

MONITORING OF SOIL BIODIVERSITY

Challenges and lessons learnt from the first year of the Biodiversa+ pilot “Soil biodiversity in protected, near-natural forests”



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Table of contents

- Executive Summary 6**
- 1 Introduction 7**
- 2 Experimental design 9**
 - 2.1 Site selection 9
 - 2.2 Protocols 10
 - 2.3 Field and lab work 11
- 3 Challenges and possible solutions 14**
 - 3.1 Study design/protocols 14
 - 3.2 Field work and site selection 15
 - 3.3 Administrative/legal/logistic issues 15
 - 3.4 Communication 17
 - 5.1 Vegetation surveys 19
 - 5.2 Soil parameters 20
 - 5.3 Invertebrate diversity 21
- Conclusions 24**



What is Biodiversa+

Biodiversa+ is the new European co-funded biodiversity partnership supporting excellent research on biodiversity with an impact for policy and society. It was jointly developed by BiodivERsA and the European Commission (DG Research & Innovation and DG Environment) and was officially launched on 1 October 2021.

Biodiversa+ is part of the European Biodiversity Strategy for 2030 that aims to put Europe's biodiversity on a path to recovery by 2030.

The Partnership aims to connect science, policy and practise for transformative change. It currently gathers 80 research programmers and funders and environmental policy actors from 40 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/>

Table of acronyms

DNA	Deoxyribonucleic Acid
eDNA	Environmental Deoxyribonucleic Acid
EU	European Union
GPS	Global Positioning System
HTS	High-throughput Sequencing
ISO	International Organisation for Standardisation
LUCAS	Land Use/Cover Area frame Survey
MTA	Material Transfer Agreement
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
RMQS	Le Réseau de Mesures de la Qualité des Sols
SISEBIO	Catalan Plot System for Terrestrial Biodiversity Monitoring
SoilBON	Soil Biodiversity Observation Network
ÖNORM	Austrian Standards
UNI EN	Italian Standards



Executive Summary

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

The main tasks of the first year of the pilot project were to:

- Develop a feasible experimental design and define common protocols for field and laboratory work;
- Test the applicability and requirements of eDNA methods in such a scheme to obtain high resolution taxonomic data;
- Identify potential administrative and logistical barriers and propose possible solutions.

Based on the results of the first year's activities, the following needs for a transnational monitoring scheme have been identified:

- a harmonised, well-defined and easy-to-use protocol,
- clear instructions to reduce the risk of contamination for eDNA analysis,
- a list of minimum infrastructure requirements for participating institutions, and
- administrative support to understand national regulations concerning the Nagoya Protocol and the sending/receiving of soil and invertebrate samples.

The next steps involve enhancing recommendations for a transnational monitoring scheme with regards to sampling coverage and frequency.

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

1 Introduction

Biodiversa+, [the European Biodiversity Partnership](#), aims at promoting and supporting transnational biodiversity monitoring, by building a transnational network of harmonised biodiversity monitoring schemes on specific priority topics¹. 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;
- To evaluate the minimum number of sites/countries to be included in the monitoring programme in order to detect changes in soil biodiversity;
- To provide an overview of already existing national soil biodiversity monitoring schemes and to extrapolate what can be learnt from them;
- To evaluate the coordination, cooperation and governance of transnational soil biodiversity monitoring.

In the long term, the results of the pilot should not only contribute to a better understanding of soil biodiversity, but also identify ways to take action to monitor soil biodiversity in order to conserve and restore it. In this way, the pilot supports actions for the forthcoming European Commission's Biodiversity Strategy and the Soil Strategy for 2030.

According to a recent study, soils are home to 59 % (± 15 %) of the known species (Anthony et al. 2023, PNAS 120: e2304663120), 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. Soil organisms are involved in a wide range of soil and ecosystem processes such as litter decomposition, nutrient cycling, water filtration and pest control and are thus essential for ecosystem functioning. Little is known about how soil organisms will be affected by human intervention and global change and how changes in community composition will affect ecosystem processes, mainly because long-term data on soil biodiversity are largely lacking. In addition, 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) level. A long-term transnational monitoring programme, following a harmonised protocol, will help to provide the necessary data to analyse the effects of global change on soil biodiversity. The selection of appropriate methods will facilitate the identification of taxa at a level necessary to find patterns and changes in soil community composition. In addition, although the rapid development of HTS technology makes it difficult to predict which

¹ Biodiversa+ priorities for biodiversity monitoring: <https://www.biodiversa.eu/biodiversity-monitoring/priorities/>



Outcomes of the Biodiversa+ pilot on soil monitoring during its first year

molecular marker or technology will be used in the future to assess changes in soil biodiversity, harmonised protocols will support future biodiversity surveys to reduce the variation introduced by inconsistent sample handling and storage.

As a way of supporting this harmonisation work to monitor soil biodiversity, the Biodiversa+ partners agreed to launch a one-year pilot on soil biodiversity in protected, near-natural forests² in January 2023. This pilot brings together nine countries: Italy - Autonomous Province of Bolzano, Belgium - Flanders, Denmark, France, Germany, Israel, Slovakia, Portugal (the Azores), and Turkey. The pilot has been extended for a further year in 2024 and will be joined by a new partner: Sweden. Additionally, Catalonia, financed by the Departament d'Acció Climàtica, Alimentació i Agenda Rural (DACCA) of the Catalonia Government (a key partner in the Biodiversa+ project), contributes with a single site.

This report describes the challenges encountered during the first year and presents the first findings of the soil biodiversity monitoring pilot.

² Biodiversa+ biodiversity monitoring pilot: <https://www.biodiversa.eu/biodiversity-monitoring/pilot/>

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) and national initiatives such as RMQS Biodiversity (France) and SISEBIO (Catalonia, Spain), both of which monitor different habitat types. The categorisation of the forest types followed the classification provided by the European Environment Agency³. The sites for each country were selected in bilateral online meetings to ensure good representation of the main 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 that it was protected and that it had a high degree of naturalness.

Table 1: Forest sites selected in each country for 2023. Numbers in the table indicate the actual number of sites.⁴

Forest types	Abbreviation	BE	DK	DE	FR	IL	BZ	AZ	SL	TU	Total
Boreal and hemi-boreal Scots pine forests	FCY			2							2
Alpine Swiss pine and larch forests	FCL						2				2
Boreal and Alpine spruce forests	FCP			2			2				4
Oak forests of Continental and Atlantic Europe	FDE	4	3		2				2		11
Beech forests of the nemoral and Alpine region	FDB	2	2	2	2				4		12
Broadleaved evergreen forest of the Black Sea region	FDC									2	2
Thermophilous deciduous forest (supramediterranean)	FDS				1		1			2	4
Broadleaved evergreen forest of the Mediterranean region	FEH				2	3					5
Broadleaved evergreen forest of Macaronesian region	FEL							4			4
Coniferous forests of the Med., Anat. and Macar. region	FCM					2				2	4

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

⁴ BE...Belgium, DK...Denmark, DE...Germany, FR...France, IL...Israel, BZ...South Tyrol (Italy), AZ...the Azores (Portugal), SL...Slovakia, TU...Turkey.



Outcomes of the Biodiversa+ pilot on soil monitoring during its first year

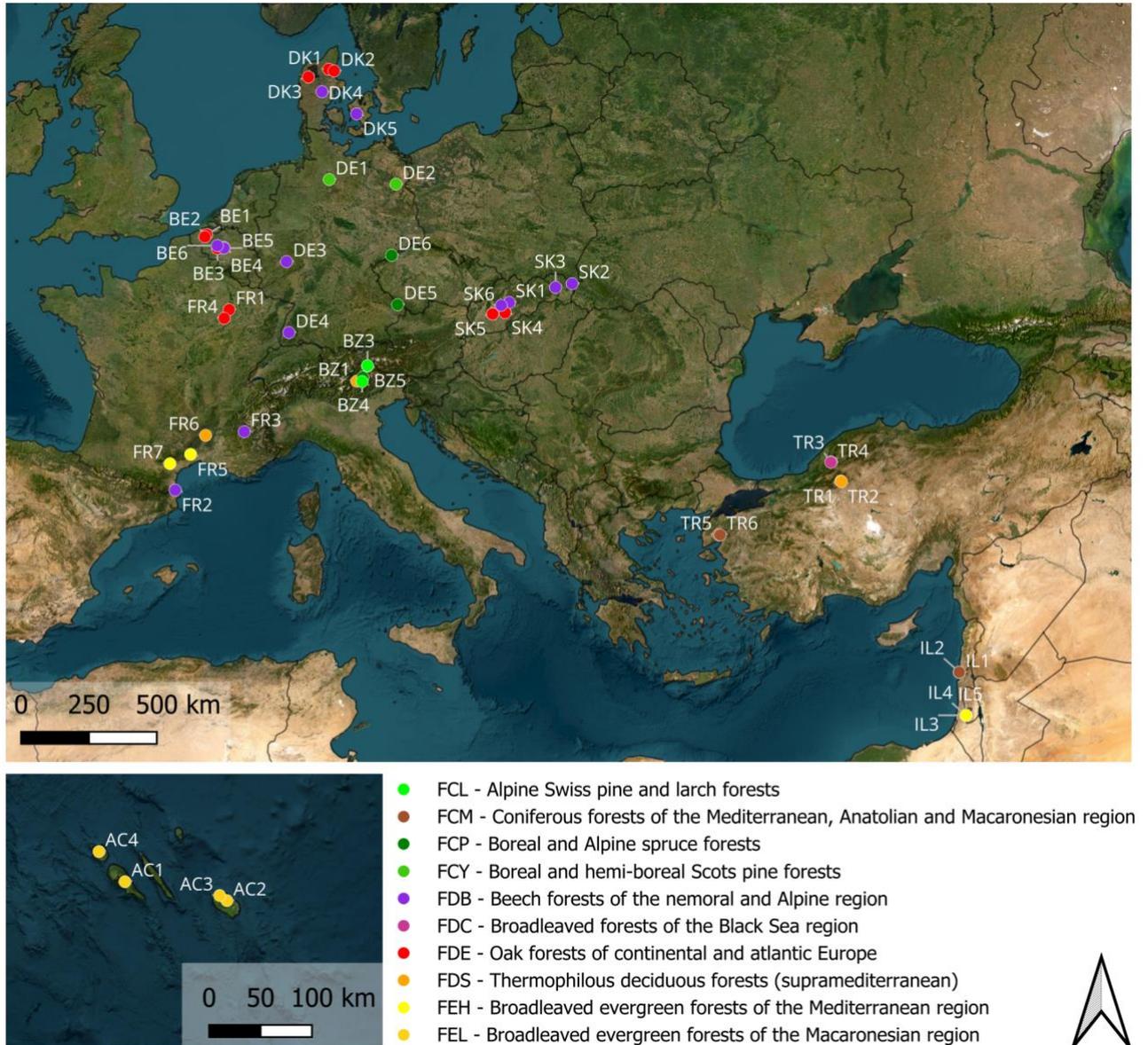


Fig 1: Map of the studied field sites.

2.2 Protocols

The SoilBON protocol was used as the starting point for the soil sampling in the field and the sample preparation in the laboratory. For DNA extraction and sequencing, this protocol also corresponds to the LUCAS protocol. For the vegetation survey, a minimal protocol to create a list of plant species present in the plots was developed. All work steps were summarised in a step-by-step protocol and a list of materials was drawn up, both of which were shared with the participants (see Appendix).

2.3 Field and lab work

In spring 2023, all participants were required to choose at least five monitoring sites. Each sampling site was established in a homogeneous part of the forest within a 30 x 30 m² grid with a central point marked by GPS. The site was photographed, and the soil profile and spring vegetation were surveyed. According to the protocol provided, two biodiversity surveys were requested at each site — one in spring and one in autumn. Each survey required the collection of (i) 9 soil cores from topsoil for eDNA analysis and estimation of soil properties, (ii) hand sorted macrofauna from two 25 x 25 cm² samples and (iii) macrofauna collected after 14 days from 3 pitfall traps. The soil samples had to be air-dried or dried in an oven at max. 40 °C. Macro-invertebrates retrieved from pitfall and hand-sorted soil core samples had to be transferred to 96 % ethanol.

All samples had to be sent to the coordinator, and data on vegetation and site characteristics had to be sent per e-mail.

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 and eDNA metabarcoding analysis, the latter in accordance with the specifications published in the LUCAS protocol⁵. 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 2. All amplicon libraries are generated using Illumina proprietary protocols (e.g. Nextera XT) to ensure high compatibility with sequencing instruments (note the deviation from the LUCAS protocol, where archaea and fungi libraries were generated using PacBio instead of Illumina). Additionally, biomass of carabid beetles will be measured by the University of Aarhus using an automatic image-based technology (BIODISCOVER⁶).

Table 2: 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 (GTACACACCGCCCGTTCG) and ITS4ngsUni (CGCCTSCSCTTANTDATATGC)
Eukaryote 18S (SSU)	Euk575F (ASCYGYGGTAAYWCCAGC) and Euk895R (TCHNHGNATTTCCACCNCT)

⁵ <https://doi.org/10.1111/ejss.13299>

⁶ <https://doi.org/10.1111/2041-210X.13428>



2.4 Material transfer agreement

The duties of the participants involve the sending of soil and invertebrate samples to the coordinator at Eurac Research in Italy. Since the coordinators will use these samples for further analysis, a Material Transfer Agreement (MTA) had to be signed by both the sending and the receiving parties. Again, using the SoilBON MTA as a starting point, and with the help of Eurac Research's legal office, a sub-pilot specific MTA was drafted and sent to all participants for signature. The signed version had to be included in the package with the soil and invertebrate samples. In addition, each participant had to check their national regulations regarding the Nagoya Protocol and contact their national contact point for further instructions (<https://absch.cbd.int/en>).

Coordinator:

Italy: Italy did not sign the Nagoya protocol and is therefore generally allowed to receive samples. However, in the case of receiving samples from Turkey, Eurac Research still encountered problems not anticipated. These samples are stuck at customs pending authorization, which can only be obtained after a visit to the laboratories by the phytosanitary agency. This procedure is intended to prevent the release of pathogenic organisms and is currently underway.

Participants:

Azores: Portugal is a party to the Nagoya Protocol. However, as the Azores are an autonomous region of Portugal and the samples are taken in protected areas, the sharing of benefits resulting from the use of the natural resources accessed or sampled is based on the MTA signed between the FRCT and Eurac Research. This agreement has been validated by the Regional Competent Authority (DRCT), preceded by the issuance of an Internationally Recognised Certificate of Compliance (IRCC) by the same authority, which regulates the legal framework for access to and use of natural resources in the Azores for scientific purposes. Both the MTA and the IRCC accompanied the shipment of samples from the Azores to Bolzano.

Belgium: Belgium has signed the Nagoya Protocol. In Flanders (region) there are no ABS requirements for genetic resources originating from Flanders forests. In Wallonia, there is a decree with benefit-sharing obligations, but no access permits are required and access is open. As not all obligations seem to be completely clear, our institute signed the MTA between INBO and Eurac Research and a copy of this document was sent with the eDNA samples.

Denmark: Since Denmark does not have regulated genetic resources there was no need to specify access conditions according to the Nagoya Protocol.

France: France did sign the Nagoya protocol. However, sampling and identification alone (not followed by further genetic research) are not in the scope of the French ABS scheme. Therefore, no French ABS certificate was needed to send samples.

Outcomes of the Biodiversa+ pilot on soil monitoring during its first year

Germany: Germany is a contracting party to the Nagoya Protocol that has not introduced Access and Benefit Sharing arrangements for its own genetic resources. Therefore, no prior consent for the sample transfer is required.

Israel: Israel is not a signatory to the Nagoya Protocol and is therefore generally allowed to send samples. If biological specimens from Israel are collected in protected areas, or if they are specimens of protected species, a collection permit and an export permit are required from the Israel Nature and Parks Authority (INPA). However, if the specimens are collected outside of protected areas, and if the specimens are not of protected species, no collection permit or export permit is required from the INPA. In this pilot project, biological specimens were collected in the natural environment, but not in areas designated as nature reserves.

Slovakia: Slovakia is signatory to the Nagoya protocol, however there are no restrictions concerning access or shipment of any sort of genetic material. Therefore, no prior consent for the sample transfer is required. Due to that, the National Contact Point for Nagoya had no authority to sign the MTA, this document was signed by the director of the institution, which applied for sampling permissions.

Turkey: Turkey has not signed the Nagoya Protocol. In the framework of the project, the prepared Material Transfer Agreement (MTA) was modified and signed between Italy and Turkey. There is no specific legislation regarding the export of soil samples for research projects. Therefore, an MTA was signed to facilitate the transfer of soil samples. A certificate is required for the export of biological samples. This certificate, known as the 'Veterinary Health Certificate for Export of Insects for Scientific Purposes' of the General Directorate of Food and Control of the Ministry of Agriculture and Forestry of the Republic of Turkey, is obtained from the relevant institution, indicating the project details, and remains valid for the duration of the project.



3 Challenges and possible solutions

On 15 November 2023, a workshop with the pilot participants took place in Bolzano (Italy), organised by the Eurac Research coordinators. The main objective of the workshop was to list the challenges encountered during the first year of the pilot and to discuss possible solutions. The challenges were grouped into four categories, which are discussed below.

3.1 Study design/protocols

- Timing of field work

Some participants found it difficult to choose the perfect time for fieldwork, as long-term weather conditions (e.g. time of snowmelt, vegetation development) are not well predictable. The main requirement is to choose a time when the vegetation is fully developed, this can be derived from experience from previous years and does not need to be completely accurate. However, the time point should be consistent between sampling years.

- Sampling for eDNA analyses

As not all participants have a lab with enough freezer space to keep the samples frozen until shipment to Bolzano, it was decided to use dried soil for eDNA analysis (this is a modification of the SoilBON protocol). It is known that the drying process alters the microbial community, although it is not well understood to what extent. The coordinators asked participants who were willing to help in this matter to also send fresh samples in order to compare the microbial community before and after drying the soil.

It was also mentioned that contamination could occur during sampling and especially during the drying process. In several participating institutions, the ovens used to dry soil samples are also used to dry soil samples from other projects and are not cleaned regularly/thoroughly in the same way as a laboratory processing eDNA samples, because these ovens are mainly used to dry samples for physico-chemical analyses. Therefore, there is a significant risk of contamination of DNA from these other soils, the step-by-step protocol will be updated in this regard.

- Amount of soil required for analyses could not be obtained during field sampling

When sampling in wet to very wet conditions, the weight of 600-700 g of dried soil after drying was not achieved. The same problem occurs in very shallow soils. In such cases the possibility of taking a larger sample mass (per core) should be considered, e.g. taking two cores on each sampling plot.

- Preservative used in pitfall traps

Outcomes of the Biodiversa+ pilot on soil monitoring during its first year

As the sampling took place in protected forests, the preservative used in the traps had to be non-toxic and non-harmful. Therefore, 10 % salt water with a few drops of bleach (to prevent vertebrates from being attracted to the traps) was used. However, the company responsible for morphological species identification reported that in arachnids (spiders, weavers) the preservative caused signs of dissolution of the distal body appendages: this affected the walking legs, the pedipalps and even the appendages of the male palpi. As a result, key identification features such as those on the male palpi, epigyne, body colouration and markings became (almost) unrecognisable, making identification more difficult. After re-checking with the company, it was decided to use 30 % salt water instead of 10 %, which should improve the preservation of individual specimens. This is of course a compromise, but salt water is easy to obtain and use, non-toxic and has good preservation properties for most taxa.

3.2 Field work and site selection

- Site selection

Some participants were concerned that their sites did not meet the requirements. The criteria for selecting a site were that it belonged to one of the selected biogeographical forest types, that it was protected and that it had a high degree of naturalness. These criteria are somewhat flexible and do not have to be met one hundred percent.

- On-field selection of sampling area

The sampling area is a 30 × 30 m² square according to the protocol. At some sites, such a regular square was not possible due to topographical irregularities and/or the site uniformity. Also, exact sampling plots were sometimes inaccessible due to rocks or crevices. In such cases the square or the sampling plot can be adapted to the existing site conditions, but care must be taken that these changes to the protocol are recorded in the 'comments' field in the site characteristics file. In addition, deviations from the sampling plots should be marked by GPS so that the plots can be found again during subsequent sampling.

3.3 Administrative/legal/logistic issues

This category contains the highest number and the most serious challenges for both participants and coordinators.

- Short period between confirmation and start of the pilot

The pilot started in January 2023, the preparation time until the start of the fieldwork was quite short and a big challenge for many participants as they had first to recruit and train staff, and purchase equipment.



Outcomes of the Biodiversa+ pilot on soil monitoring during its first year

- Nagoya protocol and material transfer agreement

All participants signed the MTA provided by Eurac Research and contacted their national contact points for the Nagoya regulations. In the case of Turkey, the MTA had to be slightly modified as the Turkish National Focal Point (NFC) refused to sign the document due to the lack of appropriate legislation. After the legal offices of both parties agreed on changes and more information on the project was provided, the Turkish NFC signed the MTA.

- Permits to sample in protected areas

Some participants encountered difficulties in obtaining permits to sample in protected areas. However, as this is a national issue, each participant will need to resolve this on their own. For a trans-national monitoring scheme, care must be taken in the selection of habitats/sites to be sampled to avoid such difficulties. Also, sufficient time should be allowed during the preparation phase to obtain legal permission in accordance with the legislation of the participating countries.

- National rules for sending samples

Many participants found it difficult to find courier companies willing to collect their ethanol samples. In the case of the Azores and of Turkey, the compromise was to send the invertebrate samples without the ethanol (but still soaked in ethanol), but this is only a short-term solution for the pilot. There was a general consensus among the participants that this must be regulated by the EU in a transnational monitoring scheme. Also, if morphological species identification is to be part of the monitoring scheme, it will be necessary to discuss whether samples will be pre-sorted by a central or national facility.

- National rules for receiving samples

Italy has not signed the Nagoya Protocol and is generally allowed to receive soil samples. The problems encountered during the pilot project depended very much on the courier company delivering the samples. Samples from the Azores, Belgium, Denmark, France, Germany and Israel were received without problems. The samples from Slovakia were held up at customs without notification and some were returned to the sender, spoiling the fresh samples. The biggest problem was with the samples from Turkey, which were held up at DHL and could only be sent to Eurac Research after clearance from the Ministry of Agriculture, Food Sovereignty and Forests. In order to receive soil samples from extra-EU countries, the receiving laboratory must first be approved by the Ministry in accordance with EU Regulation 2019/2072 on protective measures against plant pests. The laboratory must be inspected by the local phytosanitary authority. It must also provide a description of the samples it is to receive in a Letter of Authority, which must be signed by the Ministry and then attached to the samples sent. This process takes weeks and is still ongoing. Once the Eurac Research laboratory has been approved, more samples should be received. However, in order to avoid potential problems, a Letter of Authority must authorise each sample sent, unless it has been previously declared under the 'multiple shipment' section, which requires the number of shipments to be

Outcomes of the Biodiversa+ pilot on soil monitoring during its first year

received to be declared in advance. Such national regulations are often difficult to understand, and information is not always available.

- Missing infrastructure for drying and storing soil samples

As some participants are not members of a research institution, the infrastructure for drying and storing samples was not always available. In such cases, either an external facility had to be asked for help (e.g. in France) or, as in the case of the drying process, it had to be carried out at air temperature over a longer period of time (e.g. the humidity in the Azores made it difficult to air dry the samples, the process took weeks longer than expected). In a transnational monitoring scheme, care must be taken to select participants/institutions who meet certain minimum requirements for carrying out the work (e.g. ovens and fridges).

3.4 Communication

- Sharing of protocols

We provided the participants with a step-by-step protocol, but we also provided the SoilBON and SoilBON Food Web protocols for additional information and illustration. This confused some participants, as the SoilBON protocols described more steps than were required. It is therefore important to clearly communicate which protocol is to be used and ideally to integrate all necessary steps into one comprehensive protocol.



4. Next steps

Necessary changes to the protocol identified during the workshop will be implemented and the updated protocols will be shared with the active partners.

In year 2, the majority of active partners will repeat their sampling efforts in spring and autumn at the same sites as in year 1 (small changes can be discussed with the coordinators). The new partner (SEPA, Sweden) will receive all information and will be instructed during a personal online meeting.

The coordinators will continue to pre-sort the invertebrate samples and prepare all samples for further processing by the external companies for species identification (Ökoteam, Austria) and eDNA analysis (biome-id, Germany). Carabid beetles will first be sent to the University of Aarhus for biomass analysis and then directly to Ökoteam for species identification.

Specific aims for the second year of the pilot will be to

- (1) Solve practical issues in the protocol;
- (2) Understand seasonal and inter-annual variation in soil biodiversity by re-sampling the same sites in the second year. This will have important implications for monitoring as it will provide valuable data on the necessary sampling frequency for soil biodiversity and whether this differs between climates (hot, dry countries vs. cooler, humid countries);
- (3) Assess the impact of environmental conditions and phenology on the composition of soil communities and their temporal turnover, and what this might mean for the up-scaling of a potential soil biodiversity monitoring scheme in the future;
- (4) Calculate how many countries/replicates are needed to correctly assess soil biodiversity in a transnational scheme;
- (5) Review existing national soil biodiversity monitoring schemes, compare their protocols with the pilot's protocol and see what we can learn from them.

The overall aim of the pilot will be to evaluate its implementation into a transnational monitoring scheme by considering funding, sampling frequency, sampling strategy, habitat types to be included, links with the Soil Directive and existing soil monitoring strategy.

5. First results

In this section we present some initial results from the spring sampling. Please note that not all countries are shown as for logistical reasons some samples arrived too late to be included in the analyses.

5.1 Vegetation surveys

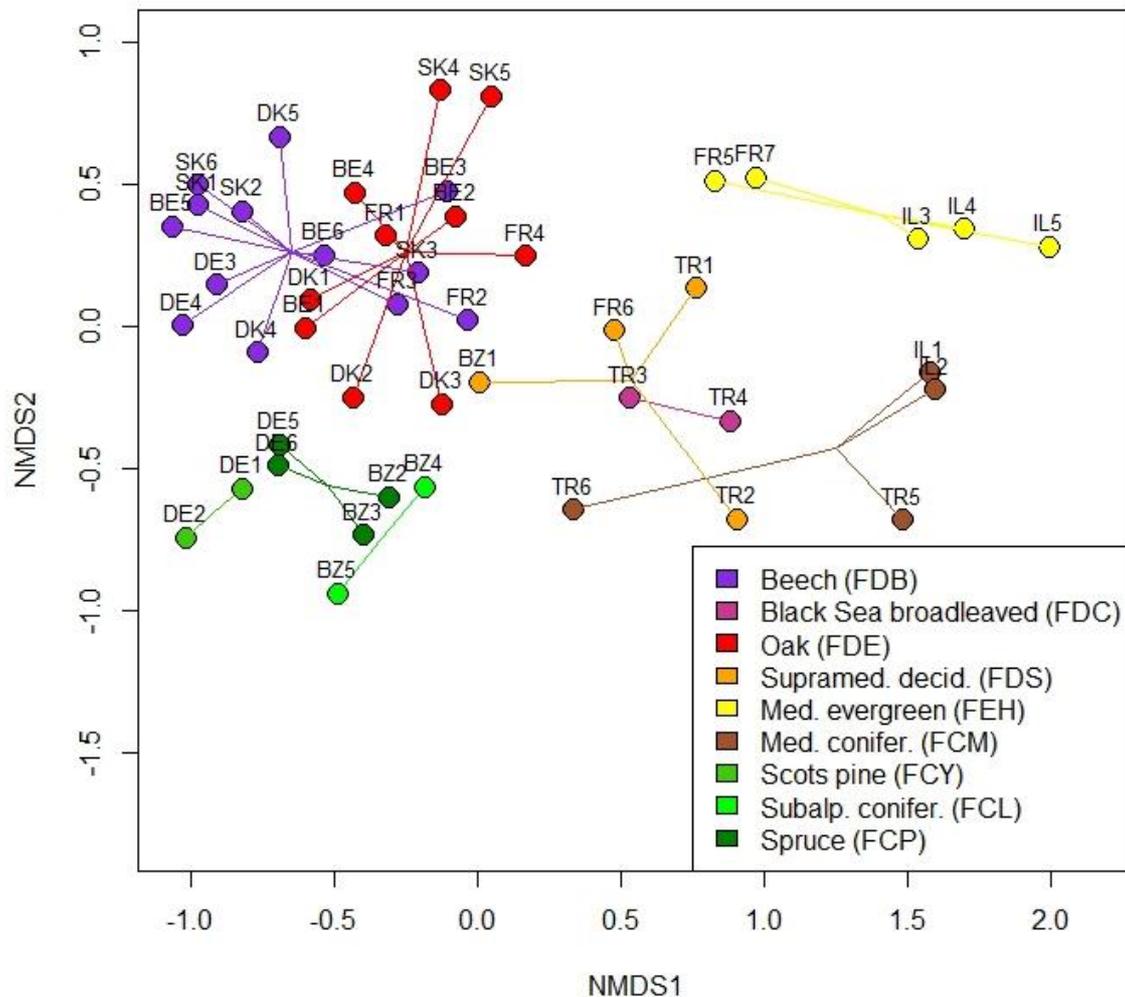


Figure 2: Non-metric multidimensional scaling (NMDS) analysis of the botanical data (percent coverage of plant species) of all sites sampled in 2023. Every point represents one single site. Sites with similar composition of vascular plants are closer to each other than sites with very different vegetation. The axes present the data in a way that best represents the dissimilarity between forest types. BE...Belgium, BZ...Province of Bolzano, DE...Germany, DK...Denmark, FR...France, IL...Israel, SK...Slovakia, TR...Turkey



All Mediterranean and supra-Mediterranean sites show a similarity and are plotted together on the right-hand side of the graph, as are Alpine and Boreal coniferous forests (lower left) and Nemoral beech and oak forests (upper left).

5.2 Soil parameters

Following the list of soil parameters analysed in the LUCAS project, we selected the following parameters for the pilot, that are relevant for forest soils:

- pH
- electric conductivity
- potassium content
- phosphorous content
- organic carbon content
- humus content
- total nitrogen content
- calcium carbonate content
- soil texture (percentage of sand, silt and clay)

Parameters were measured as described in the LUCAS protocol, following ISO, ÖNORM, and UNI EN standards, with two exceptions:

- For the analysis of potassium, ISO 11260 was replaced by ÖNORM L1086 for two reasons: the effort required for ISO 11260 is greater and ÖNORM L1086 also provides better reproducibility due to the larger sample weight.
- To measure soil texture, Eurac Research uses the automated Pario system (Meter Group, Munich), following manufacturer's instructions.

ISO and ÖNORM standards used:

- pH in CaCl₂: ISO 10390
- electric conductivity [μ S/cm]: ISO 11265
- potassium [mg K₂O/100g]: ÖNORM L 1086-1
- phosphorous [mg P/100g]: ISO 11263 (UV-Vis)
- organic carbon [%], humus [%], total nitrogen [%], CaCl₂ [%]: UNI EN 15936

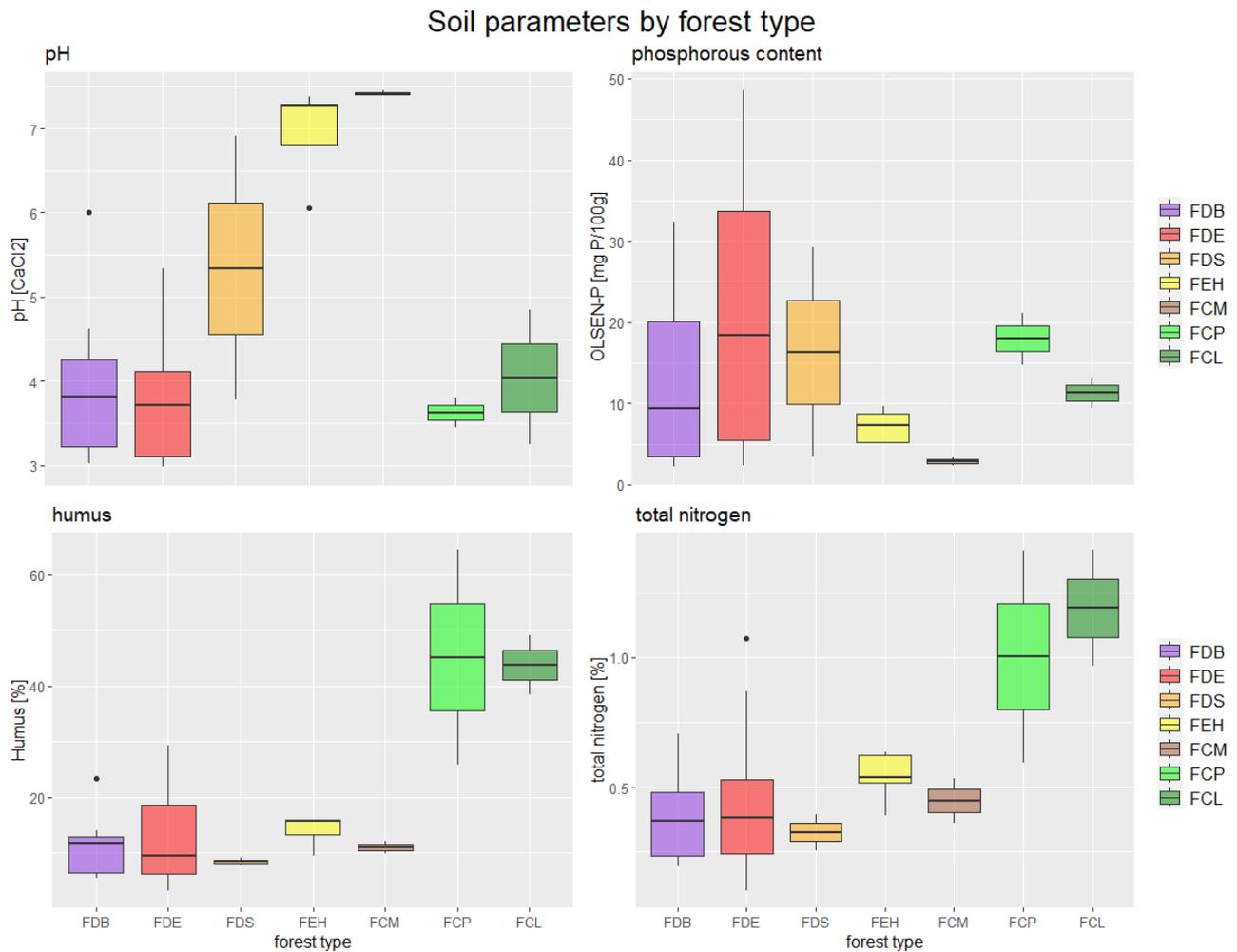


Figure 3: Selected soil parameters by forest type. FDB...beech forest, FDE...oak forest, FDS...thermophilus deciduous forest, FEH...broadleaved evergreen forest, FCM...mediterranean coniferous forest, FCP...Alpine pine forest, FCL...Alpine larch forest. Figure includes data from six countries (Belgium, Denmark, France, Israel, Italy, Slovakia).

Soil parameters vary among forest types. Specifically, the percentage of humus and total nitrogen content are significantly higher in coniferous forests (FCP, FCL). Forest type also affects pH values, with thermophilous forests (FDS, FEH, FCM) having a higher soil pH than other forest types. This increase was even significant for FEH and FCM. However, it should be noted that bedrock (silicious vs. calcareous) has not been included as a covariate yet. No differences in phosphorus content were observed among forest types.

5.3 Invertebrate diversity

Here we present the list of taxa identified at order or family level during pre-sorting and at species level by the external company. The list of taxa identified during pre-sorting is quite extensive and corresponds to the taxa identified in the national monitoring programme of the



coordinator in South Tyrol (Biodiversity Monitoring South Tyrol⁷). The taxa selected for identification at species level relate to taxa with important roles in soil processes and to the expertise of the external company. Both lists may not correspond to the final selection of taxa in a transnational monitoring scheme.

Taxa identified during pre-sorting:

- Oligochaeta (Lumbricidae, Enchytraeidae)
- Gastropoda
- Isopoda
- Arachnida (Araneae, Opiliones, Pseudoscorpiones, Acari)
- Myriapoda (Geophilomorpha, Lithobiomorpha Scolopendromorpha, Julida, Glomerida, Polydesmida, Chordeumatida, Symphyla)
- Apterygota (Collembola, Diplura, Protura, Archaeognatha)
- Coleoptera Imagines and Larvae (Brentidae, Cantharidae, Carabidae, Cerambycidae, Chrysomelidae, Cryptophagidae, Curculionidae, Dermestidae, Elateridae, Geotrupidae, Histeridae, Hydrophilidae, Latridiidae, Leiodidae, Lycidae, Monotomidae, Nitidulidae, Phalacridae, Ptiliidae, Ptinidae, Scarabaeidae, Scolytidae, Scryptidae, Silphidae, Staphylinidae)
- Diptera Imagines and Larvae (Nematocera, Brachycera)
- Formicidae
- Rhynchota (Heteroptera, Sternorrhyncha, Auchenorrhyncha)
- Blattodea
- Dermaptera
- Lepidoptera Imagines and Larvae
- Mecoptera Imagines and Larvae
- Neuroptera
- Trichoptera Imagines and Larvae
- Thysanoptera
- Psocoptera

Taxa identified to species level:

- Arachnida (Araneae, Opiliones, Pseudoscorpiones)
- Carabidae
- Heteroptera
- Dermaptera
- Blattodea

⁷ <https://biodiversity.eurac.edu/>

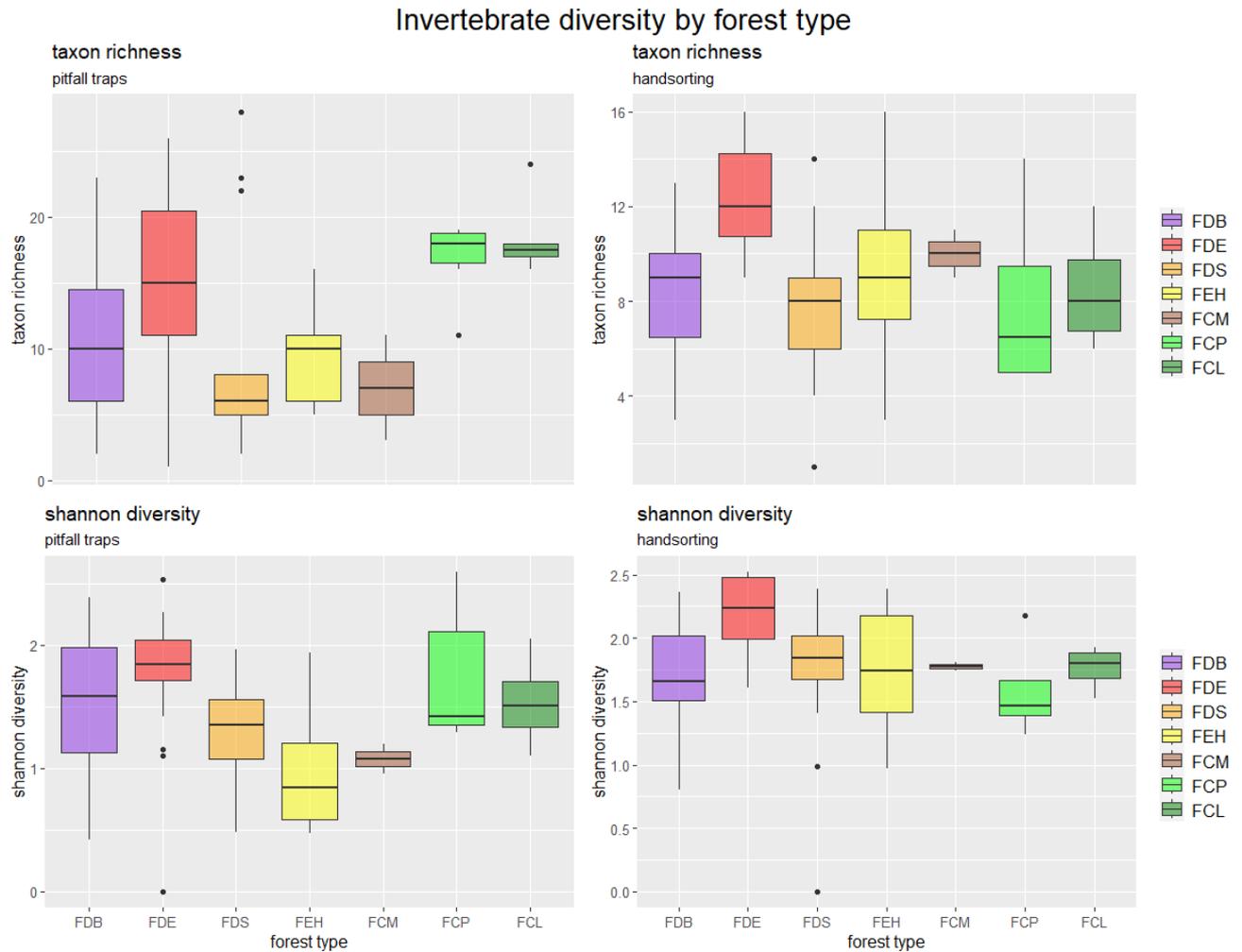


Figure 4: Invertebrate diversity (taxon richness, i.e. the number of different taxa in a sample, and Shannon diversity, i.e. taxon diversity in a community based on abundances of taxa) in pitfall traps (left upper and lower panel) and in soil cores (right upper and lower panel) by forest type. FDB...beech forest, FDE...oak forest, FDS...thermophilus deciduous forest, FEH...broadleaved evergreen forest, FCM...mediterranean coniferous forest, FCP...Alpine pine forest, FCL...Alpine larch forest. Figure includes data from six countries (Belgium, Denmark, France, Israel, Italy, Slovakia).

Forest type affects the diversity of ground-dwelling invertebrates (taxon richness and Shannon diversity) in that it is lower in thermophilous forest types (FDS, FEH, FCM) compared to beech forests (FDB), whereas oak (FDE) and coniferous forests (FCP, FCL) show significantly higher diversity. This effect is less pronounced for soil-dwelling invertebrates, only oak forests (FDE) show significantly higher invertebrate diversity.



Conclusions

The soil harbours immense biodiversity, but long-term trends in changes related to global change remain poorly understood. Therefore, a transnational monitoring scheme is urgently required to define measures for conserving and restoring soil biodiversity. This Biodiversa+ pilot study scrutinises and tests the necessary steps towards such a monitoring scheme. During the first year of the pilot, a step-by-step protocol was implemented to collect and analyse soil samples for their properties, as well as microbial and invertebrate diversity. A comparison was made between traditional and molecular species identification methods to provide methodological recommendations for a future transnational monitoring scheme. Additionally, administrative and logistical obstacles were identified, and possible solutions were suggested.

The first year of the pilot has revealed that a transnational monitoring scheme requires:

- a harmonised, well-defined, and easy-to-use protocol,
- clear instructions to reduce the risk of contamination for eDNA analysis,
- a list of minimum infrastructure requirements for participating institutions, and
- administrative support for understanding national regulations concerning the Nagoya protocol and the sending/receiving of soil and invertebrate samples.

The next steps involve enhancing recommendations for a transnational monitoring scheme with regards to sampling coverage, frequency, and habitat types. Also, links to the Soil Directive and existing national initiatives will be identified.