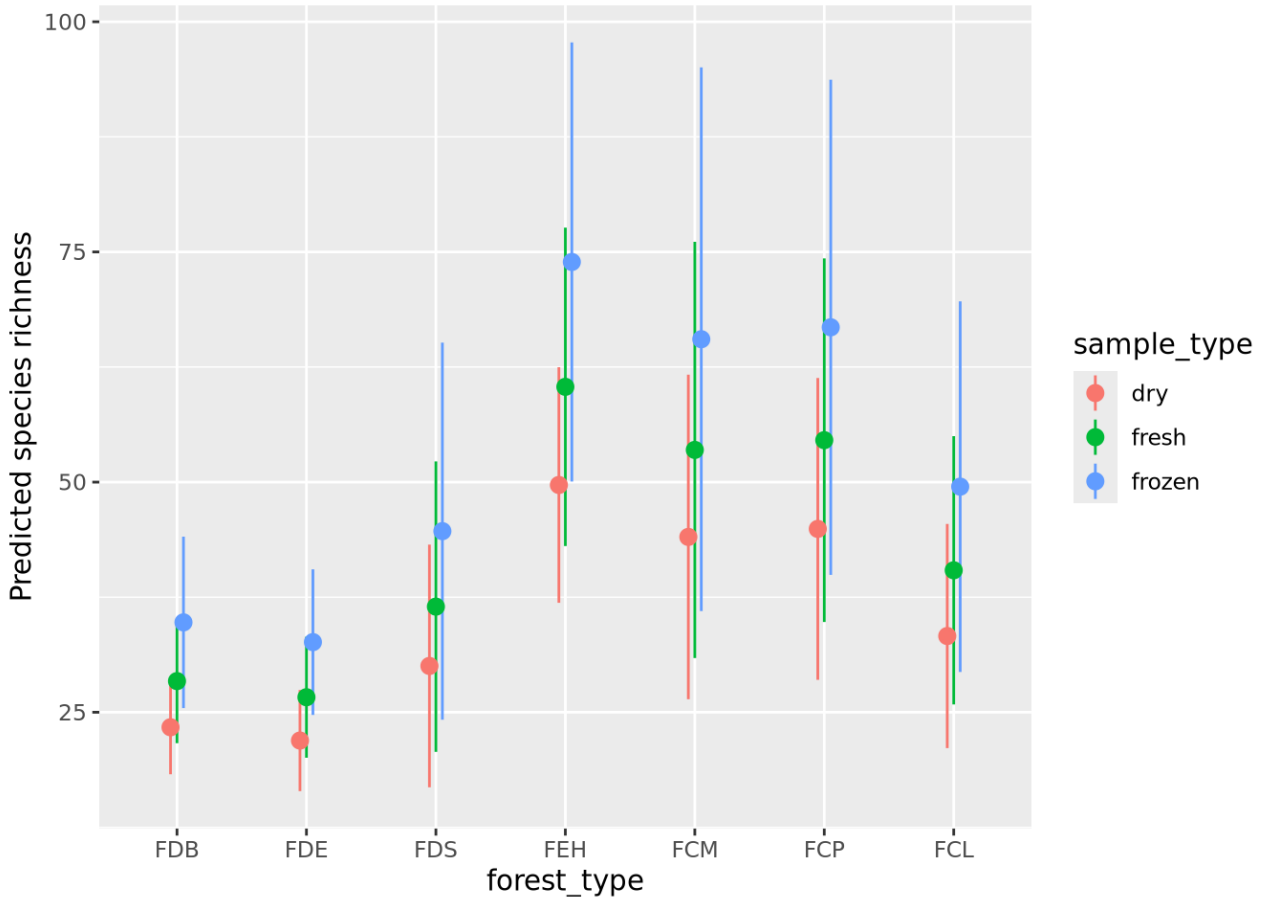

Fig. 6: Predicted species richness for arthropods (ASVs) across different forest- and sample- (dried, fresh and frozen) types. Fresh samples were frozen in the laboratory, frozen samples were put on ice in the field.

For logistical reasons, partners were asked to send dried soil samples, but two partners (Province of Bolzano and Belgium) also provided fresh and frozen samples to allow us to analyse the effect of the drying process on the soil community. Due to the small number of replicates, the results are not yet statistically meaningful, but it is already clear that drying and even freezing soil samples in the lab rather than in the field reduces the alpha diversity of soil organisms. More samples will be analysed in the pilot to look at sample handling in more detail. When analysing the effect of drying on the soil community, it must be taken into account that the duration of the drying process differs between forest types and climatic regions.

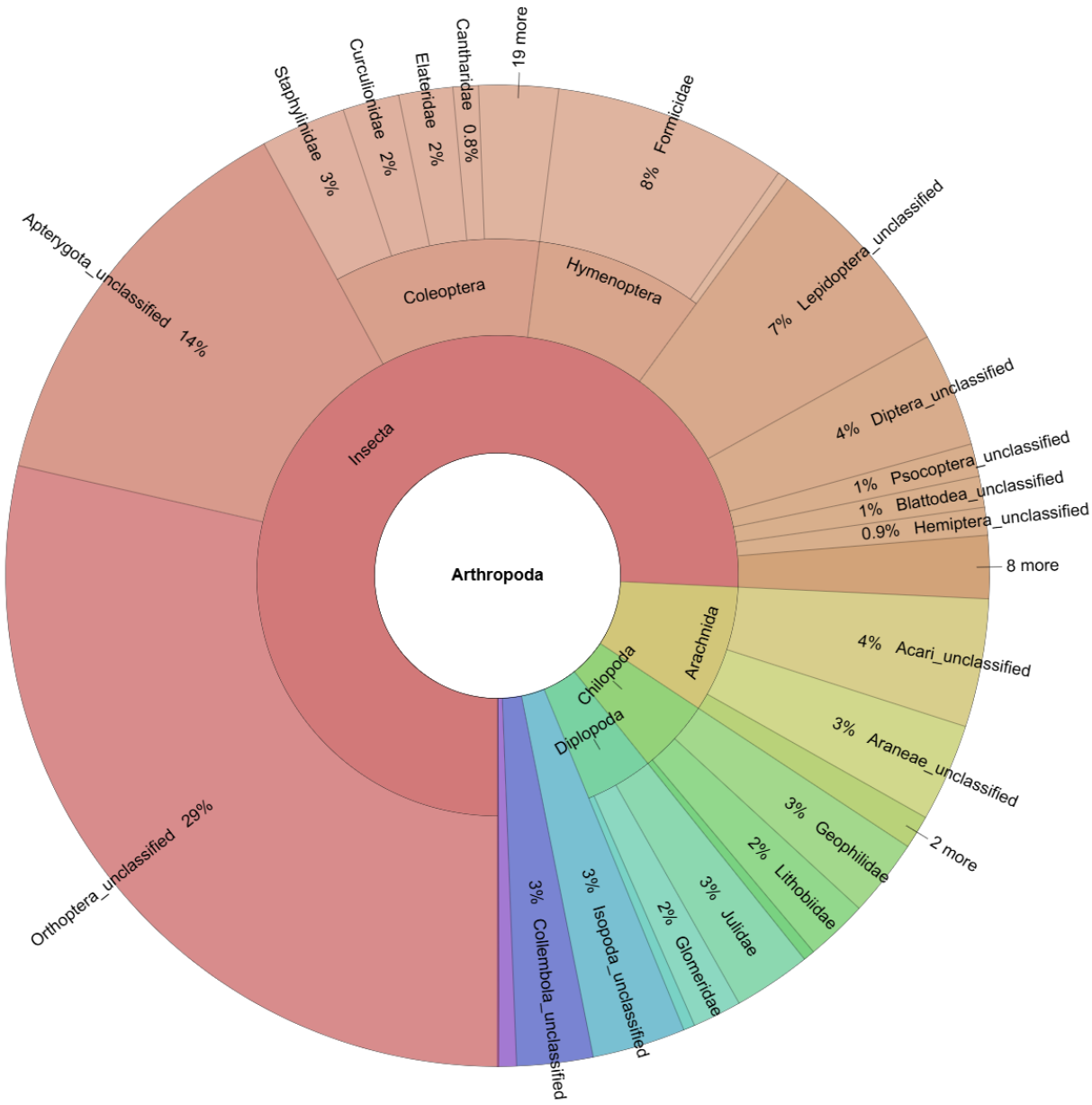

Fig. 7: Proportions of taxa identified from hand-sorted samples.

This comparison mainly shows that what we find as individuals in our samples is not the same as what we find as sequences in the bulk soil (Fig. 7). When we compare the three methods for surveying soil biodiversity—universal 18S primer-based eDNA metabarcoding, handsorting, and pitfall trapping—distinct differences in taxonomic resolution and group-specific performance were observed. Overall, the universal 18S primers used for the eDNA method identified taxa with high resolution for some specific groups, particularly mites (Acari), while handsorting and pitfall trapping identified taxa with much broader taxonomic coverage but lower taxonomic resolution. For mites, the universal 18S primers provided the highest resolution, identifying 34 specific families, whereas the traditional methods tested here recorded them only as "Acari" without family-level identification. In contrast, for beetles (Coleoptera), traditional methods detected 23 families through handsorting and 37 families through pitfall trapping, including ecologically important groups such as Carabidae and Staphylinidae, compared to only six families

identified by the universal 18S primers (Elateridae, Aphodiidae, Scirtidae, Scraptiidae, Ptiliidae, Tenebrionidae), of which three families (Elateridae, Scraptiidae, and Tenebrionidae) were shared across all methods. In addition, the universal 18S primers did not recover any spiders (Aranea), demonstrating the limitations of these primers. Both traditional and eDNA methods detected pseudoscorpions and harvestmen. For myriapods, both methods performed comparably, with the universal 18S primers identifying six families and traditional methods identifying five families. Four myriapod families were detected by all methods (Geophilidae, Julidae, Lithobiidae, Polydesmidae), while traditional methods uniquely detected one family (Glomeridae) and eDNA uniquely detected two families (Polyxenidae, Cryptopidae). For springtails (Collembola), the universal 18S primers identified four specific families (Entomobryidae, Isotomidae, Hypogastruridae, Sminthuridae), whereas traditional methods recorded them only as "Collembola" without family-level resolution. These findings provide a glimpse into the strengths and limitations of each method, with the universal 18S primers excelling in taxonomic resolution for certain groups (Acari), but exhibiting biases that limit their ability to detect others.

As future transnational monitoring programmes will likely rely mostly on molecular methods due to the huge effort and cost of traditional methods, the use of eDNA for invertebrates needs to be refined. The following problems were identified and need to be addressed:

Primer development: The universal 18S primers used by LUCAS and SoilBON to target Eukaryotes lack sufficient taxonomic resolution and predominantly amplify protists, with limited recovery of animals such as annelids and arthropods. Also, the proportion of reads attributed to these taxa is typically too small to reliably use their richness as a response variable for monitoring ecological change. Using more targeted primers that enhance amplification of key soil taxa, such as annelids and arthropods, is essential to improve the utility of eDNA for monitoring soil biodiversity (Ficetola & Taberlet., 2022; Giebner et al., 2020; Jurburg et al., 2021; Kirse et al., 2021). Even at the family level, the comparison between traditional and molecular methods shows discrepancies. A possible solution to the primer problem is the use of multiple group-specific primers (Leclerc et al., 2023), bearing in mind that costs increase with each library. For the pilot, it was decided to stick to the primers used for the first season to ensure comparability of data, but depending on the budget, other primers and pipelines (e.g. Olig01 for annelids, Bienert et al., 2012; Coll01 for collembolans, Janssen et al., 2018; Terr_EPTDr2n for annelids and arthropods, Perrelet et al., unpublished) will be tested.

Another issue is the quantity of soil used for DNA extraction. Following the LUCAS protocol for DNA extraction, 0.25 g of soil is used, which is relevant for microorganisms, but not for animals, where at least 15 g is needed (Dopheide et al., 2019; Taberlet et al., 2012).

Reference databases: Incomplete taxonomic reference libraries currently limit the potential of eDNA metabarcoding, specifically for assessing community composition and bioindicator taxa (Blackman et al., 2024, Taberlet et al., 2012). Increased investment in standardized barcoding initiatives to build comprehensive reference libraries is critical to unlocking its full capability for soil biodiversity monitoring. However, it should be noted that with major initiatives such as the International Barcode of Life or the Earth BioGenome underway, the development and expansion of such reference databases is very likely (Blackman et al. 2024).

The decision on how to store soil samples after collection (dried soil, fresh soil, frozen soil, etc.) is important as this will affect the results. Previous studies suggest that freezing the samples in the field is the best way to preserve DNA (van der Heyde et al., 2022), but this may be a challenge, especially in

very remote areas. In the pilot, tests were carried out on frozen, fresh and dried soil samples to address this issue and to understand whether storage methods affect diversity, the detection of genera or species, or the observed community structure. The effects may even be different for bacteria, fungi and other eukaryotes. As an additional method, placing the soil in ethanol to preserve DNA should be discussed.

Behaviour of DNA in the environment: Soil eDNA transport remains understudied; however, it is thought to occur primarily in a vertical direction, likely influenced by rainfall (Lyet et al., 2021; Macher et al., 2023; Valentin et al., 2021).

Data interpretation: eDNA metabarcoding currently primarily provides relative abundance data, reflecting the proportional representation of taxa across samples. Abundances may not be absolute due to PCR amplification bias between taxa. Recent advances have explored methods to move towards quantitative metabarcoding, aiming to derive true biomass or abundance of organisms from sequencing data. Several approaches have already shown promise in reducing bias and increasing the reliability of quantitative inferences (e.g. Luo et al., 2022; Shelton et al., 2022; Serrana et al., 2019).

Another discussion point identified which is not related to molecular methods is the depth of the soil layer sampled: the diversity of taxa and eDNA in the 10–30 cm soil layer is insufficient for annelids and arthropods to serve as indicators of ecological changes, making it less suitable for monitoring soil biodiversity (Lambrechts et al., unpublished). Moreover, it is hypothesized that deeper soil layers contain more relic DNA and dead organisms (necromass), inherited from former times, obscuring diversity estimates (Köninger et al., 2023; Lambrechts et al., unpublished).Therefore, efforts to monitor soil biodiversity should focus on the 0–10 cm soil layer (as has been done in the pilot). In addition, the litter layer has shown significant promise for monitoring soil biodiversity through metabarcoding (Ruppert et al., 2023; Zinger et al., 2019).

Table 2: Summary of pros and cons of methods tested in the pilot.

Method	Pros	Cons
pitfall traps	+ cheap + easy to install + suitable for all kinds of habitats + weather-proof + no observer bias	- must be emptied more often under warm weather conditions - time-consuming for remote areas - susceptible to damage by wild animals - contamination by snails and slugs possible - vertebrates might be trapped - for species identification experts are needed
hand-sorting of soil cores	+ cheap + easy method under ideal conditions (few stones and roots) + reduces destructiveness of soil methods by leaving soil in the field	- time-consuming - difficult in sites with dense roots and stony soils - observer bias - for species identification experts are needed

eDNA	+ entire community can be sequenced from a single bulk sample + sampling is easy + less invasive than traditional methods + higher detection rate of rare species and juveniles + analyses can be centralized, limiting handling bias	- Infrastructure is needed to store and process samples - contamination must be avoided - for invertebrates, DNA extraction protocol is not yet optimized - no suitable primer for invertebrates available, increasing costs by the need to use group-specific primers - reference databases not yet fully available

3.4. Questions still to be answered

Administrative, logistic, and legal issues have been identified in the Year 1 report. With the identification of methodological challenges and limitations discussed in this report, we are one step further in developing a transnational monitoring scheme to monitor soil biodiversity. Once the full data is available, the following questions can be addressed:

- Frequency of sampling to assess changes in soil biodiversity (e.g. sampling every 2 years)
- Is seasonal sampling needed to account for within-year variability in species distribution (e.g. spring and autumn sampling)?
- Do all functional groups need to be included (Bacteria, Archaea, Fungi, protists, micro-, meso-, and macro-invertebrates)?
- Is species-level resolution necessary? If not, which taxonomic level is sufficient?
- Which additional measurements would be needed for a more comprehensive list of EBVs? This could include, but is not limited to, the analysis of soil respiration, soil enzymes, soil carbon stocks, and litter decomposition.

4. Conclusions

In this report, we compared the results obtained by **traditional methods** (pitfall traps and hand-sorting of soil cores, both standard methods in soil ecology) and **molecular methods** (eDNA) in order to understand methodological possibilities and limitations for a transnational monitoring scheme. As the traditional methods target only macro-invertebrates (all invertebrates > 2 mm), the comparison is limited to this size group of soil animals. We used data obtained during the first year of the pilot study (spring and autumn 2023 for traditional methods, spring 2023 for molecular methods) for the comparison.

We found traditional methods (pitfall traps and hand-sorting of soil cores) to be cheap, simple and good at providing data suitable for evaluating and monitoring soil invertebrates (activity density or abundance data at family level across all taxa). They can be used to describe the status-quo of local soil biodiversity and to assess changes in soil biodiversity over seasons and years. However, they are time consuming, destructive and require expert knowledge if species level is required.

Molecular methods have great potential for monitoring schemes in general and are already established in aquatic sciences and soil microbiology. The big advantage is that the entire soil community can be analysed from a single sample. However, for the study of soil invertebrates, the method is rather new. We identified several limitations when analysing the data obtained during the first sampling season of the pilot, which need to be solved before using eDNA as standard method in soil biodiversity monitoring:

- Primers: The universal 18S primers used by LUCAS and SoilBON to target Eukaryotes lack sufficient taxonomic resolution and more targeted primers that enhance amplification of key soil taxa, such as annelids and arthropods, is essential to improve the utility of eDNA for monitoring soil biodiversity
- Quantity of soil used for DNA extraction. Following the LUCAS protocol for DNA extraction, 0.25 g of soil is used, which is relevant for microorganisms, but not for animals.
- Reference databases: Incomplete taxonomic reference libraries currently limit the potential of eDNA metabarcoding, specifically for assessing community composition and bioindicator taxa.
- Sample storage: In the pilot, tests were carried out on frozen, fresh and dried soil samples and we found large differences in alpha diversity between samples stored in different ways (not yet statistically significant due to low replicate numbers).
- Data interpretation: eDNA metabarcoding currently primarily provides relative abundance data, reflecting the proportional representation of taxa across samples. Abundances may not be absolute due to PCR amplification bias between taxa. Recent advances have explored methods to move towards quantitative metabarcoding, aiming to derive true biomass or abundance of organisms from sequencing data.

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