MINAGRIS

Protocol for multiscale experiments

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Version control

The protocol for multiscale experiments will be regularly updated to adapt to the latest results and resources available. The latest version can be found on the MINAGRIS SharePoint (private), and on the website (public) with the other public deliverables.

Version	Date	Description
Draft	05-31-2022	Draft prepared by Dimitrios Karpouzas, to be reviewed by relevant WP and tasks leaders.
V1	07-16-2022	Document prepared by Dimitrios Karpouzas considering contribution from partners.
V2	07-23-2022	Document updated by Dimitrios Karpouzas considering contribution from Esperanza Huerta Lwanga and Ilaria Negri.
V3	07-26-2022	Document prepared by Dimitrios considering contribution from Esperanza Huerta Lwanga. Submitted by Nicolas Beriot.

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Summary

MPs have been identified as major pollutant of agricultural soils, however their impact on the soil biota and agricultural productivity remains elusive. One of the main objective of MINAGRIS is to define the effects of MPs, either alone or in combination with other organic pollutants like pesticides and veterinary drugs which are also found in agricultural soils, and they are expected to interact with plastics, and affect the soil physical and chemical properties, the soil micro, meso and macrobiota and eventually agricultural productivity and ecosystem services. To achieve this objective, MINAGRIS will execute a multiscale experimental plan including soil laboratory microcosms, pot and mesocosm studies and field studies in three regions of Europe (Continental, Mediterranean and Atlantic). At these experiments different endpoints targeting soil micro, meso and macrobiota will be measured. These will be accompanied by measurements of soil physics, soil chemistry, plastic disintegration, and transport (biotic and abiotic), and plant productivity aiming to disentangle the overall impact of MPs on terrestrial ecosystems and ecosystem services. Following on these research activities MINAGRIS aspires to contribute to the development of a new regulatory framework regarding the plastic placement in the market. The current document (Deliverable 4.1. – Report) provides a detailed description of the experiments that will be performed at the different experimental scales. The experimental plan encompasses a series of experiments shared between partners but also separate, targeted experiments performed by specific partners. The current document will provide a description for the former (common experiments in WP4, WP5 and WP6) and it does not contain a description of the individual experiments that will be performed in specific tasks. More specifically the current document refers to the shared experiments performed in WP4 (Tasks 4.1, 4.2, 4.3, 4.5 and partially 4.5), WP5 (Tasks 5.1, 5.3, 5.4 although other specific experiments are going to be performed also in these tasks) and WP6 (Tasks 6.1 and 6.3, although other specific experiments will be also performed in these tasks). It is not subject of this document to describe experiments that will be performed separately by each partner in the frame of tasks 4.5, 5.2, 5.4, 5.5, 6.1 and 6.2. Beyond the experimental procedures the current report describes tasks per partner (who is responsible for which measurement), logistics for sample transfer between different partners, the timeline for each experimental scale and the protocols that will be used for each measurement. This is the first draft of the deliverable and will be subject to updates.

List of Abbreviations

MPs: Microplastics
NPs: Nanoplastics
AOM: Ammonia-oxidizing microorganisms
AOB: Ammonia-oxidizing bacteria
AOA: Ammonia-oxidizing archaea
AMF: Arbuscular mycorrhizal fungi
CSS: Case study sites

1. Introduction

Plastic use in agriculture has tremendously increased in the past decades resulting in soil pollution with plastic residues forming macroplastics, micro (MP) and nanoplastics (NP). MINAGRIS aims to contribute to healthy soils in Europe by providing a deeper understanding and tools to assess the impact of MP and NP in agricultural soil health and productivity. To create an overview on the actual situation across Europe, MINAGRIS will assess the use of different plastic polymers in agricultural systems in 11 case study sites (CSS) across Europe and identify the resulting types and concentrations of MPs and NPs. MINAGRIS will provide validated analytical tools that allow the quantification and identification of MPs and NPs in soils. **Based on the first results of the CSS, controlled experiments will be conducted to analyse the impact of MPs and NPs on physico-chemical soil properties, soil biodiversity, plant productivity, and ecosystem services, as well as their potential transfer to other environmental compartments and plants. Furthermore, potential synergistic effects with other stressors are assessed.** Quantification of the impacts of MPs and NPs on soil biodiversity and agricultural productivity, their transport and degradation in the environment, their impacts on socio-economic components, and synergies between all of them will make it possible to identify, in a multifactorial vision, the benefits and risks associated with the use of plastics in agriculture.

In the frame of WP4, WP5 and WP6 a series of experiments at different scales will be performed to define the effects of MPs, either alone or in combination with other organic pollutants, on the soil micro, meso and macrobiota (WP4), on the soil physicochemical properties and agricultural productivity (WP5) and on non-terrestrial organisms (WP6).

- **Soil lab microcosms** with a focus on effects on the soil microbiota
- **Soil pot experiments or mesocosm experiments** where both effects on soil micro, meso and macrobiota will be determined
- **Field experiments** where the effects on soil micro, meso and macrobiota, soil physicochemical properties, MP environmental fate (e.g. disintegration and transport) and agricultural productivity will be assessed

The data that will be produced by these multiscale experiments, along with individual experiments performed in WP4 (Task 4.5 Effects of MNP and other stressors on soil food web interactions and network configuration), WP5 (Task 5.2 Modelling transport of MNP and other stressors in the soil profile; Task 5.4 Assessment of the impact of MNP and other stressors on crop physiology and productivity; Task 5.5 NP plant uptake and risk characterization for the human food chain) and WP6 (Task 6.1 Assessment of the degradation and disintegration of MNP and other stressors in soils, Task 6.2 Microbial interactions in the plastisphere) will provide a comprehensive assessment of the effects of MPs on the soil biota and agricultural productivity.

The purpose of this report is to give a detailed overview of the experiments that will be performed at each experimental scale, who will be responsible for the experiments, how these experiments will be organized and what measurements and endpoints will be targeted at each experimental scale. Protocols for the measurements performed or citations for the methodologies followed will be provided. Finally, a timeframe for the execution of the different experiments and the deliverables and milestones associated with the multiscale experiments are given. **It should be noted that the report is going to cover only the common multiscale experimental planning and not individual experiments with a very specific focus (see above) which are the responsibility of the specific partners.**

2. Description of multiscale experiments

Regardless of the experimental scale all experiments share certain common features described below.

1. Soils

In all scales, experiments will be performed **in three soils with low background plastic pollution.** An effort will be put during the selection process so the three studied soils will be characterized by largely different physicochemical properties (e.g. organic carbon content, pH, soil texture). This will allow the manipulation of the levels of plastic pollution to the desired levels without any background plastic pollution interference. The three low plastic pollution soils will be derived from the sites where the field experiments will be performed (WU, INRAE, CHQ) and will be distributed to all partners based on their requests. The physicochemical properties of the three tested soils will be determined and efforts will be made to be representative of the main soil types in EU and vary in their physicochemical properties (mainly pH, organic matter content, texture) which are known to affect the composition of the soil microbiota but also the environmental fate of pollutants

2. Plants

The selection of plants to be used applies to the common pot and field experiment where plants will be present. Plants to be used in the pot and field experiments were selected based on the following criteria, (a) important crops for the EU agriculture and their final product is a main feed for the EU consumers, (b) mycorrhizal fungi support which will constitute a main microbial target group in our assessment of the effects of plastics on the soil microbiota (c) they are conducive with the experience of the different partners

(i) **Corn:** selected based on its universal distribution in Europe and its cultivation to all EU zones.
(ii) **Leak:** selected as a very good symbiotic mycorrhizal crop which grows well in pot and field conditions.
(iii) **Tomato, pepper or cucumber:** Horticultural crops relevant in areas where plastic mulching in agriculture and greenhouses are used (e.g. south Europe). Cucumber was proposed as a potentially good crop to check the potential uptake of MPs by the plan due to its high leaf area which induces large water uptake and higher chances for MPs uptake
(iv) **Carrot:** root crop whose edible part is underground and could be used to test potential accumulation of MPs from soil into the carrot root organ which is edible
(v) **Legumes:** crops which support both Nitrogen-fixing bacteria and arbuscular mycorrhizal fungi and we could assess the specific effect of MPs on those two important functional microbial groups and maybe on their interplay

In the pot experiments the partners could select from the range of crops above based on the question they would like to ask. The field experiments will be ideally performed with the same crop (e.g. Corn) unless otherwise decided or it is not technically feasible in a certain region (Crete, France, The Netherlands).

3. Size and type of plastics

Three types of plastics will be studied. All three will be commercial films and will be bought from the market and fragmented. The three types of plastics decided to be used in MINAGRIS will be

- **LDPE-based:** the most frequently used plastic type in agriculture as deduced from the plastic inventory of MINAGRIS (LDPE based mulch film, black, only virgin feedstock (i.e. no regenerated/recycled plastics incorporated), thickness 30 micron, from Oerlemans).
- **PBAT-based biodegradable plastic**: as one of the most common biodegradable plastic type that are coming in the market quite strongly (PBAT-based with some other polyesters,

including a low level of PLA, black (using carbon black), thickness 15 micron, certified EN17033 from Oerlemans).

- **Starch-based biodegradable plastic:** as one of the most common biodegradable plastic type that are coming in the market quite strongly (Starch based, PLA due to its low content is not considered as a constituent, black, thickness 15 microns

The plastic films will be purchased from the market with consultation by NOVAMONT, who is a partner in the project, and local producers of plastic films in the Netherlands (Partner Responsible: Wageningen Research). The plastic films will be fragmented by a commercial company and a large pool of each plastic will be produced and distributed to all partners involved based on their demands for the lab, pot and field experiments. The size of the fragments generated will be <300 µm.

Prior to their incorporation in soil in the different experiments, MPs should be washed with ethanol and sterile distilled water to avoid any carryover of microbes in the soil.

4. Other pollutants tested in combination with MPs

The effects of MPs on the soil micro, meso- and macro-biota will be looked at all experimental scales either individually or in combination with other organic pollutants known to be present in agricultural soils and expected to interactively affect or not the soil biota. In this frame one pesticide and one veterinary drug were selected to be included in the different experiments (as it is explained below).

Pesticides: A wide range of pesticides was considered for inclusion in MINAGRIS. Pesticides are organic pollutants that are intentionally released in agricultural soils and they were prioritized as organic pollutants interacting with MPs in agricultural soils. The pesticide selection was based on the following criteria (i) is registered and heavily used in agriculture in Europe (ii) there are data for its interaction with plastic surfaces in soil (iii) it shows an intermediate sorption on plastic surfaces (40-60%) so as its interaction with plastics will alter its availability to a certain extent but not fully and at the same time will interact with the plastic directly (iv) there are evidence of toxicity to soil microbial endpoints. Based on these criteria PYRACLOSTROBIN was selected (fungicide belonging to strobilurins).

Veterinary drugs: A second group of organic pollutants that are expected to end up in agricultural soils are veterinary antibiotics and anthelmintic compounds. Both groups end up in soil through application of manures. There are a few studies about the interaction of antibiotics with plastics in soil (e.g. tetracycline, sulfonamides, ciprofloxacin, amoxicillin, tiamulin, tylosin, trimethoprim) but nothing is known about anthelmintic compounds like benzimidazoles and macrocyclic lactones. Based on prior evidence for the toxicity of anthelmintic compounds on the soil microbiota and the expected potential toxicity on fauna, ALBENDAZOLE, a benzimidazole anthelmintic compound was selected to be included in the study (pros: lower adsorption on plastic surfaces is expected based on its LogKow, preliminary evidence for higher toxicity to soil microbes, nothing in the literature about these group of veterinary drugs and their interactions with plastics in soil)

2.1. Soil laboratory experiments

A series of soil laboratory microcosm scale experiments will be performed in the frame of WP4. The focus will be restricted on effects of MPs, either individually or in combination with other soil stressors, on the soil microbiota. No plants will be used at this scale and no soil fauna measurements will be performed at this scale. **The focus will be entirely on soil microbiota**.

It is important to note that all soil laboratory experiments should be performed in glass jars and not in plastic containers to avoid cross contamination during the experiment by wearing off.

Aim: To determine the effects of MPs, either individually or in combination with other soil stressors on the structure and function of the soil microbial community under highly controlled conditions where other confounding factors are excluded

Partners (PI in charge of the experiments): FUB (M. Rillig), UTH (D. Karpouzas), INRAE (F. Martin-Laurent), WU (S. Geisen)

Brief experimental description: microcosms of 200 g of soil will be prepared (in glass and not plastic containers), without plants (no AMF therefore), where higher number of treatments (MPs, organic pollutants, soils) could be used under a very controlled environment (not as realistic though as pot experiments allowing assessment of the effects of MPs in the absence of Plants excluding feedback effects of plants)

Treatments: For each of the three soils tested there will be the following treatments
Plastics: 4

- MP1, LPDE-based
- MP2, PBAT-based
- MP3, Starch-based
- MP4, Mixture of all 3 plastics: In this treatment the three different plastic types will be applied in one third of the final dose rate (0.01 or 0.1% w/w) in order all three to sum to a dose rate equivalent to the individually applied plastic treatments

Dose rates: 2

- 0.01% w/w
- 0.1% w/w

There will be a control treatment (no plastics) but this will be common for all plastic types. Before incorporation in soil, MPs should be washed with ethanol and sterile distilled water to avoid microbial carryover to the studied soils.

Organic Pollutants: 4

- ALBENDAZOLE^{+}
- PYRACLOSTROBIN^{+}
- ALB^{+}, PYR^{+}
- No pesticide/veterinary drug application

Albendazole and Pyraclostrobin will be applied at environmental relevant dose rates which according to previous measurements and calculations of UTH will be 1 mg/kg soil (dw). Organic pollutants will be applied to soil 24 h before the incorporation of plastics in soil.

Replicates per treatment: 5

<u>The above treatments sum up to the following (per soil):</u>

4 plastic treatments x 2 dose rates x 4 organic pollutants x 5 replicates = 160 experimental units

There will be also **one control treatment** (no plastics) x 4 organic pollutants x 5 replicates = 20 samples

Each of the partners will undertake one lab experiment with the three soils available for experimentation. The soil microcosms will be incubated at 20°C for 90 days in the dark and the moisture content will be adjusted and maintained to 40% of the water holding capacity of the soils.

Measurements: The following measurements will be performed at soil microcosm scale to determine the effects of MPs on the soil microbiota

- Abundance of AOM via q-PCR
- Abundance of denitrifying bacteria via q-PCR
- Inorganic Nitrogen pools in soil (NH_4^+, NO_3^-),
- Potential nitrification
- Abundance of *catA* and *pcaH* genes representing microorganisms degrading natural and xenobiotic aromatic compounds
- Activity of soil microbial enzymes via fluorometric methods
- Amplicon sequencing analysis of bacteria and fungi

Considering that one of the main microbial endpoints will be related to N cycling, ammonium fertilization will be added at the start of each experiment (in the form of NH_4Cl or urea) to stimulate nitrification.

For all microbial measurements DNA extraction will be performed by the local partners using the Qiagen DNAeasy PowerSoil kit which has been the cornerstone commercial kit for DNA extraction in soil microbial ecology in the last 20 years. This will ensure a uniformity in the DNA extraction process between different lab experiments. Soil DNA will be quantified via Nanodrop or Qubit fluorometric assay (preferred). Soil DNA extracts (20-30 µl) will be sent to the following partners for performing downstream measurements as follows:

- q-PCR analysis of the abundance of the *amoA* gene (UTH)
- q-PCR analysis of the abundance of the denitrification genes (INRAE)
- q-PCR analysis of the abundance of the aromatic carbon degraders (INRAE)
- amplicon sequencing analysis of bacteria and fungi (INRAE, FUB, UTH). WU should sent DNA samples to UTH for the bacterial analysis and to FUB for the fungal analysis

The measurements of PNT and inorganic N pools will be performed by each of the partners using the established protocols described in Section 5.

The enzymatic activity measurements will be performed by UCSC (1 g of moist soil per sample shipped in ice backets to UCSC, see protocol in Section 5)

The protocols for each of the above measurements will be shared by all partners and are given in Section 5 of this report.

2.2 Pot experiments

2.2.1. Microbiome targeted pot experiments

Pot experiments will be also employed in the frame of MINAGRIS. The presence of plants in pot experiments gives the opportunity to expand our endpoints to symbiotic microorganisms like arbuscular mycorrhizal fungi (AMF) which constitute an important functional microbial group in soil with a significant role in plant nutrition and productivity, and also in soil structure and porosity. Potential effects on the structure and on the function of AMF would have strong negative effects on ecosystem services.

Aim: To determine the effects of MPs, either individually or in combination with other soil stressors, on the structure and function of the soil microbial community in the presence of plants under semi-field conditions.

Partners: FUB (M. Rillig), UTH (D. Karpouzas), UCSC (E. Puglisi)

Brief experimental description: The experiments will be established in 5 L pots (or larger if needed depending on the plant selected). In order to avoid using large amounts of soil peat or sand : soil (1:1 v/v) will be used. In total 6 pot experiments will be performed (two per partner). Each partner will perform pot experiments in two different soils (2 of the 3 pristine soils available). This setup will end up having two pot experiments per soil.

Treatments: For each of the three soils tested there will be the following treatments:

Plastics: 3

- MP1, LPDE based
- MP2, PBAT based
- MP3, Starch based

Dose rates: 2

- 0.01% w/w
- 0.1% w/w

Organic Pollutants: 2

- ALB^{+}, PYR^{+} (Applied dose rate: 1 mg/kg soil dw each of the compounds)
- No pesticide/veterinary drug application

Pesticides will be applied a day before the plastics incorporation in soil

Replicates per treatment: 6

<u>The above treatments sum up to the following (per soil):</u>
3 plastic treatments x 2 dose rates x 2 organic pollutants x 7 replicates = 84 experimental units

There will be also **one control treatment** (no plastics) x 2 organic pollutants x 7 replicates = 14 experimental units

There will be also a **second control treatment** to be a comparable control to biodegradable plastics considering the amount of biodegradable carbon added in soil through their application. The **biodegradable plastic control treatment** will involve addition in soil an amount of straw which will provide comparable C amounts in the soil ecosystem: 2 organic pollutants x 7 replicates = 14

In the pot experiments there is the option of getting time series samples after the application of the MPs (and organic pollutants). This could be done using a sterile cork borer which will allow the collection of a small amount of soil near the plant root with minimum disturbance. We propose the collection of samples at three time points 10, 45, 90 days after application of plastics in soil (can be adjusted based on the crop used). From each pot at each sampling three samples will be collected with the core borer and homogenized leading to the collection of one composite soil sample per pot at each sampling time.

The amounts of soils needed for the different measurements are as follows

- max 5 g for DNA extraction
- 5-10 g for potential nitrification
- 2-5 g for ammonium and nitrate soil measurements,
- 0.4-1 g for enzymatic activities

Measurements: The following measurements will be performed to determine the effects of MPs on the soil microbiota:

- Abundance of AOM via q-PCR
- Abundance of denitrifying bacteria via q-PCR
- Inorganic N pools in soil (NH_4^+, NO_3^-)
- Potential nitrification
- Abundance of *catA* and *pcaH* genes via q-PCR
- Activity of soil microbial enzymes via fluorometric analysis
- Soil microbial respiration via MicroRespTM
- Amplicon sequencing analysis of bacteria, fungi, protists, AOM (AOA, AOB, Comammox bacteria)
- Amplicon sequencing analysis of AMF (intraradical)
- AMF colonization and P concentration in plant roots
- Abundance of Plant Growth Promoting Microorganisms (PGPM) via q-PCR of the genes *nifH, ipdC, ppdC, gcd, gad, phnX*

Plant Cultivation Conditions:

Standard fertilization, relevant for the crop selected in the experiment, will be provided to the plants. Since a significant endpoint will be ammonia-oxidizing microorganisms, the partners should ensure that ammonium N would be applied through fertilization (to stimulate AOM).

Plant growth conditions (light and dark period, temperature) will be adjusted according to the selection of plant in case experiments are performed in growth cabinets, while this will not be necessary if experiments are performed in greenhouses.

Growth conditions should be optimum to limit abiotic stress only to the plastic and other organic pollutants applied.

For all microbial measurements DNA extraction will be performed by the local partners using the Qiagen DNAeasy PowerSoil kit which has been the cornerstone commercial kit for DNA extraction in soil microbial ecology in the last 20 years. This will ensure a uniformity in the DNA extraction process between different lab experiments. Soil DNA will be quantified via Nanodrop or Qubit fluorometric assay (preferred). Soil DNA extracts (20-30 µl) will be sent to the following partners for performing downstream measurements as follows

- q-PCR analysis of the abundance of the *amoA* gene (UTH)
- q-PCR analysis of the abundance of the denitrification genes (INRAE)
- q-PCR analysis of the abundance of the aromatic carbon degraders (INRAE)
- q-PCR analysis of the PGPM (UCSC)
- Amplicon sequencing analysis of bacteria and fungi (UCSC, FUB and UTH)
- Amplicon sequencing of AOM (UTH)
- Amplicon sequencing of the intraradical community of AMF (UTH, FUB). A common protocol for root DNA extraction will be used. All partners including UCSC will extract DNA from roots following the common protocol and will send DNA to FUB for amplicon sequencing analysis
- Amplicon sequencing of protists (WU)

The measurements of PNT and inorganic N pools will be performed by each of the partners following the protocols given in Section 5 (no shipment of samples is required)

The enzymatic activity measurements will be performed by UCSC (1 g of moist soil per sample will be shipped with ice backets to UCSC)

The measurements in MicroResp will be performed by FIBL-CH. At final harvest of the pot experiments, 150-200 g of root-free soil is collected, sieved to 2mm, stored at 4°C and shipped on ice to FiBL-CH as soon as possible. Please ship to Dominika Kundel/Andreas Fliessbach, Department of soil sciences, Ackerstrasse 113, CH-5070 Frick, Switzerland. Ship samples only after getting in touch with FiBL CH people.

The protocols for each of the above measurements will be shared by all partners and are given in Section 5 of this report.

2.2.2. Micro-, and meso-fauna targeted experiments

Aim: To determine the effects of MNPs, either individually or in combination with other soil stressors, on edaphic, hemiedaphic and epiedaphic collembola (mesofauna) (UCSC)

Brief experimental description: The experiments will be established by UCSC in arenas with the same soil as WP5 to check effects on edaphic, hemiedaphic and epiedaphic collembola species. Plastic fragments will be delivered to UCSC by WU.

Treatments:
Soils: 1 (one of the three soils will be selected)

Plastics: 3
- MP1, LPDE based
- MP2, PBAT based
- MP3, Starch based

Dose rates: 2
- 0.01% w/w
- 0.1% w/w

Organic Pollutants: 2
- ALB^{+}, PYR^{+} (dose rate 1 mg/kg soil dw each)
- No pesticide/veterinary drug application

Species of collembola: 3

Replicates per treatment: 3

The above treatments sum up to the following (see Table S2 Annex I for more details):
1 soil x 3 plastic treatments x 2 doses rate x 2 OP (with and without organic pollutants) x 3 species (edaphic, hemiedaphic and epiedaphic) x 3 replicates = 108 experimental units
There will be **one control treatment** (no plastics)/each, thus:
1 soil x 3 NO plastic treatments x 2 doses rate x 2 OP (with and without organic pollutants) x 3 species (edaphic, hemiedaphic and epiedaphic) x 3 replicates = 108 experimental units

Measurements: The following measurements will be performed to determine the effects of MNPs on edaphic, hemiedaphic and epiedaphic collembola species:

- Ingestion of MNPs by edaphic species
- Ingestion of MNPs by hemiedaphic species
- Ingestion of MNPs by epiedaphic species
- Mortality of edaphic species
- Reproduction rate of edaphic species
- ovideposition of edaphic species
- Behaviour of edaphic species
- Mortality of hemiedaphic species
- Reproduction rate of hemiedaphic species
- ovideposition of hemiedaphic species
- Behaviour of hemiedaphic species
- Mortality of epiedaphic species
- Reproduction rate of epiedaphic species
- ovideposition of epiedaphic species
- Behaviour of epiedaphic species
- Body size of edaphic species
- Body size of hemiedaphic species
- Body size of epiedaphic species

2.2.3. Macrofauna targeted experiments

Aim: To determine the effects of MPs (combined or separate), on either individually or in combination with other soil stressors, on epigeic, anecic and endogeic earthworms (ecological categories according to Bouche 1977) (WU)

Brief experimental description: The experiments will be established by WU in pots and mesocosms/columns according to the ecological category of the earthworm (Table S1) with one or several plastics together or without organic stressors, with one or several earthworms species.

Treatments:
Thirty nine to forty eight treatments per soil type are expected (Table S1, annex), with 3 combined or separate microplastic types, with 2 concentrations (0.01%, 0.1% w/w), 3 earthworms categories plus 1 combination, 2 organic stressors (1 pesticides and 1 veterinary drug), and 5 replicates per treatment, given a total of 195 to 240 experimental units per soil type. Due to several studies are already performed with single plastic types on single species, this experiment will give focus on the effect of combined MPs (following the proportions found on field) on combined species (following the proportions found on field).

Measurements and Endpoints:
Every 7 days superficial casts will be collected from each pot for determining the ingestion rate, earthworms' biomass will be recorded at time 0, 14, and 60 d. Mortality and reproduction will be also assessed after 60 days of experimentation. In case of anecic earthworms avoidance behaviour together with microplastics transport in burrows will be also measured.

2.3 Field experiments

Field experiments will be also employed in the frame of MINAGRIS in the years 2024 and 2025. Field experiments will be performed at three field sites as follows:
- Dijon (France, Continental region) employed by INRAE
- Wageningen (The Netherlands, Atlantic region) employed by WU
- Crete (Greece, Mediterranean region) employed by CHQ

Aim: To determine the effects of MPs, either individually or in combination with other soil stressors on the structure and function of the soil microbiota and soil invertebrate community under realistic soil field conditions.

Partners involved and their roles:
- Field Experiment Execution (INRAE, WU, CHQ)
- Soil microbiota measurements (UTH, UCSC, INRAE, FUB, WU)
- Soil meso- and macro-biota measurements (WU, UCSC)
- Physical and chemical soil measurements (UL, WU, GIUB)
- Plant productivity measurements (INRAE, CHQ, WU coordinated by FIBL-CH)
- Plastic disintegration measurements (UBERN, POLITO, WU)

Brief experimental description:

Treatments: For each of the three soils tested there will be the following treatments:

Plastic treatments: 3*
- LPDE based
- PBAT based
- Mixture of two plastic types

** plastic types that showed effects on the soil biota in the lab and pot experiments will be selected to be further assessed in the field study*

Dose rates: 2*
- 0.01%
- 0.10%

There will be a control treatment (no plastics) but this will be common for all plastic types. Application of plastic fragments will be performed at the top 10 cm of the soil and incorporated in the soil surface.

Organic Pollutants: 2
- ALB^{+} or PYR^{+*}
- No pesticide/veterinary drug application

** final selection based on the pot and lab experiments, and the most toxic one will be selected*

Replicates: 5

The above treatments sum up to the following (per soil):
3 plastic treatments x 2 dose rates x 2 organic pollutants x 5 replicates = 60 plots
There will be a control treatment (no plastics) x 2 organic pollutants x 5 replicates = 10 control plots

The field experiments will follow a complete randomized design. A tentative organization of treatments can be seen in Figure 1.

Field experiments will last for two years (cultivation seasons 2024 and 2025). In 2024 the selected plastics will be applied to the field plots as described above. In 2025 all plots will be separated in two sub-plots A and B. All sub-plots A will be treated again with the same plastic type and the same amount while sub-plots B will remain untreated. This experimental setup will compare effects between continuous treatment and intermittent treatment of soils with plastics and enable to observe potential long-term recovery of the soil biota and the whole ecosystem (see Figure 2)

Similar to the pot experiments, in the field experiments there is the option of getting time series samples after the application of the MPs (and organic pollutants). Regarding soil micro, meso and macro-invertebrates, measurements will be performed in samples collected at the beginning and at the end of the experimentation. Regarding the soil microbiota measurements, we propose the collection of soil samples at two time points at 30 and 120 days post application (or adjusted depending on the lifecycle of the crop studied. The philosophy behind these two time points is to identify any possible effects close to the day of application and potential recovery from effects at later stages of the experiment and crop growth. At each sampling time point soil samples could be collected using a soil auger which will allow the collection of soil near the plant root with minimum disturbance. From each treatment plot we suggest the collection of 5 subsamples which would be homogenized. This sampling strategy will lead to the collection of 5 bulk samples per plot for each treatment at each sampling time (see Figure 3).

70 plots * 2 time points * 5 samples = 700 samples (year 1)
70 plots * 2 time points * 5 samples * 2 sub-plots = 1400 samples (year 2)

Standard fertilization and crop practices will be provided to the plants.

Figure 1. A completely randomized plot design for the field experiment (tentative based on the treatments described above)

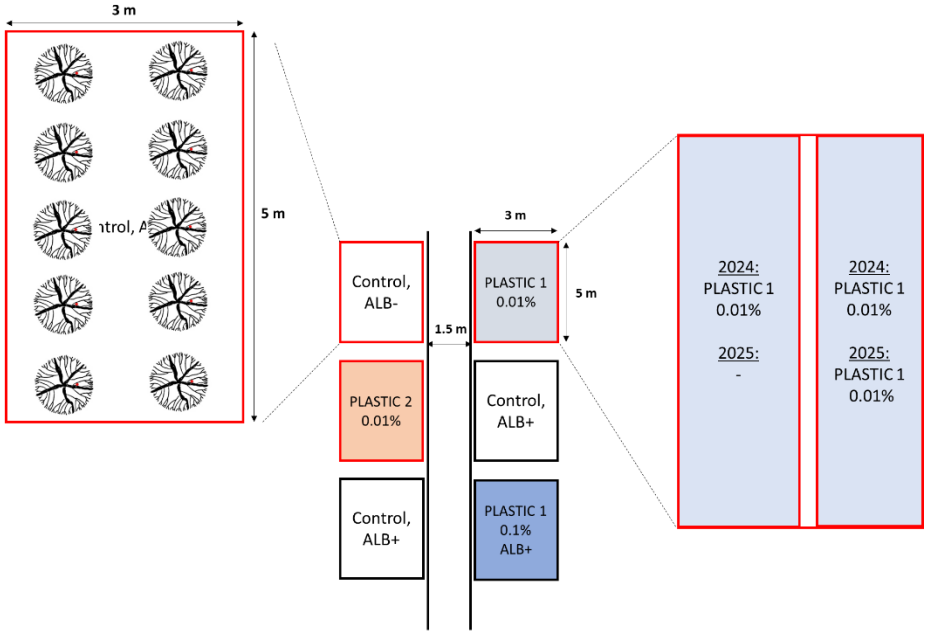

Figure 2. A schematic representation of the position of the plants in each plot (left) and of the treatment planning in each plot in the two years of experimentation 2024 and 2025 (right, an example for a plot treated in 2024 with plastic type 1 at the dose rate of 0.01%).

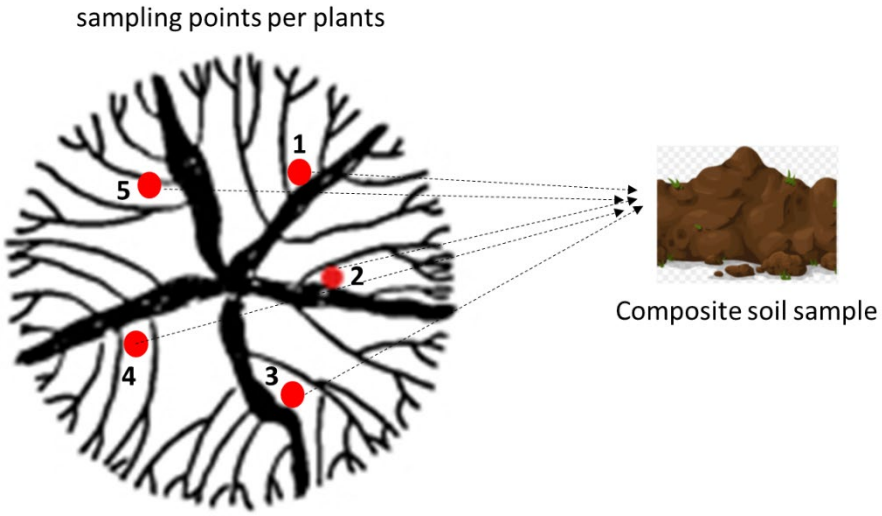

Figure 3. A representation of the sampling scheme that would be followed for collecting composite soil samples from the root zone of each plot.

2.3.1. Microbiome measurements

To avoid the transportation of large amounts of soil it is proposed that soil DNA extraction will be performed by the local partners responsible for the three field experiments meaning INRAE, CHQ and WU. The amounts of soils needed for soil DNA extraction will be 5 g. Soil DNA extraction will be performed using the Qiagen DNAeasy PowerSoil kit which has been the cornerstone commercial kit for DNA extraction in soil microbial ecology in the last 20 years. This will ensure a uniformity in the DNA extraction process between different lab experiments. Soil DNA will be quantified via Nanodrop

or Qubit fluorometric assay (preferred). Soil DNA extracts (20 µl) will be sent to the relevant partners (See below) for performing downstream measurements:

- q-PCR analysis of the abundance of the *amoA* gene
- q-PCR analysis of the abundance of the denitrification genes
- q-PCR analysis of the abundance of the aromatic carbon degraders
- Amplicon sequencing analysis of bacteria, fungi, protists, and AOM

Regarding AMF, amplicon sequencing of the intraradical AMF community, AMF colonization levels and P concentration levels will be measured in plant roots. A common protocol for root DNA extraction will be used (see section 5). Local partners will extract DNA from roots following the common protocol and will send DNA to FUB and UTH for amplicon sequencing analysis. The rest of the measurements will be performed either in house (in case the partners have relevant expertise like INRAE) or root fragments will be sent fresh to FUB (by WU) and UTH (by CHQ) for measuring colonization and P levels in plant roots. The protocols for each of the above measurements will be shared by all partners and are given in Section 5 of this report.

To distribute evenly the workload for the different partners and support the partners organizing the field experiments with regards to the microbiota measurements a first plan of the tasks for each of the partner has been issued.

- **Dijon Field Experiment:** INRAE and FUB will be responsible for running all the different microbiota analysis listed above
- **Crete Field Experiment:** UTH and UCSC will be responsible for running all the different microbiota analysis listed above
- **Wageningen Field Experiment:** UTH, FUB and WU will be responsible for running all the different microbiota analysis listed above

The specific distribution of the measurements between the partners above will be finalized upon discussion between the partners and if there is a conflict the final decision will be made by the scientific coordinator.

2.3.2. Soil meso- and macro-fauna measurements

All soil fauna measurements will be performed by WU and UCSC

Effects of MPs on the community of soil micro-arthropods will be analyzed by UCSC by choosing a sample area representative of the three field sites (Dijon, Wageningen, and Crete).

Three soil samples for each field site will be collected by UCSC away from dry seasons and periods and at least 5 m from the margin. Soil fauna will be extracted within 48h by using Berlese-Tüllgren extractors for at least 5 days. The biodiversity of soil micro-arthropods community will be analyzed. The following measurements will be performed:

- Determination of biological forms in soil samples from Dijon
- assignment of the Ecological-Morphological Index in soil samples from Dijon
- QBS-ar index computation in soil samples from Dijon
- Determination of biological forms in soil samples from Wageningen
- assignment of the Ecological-Morphological Index in soil samples from Wageningen
- QBS-ar index computation in soil samples from Wageningen
- Determination of biological forms in soil samples from Crete
- assignment of the Ecological-Morphological Index in soil samples from Crete
- QBS-ar index computation in soil samples from Crete

Macroinvertebraes will be assessed by the TSBF method (Anderson and Ingram 1993) at the beginning and end of the experiment, for determining abundance, biomass, diversity and microplastics in the gut and casts of earthworms.

2.3.3. Soil physical and chemical properties measurement

Soil physical properties related to soil water in the field experiments will be determined by UL. The impact of the plastic particle size, their concentration and type of plastic additives on soil will be analyzed. The following physical properties will be assessed:

- Soil aggregate stability and aggregate size distribution (UCSC)
- Texture structure, bulk density, hydraulic conductivity, pore size distribution, infiltration, soil water repellency and soil water retention (UL).

The protocols for each of the above measurements are given in Section 5 of this report.

2.3.4 Plant productivity measurements

Plant productivity measurements in the field experiments will be performed by the local partners e.g. INRAE, WU and CHQ following protocols and directions given by FIBL-CH.
Plant productivity will be assessed according to the following crop-specific metrics:

Maize:

At flowering: Per experimental unit, mark three plants and measure

- growth stage (following the BBCH scale see BBCH folder in WP4)
- Plant height (measured from the soil surface to the maximum height)
- crop nitrogen status using SPAD-measurements: for each of the three selected plants, measure the crop nitrogen status using a SPAD device. Always measure on the top fully expanded leaf. Take six replicated measures per leaf (3 at each of the leaf axis)
- Observation on plant diseases

At harvest:

Start with the three marked plants and record:

- growth stage (following the BBCH scale)
- Plant height (measured from the soil surface to the maximum height)
- Observation on plant diseases
- Cut the plants at 2cm above the soil surface, cut the corncob and determine weight of shoot and corncobs. Determine the dry weight of corncobs, shoot and roots after drying the samples at 40°C to constant weight.
- Using a shovel, dig out the root system to a depth of 20 cm. Remove the soil from the roots by washing the root system over a 2mm sieve, dry at 40°C to constant weight and record the dry weight.

Leek/Tomato/Pepper/Carrot:

At harvest, above-ground biomass and/or crop yield along with estimates of root biomass is assessed and observations on plant diseases are recorded.

2.3.5. Plastic disintegration measurement

During the two-year field experiments soil samples will be collected from the three field experiments and analyzed for plastic disintegration by UBERN, POLITO and WU as describe in Deliverable 3.2.

A summary of the measurement tasks per field site follows

Wageningen Field Experiment

- Microbiota measurements: UTH, FUB, (tasks 4.2, 4.3, 4.5)
- Soil meso- and macro-biota : WU, UCSC (task 4.4)
- Soil physics: UL (task 5.1)
- Soil chemistry: WU (task 5.4)
- Plant measurements: FIBL-CH, Plant growth and plant health measurements: local partner (FIBL will provide a detailed protocol on how these measurements will be done) (task 5.4)
- Plant disintegration measurements: WU, UBERN, POLITO (task 6.1)

Dijon Field Experiment

- Microbiota measurements: FUB, INRAE
- Soil meso- and macro-biota : WU, UCSC
- Soil physics: UL,
- Soil chemistry: WU,
- Plant measurements: FIBL-CH, Plant growth and plant health measurements: local partner (FIBL will provide a detailed protocol on how these measurements will be done) (task 5.4)
- Plastic disintegration measurements: WU, UBERN, POLITO (task 6.1)

Crete Field Experiment

- Microbiota measurements: UTH, UCSC
- Soil meso and macrobiota : WU, UCSC
- Soil physics: UL,
- Soil chemistry: WU,
- Plant measurements: FIBL-CH, Plant growth and plant health measurements: local partner (FIBL will provide a detailed protocol on how these measurements will be done) (task 5.4)
- Plastic disintegration measurements: WU, WR, UBERN, POLITO (task 6.1)

2.4. Aerial transport and effects of MNPs on honeybees (Task 6.3.2)

2.4.1. Effects of MNPs on honeybees (Mesocosm/Microcosm experiments)

Aim: To determine the effects of MNPs on worker honeybees under laboratory conditions (UCSC).

Brief experimental description: Study the effects on worker honeybees following acute and chronic oral exposure to MNPs. Young worker bees will be exposed to aqueous sucrose solution prepared with MNPs suspensions by continuous and *ad libitum* feeding over a period of 10 days. The experiments will be carried out in the ecotoxicological lab (UCSC).

Treatments:

Plastics: the most common polymer type found in CSS sites without organic stressor

Dose rates: 0.01%. A preliminary test of palatability will be carried out. In case no palatability occurs, we will test the palatability of lower concentrations with or without organic stressors (ALB^+, PYR^+).

Exposure:

- Acute 1 day
- Chronic 10 days

Numbers of bees per replicate:

- At least 16 specimens for acute test x3
- At least 16 specimens for chronic test x3
- At least 16 specimens for control x3

Analyses:

Acute exposure analyses:

- dissection of the gut for SEM-EDX analysis Papa et al., 2021;
- extraction of haemolymph for SEM-EDX analysis Papa et al., 2021;

Chronic exposure analyses:

- dissection of the gut for SEM-EDX analysis Papa et al., 2021;
- extraction of haemolymph for SEM-EDX analysis Papa et al., 2021;
- dissection of the gut for molecular analysis (gut microbiota) Papa et al., 2021;
- dissection of the gut for histological analysis

Measurements:

Acute exposure:

- absence or presence of MNPs in faeces;
- absence or presence of MNPs in haemolymph;
- mortality.

Chronic exposure:

- absence or presence of MNPs in faeces;
- absence or presence of MNPs in haemolymph;
- effects on gut microbiota;
- histological effects on the gut;
- mortality;
- behavioural abnormalities.

2.4.2. Aerial transportation of MNPs through honeybees (field experiments)

Aim: To determine the potential for aerial transportation of MNPs through worker honeybees in the field (UCSC).

Brief experimental description:
Bees living in MNPs-polluted agroecosystems (CSS at WU) will be used as bio-monitors by UCSC. MNP aerial transport will be assessed on the bee body and through translocation in tissues/organs.

Treatments:
Forager honeybees will be sampled during the reproductive season (from June to August) from hives exposed to environmental MNP levels (CSS at WU). The following analyses will be carried out:
- dissection of the body (e.g., forewings, heads; Negri et al., 2015), and optic and SEM/EDX analysis (Figure 4).
- dissection of the gut (Negri et al., 2015; Papa et al., 2021), and optic and SEM/EDX analysis
Negative control bees will consist of newly enclosed individuals from a brood frame kept in a growth chamber.

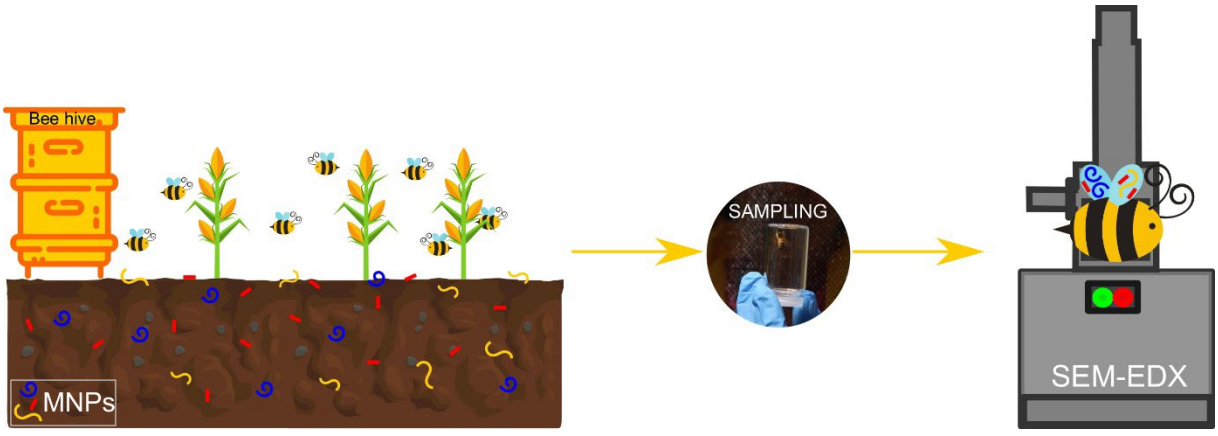

Figure 4. A representation of the field-based experiment addressing MNPs aerial transportation by pollinators.

3. Deliverables and Milestones associated with multiscale experiments

Table 1. List of deliverables associated fully or partially with the multiscale experiments

No.	Title	WP	Partner	Type	Level	Month
4.1	Protocol for multi-scale experiments	4	UTH	R	PU	M11
4.2	Guidelines on the assessment of the toxicity of MNP and other stressors on soil microbial functioning	4	FUB	R	PU	M46
4.3	Guidelines on the assessment of the toxicity of MNP and other stressors on soil microbial diversity	4	INRAE	R	PU	M49
4.4	List of soil biota indicators affected by MNP and other stressors	4	WU	R	PU	M52
4.5	Guidelines on the assessment of the toxicity of MNP and other stressors on soil fauna	4	UCSC	R	PU	M56
4.6	Guidelines for assessing effects of MNP and other stressors on the entire soil food web	4	WU	R	PU	M60
5.1	Effects of MNP on the soil physical properties related to soil water	5	UL	R	PU	M48
5.3	Effects of MNP on the soil chemical properties	5	WU	R	PU	M54
5.4	Impact of MNP on crop productivity and plant physiology	5	FIBL-CH	R	PU	M54
6.1	Degradability report of MNP in soils	6	UBERN-GIUB	R	PU	M48
6.4	Biotic and abiotic MNP transportation characterization and monitoring of sublethal effects on bees	6	WU-UCSC	R	PU	M56

Table 2. A list of milestones associated with the multiscale experiments

#	Milestone name	WP	Date	Means of verification
4.1	Functional microbial endpoint values and diversity data derived from pot and soil microcosms are gathered	4	M36	Data available on project database
4.2	Diversity indices and toxicity endpoints for soil fauna from soil microcosms to pot experiments	4	M40	Data available on project database
4.3	Diversity data from all trophic levels of the soil food web gathered and analysed	4	M48	Data available on project database

4. Timeline of the multiscale experiments

WP4	Scale	Partners perform exp.	Partners measuring	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54	57	60
Task 4.2 - effects microbial function (FUB)	lab	FUB, UTH, INRAE	INRAE, UCSC, FIBL-CH																				
	pot	FUB, UCSC, UTH	INRAE, UCSC, FIBL-CH, UTH																				
	field	INRAE, WU, CHQ	INRAE, FUB, UTH, UCSC																				
Task 4.3 - effects microbial diversity (INRAE)	lab	FUB, UTH, INRAE	INRAE, FUB, UTH, UCSC																				
	pot	FUB, UCSC, UTH	UTH, FUB, INRAE, UCSC, WU																				
	field	INRAE, WU, CHQ	INRAE, FUB, UTH, UCSC, WU																				
Task 4.4 - effects fauna (WU)	pot	WU, UCSC	WU, UCSC																				
	field	INRAE, WU, CHQ	WU, UCSC																				
Task 4.5. - effects on soil food web (WU)	pot	FUB, UCSC, UTH	UTH, FUB, INRAE, UCSC																				
	field	INRAE, WU, CHQ	UTH, FUB, INRAE, UCSC, WU																				
	in vitro	WU	WU, UTH																				

The timeline of the lab, pot and field experiments regarding WP4 activities is presented in the Gantt chart above. Yellow color: Lab soil microcosms; Orange color: pot experiments; Green color: Field experiments.

5. Methods and protocols used in the different experimental scales

5.1. Soil microbiota

Soil DNA extraction: The type of protocol used for soil DNA extraction is known to affect the quality and the quantity of the DNA derived and has strong reciprocal effects on the results of downstream PCR-based measurements. Soil DNA extraction will be performed using the Qiagen DNAeasy PowerSoil kit which has been the cornerstone commercial kit for DNA extraction in soil microbial ecology in the last 20 years.

5.1.1. Functional microbial endpoints

q-PCR measurement of the abundance of ammonia oxidizing bacteria, archaea and comammox bacteria: The abundance of AOB, AOA and Comammox bacteria will be determined as described in Vasileiadis et al., (2018) and Papadopoulou et al., (2022). The primers used and thermocycling conditions are described in Table 3. It should be noted that for the Comammox bacteria an in house optimized protocol of UTH will be used (not published yet) which is based on the original primers and protocols of Pjevac et al., (2017) and quantifies both Clade A and Clade B separately. Alternatively, UTH has also tested two other primer sets for q-PCR quantification of the Comammox bacteria:

- Ntsp-amoA 162F (5'-GGATTTCTGGNTSGATTGGA-3') /359R (5'-WAGTTNGACCACCASTACCA-3') by Fowler et al., (2018) which are highly degenerate and provides a quantification of the whole Comammox bacteria population without differentiation between Clade A and Clade B, the two dominant clades of Comammox in soil environments
- CA377f (5'-GTGGTGGTGGTCBAAYTA-3') / C576r (5'- GAAGCCCATRTARTCNGCC-3') for Clade A and CB377f (5'-GTACTGGTGGGCBAAYTT) / CB576r (5'-GAAGCCCATRTAR TCNGCC-3') for Clade B by Jiang et al., (2020). These set of primers have been tested by UTH and work well in most soils with both clades providing comparative results to the primers of Pjevac et al., (2017) and they will be the first alternative.

Table 3. The primers used for the determination of the abundance of ammonia oxidizing bacteria, archaea, comammox bacteria and nitrite oxidizing bacteria

Target gene	Primer name	Primers Sequence	Thermal cycling conditions	Reference
AOB *amoA*	amoA-1F /amoA-2R	5′-GGGGTTTCTACTGGTGGT-3′ 5′-CCCCTCKGSAAAGCCTTCTTC-3’	95ºC for 5, 57ºC for 10s, 72ºC for 30s (40 cycles)	Rotthauwe et al., 1997
AOA *amoA*	Arch-amoAF Arch-amoAR	5'- STAATGGTCTGGCTTAGACG-3′ 5'-GCGGCCATCCATCTGTATGT-3’	95ºC for 5s, 53ºC for 10s, 72ºC for 30s (45cycles)	Francis et al., 2005*
comammox *amoA*	comaA-244f a-f comaA-659r a-f comaB-244f a-f comaB-659r a-f	See Pjevac et al., 2017	95°C for 30 s, 48 or 52°C for 45s 1 min at 72°C (45 cycles)	Pjevac et al., 2017
NOB-*Nitrospira* *nxrB*	nxrB169f nxrB638r	5′- TACATGTGGTGGAACA -3′ 5′- CGGTTCTGGTCRATCA -3’	95ºC for 5s, 56.2ºC for 30s, 72ºC for 30s (40cycles)	Pester et al., 2014
NOB-*Nitrobacter* *nxrB*	nxrB-1F nxrB-1R	5’-ACGTGGAGACCAAGCCGGG-3’ 5’-CCGTGCTGTTGAYCTCGTTGA-3’	95ºC for 5s, 57ºC for 20s, 72ºC for 30s (40cycles)	Vanparys et al., 2007

* CrenamoA23f-CrenamoA616r by Tourna et al., (2008) could be also used and given comparative results

q-PCR measurement of the abundance of denitrifying bacteria: The abundance of the genes *narG, nirK, nirS, nosZ* involved in different steps of the denitrification process will be determined via q-PCR following the protocols and primers described in Table 4.

Table 4. The primers used and relevant citations for the determination of the abundance of denitrifying bacteria via q-PCR

Target gene	Primer set	Thermocycling conditions	Reference
Nitrate reductase (*narG*)	narG f-narG r 5'- TCG CCS ATY CCG GCS ATG TC -3' 5'-GAGTTGTACCAGTCRGCSGAYTCSG -3'	Initial denaturation at 95°C for 3 min; 6 cycles at 95°C for 15 sec, 63°C to 58°C with 1°C decrease per cycle for 30 sec, 72°C for 11 sec; 34 cycles at 95°C for 15 sec, 60°C for 30 sec, 72°C for 11 sec	Bru et al., (2007)
nitrite reductase (*nirK*)	nirK876-nirK1040 5'-ATYGGCGGVCAYGGCGA-3' 5'–GCCTCGATCAGRTTRTGGTT-3'	See Henry et al., (2004)	Henry et al., (2004)
nitrite reductase (*nirS*)	nirSCd3aFm-nirSR3cdm 5'- GAS TTC GGR TGS GTC TTS AYG AA-3' 5'- AAC GYS AAG GAR ACS GG -3'	Initial denaturation at 95°C for 3 min; 6 cycles at 95°C for 15 sec, 63°C to 58°C with 1°C decrease per cycle for 30 sec, 72°C for 30 sec; 35 cycles at 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec	Throback et al., (2004)
nitrous oxide reductase (*nosZ*)	nosZ2F-nosZ2R 5'- CGCRACGGCAASAAGGTSMSSGT -3' 5'- CAK RTG CAK SGC RTG GCA GAA -3'	Initial denaturation at 95°C for 3 min; 6 cycles at 95°C for 15 sec, 63°C to 60°C with 1°C decrease per cycle for 30 sec, 72°C for 30 sec; 36 cycles at 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec	Henry et al., (2006)

q-PCR measurement of the abundance of *pcaH* and *catA* genes associated with the degradation of aromatic compounds: The abundance of genes *pcaH* and *catA* encoding protocatechuate dioxygenase and catechol dioxygenase which are involved in the *meta* and *ortho*-cleavage pathways of biogenic and xenobiotic aromatic compounds in soil will be determined as a measure of the biodegradation and natural attenuation capacity of the soil microbiota. The abundance of these two genes will be determined following protocols and primers given in Table 5 and have been developed by INRAE.

Table 5. The primers used and relevant citations for the determination of the abundance of catA and pcaH genes involved in the degradation of aromatic compounds in soil via q-PCR

Microbial group	Gene target	Primers	Primer sequences (5' to 3')	Thermocycling conditions	References
Protocatechuate dioxygenase	*pcaH*	PCAHf PCAHr	GAGRTSTGGCARGCSAAY CCGYSSAGCACGATGTC	Initial denaturation 95°C for 3 min; 6 cycles at 95°C for 15 sec, 60°C for 30 sec (0.5°C increase per cycle), 72°C for 30 sec, 80°C for 15 sec and 30 cycles at 95°C for 15 sec, 57°C for 30 sec, 72°C for 30 sec and 80°C for 15 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec	El Azhari *et al*., 2008
1,2-catechnol dioxygenase	*catA*	CATAf	ACVCCVCGHACCATYGAAGG	Initial denaturation 95°C for 3 min; 8 cycles at 95°C for 15 sec, 62°C for 30 sec (0.5°C increase per cycle), 72°C for 45 sec and 30 cycles at 95°C for 15 sec, 58°C for 30 sec and 72°C for 45 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec	El Azhari *et al.,* 2010

q-PCR measurements of the abundance of PGPM in soil:

Table 6. The primers used and relevant citations for the determination of the abundance of PGPM in soil via q-PCR

Target gene	Primer set	Thermocycling conditions	Reference
Dinitrogenase reductase (NifH)	NifHF- NifHR 5'- TGCGATCCSAATGCBGACTC -3' 5'- ATSGCCATCCTYTCRCCGGA -3'	Initial denaturation at 95 °C for 5 min; 50 cycles of denaturation at 95 °C for 30 s, the appropriate annealing temperature for 30 s, and elongation at 72 °C for 30 s;	(Thomas et al., 2013)
Indole-3-Pyruvate Decarboxylase (IpdC)	IpdCF-IpdcR 5'- TCGAACTCAGCAAACAGCAC -3' 5'– AGGTTTGCAACGTTCTCCAG -3'	2 min at 50°C; 2 min at 95°C; and 45 cycles of 95°C for 15 s, 60°Cfor 30 s, and 72°C for 10	(Ryu et al., 2008)
Phenylpyruvate decarboxylase (ppdC)	ppdCF-ppdCR 5'- GGCGACTGCCTGTTCACC -3' 5'- CCAGCTGGCGTTGTTGAAC -3'	Initial 5-min denaturation at 94 °C, followed by 35 cycles of 1 min at 94 °C, 35 s at 52–62 °C, and 35 s at 72 °C. A final elongation step of 1 min at 72 °C was performed	(Gruet et al., 2022)
Glucose dehydrogenase (gcd)	gcdF-gcdR 5'- CGG CGT CAT CCG GGS NTN YRA YRT -3' 5'- GGG CAT GTC CAT GTC CCA NAD RTC RTG -3'	Initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 55°C for 1 min.	(Cleton-Jansen et al., 1990; Wan et al., 2021)
Gluconate dehydrogenase (gad)	gadF-gadR 5'- CGCGGATCCTGAAGGACGACATTCGTTAAG CAAGGAGAAAGATATGCCTGAGCATGCCCC AGA -3' 5'- CGCAAGCTTTCAGCGCAGGCTCTTTACATC ACCTGCGGT -3'	Initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 55 °C for 1 min..	(Yu et al., 2018)
Phosphonoacetaldehyde hydrolase (phnX)	phnXF-pnhXR 5'- CGTGATCTTCGACTGGGCNGGNAC -3' 5'- GTGGTCCCACTTCCCCADNCCCATNGG -3'	Initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 55 °C for 1 min.	(McGrath et al., 2013)

Potential nitrification: Potential nitrification will be determined by the method of Kandeler (1995). Briefly, 5-g soil sample are amended with 20 ml of 1 mM $(NH_4)_2SO_4$ and 0.1 ml of 1.5 M $NaClO_3$ and incubated under constant agitation at 20°C for 5 h, while triplicate control samples were treated in the same way and incubated at -20°C for the same period. At the end of the incubation period, NO_2^- was extracted from all samples with 2 M KCl. The extract (5 ml) was amended with 3 ml of 0.19 M NH4Cl and 2 ml of a colorimetric indicator prior to final determination of its adsorption at 520 nm. The potential nitrification in the soil samples was then determined with an external calibration curve prepared by measurement of the adsorption of a series of $NaNO_2$ solutions.

MicroResTM: the soil's microbial community level physiological profiling will be assessed at FiBL CH for samples derived from the WP4 pot experiments using the Microresp test assay as described previously (Cameron 2007, Creamer et al. 2016, Bongiorno et al. 2020) with a total of eight substrates (water, glucose, alanine and gamma-amino butyric acidn-acetyl-glucosamine, oxalic acid and alpha-ketoglutaric acid and lignin). Functional diversity will be expressed calculating the multiple substrate induced respiration (MSIR), the microbial catabolic profile expressed as absolute and relative utilization rate, and the Shannon microbial functional diversity index (H') following Bongiorno et al 2020.

Ammonium and Nitrate Concentrations in Soil: NH_4^+-N and NO_3^--N levels in soil samples, soils (2 g) were extracted with 20 ml of 1M KCl, shaken on a horizontal shaker, at 300 rpm, at room temperature for 30 min, and filtered gravimetrically through Whatman filter paper. $NH4^+$-N and NO_3^--N were determined in KCl extracts using a modified indophenol method based on the classical Berthelot reaction (Kandeler and Gerber 1998), and on the VCl_3/Griess method (Doane and Horwath 2003), respectively.

Activity of microbial enzymes: Soil microbial activity will be determined via measurements of the activity of microbial enzymes involved in key steps of P, S, N and C cycling like (i) acid (AcP, EC.3.1.3.2) and alkaline phosphomonoesterase (AlkP, EC.3.1.3.1), phosphodiesterase (BisP, E.C.3.1.4.4.1.), pyrophosphodiesterase (PiroP, EC.3.6.1.9.) involved in P cycling, (ii) arylsulfatase (AryS, EC.3.1.6.1.) involved in S cycling, (iii) N-acetyl-b-D-glucosaminidase (Chit, EC 3.2.1.14), leucine aminopeptidase (Leu, EC.3.4.11.1.) involved in N cycling, (iv) betaglucosidase (Bgluc, EC.3.2.1.21) involved in C cycling and (v) esterase (3.1.) involved in the hydrolysis of ester bonds. Enzymatic activities will be measured by a heteromolecular exchange procedure (Fornasier and Margon, 2007), using a 3% solution of lysozyme as desorbent and bead-beating to disrupt soil aggregates and microbial cells (Cowie et al., 2013). Briefly, 0.4 g of moist soil was placed into 2-mL tubes, together with 1.4 mL of a solution containing 3% lysozyme and glass beads. Tubes were then shaken at 30 strokes s−1 for 3 min, using a Retsch 400 MM beating mill, then centrifuged at 20,000g for 5 min. The supernatant containing desorbed enzymes was dispensed into 384-well white microplates with the appropriate buffer to fluorometrically quantify the enzymatic activities using fluorescent substrates 4-methyl-umbelliferyl (MUF) and 7-amino-4-methyl coumarin (AMC, for Leu) linked to appropriate enzyme substrates (Bardelli et al., 2017). Activities are corrected for soil moisture content and expressed as nanomoles of MUF (or AMC) h^{-1} g^{-1} dry soil.

AMF colonization in plant roots: the roots of plants (3 plants per treatment) will be collected, washed free of soil, and a subsample (2 g fresh weight) is going to be used for determination of the colonization levels. Plant roots will be immersed in 10% KOH for 45 min at 80 °C, rinsed with tap water, and immersed in 2.5% HCl for 30 min. Subsequently, roots are stained with trypan blue (Sylvia 1994). Mycorrhizal colonization was estimated on slides according to McGonigle et al. (1990), placing at least 20 root segments of 2 cm length (the length of the coverslip) and measuring at least 100 intersections.

The oven dry masses of the remaining roots were measured, with appropriate adjustment to account for the root portion removed for staining.

Tip for field and pot experiments: In the field experiments the colonization level of AMF would be followed along the growth season by uprooting of 3 plants per selected time point. Regarding the pot experiment this would be possible only for two sampling time points (45 and 90 days) and considering either (a) doubling the number of pots and employing destructive sampling or (b) planting more than one plant per pot to allow removal of one plant per sampling point from each pot.

Nutrient content in plant roots and shoots (including N, P and micronutrients): At the end of the experimental period , subsamples of the dried shoots and roots of all plants per treatment will be finely ground. Concentrations of mineral nutrients will be determined in the ground samples on a dry weight basis. Total N will be determined colorimetrically following wet digestion (Kjeldahl method) of a subsample of 0.1 g of dry tissue (Gaines and Mitchell 1979). Another subsample of 1 g will be dried to ash at 550 °C, solubilized with 5 ml of 20% HCl, and diluted to 25 ml. The solution was then dispersed into 2 vessels, each one used for determination of the following: (a) total concentration of P, K, Ca, Mg, Fe, Zn, Mn, and Cu, using a dual-view ICP-OES, and (b) total Na concentration using a flame photometer.

5.1.2. Diversity microbial endpoints

For amplicon sequencing analysis the protocols described below are applicable to Illumina sequencing platforms using 2x250 (NovaSeq) or 2x300 bp (Miseq) platforms, unless otherwise stated.

Amplicon sequencing analysis of bacteria: Prokaryotic 16S rRNA (bacteria and archaea) will be amplified with the updated version of the primers 515f-806r (Parada et al., 2016) suggested by the Earth Microbiome Project (Caporasso et al., 2018). All samples are initially amplified (28 amplification cycles) using the domain-specific primers mentioned above, followed by a PCR (7 amplification cycles) using the same primers carrying indexes for sample-wise multiplexing of PCR products. Primer sequences and PCR conditions used in amplicon sequencing analysis are presented in detail in Katsoula et al., (2021) and also in Table 7. A detailed description of the multiplexing protocol used is given by Vasileiadis et al., (2015). This protocol is applicable to 2x250 bp paired-end sequencing in Illumina NovaSeq, Hiseq or MiSeq platforms.

Amplicon sequencing analysis of fungi: Amplification of the fungal ITS2 will be performed with the primers ITS7-ITS4 (Ihrmark et al., 2012). Primer sequences and PCR conditions used in amplicon sequencing analysis are presented in detail by Katsoula et al., (2021) and also in Table 7. A detailed description of the multiplexing protocol used is given by Vasileiadis et al., (2015). This protocol is applicable to 2x250 bp paired-end sequencing in Illumina NovaSeq, Hiseq or MiSeq platforms.

Amplicon sequencing analysis of protists: The community of protists in soil will be determined using the protocol described in Guerra et al (2020). The V4 region of the 18S rRNA gene is amplified with universal eukaryotic primers Euk575Fngs (ASCYGYGGTAAYWCCAGC) and (Euk895Rngs; TCHNHGNATTTCACCNCT) on an Illumina Novaseq 2x300 bp.

Amplicon sequencing analysis of ammonia-oxidizing bacteria, archaea and comammox: Regarding AOB and AOA an amplicon sequencing protocol described in detail by Papadopoulou et al., (2022) and Vasileiadis et al., (2018) will be used. Primers and thermocycling conditions for AOA and AOB amplicon sequencing amplification are given Table 4. The sequencing of amplicons for AOB is done in Miseq (2x300 bp), whereas for AOA amplicon sequencing is performed in Illumina Hiseq or Novaseq platforms (2x250 bp).

Regarding amplicon sequencing of comammox bacteria it will be performed based on the protocol of Pjevac et al., (2017) which is under optimization from UTH. The optimized protocol will be available by UTH to the partners of the project before the end of 2022. Alternatively, UTH has also tested two other primer sets

- Ntsp-amoA 162F (5'-GGATTTCTGGNTSGATTGGA-3') /359R (5'-WAGTTNGACCACCASTACCA-3') by Fowler et al., (2018) which are highly degenerate and provides a quantification of the whole Comammox bacteria population without differentiation between Clade A and Clade B, the two dominant clades of Comammox in soil environments
- CA377f (5'-GTGGTGGTGGTCBAAYTA-3') / C576r (5'- GAAGCCCATRTARTCNGCC-3') for Clade A and CB377f (5'-GTACTGGTGGGCBAAYTT) / CB576r (5'-GAAGCCCATRTAR TCNGCC-3') for Clade B by Jiang et al., (2020). These set of primers have been tested by UTH and work well in most soils with both clades providing comparative results to the primers of Pjevac et al., (2017) and they will be the first alternative.

Amplicon sequencing analysis of AMF: DNA from plant roots will be extracted using a standard commercial kit like NucleoSpin Plant II kit (Macherey Nagel) or the DNeasy Plant Pro Kit (Qiagen). Quantification of the extracted DNA could be done either with Nanodrop or with the Qubit assay.

The intraradical community of AMF will be determined using the multiplex protocol described by Tsiknia et al., (2021). Briefly, the intraradical AMF communities will analysed via Illumina MiSeq 2×300 bp pair-end sequencing, using a semi-nested approach targeting the SSU region of the 18S rRNA gene. The Glomeromycota-specific AML1-AML2 primer pair (Lee et al., 2008) is used initially, followed by WANDA-AML2 (Dumbrell et al. 2011) as this provides a shorter informative amplicon. PCR conditions were as follows. For AML1-AML2: 3 min initial denaturation at 94◦C, followed by 30 cycles of 1 min denaturation at 94◦C, 1 min primer annealing at 50◦C and 1 min extension at 72◦C, followed by a final extension period of 10 min at 72◦C. For nesting, using WANDA-AML2 primers and the following thermocycling conditions 95◦C for 5min; 10 cycles at 94◦C for 0.5 min, 56◦C for 0.5 min and 72◦C for 1 min; and 72◦C for 10 min.

To further improve phylogenetic resolution of our diversity analysis regarding AMF, a novel sequencing protocol based on long-read sequencing using Pacific Biosciences platform as described by Kolarikova et al., (2021).

Table 7. The primers, thermocycling conditions and references for the amplification sequencing analysis of bacteria, fungi, AOB and AOA

Target gene	Primers	Thermal cycles	Reference
16S rRNA Bacteria	343f NNNNNNN**TA**TACGGRAGGCAGCAG 802r TACNVGGGTWTCTAATCC	94 °C 30s, 50°C 30 s, 72°C 30 s (25+7 cycles)	Vasileiadis et al., (2015)
ITS1 Fungi	ITS-1 NNNNNNN**AA**TCCGTAGGTGAACCTGCGG ITS-2 GCTGCGTTCTTCATCGATGC	94 °C 30s, 56°C 30 s, 72°C 60 s (28+7 cycles)	Adapted with linker and tags from White et al. (1990)
amoA AOA	amoA-1f NNNNNNN**AA**GGGGTTTCTACTGGTGGT amoA-2r CCCCTCKGSAAAGCCTTCTTC	94 °C 30s, 54°C 30 s, 72°C 60 s (25+5 cycles)	Adapted with linker and tags from Rotthauwe et al. (1997)
amoA AOA	amoA-310f NNNNNNNGGTGGATACCBTCWGCAATG amoA-529r GCAACMGGACTATTGTAGAA	94 °C 30s, 54°C 30 s, 72°C 60 s (25+5 cycles)	Adapted with linker and tags from Marusenko et al. (2013)

5.2. Macro-fauna

5.1.1. Earthworms endpoints

LD_{50} mortality, growth rate, ingestion rate, and MPs accumulation will be assessed as described by Huerta Lwanga et al. 2016, biomass change, ingestion rate and reproduction will be evaluated in all experiments. Avoidance behavior, microplastic transport in burrows, and burrows volume (Huerta Lwanga et al. 2017) will be assessed in mesocosms with anecics and endogeic earthworms. Microplastics in earthworms guts and casts will be also collected.

Mortality will be assessed by percentage of dead earthworms per treatment at the end of 14 and 60 day pot and mesocosm experiments.

Growth rate (kgr, Huerta Lwanga et al. 2016) will be determined by weight per individual (gain or loss), per day (mg g^{-1} day^{-1}). The growth rate (kgr) will be calculated by the following equation:

$$Kgr = \frac{\frac{Morg2 - Morg1}{Morg1}}{t}$$

where Morg,1 and Morg,2 are the initial and final earthworm body weights (g), and *t* is the time exposure (days).

The ingestion rate (IRt, mg cast g^{-1} worm–1 day^{-1}) will be determined by

$$IRt = \frac{\frac{MC}{Morg2}}{t}$$

where MC is the weight of the casts (mg DW).

The fraction of plastic in the casts (SPL) will be calculated by

$$SPL = MPL/MC$$

where MPL (mg) is the DW of the plastic in the casts. The cast concentration factor (CF) in for earthworms casts was calculated by

$$CF = SPL/Ss$$

Ss is the plastic fraction at the initial substrate based on the CF proposed by Mosleh et al. 2003.

Reproduction will be determined by the number of cocoons produced per adult per pot/mesocosm.

When coleoptera will be used the same endpoints will be evaluated (as for earthworms).

At field experiments, abundance, biomass and diversity will be evaluated at the begin and end of the experiment, following the TSBF method (Anderson and Ingram 1993), collecting the earthworms by hand-sorting, with 2 25x25x30cm monoliths per experimental plot.

5.1.2 Collembola

Three collembola species adapted to different soil layers (i.e., an edaphic Onychiuridae, a hemiedaphic Isotomidae, and an epiedaphic Entomobryidae; Negri 2004), having different roles in soil nutrient cycling, will be considered for the experiments (Table S2). A suitable breeding environment will be established in room chambers at 20°C and 90% relative humidity. As food, brewer's yeast will be provided ad libitum. Substrate moisture content will be maintained by adding distilled water. To obtain age-synchronized individuals, Petri dishes with a layer of moist plaster of Paris/activated charcoal mixture (ratio 8:1 w/w) with eggs will be prepared (ISO protocol 11267:1999). Eggs will be incubated in room chambers at about 20°C and 90% relative humidity. Before MPNs exposure, ten individuals of 11-12-day-old collembolan juveniles will be collected and transferred into the experimental arenas for testing lethal and sub-lethal effects, as specified below.

Pristine soil and plastic fragments will be delivered to UCSC by WU. Soil moisture content will be maintained by adding distilled water. The experimental substrate will be prepared by mixing pristine soil with fragments of LPDE based, PBAT based and starch based plastic fragments (Table S2). Three additional treated soils will be prepared by mixing pristine soils with LPDE based, PBAT based and starch based plastic fragments, previously treated with albendazole and pyraclostrobin, as in Task 4.1., 4.2, WP5. Tests will be carried out by exposing 10 synchronized individuals of each species in Petri dishes for 4 weeks. As food, brewer's yeast will be provided when needed and substrate moisture content will be maintained by adding distilled water. At the end of the experiments, collembola will be collected by adding water (flotation method) and mortality, behaviour, specimens with full/empty gut recorded. Data on juveniles, eggs and body size of alive specimens will be also recorded. Specimens with full gut from control and treatments will be randomly collected and the gut content prepared to analyze MNPs presence (Table S2).

Data will be subject to statistical analysis to define significant differences between control groups and groups exposed to soil contaminated by MNPs treated with or without albendazole and pyraclostrobin.

5.3. Soil physical properties

5.3.1 Soil aggregate stability & Aggregate size distribution (UCSC)

Methods: The wet sieving apparatus is used with dry samples (from min 4 up to max 8 g per sample) to determine water aggregate stability (WSA) and aggregate size classes distribution. Sieves of 0.250 mm mesh size and 0.053 mm are used to obtain dry weight of macroaggregates (2-0.250 mm), microaggregates (0.250-0.053mm) and free silt+clay fraction (<0.053mm). WSA and mean weight diameter (MWD) are calculated from dry weights of aggregate size classes.

5.3.2 Soil texture (Partners conducting experiments)

Methods: Bouyoucos Hydrometer Method, already described in Annex 14 in MINAGRIS Monitoring Plan (.docx). Copied from MINAGRIS Monitoring Plan: **Bouyoucos Hydrometer Method**

Materials:

1. Air-Dry < 2mm fraction (50 g fine-textured, 100 g if sandy)
2. Electronic balance, ±1-mg sensitivity.
3. Electric mixer and cup
4. Sedimentation cylinder (1000 mL)
5. Bouyoucos hydrometer
6. Thermometer (-20° - 110°C)
7. Sodium hexametaphosphate, 1N

Drying Oven with forced air ventilation, 35 ±5 °C

Procedure: Place 50-100 g of dry soil (50 g if fine-textured, 100 g if sandy) into a soil dispersing cup. The soil should be sieved first to remove any fragments larger than sand (>2mm). Record the weight to at least 0.1g.

8. Fill cup to within 5 cm of the top with distilled water at room temperature
9. Add 5 ml of 1N sodium hexametaphosphate.
10. Allow to soak for 15 minutes (high-clay soils only).
11. Attach cup to mixer; mix 5 minutes for sandy soils, 15 minutes for fine-textured soils.
12. Transfer suspension to sedimentation cylinder; use water to get all of sample from mixing cup.
13. Fill cylinder to 1000-mL mark with distilled water.
14. Insert plunger into cylinder, carefully mixing suspension thoroughly by pulling plunger upwards in short jerks. After removing plunger, begin timing. Carefully place hydrometer into suspension; note reading at 40 seconds. Repeat this procedure 2-3 times to obtain an average of 40 sec reading.
15. After final 40-second reading, remove hydrometer, carefully lower a thermometer into the suspension and record the temperature (°C). Mixing raises temperature by 3-5°C, so it is important to record the temperature for both hydrometer readings (40 sec and 2 hr).
16. After the final reading, do not disturb the cylinder. It should stand undisturbed for two hours.
17. Make up a blank cylinder with water and sodium hexametaphosphate. Record the blank hydrometer reading.

Take a hydrometer reading at 2 hours, followed by a temperature reading

Calculations:

18. Temperature correction factor, T (may be different for each reading):
19. T = (Observed temperature - 20°C) * 0.3
20. Corrected 40-second reading:
21. 40-sec (corr) = 40-sec reading - Blank + T

22. Corrected 2-hour reading:
23. 2-hr (corr) = 2-hr reading - Blank + T
24. %Sand=Dry soil weight−corr 40 sec readingDry soil weight∗100%
25. %Clay=corr 2 hr readingDry soil weight∗100%
26. %Silt=100%−(%Sand+%Clay)

Note: The Textural classification should be made according to the USDA soil taxonomy.

5.3.3 Soil water retention (Partners conducting experiments (T4.1), supported by UL)

Methods:

1. 0 to about -100 kPa

Soil water retention at matric potential range of 0 to about -100 kPa is determined using HYPROP® (Hydraulic Property Analyzer, METER Group, USA). Metal soil sample holders of cylindrical shape and known volume (250 cm3) are pressed vertically into the soil to fill the sampler without compaction or deflection. The measurement procedure is described in more detail in Shokrana and Ghane (2020).

2. -100 to about -1500 kPa

Soil water retention at matric potential range of -100 to about -1500 kPa should be determined using a method with a pressurized gas and a pressure plate extractor on disturbed soil samples (ISO 11274, 1998). Disturbed soil samples should be sieved through a 2 mm mesh. To determine the volumetric water content, the gravimetric water content must be multiplied by the soil bulk density. Four additional points (-200, -500, -1000, -1500 kPa) are determined using this method.

Notes: Between sampling and measurement, samples must be stored in a cold, dark place, e.g., refrigerator. After the procedure of determining soil water retention with HYPROP® has been completed, the same sample can also be used for determination of soil bulk density and later for the soil water repellency.

5.3.4 Hydraulic conductivity(Partners conducting experiments (T4.1), supported by UL)

Methods:

1. Saturated

Soil samples must be collected by pushing sampling metal cylinders vertically into the soil. The saturated hydraulic conductivity is measured by the constant or falling head method, depending on the permeability of the soil sample. This is done with a laboratory permeameter (Eijkelkamp Soil & Water, Netherlands) according to the manual on the website:
https://en.eijkelkamp.com/products/laboratory-equipment/soil-water-permeameters.html

2. Unsaturated

Soil samples must be collected by pushing sampling metal cylinders vertically into the soil. The unsaturated hydraulic conductivity should be determined with a HYPROP® measuring system, simultaneously with the determination of the soil water retention curve.

Notes: Between sampling and measurement, samples must be stored in a cold, dark place, e.g., refrigerator.

5.3.5 Soil bulk density (Partners conducting experiments (T4.1), supported by UL)

Method: Metal soil sample holders of cylindrical shape and known volume (between 100 cm^3 and 400 cm^3) are pressed vertically into the soil to fill the sampler without compaction or deflection. Metal samplers with soil should be carefully removed and excess soil trimmed to the edge so that the soil in the sampler has a known volume. The mineral soil samples are then dried in an oven at 105 ˚C until the mass is constant (at least 48 hours). After drying, allow the sample to cool in the desiccator and then weigh it. Calculate the bulk density of the dry soil using the equation (ISO 11272: 1998):

Notes: Between sampling and measurement, samples must be stored in a cold, dark place, e.g., refrigerator.

5.3.6 Soil water repellency (Partners conducting experiments (T4.1), supported by UL)

Method: The Water Drop Penetration Time (WDPT) test is used to determine the water repellency of the soil. Potential water repellency is used, the WDPT test is performed on an oven-dried soil sample. The mineral soil samples are oven-dried at 105 ˚C until the mass is constant (at least 48 hours). The oven-dried samples must reach ambient temperature before measurement, so they should stand at room temperature for at least 24 hours. Three drops of distilled water from a plastic pipette (all organisations should use the same type) are placed on the smoothed surface of the soil sample and the time taken for the drops to infiltrate into the soil is measured (Dekker and Ritsema, 1994; Ritsema and Dekker, 1996).
Notes: Dried soil samples from the determination of other parameters can be used to determine the water repellency of the soil.

5.3.7 Infiltration (Partners conducting experiments (T4.1), supported by UL)

Method: The measurement of cumulative infiltration is performed with a mini disc infiltrometer - MDI (METER Group, USA). Manual: http://publications.metergroup.com/Manuals/20421_Mini_Disk_Manual_Web.pdf
Before the measurement, the soil surface must be prepared, plant debris must be removed, but at the same time the soil surface should not be damaged or changed. Record the initial amount of water at time *t*=0. Place the MDI on the soil surface and verify that the contact of the porous disc with the soil is complete. Note the amount of water at a specific time interval, e.g., 30 s.
Notes:

- Infiltration must be measured *in situ*.
- For pot experiments, the MDI should not be placed too close to the edge of a container.
- Initial soil water content conditions prior to measurement should be as homogeneous as possible - at least when different treatments are measured at the same location (Bhave & Sreeja, 2013).

5.4. Aerial transport and effects of MNPs on honeybees (Task 6.3.2)

5.4.1. Effects of MNPs on honeybees

At least 16 young bees (max. 2 days old) reared out from brood combs taken from queen-right colonies with no disease symptoms will be used for the experiments. No chemical substances (such as antibiotics, anti *varroa* treatments, etc.) must have been used in the hive for at least one month before the test. If one colony cannot provide the appropriate number of bees, combs from several colonies will be used. In this case, it is ensured that the bees are equitably distributed across the treatments.
Brood frames with capped cells from a colony will be selected and kept in a climatic chamber at ~36 °C and ~60% relative humidity for approximately 16 hours. No starvation period is necessary.

For acute exposure: bees will be collected from the combs and fed with 1 µL of MNPs solution and then placed into plastic cages containing supplies with sucrose solution prepared without MNPs for 1 day. Mortality will be recorded. After 24 hours the bees will be collected to perform SEM-EDX analyses.

For chronic exposure: bees will be collected from the combs and fed with 1 µL of MNPs solution and then placed into plastic cages containing supplies with the same solution for 10 days.
Mortality and behavioural abnormalities will be observed and recorded daily.
The last day of the test, the bees will be collected to perform SEM-EDX, gut microbiota, and histological analyses.
The experiment will be based on Papa et al., 2021, and OECD 2017 guidelines conveniently modified**.**

5.4.2. Aerial transport of MNPs through honeybees

Beehives will be installed in the study area (CSS at WU) and worker bees will be collected from June to August during warm weather when they are more active. Worker bees will be collected with a butterfly net while exiting/entering the hive. Older bees at the end of their life will be chosen for the analysis, euthanized. Since previous studies have shown that pollutant PM on the bees is mostly concentrated along the costal margin of the forewings, medial plane of the head, and inner surface of the hind legs (Negri et al., 2015; Edo et al., 2021), preliminary observation under stereoscope of the body parts will be carried out to check for the presence of millimetric and micrometric-sized plastics. Plastic fragments will be classified according to the size, shape (e.g., fragments, films, fibers, and filaments), and colour (Edo et al., 2021), and the total number recorded.

Both the forewings and the gut will be dissected and prepared for SEM/EDX analyses to characterize the size, morphology and chemical composition of the particles, with special reference to nanosized ones, and the total number recorded.

Negative control bees will consist of newly eclosed individuals from a brood frame kept in a growth chamber. Briefly, a brood frame will be selected and placed in an incubator at 36 °C and 60% relative humidity for approximately 16 h. Newly eclosed adults will be randomly selected and prepared for optic and SEM/EDX analyses. Once collected, worker bees will be placed in soda-glass capped vials, stored on ice to keep them inactive, and rapidly brought to the lab for sample preparation (Negri et al., 2015). The bees' wings will be cut under a stereoscope with a scalpel and mounted onto SEM stubs using double adhesive carbon tape. Before the SEM/EDX investigation, because of the insulating properties of the samples, the stubs will be carbon coated to make them conductive to avoid charging flare during the observations.

6. References

- Anderson, J. M., & Ingram, J. S. I. (1993). A handbook of methods. CAB International, Wallingford, Oxfordshire, 221, 62-65.
- Bardelli, T., Gomez-Brandon, M., Ascher-Jenull, J., Fornasier, F., Arfaioli, P., Francioli, D., Egli, M., Sartori, G., Insam, H., Pietramellara, G., 2017. Effect of slope exposure on
- Bhave, S., & Sreeja, P. (2013). Influence of initial soil condition on infiltration characteristics determined using a disk infiltrometer. ISH Journal of Hydraulic Engineering, 19(3), 291–296. https://doi.org/10.1080/09715010.2013.808445
- Bongiorno, G., Bünemann, E. K., Brussaard, L., Mäder, P., Oguejiofor, C. U., & de Goede, R. G. (2020). Soil management intensity shifts microbial catabolic profiles across a range of European long-term field experiments. *Applied Soil Ecology*, *154*, 103596.
- Bru, D., Sarr, A., Philippot, L. (2007) Relative abundances of proteobacterial membrane-bound and periplasmic nitrate reductases in selected environments. Appl Environ Microbiol 73: 5971–5974.
- C. Cameron (2007). **M**icroResp Technical Manual - A Versatile Soil Respiration System, Macaulay Insitute, Aberdeen, Scotland, UK
- Caporaso JG, Ackermann G, Apprill A, Bauer M, Berg-Lyons D, Betley J, Fierer N, Fraser L, Fuhrman JA, Gilbert JA, Gormley N, Humphrey G, Huntley J, Jansson JK, Knight R, Lauber CL, Lozupone CA, McNally S, Needham DM, Owens SM, Parada AE, Parsons R, Smith G, Thompson LR, Thompson L, Turnbaugh PJ, Walters WA, Weber L (2018) Earth microbiome project: EMP 16S Illumina amplicon protocol . In: protocols.io. https://www.protocols.io/view/emp-16s-illuminaamplicon-protocol-nuudeww
- Cleton-Jansen, A. M., Goosen, N., Fayet, O., and van de Putte, P. (1990). Cloning, mapping, and sequencing of the gene encoding Escherichia coli quinoprotein glucose dehydrogenase. J. Bacteriol. 172, 6308–6315. doi: 10.1128/jb.172.11.6308-6315.1990
- Cowie, A., Lonergan, V.E., Rabbi, F.S.M., Fornasier, F., Macdonald, C., Harden, S., Akitomo Kawasaki, A., Brajesh, K., Singh, B.K., 2013. The impact of carbon farming practices on soil carbon in northern New South Wales. Soil Res. 51, 707–718.
- R.E. Creamer, D. Stone, P. Berry, I. Kuiper. Measuring respiration profiles of soil microbial communities across Europe using MicroResp™ method, Appl. Soil Ecol., 97 (2016), pp. 36-43, 10.1016/j.apsoil.2015.08.004
- Dekker, L. W., & Ritsema, C. J. (1994). How water moves in a water repellent sandy soil: 1. Potential and actual water repellency. Water Resources Research, 30(9), 2507–2517. https://doi.org/10.1029/94WR00749
- Doane TA, Horwath WR. 2003. Spectrophotometric determination of nitrate with a single reagent. Anal Lett 36: 2713–2722. https://doi.org/10.1081/AL-120024647.
- Dumbrell AJ, Ashton PD, Aziz N et al. Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. New Phytol 2011;190:794–804
- Edo, C., Fernández-Alba, A.R., Vejsnæs, F., van der Steen, J.J.M., Fernández-Piñas, F., Rosal, R., 2021. Honeybees as active samplers for microplastics. Science of The Total Environment 767, 144481. https://doi.org/10.1016/j.scitotenv.2020.144481
- N. El Azhari, M. Devers-Lamrani, G. Chatagnier, N. Rouard, F. Martin-Laurent, Molecular analysis of the catechol-degrading bacterial community in a coalwasteland heavily contaminated with PAHs. J. Haz. Mater. 177 (2010) 593-601
- N. El Azhari, D. Bru, A. Sarr, F. Martin-Laurent, Estimation of the density of the protocatechuate degrading bacterial community in soil by real-time PCR. Eur. J. Soil Sci. 59 (2008) 665-673
- Fornasier, F., Margon, A., 2007. Bovine serum albumin and Triton X-100 greatly increase phosphomonoesterases and arylsulphatase extraction yield from soil. Soil Biol. Biochem. 39, 2682–2684.

- Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water column sand sediments of the ocean. PNAS USA 102: 14683–14688. https://doi.org/10.1073/pnas.0506625102.
- Fowler SJ, Palomo A, Dechesne A, Mines PD, Smets BF. 2018. Comammox Nitrospira are abundant ammonia oxidizers in diverse groundwater-fed rapid sand filter communities. Environ Microbiol 20:1002–1015. doi: 10.1111/1462-2920.14033
- Gaines TP, Mitchell GA (1979) Boron determination in plant tissue by the azomethine H method. Commun Soil Sci Plant 10:1099–1108. https://doi.org/10.1080/00103627909366965
- Guerra et al., (2021) Tracking, targeting and conserving soil biodiversity. Science 371 (6526): 239-241
- Gruet, C., Oudot, A., Abrouk, D., Moënne-Loccoz, Y., & Muller, D. (2022). Rhizophere analysis of auxin producers harboring the phenylpyruvate decarboxylase pathway. Applied Soil Ecology, 173, 104363. https://doi.org/https://doi.org/10.1016/j.apsoil.2021.104363
- Henry, S., Baudoin, E., Lopez-Gutierrez, J.C., Martin-Laurent, F., Brauman, A., and Philippot, L. (2004) Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. J Microbiol Methods 59: 327-335.
- Henry, S., Bru, D., Stres, B., Hallet, S.,Philippot, L. (2006) Quantitative Detection of the nosZ Gene, Encoding Nitrous Oxide Reductase, and Comparison of the Abundances of 16S rRNA, narG, nirK, and nosZ Genes in Soils. Appl Environ Microbiol 72: 5181–5189. doi: 10.1128/AEM.00231-06.
- Huerta Lwanga, E., Gertsen, H., Gooren, H., Peters, P., Salánki, T., Van Der Ploeg, M., ... & Geissen, V. (2016). Microplastics in the terrestrial ecosystem: implications for Lumbricus terrestris (Oligochaeta, Lumbricidae). Environmental science & technology, 50(5), 2685-2691.
- Huerta Lwanga, E. H., Gertsen, H., Gooren, H., Peters, P., Salánki, T., van der Ploeg, M., ... & Geissen, V. (2017). Incorporation of microplastics from litter into burrows of Lumbricus terrestris. Environmental Pollution, 220, 523-531.
- Ihrmark K, Bödeker ITM, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, Strid Y, Stenlid J, Brandström-Durling M, Clemmensen KE, Lindahl BD (2012) New primers to amplify the fungal ITS2 region - evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiol Ecol 82:666–677
- ISO 11272 (1998). Soil quality — determination of dry bulk density. International Organization for Standardization, Genève, Switzerland.
- ISO 11274 (1998). Soil quality — determination of the water retention characteristics, laboratory methods. International Organization for Standardization, Genève, Switzerland.
- ISO 11267 (1999). Soil quality — Inhibition of reproduction of Collembola (Folsomia candida) by soil pollutants
- Jiang, R., Wang, J. G., Zhu, T., Zou, B., Wang, D. Q., Rhee, S. K., An, D., Ji, Z. Y., & Quan, Z. X. (2020). Use of Newly Designed Primers for Quantification of Complete Ammonia-Oxidizing (Comammox) Bacterial Clades and Strict Nitrite Oxidizers in the Genus Nitrospira. Applied and environmental microbiology, 86(20), e01775-20. https://doi.org/10.1128/AEM.01775-20
- Kandeler E, Gerber H. 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils 6: 68–72. https://doi.org/10.1007/BF00257924.
- Kandeler E. 1995. Potential nitrification, p 146–149. In Schinner F, Ohlinger R, Kandeler E, Margesin R (ed), Methods in soil biology. Springer, Heidelberg, Germany
- Katsoula A., Vasileiadis S., Karamanoli K., Vokou D., Karpouzas D.G. (2021) Factors structuring the epiphytic archaeal and fungal community in a semi-arid Mediterranean ecosystem Microbial Ecology https://doi.org/10.1007/s00248-021-01712-z
- Kolarikova Z., Slavikova R., Kruger C., Kruger M., Kohout P. (2021) PacBio sequencing of Glomeromycota rDNA: a novel amplicon covering all widely used ribosomal barcoding regions and its applicability in taxonomy and ecology of arbuscular mycorrhizal fungi. New Phytologist 231(1): 490-499

- Lee J, Lee S, Young JPW. Improved PCR primers for the detection and identification of arbuscularmycorrhizal fungi. FEMS Microbiol Ecol 2008;65:339–49.
- Menta, C., Conti, F.D., Pinto, S., Bodini, A., 2018. Soil Biological Quality index (QBS-ar): 15 years of application at global scale. Ecological Indicators 85, 773–780. https://doi.org/10.1016/j.ecolind.2017.11.030
- Marusenko, Y., Bates, S. T., Anderson, I., Johnson, S. L., Soule, T., & Garcia-pichel, F. (2013). Ammonia-oxidizing archaea and bacteria are structured by geography in biological soil crusts across North American arid lands. Ecological Processes *2*(9): 1–10.
- McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan J (1990) A new method which gives an objective measure of colonization of roots by vesicular—arbuscular mycorrhizal fungi. New Phytol 115: 495–501
- McGrath, J.W., Chin, J.P., Quinn, J.P., 2013. Organophosphonates revealed: new insights into the microbial metabolism of ancient molecules. Nature Review Microbiology 11, 412–419.
- Mosleh, Y. Y.; Paris Palacios, S.; Couderchet, M.; Vernet, G. Effects of the herbicide isoproturon on survival, growth rate, and protein content of mature earthworms (Lumbricus terrestris L.) and its fate in the soil Appl. Soil Ecol. 2003, 23 (1) 69– 77
- Negri, I., 2004. Spatial distribution of Collembola in presence and absence of a predator. Pedobiologia 48, 585–588. https://doi.org/10.1016/j.pedobi.2004.07.004
- Negri, I., Mavris, C., Di Prisco, G., Caprio, E., Pellecchia, M., 2015. Honey Bees (Apis mellifera, L.) as Active Samplers of Airborne Particulate Matter. PLoS ONE 10, e0132491. https://doi.org/10.1371/journal.pone.0132491
- OECD (2017) Test No. 245: Honey Bee (Apis Mellifera L.), Chronic Oral Toxicity Test (10-Day Feeding)
- Papa, G., Di Prisco, G., Spini, G., Puglisi, E., Negri, I., 2021. Acute and chronic effects of Titanium dioxide (TiO2) PM1 on honey bee gut microbiota under laboratory conditions. Sci Rep 11, 5946. https://doi.org/10.1038/s41598-021-85153-1
- Papadopoulou ES., Bachtsevani E., Papazlatani C., Rousidou C., Brouziotis A., Lambronikou E., Tsiknia M., Menkissoglou-Spiroudi U., Ehaliotis C., Philippot L., Nicol G.W., Karpouzas D.G., (2022) Quinone imine, dicyandiamide (DCD) and nitrapyrin effects on target and non-target microbial groups under contrasting soil pH. Microbiology Spectrum accepted
- Parada AE, Needham DM, Fuhrman JA (2016) Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environ Microbiol 18:1403–1414
- Pester M, Maixer F, Berry D, Rattel T, Koch H, Lucker S, Nowka B, Richter A, Spieck E, Lebedeva E, Loy A, Wagner M, Daims H. 2014. NxrB encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing Nitrospira. Environ Microbiol 16:3055–3071. https://doi.org/10.1111/1462-2920.12300.
- Pjevac P, Schauberger C, Poghosyan L, Herbold CW, van Kessel MAHJ, Daebeler A, Steinberger M, Jetten MSM, Lücker S, Wagner M, Daims H. 2017. AmoA-targeted polymerase chain reaction primers for the specific detection and quantification of comammox Nitrospira in the environment. Front Microbiol 8:1508. https://doi.org/10.3389/fmicb.2017.01508.
- Ritsema, C. J., & Dekker, L. W. (1996). Water repellency and its role in forming preferred flow paths in soils. Soil Research, 34(4), 475–487. https://doi.org/10.1071/sr9960475
- Ryu, R. J., & Patten, C. L. (2008). Aromatic amino acid-dependent expression of indole-3-pyruvate decarboxylase is regulated by tyrr in Enterobacter cloacae UW5. Journal of Bacteriology, 190(21), 7200–7208. https://doi.org/10.1128/JB.00804-08
- Rotthauwe, J.-H., Witzel, K.-P., &Liesack, W. (1997). The ammonia monooxygenase structural gene amoA as a functional marker: Molecular fine-scale analysis of natural Ammonia-Oxidizing populations. Applied and Environmental Microbiology, 63(12): 4704–4712.
- Shokrana, M. S. B., & Ghane, E. (2020). Measurement of soil water characteristic curve using HYPROP2. MethodsX, 7, 100840. https://doi.org/10.1016/j.mex.2020.100840

- Sylvia DM (1994) Vesicular-arbuscular mycorrhizal (VAM) fungi. In: Weaver RW, Angle JS, Bottomley PJ, Bezdicek D, Smith S, Tabatabai A, Wollum AG (eds) Methods of soil analysis, part 2. Microbiological and biochemical properties. Soil Science Society of America, Madison, pp 351–378
- Thomas F. Ducey, James A. Ippolito, Keri B. Cantrell, Jeffrey M. Novak, Rodrick D. Lentz, Addition of activated switchgrass biochar to an aridic subsoil increases microbial nitrogen cycling gene abundances,Applied Soil Ecology, Volume 65, 2013, Pages 65-72,ISSN 0929-1393, https://doi.org/10.1016/j.apsoil.2013.01.006
- Throbäck, I.N., Enwall, K., Jarvis, A., Hallin, S. (2004) Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiology Ecology 49: 401–417. https://doi.org/10.1016/j.femsec.2004.04.011
- Tourna M, Freitag TE, Nicol GW, Prosser JI. 2008. Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environ Microbiol 10:1357–1364. https://doi.org/10.1111/j.1462-2920.2007.01563.x.
- Tsiknia M., Skiada V., Ipsilantis I., Vasileiadis S., Kavroulakis N., Genitsaris S., Papadopoulou K., Hart M., Klironomos J., Karpouzas D.G., Ehaliotis C (2021) Strong host selection and over-dominance characterize arbuscular mycorrhizal fungal colonizers of coastal sand dune plants of the Mediterranean region. FEMS Microbiology Ecology 97: fiab109, https://doi.org/10.1093/femsec/fiab109
- Vanparys B, Spieck E, Heylen K, Wittebolle L, Geets J, Boon N, De Vos P. 2007. The phylogeny of the genus Nitrobacter based on comparative rep-PCR, 16S rRNA and nitrite oxidoreductase gene sequence analysis. Syst Appl Microbiol 30: 297–308.https://doi.org/10.1016/j.syapm.2006.11.006.
- Vasileiadis, S., Puglisi, E., Trevisan, M., Scheckel, K.G., Langdon, K.A., McLaughlin, M.J., Lombi, E., Donner, E. (2015). Changes in soil bacterial communities and diversity in response to long-term silver exposure. FEMS Microbiology Ecology 91: 11
- Vasileiadis S., Puglisi E., Papadopoulou E.S., Pertile G., Suciu N., Papolla, A., Tourna M., Karas P.A., Papadimitriou F., Kasiotakis A, Ipsilanti N., Ferrarini A., Sulowic S., Fornasier F., Nicol G.W, Trevisan M., Karpouzas D.G (2018) Blame it on the metabolite: 3,5-dichloraniline rather than the parent compound iprodione induces strong effects on the diversity and function of soil microorganisms. Applied and Environmental Microbiology 84(22): e01536-18
- Wan, W., Qin, Y., Wu, H., Zuo, W., He, H., Tan, J., Wang, Y., & He, D. (2020). Isolation and Characterization of Phosphorus Solubilizing Bacteria With Multiple Phosphorus Sources Utilizing Capability and Their Potential for Lead Immobilization in Soil. Frontiers in Microbiology, 11(April), 1–15. https://doi.org/10.3389/fmicb.2020.00752
- White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* 18: 315-322.
- Yu, S., Lai, B., Plan, M. R., Hodson, M. P., Lestari, E. A., Song, H., & Krömer, J. O. (2018). Improved performance of Pseudomonas putida in a bioelectrochemical system through overexpression of periplasmic glucose dehydrogenase. Biotechnology and Bioengineering, 115(1), 145–155. https://doi.org/10.1002/bit.26433

ANNEX 1

Table. S1. Microplastics treatments, effect on soil macrofauna. OP: organic pollutants (ALB, PYR), VD: Veterinary drug, An: anecic earthworm, EP: epigeic earthworm, CO: coleoptera, EN: endogeic earthworm, COM: combined macroinvertebrates (multispecies approach). The individual effect of plastics on individual species is already tested in several studies, in this experiment priority is given to test the combined effect of microplastics on multispecies.

	microplastics				OP		earthworms and coleoptera			
Treat	concentration	LDPE based	PBAT based	Starch based	ALB	PYR	AN	EP or CO	EN	COM
1	0%	x					x			
2	0.01%	x	x	x						x
3	0.10%	x	x	x			x			
4	0%	x	x	x				x		
5	0.01%	x	x	x						x
6	0.10%	x	x	x				x		
7	0%	x	x	x					x	
8	0.01%	x	x	x						x
9	0.10%	x	x	x					x	
10	0%	x	x	x						x
11	0.01%	x	x	x						x
12	0.10%	x	x	x						x
13	0.10%	x	x	x	x		x			
14	0.10%	x	x	x	x			x		
15	0.10%	x	x	x	x				x	
16	0.10%	x	x	x	x					x
17	0.01%	x	x	x	x					x
18	0.10%	x	x	x		x	x			
19	0.10%	x	x	x		x		x		
20	0.10%	x	x	x		x			x	
21	0.10%	x	x	x		x				x
22	0.01%	x	x	x		x				x
23	0.10%	x	x	x			x			
24	0.10%	x	x	x				x		
25	0.10%	x	x	x					x	
26	0.10%	x	x	x						x
27	0.01%	x	x	x						x
28	0.10%	x								x
29	0.10%		x							x
30	0.10%			x						x
31	0.10%	x			x					x
32	0.10%		x		x					x
33	0.10%			x	x					x
34	0.10%	x				x				x
35	0.10%		x			x				x
36	0.10%			x		x				x
37	0.10%	x								x
38	0.10%		x							x
39	0.10%			x						x

Table S2. Microfauna targeted experiments. MNPs: micro-nanoplastics; OP: organic pollutants (ALB, PYR). After 4 weeks of treatments data on mortality, body size, presence of juvenile stages, eggs, behaviour will be collected.

Collembola	Treat	MNPs in soil				PO
		concentration	**LPDE based**	**PBAT based**	**STARCH based**	**ALB+PYR**
EDAPHIC	1	0%	✓			
	2	0%	✓			
	3	0%	✓			
	4	0.01%	✓			
	5	0.01%	✓			
	6	0.01%	✓			
	7	0%	✓			
	8	0%	✓			
	9	0%	✓			
	10	0.01%	✓			✓
	11	0.01%	✓			✓
	12	0.01%	✓			✓
	13	0%		✓		
	14	0%		✓		
	15	0%		✓		
	16	0.01%		✓		
	17	0.01%		✓		
	18	0.01%		✓		
	19	0%		✓		
	20	0%		✓		
	21	0%		✓		
	22	0.01%		✓		✓
	23	0.01%		✓		✓
	24	0.01%		✓		✓
	25	0%			✓	
	26	0%			✓	
	27	0%			✓	
	28	0.01%			✓	
	29	0.01%			✓	
	30	0.01%			✓	
	31	0%			✓	
	32	0%			✓	
	33	0%			✓	
	34	0.01%			✓	✓
	35	0.01%			✓	✓
	36	0.01%			✓	✓
	37	0%	✓			
	38	0%	✓			
	39	0%	✓			
	40	0.1%	✓			

	41	0.1%	✓			
	42	0.1%	✓			
	43	0%	✓			
	44	0%	✓			
	45	0%	✓			
	46	0.1%	✓			✓
	47	0.1%	✓			✓
	48	0.1%	✓			✓
	49	0%		✓		
	50	0%		✓		
	51	0%		✓		
	52	0.1%		✓		
	53	0.1%		✓		
	54	0.1%		✓		
	55	0%		✓		
	56	0%		✓		
	57	0%		✓		
	58	0.1%		✓		✓
	59	0.1%		✓		✓
	60	0.1%		✓		✓
	61	0%			✓	
	62	0%			✓	
	63	0%			✓	
	64	0.1%			✓	
	65	0.1%			✓	
	66	0.1%			✓	
	67	0%			✓	
	68	0%			✓	
	69	0%			✓	
	70	0.1%			✓	✓
	71	0.1%			✓	✓
	72	0.1%			✓	✓
Collembola	Treat	**MNPs in soil**				**PO**
		concentration	**LPDE based**	**PBAT based**	**STARCH based**	**ALB+PYR**
HEMIEDAPHIC	1	0%	✓			
	2	0%	✓			
	3	0%	✓			
	4	0.01%	✓			
	5	0.01%	✓			
	6	0.01%	✓			
	7	0%	✓			
	8	0%	✓			
	9	0%	✓			
	10	0.01%	✓			✓
	11	0.01%	✓			✓

	12	0.01%	✓			✓
	13	0%		✓		
	14	0%		✓		
	15	0%		✓		
	16	0.01%		✓		
	17	0.01%		✓		
	18	0.01%		✓		
	19	0%		✓		
	20	0%		✓		
	21	0%		✓		
	22	0.01%		✓		✓
	23	0.01%		✓		✓
	24	0.01%		✓		✓
	25	0%			✓	
	26	0%			✓	
	27	0%			✓	
	28	0.01%			✓	
	29	0.01%			✓	
	30	0.01%			✓	
	31	0%			✓	
	32	0%			✓	
	33	0%			✓	
	34	0.01%			✓	✓
	35	0.01%			✓	✓
	36	0.01%			✓	✓
	37	0%	✓			
	38	0%	✓			
	39	0%	✓			
	40	0.1%	✓			
	41	0.1%	✓			
	42	0.1%	✓			
	43	0%	✓			
	44	0%	✓			
	45	0%	✓			
	46	0.1%	✓			✓
	47	0.1%	✓			✓
	48	0.1%	✓			✓
	49	0%		✓		
	50	0%		✓		
	51	0%		✓		
	52	0.1%		✓		
	53	0.1%		✓		
	54	0.1%		✓		
	55	0%		✓		
	56	0%		✓		
	57	0%		✓		

	58	0.1%		✓		✓
	59	0.1%		✓		✓
	60	0.1%		✓		✓
	61	0%			✓	
	62	0%			✓	
	63	0%			✓	
	64	0.1%			✓	
	65	0.1%			✓	
	66	0.1%			✓	
	67	0%			✓	
	68	0%			✓	
	69	0%			✓	
	70	0.1%			✓	✓
	71	0.1%			✓	✓
	72	0.1%			✓	✓
Collembola	Treat	**MNPs in soil**				**PO**
		concentration	**LPDE based**	**PBAT based**	**STARCH based**	**ALB+PYR**
EPIEDAPHIC	1	0%	✓			
	2	0%	✓			
	3	0%	✓			
	4	0.01%	✓			
	5	0.01%	✓			
	6	0.01%	✓			
	7	0%	✓			
	8	0%	✓			
	9	0%	✓			
	10	0.01%	✓			✓
	11	0.01%	✓			✓
	12	0.01%	✓			✓
	13	0%		✓		
	14	0%		✓		
	15	0%		✓		
	16	0.01%		✓		
	17	0.01%		✓		
	18	0.01%		✓		
	19	0%		✓		
	20	0%		✓		
	21	0%		✓		
	22	0.01%		✓		✓
	23	0.01%		✓		✓
	24	0.01%		✓		✓
	25	0%			✓	
	26	0%			✓	
	27	0%			✓	
	28	0.01%			✓	

	29	0.01%			✓	
	30	0.01%			✓	
	31	0%			✓	
	32	0%			✓	
	33	0%			✓	
	34	0.01%			✓	✓
	35	0.01%			✓	✓
	36	0.01%			✓	✓
	37	0%	✓			
	38	0%	✓			
	39	0%	✓			
	40	0.1%	✓			
	41	0.1%	✓			
	42	0.1%	✓			
	43	0%	✓			
	44	0%	✓			
	45	0%	✓			
	46	0.1%	✓			✓
	47	0.1%	✓			✓
	48	0.1%	✓			✓
	49	0%		✓		
	50	0%		✓		
	51	0%		✓		
	52	0.1%		✓		
	53	0.1%		✓		
	54	0.1%		✓		
	55	0%		✓		
	56	0%		✓		
	57	0%		✓		
	58	0.1%		✓		✓
	59	0.1%		✓		✓
	60	0.1%		✓		✓
	61	0%			✓	
	62	0%			✓	
	63	0%			✓	
	64	0.1%			✓	
	65	0.1%			✓	
	66	0.1%			✓	
	67	0%			✓	
	68	0%			✓	
	69	0%			✓	
	70	0.1%			✓	✓
	71	0.1%			✓	✓
	72	0.1%			✓	✓