Selection of a bioindicator toolbox for monitoring effects of plant protection product residues

Part 2 – Scoring of actors and bioindicator methods

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Photo header: Swiss Agricultural landscape - Mathieu Renaud, Swiss Centre for Applied Ecotoxicology

Summary

The Swiss Action Plan on Plant Protection Products (AP-PPP) includes a measure for long-term monitoring of PPP residues in agricultural soils and their effects on soil fertility. Within this measure the ConSoil project proposes an integrated approach for combining risk-based reference values with an ecological and ecotoxicological bioindicator toolbox. The current report details the different steps and considerations for selecting and proposing an initial toolbox of bioindicators.

The selection, scoring and ranking of organism groups is necessary for their prioritization as bioindicators. The scoring includes previously established links of in-soil organisms and plants to ecological soil functions and also integrates the importance of these links for soil fertility according to various Swiss stakeholders. The stakeholder evaluation was conducted through a questionnaire and the results as well as the scoring of organisms are presented and described. From this scoring, a priority list was established and possible bioindicator methods for each organism group were discussed in a workshop with several national and international experts in soil ecology and ecotoxicology. Finally, an initial toolbox of bioindicators is proposed considering different sampling strategies and limitations.

The bioindicator toolbox will be tested in pilot studies to evaluate the performance, variability and sensitivity of the proposed methods. The pilot studies also aim to refine and establish tools for the interpretation of effects such as toxicity thresholds. Based on these pilot studies and in collaboration with project partners and the federal offices the toolbox will be further refined.

Content

1 ConSoil project

1.1 Mandate

In September 2017, the Swiss Federal Council approved an Action Plan for the reduction and sustainable use of Plant Protection Products (PPP) (Conseil Fédéral Suisse 2017). In the Action Plan, Objective 5.7 “aims at ensuring that the use of PPP has no long-term adverse effects on soil fertility and at reducing the use of PPP with a high-risk potential for soil". Within Objective 5.7, Measure 6.3.3.7 requires the development of a long-term monitoring of PPP residues in agricultural soils. Specific objectives are the establishment of a chemical monitoring of PPP residues, the development of ecotoxicological risk-based reference values and the proposal of (bio)indicators for assessing the effects of PPP residues on soil fertility.

In 2019, a collaboration between the Swiss Soil Monitoring Network (NABO), the Ecotox Centre and EnviBioSoil was established to address the objectives set out in Measure 6.3.3.7 (Godbersen et al. 2019). NABO is responsible for the analysis of PPP residues since 2018 and will implement a chemical monitoring of PPP residues in in-crop soils by 2024. The Ecotox Centre and EnviBioSoil developed the ConSoil project which aims to derive risk-based reference values by 2025 and bioindicators for the effects of PPP residues on long-term soil fertility by 2027.

1.2 Conceptual framework

The ConSoil project proposes a combined approach that integrates chemical monitoring with risk-based references values and ecological and ecotoxicological indicators. Monitoring of PPP residues by NABO will be compared with Soil Guideline Values (SGV) for site screening and for identification and prioritization of potentially at-risk sites. The SGV include thresholds for individual substances but a mixture approach is also being developed. For sites exceeding reference values, detailed monitoring is performed and will include the refinement of the SGV for site-specific properties and the assessment of effects using an ecological and ecotoxicological bioindicator toolbox (Figure 1). Further information on the risk-based reference values for soil and SGV is available in two reports, a literature review (Marti-Roura et al. 2023a) and the recommendations for the derivation of SGV (Marti-Roura et al. 2023b).

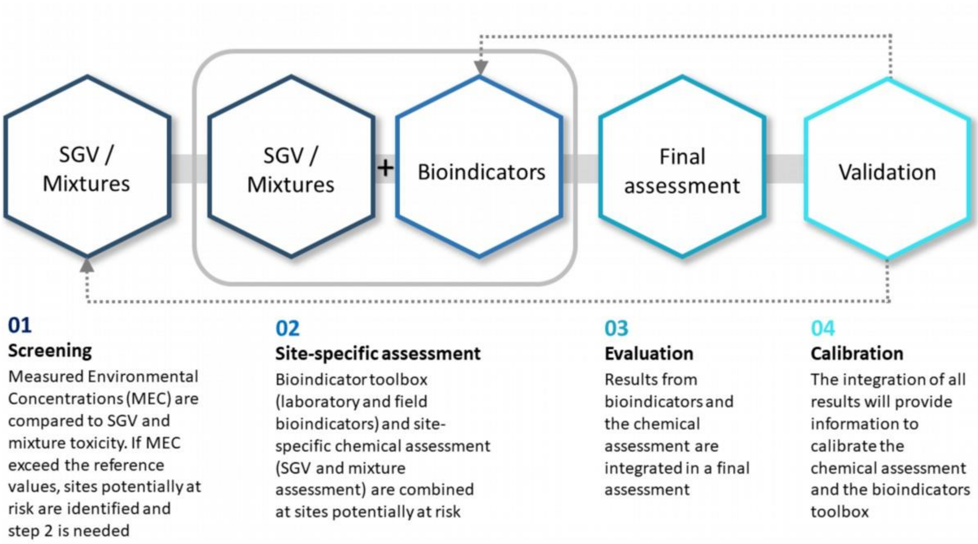


Figure 1. Conceptual framework integrating risk-based reference values and bioindicator tools for the long-term monitoring of PPP residues (Marti-Roura et al. 2023b).

The combination of chemical, ecotoxicological and ecological indicators should provide a comprehensive understanding of the effects and risks of the PPP residues and help distinguish these effects from those related to soil and its properties, management practices and/or environmental factors. The approach of combining chemical, ecological and ecotoxicological data is inspired by the TRIAD approach (ISO 19204 2017) with specific adaptations for a monitoring scheme. Adaptations to the TRIAD approach include the screening of potentially at-risk sites using a generic SGV prior to the detailed monitoring. In the detailed monitoring rather than the tiered approach used in the TRIAD, a fixed bioindicator toolbox is proposed.

In the TRIAD approach for site-specific risk assessment, effects are measured against a reference site or response (ISO 19204 2017). However, when monitoring agricultural land, it is unlikely that a representative reference site can be established for each monitoring site. Therefore, under the current project, where possible, the natural variability of each bioindicator will be evaluated to define normal operating ranges (NOR) and effect thresholds. In addition, it is proposed to monitor the same indicators over time (e.g., 5 years) to allow the interpretation of data trends rather than single observations, thus improving the assessment of long-term risks. Over time the monitoring data can help to further establish NOR and effect thresholds.

Currently, the proposed duration of the detailed monitoring is 5 years with annual sampling, but an adapted timeframe might be proposed later in agreement with project partners and the regulatory authorities.

1.3 Protection goal

Following the mandate of Measure 6.3.3.7, the project focuses on the effects of **PPP residues**, in the **in-crop area** and has the protection goal of **long-term soil fertility**.

Excluding human and animal health aspects, a soil is considered fertile when, as presented in the Swiss National Soil Strategy (Swiss Federal Council 2020):

- The active biotic community, soil structure and composition, and soil depth are typical of its site, and its degradation capabilities have been unaffected;
- Natural plants and plant communities, and those that have been subject to human influence, are able to grow and develop unhindered with their characteristic properties intact.

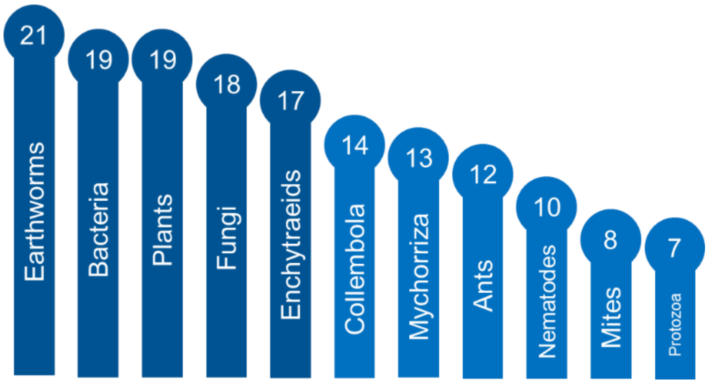
It was therefore decided that the monitoring of PPP residues under Measure 6.3.3.7 should focus on in-soil organisms and plants that play an important role in maintaining soil fertility (Godbersen et al. 2019). More specifically, in-soil organisms and plants that play an important role in the three following ecological soil functions that support soil fertility (Swiss Federal Council 2020):

- **Habitat function**: The ability of soil to sustain organisms and to maintain the diversity of ecosystems, species and their gene pool. The habitat function also covers soil's suitability as a habitat for organisms and as a location for plants.
- **Regulating function**: The ability of soil to regulate, buffer or filter water and energy cycles, as well as to transform substances.
- **Production function**: The ability of soil to produce biomass, i.e. food and feedstuffs, as well as wood and other fibres.

1.4 Which soil organisms contribute to soil fertility?

Soils are inhabited by different organisms, such as microorganisms (e.g., fungi, bacteria), mesofauna (e.g., soil microarthropods, enchytraeids), macrofauna (e.g., earthworms, isopods) as well as plants, which interact together in intricately connected networks. Based on the above protection goal (see section 1.3), it was important to identify the key soil organisms or "actors" which drive ecological soil functions that support soil fertility.

To identify those specific organisms or "actors", the ecosystem services (ES) framework (CICES 2023) and key references from the scientific literature were used (Creamer et al. 2022; Faber et al. 2021; Ockleford et al. 2017). Further information is provided in a technical report (Dell'Ambrogio et al. 2023), where links between actors, processes and ecological soil functions are compiled from key references. The main actors are summarized in figure 2, which depicts the number of processes linking the actors to the three ecological soil functions that support soil



fertility.

Figure 2. Soil organism groups and the number of linked processes relevant for ecological soil functions (habitat, regulating, production) that support soil fertility. For further details, see Dell'Ambrogio et al. 2023.

In this report, the selection and prioritization of actors for the bioindicator toolbox is presented. These considered the above-mentioned number of processes linking actors to ecological soil functions but also results of the evaluation of ES importance by stakeholders from the Swiss soil and agricultural context (section 2). For the prioritized organism groups, a selection of appropriate bioindicator methods supported by a workshop with national and international experts is presented (section 3).

2 Stakeholder evaluation and scoring of actors for soil fertility

2.1 Stakeholder and expert evaluation of ecosystem services for long-term fertility of agricultural soil

Using key scientific references, Dell'Ambrogio et al. (2023) identified the links between soil organisms, processes, ES and finally ecological soil functions. However, not all ES contribute equally to soil fertility according to various stakeholders. Therefore, in order to improve the prioritization of the actors for the selection of bioindicators, a questionnaire was conducted with stakeholders/experts from the soil and agricultural context, covering scientists (research/academia) policy makers (policy/government) and land users (farmers). Stakeholders were asked to value 13 ES classes (Dell'Ambrogio et al. 2023, Figure 3) in terms of their relative importance for long-term soil fertility, considering the protection goal described above (Section 1.3).

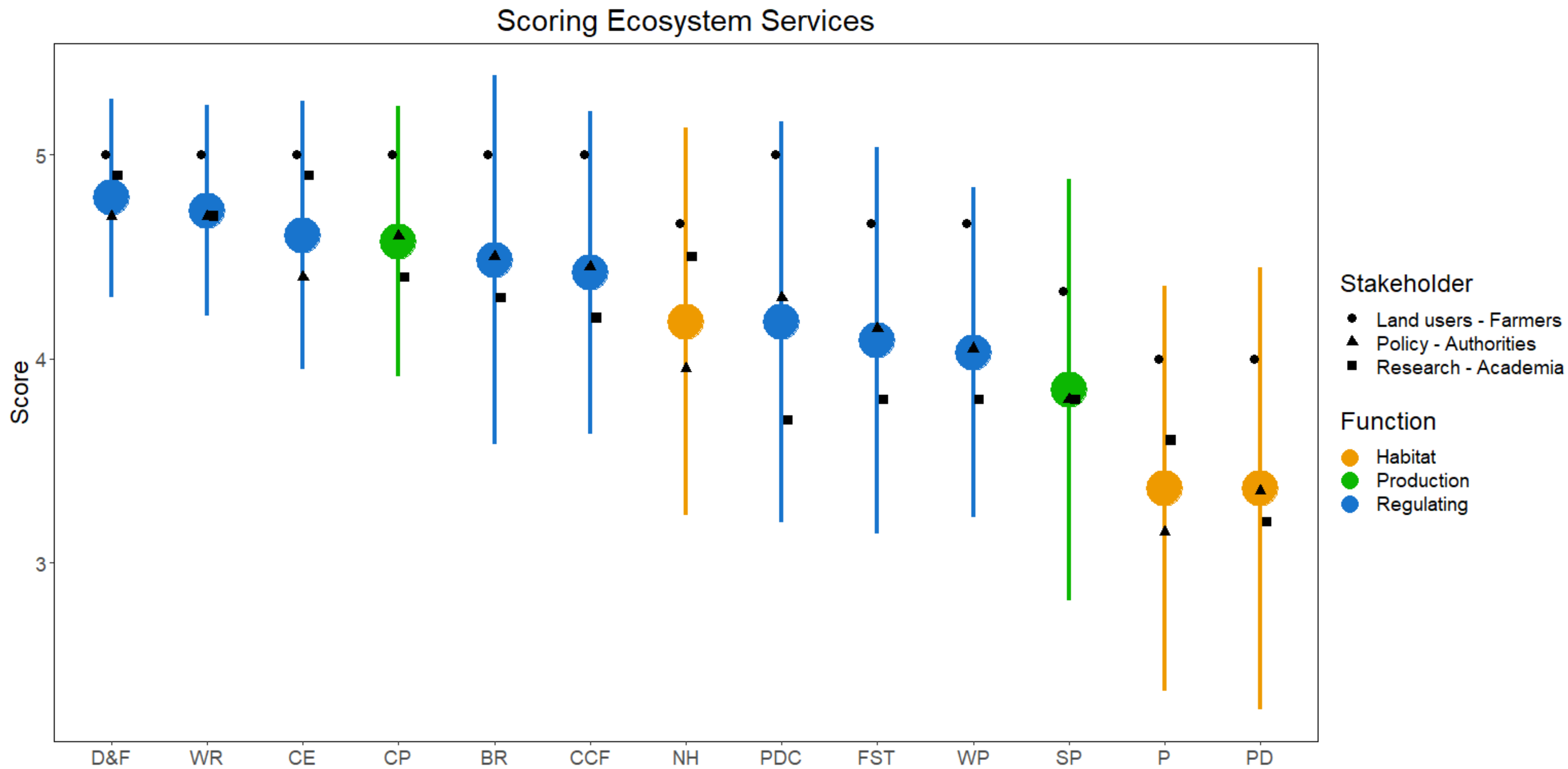
For this assessment, the stakeholders/experts were given a short description of the ES together with a concrete example and were asked to rate its relative importance to long-term soil fertility on a scale of 1 to 5, from very low to very high. The questionnaire was administered anonymously, with only the stakeholder class requested as a mandatory field to fill in (research/academia, policy/government, farmers). An optional field was provided to gather the institution for each stakeholder. The questionnaire was made available in English as well as in three Swiss national languages (French, German and Italian). The English questionnaire is available in the supplementary material.

The list of stakeholders was compiled in collaboration with the Federal Office for the Environment and the Federal Office for Agriculture, resulting in 78 invited participants. A total of 70 participants visited the questionnaire, 35 started the questionnaire and 33 completed it, giving a participation rate of 50% and a completion rate of 93% for those actively participating. Of the 33 participants who completed the questionnaire, 61% (20 participants) identified themselves as representing policy/authorities, 30% (10 participants) as representing research/academia and 9% (3 participants) as land users. In addition, four participants (2 representing land users/ and 2 representing research/academia) emailed their inability to participate in the questionnaire due to lack of expertise and/or their inability to objectively assess the ES.

The participants' institution was gathered as an optional category and was therefore filled in with varying levels of specificity, rendering a detailed analysis impossible. 5 participants chose not to disclose their institution (3 from policy/authorities and 2 from research/academia). From the available information for the policy/government stakeholders there was balanced participation from environmental protection offices (8 participants) and from agriculture offices (7 participants). Regarding research/academia, participants represented research institutions (5), universities of applied sciences (2), one university and one foundation. For land users, all three participants came from different institutions. The raw results of the questionnaire can be found in Annex 1.

The average response across all stakeholders and for each stakeholder group is shown in Figure 3. ES scores when averaged across stakeholders ranged from 4.8 to 3.4, but with most (10/13) ES scoring above 4. Also, as the score decreases, the deviation from the average increases, indicating a lower level of agreement between stakeholder classes on the valuation of these ES. Interestingly, land users/farmers gave a higher scoring than the other stakeholder groups for all ES, but the results should be treated with caution due to the low participation rate of this stakeholder group (9%). The low participation rate is also reflected in the very high standard deviations where there was disagreement within this group (e.g. pollination).

Even if only slightly, some regulating services (decomposition & fixing processes, hydrological cycle & water flow regulation and control of erosion rate) outscored the production of crops in a strictly agricultural context. These results highlight the growing interest in maintaining critical soil ES which – while not a direct measure of production – support, regulate and allow sustainable crop production and soil fertility. Interestingly, the regulation of the hydrological cycle and water flow, which is not exclusive to soil but links soil and water compartments, was the second highest scoring ES. The lowest scoring ES were those where a lesser role of soil organisms is described, such as pollination (stimulation of pollination or attraction of pollinators), or those with lower perceived relevance in an agricultural context, such as propagule dispersal (dispersal of seeds and spores in agricultural soils) where sowing takes place.



D&F - Decomposition and fixing processes (CICES code: 2.2.4.2)
WR - Hydrological cycle and water flow regulation (CICES code: 2.2.1.3)
CE - Control of erosion rates (CICES code: 2.2.1.1)
CP - Cultivated terrestrial plants, fibres or other materials from cultivated plants grown for nutritional purposes, for direct use or processing, or as a source of energy (CICES codes: 1.1.1.1; 1.1.1.2; 1.1.1.3) *
BR - Bioremediation (CICES code: 2.1.1.1)
CCF - Regulation of the chemical condition of freshwaters (CICES code: 2.2.5.1)
NH - Maintaining nursery populations and habitats (Including gene pool protection) (CICES code: 2.2.2.3)
PDC - Pest and disease control (CICES code: 2.2.3.1; 2.2.3.2)
FST - Filtration/ sequestration/storage/accumulation of toxic substances (CICES code: 2.1.1.2)
WP - Weathering processes (CICES code: 2.2.4.1)
SP - Seeds, spores and other plant materials collected for maintaining or establishing a population (CICES code: 1.2.1.1)
P - Pollination (CICES code: 2.2.2.1)
PD - Propagule dispersal (CICES code: 2.2.2.2)

Figure 3 – Average scoring of ecosystem service classes for all stakeholders (large bullets) with standard deviation (large error bars) with their associated ecological soil functions (green: production, yellow: habitat, blue: regulating) and the average scoring for each of the stakeholder groups (small black symbols): Policy/Authorities (triangles – n=20), Research/Academia (squares, n=10) and Land users/Farmers (circles, n=3)).

2.2 Scoring of actors

The scoring of actors for the bioindicator toolbox was based on their number of links (i.e., number of processes) to ecological soil functions and the relative importance of those links according to the stakeholder evaluation of ES. In a first step, the average score of each ES across all stakeholders was associated for all processes that are linked to the ES. In a second step, the scores of these processes were added up to provide the score for each of the related soil organisms groups or actors (Figure 4).

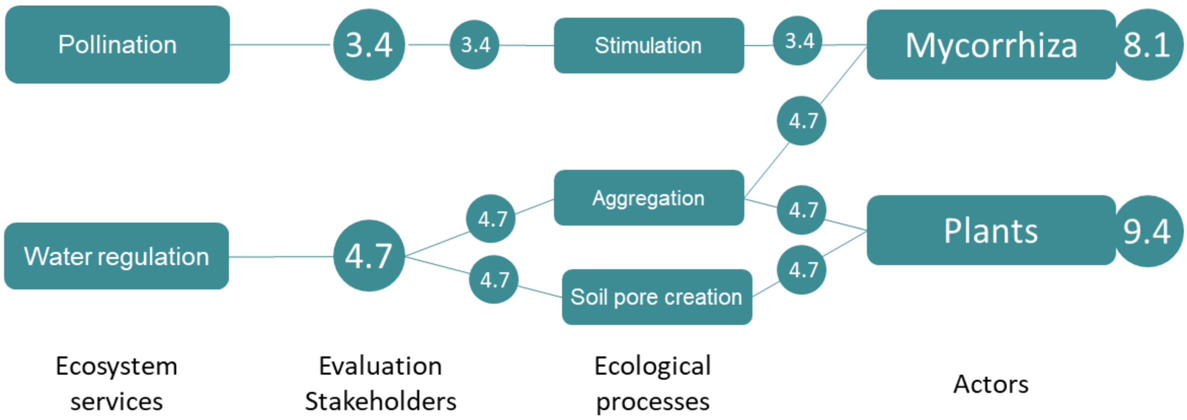


Figure 4 –Example of the final score attribution from ecosystem services to soil organism groups (actors) with two example ES.

Table 1 shows the number of processes that link each organism group to ES and the overall score, which is the number of links weighted by the stakeholder evaluation (ES score given by the stakeholders). Overall, there were very few differences in actor ranking between the level of linkage and the overall score after including the stakeholder assessment (ES scores). The lack of large changes for the overall score compared to the level of linkage is due to the fact that the stakeholder valuation of ES was balanced (see Section 2.1 Figure 3) and that some key processes are shared between different ES.

For the selection of bioindicators the top 11 highest scoring actors were selected (Table 1: those above the red cut-off line). The initial selection was for the 10 highest scoring actors but the spacing in overall scoring between acari and protozoa compared to protozoa and microalgae justified the inclusion of protozoa.

It is, however, important to highlight that these scores do not reflect the de-facto contribution of organisms to ecological soil functions. The scores are subject to important biases in terms of research priorities for ecological processes (more studied organisms will tend to be better understood and score higher), and stakeholder understanding of the importance of ES. Specifically, the evaluation by stakeholders reflects cultural and societal perceptions and the views of stakeholder groups and professions therein (individual stakeholder scoring is presented in Annex 1 and scoring by stakeholder group in Annex 2).

Table 1 – Number of processes linking actors to ecological soil functions and scoring of actors after integration of ES scores given by stakeholders.

Linkage Actor Ranking	Processes	Overall Actor Ranking	Score
Earthworms	21	Earthworms	93
Bacteria	19	Plants	85
Plants	19	Bacteria	83
Fungi	18	Fungi	79
Enchytraeids	17	Enchytraeids	76
Collembola	14	Collembola	62
Mycorrhiza	13	Mycorrhiza	56
Ants	12	Ants	54
Nematodes	10	Nematodes	43
Acari (mites)	8	Acari (mites)	35
Protozoa	7	Protozoa	30
Coleoptera	4	Microalgae	18
Diplopoda	4	Diplopoda	17
Isopods	4	Isopods	17
Microalgae	4	Coleoptera	16
Archaea	3	Archaea	13
Gastropods	3	Gastropods	13
Insects	3	Insects	13
Spiders	2	Spiders	8
Viruses	2	Viruses	8

3 Bioindicator method selection

For the proposed biomonitoring framework (see section 1.2 and figure 1), ecological and ecotoxicological indicator-based methods need to be selected to assess the effects of PPP residues on key actors for soil fertility (see section 2). Ecotoxicological and ecological indicators are defined as follows:

Ecotoxicological indicators – Tests or bioassays with organism groups or species conducted in the laboratory to measure the actual toxicity present in environmental samples from the monitoring site.

Ecological indicators - Field observations of vegetation, soil fauna and microorganisms that provide a measure of ecosystem structure and functioning.

As the focus of the ConSoil project is on PPP residues in soil, the indicators should be sensitive to these chemical stressors and allow for the measurement of small effect sizes consistent with the protection goal (see section 1.3). In addition, due to the applied nature of the ConSoil project, the proposed biomonitoring approach should be able to be implemented in case a routine monitoring is implemented. Therefore, priority is given to well-established or standard methods, which facilitates the knowledge transfer to public or private laboratories.

To assist in the creation of the indicator toolbox, a workshop with national and international experts was organised to help select appropriate bioindicator methods for each key organism group for the three different sampling options described below (see section 3.1). Section 3.2

reflects the key considerations on indicator methods and toolbox selection and is supported by the discussions with experts for each organism group.

3.1 Sampling

The focus of the current project is on PPP residues and must not target the effects of PPP during the application periods (see section 1 for further context). Therefore, soil sampling will take place in the winter period (November – March) when no PPP application is expected and when only the residues from previous applications will be assessed.

The sampling sites are agricultural lands, privately owned and cultivated by Swiss farmers. Their participation in the biomonitoring research project is voluntary. Negotiations with farmers are still ongoing, which may impose some restrictions on the extent, type of sampling, number of interventions and level of site disturbance.

In view of the upcoming negotiations with farmers and for the purpose of expert discussions and initial toolbox proposal, three scenarios for the size and type of sampling were proposed for discussion:

1. Option 1 "Soil samples provided": Composite soil samples representative of the site (up to 10kg) provided by project partners.
2. Option 2 "Soil sampling + Site assessment": Composite soil samples (up to 10kg) and functional non-invasive measurements or visual assessments. Site assessment can be carried out by project partners or the farmer.
3. Option 3 "Full soil and ecological sampling": Composite soil samples (up to 10kg) and on-site ecological sampling. Experts have access to the site for sampling.

From the three proposed sampling options, only one will be selected for the biomonitoring framework after negotiations with farmers.

The sampling period is particularly important for the ecological indicators, and the proposed winter period (November to March) may impose technical and ecological constraints on the sampling and activity of some groups of soil organisms. Therefore, for ecological indicators, sampling in the later period of February to March will be preferred. To reduce confounding factors, the environmental conditions should be carefully assessed prior to sampling and adequate soil moisture levels should be observed (i.e. no drought). It is also important that an appropriate sampling effort (e.g. sampling design and number of samples) is applied to obtain representative samples, which allow an appropriate statistical analysis to monitor adequate effect sizes, particularly for sampling options 2 or 3.

Finally, for long-lived species, delayed effects of PPP applications during the summer may still be observed during sampling in winter. However, with the proposed monitoring framework with sampling over time (see section 1.2), an evaluation of trend data and of long-term effects should still be possible. In fact, these limitations and potential delayed effects highlight the importance of the annual sampling strategy for understanding long-term effects.

3.2 Indicator methods

For each of the top 11 highest scoring actors (Table 1), different options and suitable methods are presented and discussed supported by information obtained from the national and international experts during the workshop (general minutes – annex 4). In the workshop, discussion was based on the 3 sampling options defined above for both the ecotoxicological and the ecological indicators, which are included in the next sections. The ecological and ecotoxicological bioindicator methods that are recommended for each sampling option are presented in table 2. Standardized methods mentioned in the text, as well as all currently available standardized methods are listed in Annex 3. Non-standard methods are included in the reference list.

3.2.1 Earthworms

Earthworms are one of the highest scoring actors but their use as indicators presents some challenges.

For an ecotoxicological assessment, for all three sampling options the earthworm reproduction test (ISO 11268-2; OECD 222) can be used. Most available research and standards – including those used for the authorization of chemicals such as PPP – use compost worms, *Eisenia andrei* and *Eisenia fetida,* However, these species are not commonly found in agricultural soils (Environment and Climate Change Canada 2022; ISO 11268-2; OECD 222) and, as epigeic species, they are less representative compared to anecic and endogeic species when considering the highest overall contribution of earthworms to soil functions (Creamer et al. 2022). Because of these limitations, tests using other more representative species (*Aporrectodea caliginosa* and *Dendrodrilus rubidus*) have recently been included in guidelines (Environment and Climate Change Canada 2022; ISO 11268-2). However, those require greater technical effort and the species are not commercially available, which could be an important limitation for their implementation in routine testing.

Despite the above limitations, *Eisenia fetida/andrei* as the most commonly tested species allow for a direct comparison with most (including regulatory) ecotoxicological data and both species can be purchased commercially. These species are routinely used and readily available from ecotoxicological laboratories and companies. However, suppliers for compost use should be used with caution in ecotoxicological testing as there is no guarantee of species accuracy or quality. From a technical point of view, the amount of soil required (e.g. 500g soil per replicate) and the long duration of the earthworm reproduction test (e.g. 2 months) may also be limiting factors.

One option to overcome these technical limitations could be the use of the enchytraeid reproduction test (ISO 16387 2014; OECD 220 2016) as a surrogate for oligochaetes. The enchytraeid reproduction test has much lower technical requirements (see section on enchytraeids) and, according to Jarratt & Thompson, (2017), there are no consistent differences in sensitivity to PPP between the Lumbricidae and Enchytraeidae families and they have a similar overall sensitivity for effects on reproduction. A lack of difference in sensitivity to pesticides between Enchytraeus (*Enchytraeus albidus/crypticus*) and Eisenia (*Eisenia fetida/andrei*) was also observed in a metanalysis (personal communication, Pelosi et al. unpublished). Based on these observations, the use of enchytraeids as representatives for all oligochaetes is proposed for the test battery for all three sampling options (see section 3.2.2 for more information on the Enchytraeus reproduction test).

For the ecology, no recommendations are made for sampling option 1 as the prerequisites for selecting bioindicator methods are not met (no well-established or standardized methods are currently available; see section 3.0). For sampling option 2, the biomass of surface casts could be an important indicator of earthworm abundance where earthworm sampling is not possible (Bayon et al. 2022; Escudero, Domínguez, and Bedano 2023). For sampling option 3, field sampling of earthworms (ISO 23611-1) is suggested. However, in addition to the aforementioned ecological challenges associated with the winter sampling period, earthworm field sampling can be technically challenging due to the large effort required to obtain a representative sample size that allows for the detection of adequate minimum detectable differences and effect sizes. Ideally, identification to the species level should be performed but, if this is not possible, the minimum data criteria would be biomass, ecological category and proportion of juveniles/adults. Molecular approaches are promising to enable the identification of earthworms (and other groups) to the species level, either by analysing the DNA of extracted individuals or by analysing the environmental DNA, but more research is still necessary and no standard guidelines exist so far.

3.2.2 Plants

Several tests are available for plant ecotoxicology (see Appendix 1) but a potential limitation of these is the relatively high amount of soil required by some standard protocols. Plant germination and seedling growth tests with crop species appear to be well suited to the protection goal of long-term soil fertility and could be included for all three sampling options. Several standards are available (ISO 11269-2, ISO 17126, ISO 18763, OECD 208, Env Canada method), which allow several different plant species (typically crops but also cover crop species) to be used. Overall, ISO 18763 is proposed, which evaluates seed germination as well as root elongation, two critical endpoints well-linked to the protection goal. In addition, shoot height can be measured. Compared to other methods on similar endpoints (e.g. ISO 11269-2, OECD 208), ISO 18763 is a more suitable cost-effective screening option to consider phytotoxicity. This standard can be rapidly implemented and, optionally, it can be purchased as a commercial kit.

For the ecology, plants are the perfect group for integrating farmers into the biomonitoring, especially when the measurement of other ecological indicators is not possible or feasible. The assessment by farmers of the productivity of crops or of percentages of land cover after early growth could, in combination with the proposed ecotoxicological test, provide valuable information on effects on plants.

3.2.3 Microorganisms (Bacteria, Fungi, Mycorrhiza, Protozoa)

Bacteria and fungi are extensively studied and there is a wealth of information for Swiss soils, with several research institutions such as Agroscope and Swiss Federal Institute for Forest, Snow and Landscape Research working on these soil microorganisms. In addition, some microorganism indicators are monitored by the NABO Biological Group. In terms of standard tests, functional or diversity methods (18 methods listed in appendix 3) can be used in ecotoxicology or ecology (Karpouzas, Vryzas and Martin-Laurent 2022; Niemeyer et al. 2015).

For ecotoxicity testing, functional methods measuring enzymatic activities or rates of microbial processes are commonly used (e.g. Niemeyer et al. 2015). While soil microbial respiration tests do not always show a consistent response to PPP exposure, processes linked to nitrogen transformation (i.e. nitrification) were found to be more sensitive(Karpouzas, Vryzas, and Martin-Laurent 2022). Nitrogen transformation also provides a clear link to ecosystem function and is well aligned with the protection goal in the context of the project. Several standards are available to assess nitrification by soil microbiota (ISO 14238; ISO 15685; OECD 216). Among these, ISO 15685, which details the quantification of nitrification and inhibition of nitrification, is proposed for the toolbox as the method can be more easily implemented compared to the others.

For the ecological testing, DNA-based methods that assess the composition of the microbial community (microorganisms diversity) allowing to link soil microbiome signatures to pesticide residues (Walder et al. 2022) or to identify microbial groups with specific functions (e.g. nitrifying microorganisms) are relevant for the bioindicator toolbox (Karpouzas et al. 2022). Some standardized methods are already available allowing such kind of measurements. ISO 11063 provides a procedure for the direct extraction of DNA from soil samples, which will in turn allow the further study of abundance and composition of the microbial community (e.g. real time quantitative PCR (qPCR), next generation sequencing of ribosomal amplicons (NGS)). Methods proposed for this ecological line of evidence generally allow the use of frozen soil samples, but these must be stored between -40°C and -80°C as higher temperatures do not suit optimal DNA preservation. Furthermore, soil DNA extracts could be stored instead of the collected soil samples, which allows for the reduction of storage space.

Mycorrhizal fungi, for which an increasing amount of research is available, play a very important role in soil fertility and are sensitive to pesticides. Regarding ecotoxicological tools, there is currently one standard ecotoxicity test available, which evaluates the spore germination of *Glomus mosseae* (ISO/TS 10832) on collected soil samples. Some additional ecotoxicological approaches with mycorrhizal fungi are being developed with other species and endpoints but are not yet standardized (Klauberg-Filho et al. 2023; Mallmann et al. 2018).

Regarding ecological testing, no standard methods exist but some established research groups working on mycorrhiza have focussed on the impacts caused by chemical exposure (Mozafar et al. 2002) including pesticides (Edlinger et al. 2022; Riedo et al. 2021). Root colonization by mycorrhiza is an interesting indicator but is dependent on the type of crop in the field and therefore on crop rotation. Increasingly molecular tools can be used to study and evaluate mycorrhiza diversity (Edlinger et al. 2022; Hartman et al. 2023; Lutz et al. 2023).

Protozoa have been identified as playing an important role in soil fertility and there is some evidence of their importance as bioindicators (Fournier et al. 2022). However, this group is under-represented in the scientific literature and standardization compared to other organism groups. There are currently no standardized guidelines for ecotoxicological or ecological tests with protozoa.

Whereas protozoa can currently not be recommended for ecotoxicological testing, the methods developed to assess microflora communities (bacteria and fungi) would also be applicable to measure protozoa diversity as an ecological indicator. Gaining insight into protozoa diversity for the current project will help complete the information obtained for microorganisms. Protozoa can be just as sensitive or even more sensitive and responsive to PPP exposure than bacteria and or fungal communities through changes in diversity or indicator taxa (Foissner 1997; Fournier et al. 2022).

3.2.4 Enchytraeids

For ecotoxicological methods, the enchytraeid reproduction test with *Enchytraeus crypticus* is proposed (ISO 16387 or OECD 220, following the adaptations of Castro-Ferreira et al. (2012)). The method is well established and, using *E. crypticus* as test species with the adaptations proposed by Castro-Ferreira et al. 2012 instead of *E. albidus*, the test duration can be reduced from 6 to 3 weeks due to the species' shorter generation time. Another advantage is that the amount of soil required for the test is small (20g dry weight per replicate). The enchytraeid reproduction test can be performed for all three sampling options.

No ecological indicators can be recommended for sampling options 1 and 2 because no feasible standard method has been identified that could be applied. For sampling option 3, field sampling of enchytraeids (ISO 23611-3) is proposed. Ideally, the enchytraeids should be identified to the species level. If this is not possible, identification to at least the genus level is necessary. The total abundance of enchytraeids is not sufficiently informative and, unlike for earthworms, ecological categories other than the classification as R (opportunist) or K (equilibrium) strategists are not well established for enchytraeids.

3.2.5 Microarthropods (Collembola and Mites)

As part of the ecotoxicological testing, the collembola reproduction test is recommended. Collembolans are one of the most sensitive groups of soil fauna to PPP (Joimel et al. 2022) and are generally more sensitive to PPP than mite test species (de Lima e Silva et al. 2017; Natal-da-Luz et al. 2019). There are two commonly tested and well-established collembolan species, *Folsomia candida* and *Folsomia fimetaria*. *F. fimetaria* occurs more commonly in agricultural soils and would therefore be a more ecologically relevant choice compared to *F. candida* but the test with *F. fimetaria* has higher technical requirements (selection of males and females) and produces results with a higher variability due to the species' sexual mode of reproduction. Also, there are no overall differences in sensitivity between the two species (Scott-fordsmand and Krogh 2005). For these reasons, the collembolan reproduction test with *F. candida* (ISO 11267 or OECD 232) is recommended for all three sampling options.

For the ecology, no specific recommendations can be made for sampling option 1 and 2 due to the lack of well-established or standard methods. For option 3, the assessment of the microarthropod community (ISO 23611-2) is recommended. For microarthropod sampling, the focus should preferably be on collembola, as the taxonomic identification of mites is much more difficult. Regarding the level of identification, the collembola should ideally be identified to the species level. The use of DNA for species identification is only partially possible as genetic libraries are improving but not yet complete. The use of morphospecies for monitoring is also possible and shows a good correlation with species richness but still requires further research (Reis et al. 2016).

3.2.6 Ants

So far, no ecotoxicological or ecological standard methods exist for ants. Some research has been conducted on the effect of chemicals on ants, but the experimental designs are complex and, so far, not standardized. Similarly for the ecology, some research exists but tests remain very complex to conduct and have not been standardized (Sakamoto and Goka 2023; Seidenath et al. 2021). For routine monitoring, the implementation of indicators based on ants does not currently seem feasible but could be included in a future revision of the toolbox if the methods become more established.

3.2.7 Nematodes

As an ecotoxicological indicator, the standardized *Caenorhabditis elegans* ecotoxicity test (ISO 10872) is recommended for all three sampling options. The test is sensitive to chemical stressors, appropriate for different soil types and toxicity thresholds have been established (Höss et al. 2009), which could be refined for Swiss soils. However, the test may give poor results for soils with a high clay content.

Regarding ecological indicators, there is a wealth of information on nematode communities and some well-established community indices (e.g. maturity index and plant parasite index) that could provide important information on soil quality (Yeates 2003). Nematode communities and their indices, in particular the maturity index, are sensitive indicators of chemical stress (Höss et al. 2021), including PPP (Haegerbaeumer et al. 2019; Höss et al. 2022). In addition, nematode community sampling has been standardized (ISO 25611-4) and is potentially suitable for all sampling options but requires the use of fresh soil or its fixation with diluted formalin (4% formaldehyde).

3.2.8 General functional indicators

Some important indicators do not represent a single group of key organisms but measure the overall functioning of the ecosystem. Functional indicators are important to complement the diversity and ecotoxicity focused tools suggested above.

For soil invertebrates, the bait-lamina test (ISO 18311 2016) is a quick and accessible tool to use in sampling option 2 and to complement specific community endpoints in sampling option 3. It provides an indication of the decomposition of organic matter and the feeding activity of soil invertebrates and has already been used to measure the effects of PPP in the field (e.g. Larink and Sommer 2002; Niemeyer et al. 2018). Another option to measure the decomposition is the litter bag approach (OECD 2006), which is much more time consuming to prepare, implement and process compared to the bait-lamina test.

For microorganisms, the tea bag test using two different types of tea with different degradation rates can be used to evaluate the breakdown of organic matter by soil microorganisms (Keuskamp et al. 2013). The tea bag test has also been used previously to evaluate the effect of PPP on the organic matter decomposition by microorganisms (Maderthaner et al. 2020; Zaller et al. 2016) and would be a suitable ecological indicator for sampling options 2 and 3.

A general limitation for functional indicators is the lower activity that can be observed in the winter period. For instance, it is possible that the minimum feeding rate and organic matter breakdown might not be attained in a reasonable exposure timeframe.

4 Bioindicator toolbox and outlook

Based on the indicator methods discussed in section 3.2., the bioindicator toolbox in Table 2 is proposed. The toolbox is organised according to the different sampling options presented above. For the ecotoxicological indicators, initial tests will be carried out on a number of Swiss soils to measure natural variability and to establish toxicity thresholds similar to those already established for the nematode ecotoxicity test (Höss et al. 2009). The full bioindicator toolbox will be tested in a pilot study to assess the variability and sensitivity of the different tools and the integration of data. Based on these initial experiments and the pilot study, and in collaboration with the project partners and the federal authorities, the toolbox can be further refined. Finally, as described in the conceptual framework, the refined toolbox can be implemented, under the sampling option agreed with farmers, at NABO monitoring sites identified as potentially at-risk using soil guideline values.

Over time, the bioindicator toolbox can be further refined as new methods are developed and standardized. The refinement could allow for the inclusion of indicators for which standards do currently not exist (e.g. Ants) or are unfeasible under certain sampling options (e.g. sampling option 1 for ecological indicators – Earthworms, Enchytraeus, Collembola). In addition to the improvement of methods, the interpretation of effects will be refined and improved when more data and information on the different bioindicators become available allowing for refined effect thresholds and more accurate risk evaluation.

Table 2 –Ecological and Ecotoxicological bioindicator toolbox recommendations under three sampling options.

Organism group	Indicator type	Sampling option 1 Soil samples provided	Sampling option 2 Soil sampling + site assessment	Sampling option 3 Full sampling
Earthworms	Ecotox.	Enchytraeus reproduction (ISO 16387 / OECD 220)	Enchytraeus reproduction (ISO 16387 / OECD 220)	Enchytraeus reproduction (ISO 16387 / OECD 220)
	Ecol.	No recommendation	Biomass of surface casts	Earthworm hand sampling (ISO 23611-111-1)
Enchytraeus	Ecotox.	Enchytraeus reproduction (ISO 16387 / OECD 220)	Enchytraeus reproduction (ISO 16387 / OECD 220)	Enchytraeus reproduction (ISO 16387 / OECD 220)
	Ecol.	No recommendation	No recommendation	Enchytraeid sampling (ISO 23611-3)
Microarthropods	Ecotox.	Collembolan reproduction (ISO 11267 / OECD 232)	Collembolan reproduction (ISO 11267 / OECD 232)	Collembolan reproduction (ISO 11267 / OECD 232)
	Ecol.	No recommendation	No recommendation	Microarthropod sampling (ISO 23611-2)
Nematodes	Ecotox.	Nematode toxicity test (ISO 10872)	Nematode toxicity test (ISO 10872)	Nematode toxicity test (ISO 10872)
	Ecol.	Nematode soil community (ISO 23611-4)	Nematode soil community (ISO 23611-4)	Nematode soil community (ISO 23611-4)
Bacteria, Fungi, Mycorrhiza and Protozoa	Ecology	Microbial community composition (ISO 11063)	Microbial community composition (ISO 11063)	Microbial community composition (ISO 11063)
Bacteria and Fungi	Ecotox.	Potential nitrification (ISO 15685)	Potential nitrification (ISO 15685)	Potential nitrification (ISO 15685)
Mycorrhiza	Ecotox.	Spore germination (ISO/TS 10832)	Spore germination (ISO/TS 10832)	Spore germination (ISO/TS 10832)
Plants	Ecotox.	Germination and early growth (ISO 18763)	Germination and early growth (ISO 18763)	Germination and early growth (ISO 18763)
	Ecol.	No recommendation	Land cover / Yield winter or cover crops	Land cover / Yield winter or cover crops
General	Ecol.	No recommendation	Bait-lamina (ISO 18311)	Bait-lamina (ISO 18311)

5 Supplementary material

The stakeholder questionnaire in English is available as supplementary material - http://www.oekotoxzentrum.ch/media/olaphygb/questionnaire_eng_vf.pdf

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7 Abbreviations

Action Plan on Plant Protection Products (AP-PPP)

Common international classification of ecosystem services (CICES)

Deoxyribonucleic acid (DNA)

Ecosystem services (ES)

Federal Office for the Environment (FOEN)

Federal Office for Agriculture (FOAG)

International Organization for Standardization (ISO)

Next-generation sequencing (NGS)

Normal operating range (NOR)

Organization for Economic Co-operation and Development (OECD)

Polymerase chain reaction (PCR)

Plant protection products (PPP)

Soil guideline values (SGV)

Swiss Soil Monitoring Network (NABO)

Annex 1 – Questionnaire results

Table A1- Raw data from the questionnaire evaluation and scoring of Ecosystem services by stakeholders

Stakeholder	Institution:	CP	SP	BR	FST	CE	WR	P	PD	NH	PDC	WP	D&F	CCF
Land users	Mandaterre	5	5	5	5	5	5	5	5	5	5	5	5	5
Land users	Agridea	5	3	5	5	5	5	2	4	4	5	4	5	5
Land users	Union suisse des paysans USP	5	5	5	4	5	5	5	3	5	5	5	5	5
Policy	BAFU	5	4	5	5	5	5	3	4	5	5	5	5	5
Policy	Bundesamt für Umwelt	5	5	4	5	5	4	5	5	4	5	4	5	4
Policy	Landwirtschaftsamt	5	3	5	4	5	5	3	2	3	5	4	5	5
Policy	OFEV	5	4	5	5	4	5	4	5	5	5	4	5	5
Policy		3	3	4	4	3	4	3	3	3	3	4	3	4
Policy	Fachstelle Pflanzenschutz Kanton Bern	5	5	5	4	4	4	3	3	4	3	4	5	5
Policy	DGAV	4	4	3	4	4	3	3	4	3	5	3	4	3
Policy		5	4	2	5	5	5	3	4	4	4	4	5	5
Policy	BAFU	5	4	5	2	5	5	4	4	5	5	5	5	5
Policy	Amt für Umwelt - Bodenschutz	3	3	5	5	5	5	3	4	5	5	4	5	4
Policy		5	4	5	4	5	5	1	2	3	4	4	5	4
Policy	Fachstelle Kanton	5	5	5	5	3	5	3	3	5	5	4	5	5
Policy	Amt für Landwirtschaft	5	4	3	3	4	5	3	3	3	4	4	4	3
Policy	OFEV	5	3	5	5	3	5	3	2	2	5	4	5	5
Policy	Arenenberg	4	1	5	3	5	5	4	1	4	1	3	5	5
Policy	BLW	5	3	4	3	4	4	3	2	3	4	3	4	3
Policy	Grangeneuve - Section Agriculture - Etat de Fribourg	5	5	5	3	5	5	4	4	5	5	4	5	5
Policy	Amt für Natur und Umwelt Graubünden	5	5	5	5	5	5	3	4	5	5	5	5	5
Policy	Kanton Luzern - Landwirtschaftsamt	4	4	5	5	5	5	3	5	4	4	5	5	4
Policy	Servizio di protezione dell'ambiente	4	3	5	4	4	5	2	3	4	4	4	4	5
Research		5	5	2	2	5	4	5	4	5	4	3	5	3
Research	Agroscope	5	5	5	3	5	4	3	3	5	4	4	5	3
Research	agroscope	5	2	3	3	5	5	5	1	2	4	5	5	5
Research		5	5	5	5	5	5	5	5	5	5	5	5	5
Research	Université de Neuchâtel	3	2	4	3	5	5	4	3	5	2	3	5	5
Research	HAFL-Zollikofen	4	4	5	4	5	5	2	4	5	4	2	5	4
Research	HES Changins	4	3	5	5	5	5	3	3	5	4	5	5	5
Research	Agroscope	5	4	5	4	5	5	3	3	4	3	3	5	4
Research	Agroscope	4	4	5	5	5	5	3	3	5	3	5	5	5
Research	Fondation rurale interjurassienne	4	4	4	4	4	4	3	3	4	4	3	4	3

(see codes below).

CP- Cultivated terrestrial plants, fibres or other materials from cultivated plants grown for nutritional purposes, for direct use or processing, or as a source of energy; SP - Seeds, spores and other plant materials collected for maintaining or establishing a population (CICES code: 1.2.1.1); BR - Bioremediation (CICES code: 2.1.1.1); FST - Filtration/ sequestration/storage/accumulation of toxic substances (CICES code: 2.1.1.2); CE - Control of erosion rates (CICES code: 2.2.1.1); WR - Hydrological cycle and water flow regulation (CICES code: 2.2.1.3); P - Pollination (CICES code: 2.2.2.1); PD - Propagule dispersal (CICES code: 2.2.2.2); NH - Maintaining nursery populations and habitats (Including gene pool protection) (CICES code: 2.2.2.3); PDC - Pest and disease control (CICES code: 2.2.3.1; 2.2.3.2); WP - Weathering processes (CICES code: 2.2.4.1); D&F - Decomposition and fixing processes (CICES code: 2.2.4.2); CCF - Regulation of the chemical condition of freshwaters (CICES code: 2.2.5.1).

Annex 2 – Overall scoring of actors and by stakeholder group

Table A2 – Level of linking and scoring of actors by integrating relative importance of ecosystem services to soil fertility for different stakeholder groups and overall for all stakeholders combined.

Linkage		Land users - Farmers		Policy - Authorities		Research - Academia		Overall	
Actor Ranking	Nº Processes	Actor Ranking	Score	Actor Ranking	Score	Actor Ranking	Score	Actor Ranking	Score
Earthworms	21	Earthworms	102	Earthworms	92	Earthworms	91	Earthworms	93
Bacteria	19	Bacteria	93	Plants	84	Plants	84	Plants	85
Plants	19	Plants	93	Bacteria	82	Bacteria	80	Bacteria	83
Fungi	18	Fungi	89	Fungi	79	Enchytraeids	76	Fungi	79
Enchytraeids	17	Enchytraeids	83	Enchytraeids	75	Fungi	76	Enchytraeids	76
Collembola	14	Collembola	68	Collembola	61	Collembola	61	Collembola	62
Mycorrhiza	13	Mycorrhiza	63	Mycorrhiza	56	Mycorrhiza	55	Mycorrhiza	56
Ants	12	Ants	59	Ants	53	Ants	54	Ants	54
Nematodes	10	Nematodes	49	Nematodes	43	Nematodes	40	Nematodes	43
Acari	8	Acari	39	Acari	35	Acari	35	Acari	35
Protozoa	7	Protozoa	35	Protozoa	31	Protozoa	29	Protozoa	30
Coleoptera	4	Microalgae	20	Microalgae	18	Microalgae	19	Microalgae	18
Diplopoda	4	Diplopoda	19	Diplopoda	17	Diplopoda	17	Diplopoda	17
Isopods	4	Isopods	19	Isopods	17	Isopods	17	Isopods	17
Microalgae	4	Coleoptera	18	Coleoptera	16	Coleoptera	16	Coleoptera	16
Archaea	3	Archaea	15	Archaea	13	Archaea	13	Archaea	13
Gastropods	3	Insects	15	Gastropods	13	Gastropods	13	Gastropods	13
Insects	3	Gastropods	14	Insects	13	Insects	12	Insects	13
Spiders	2	Spiders	10	Spiders	8	Spiders	8	Spiders	8
Viruses	2	Viruses	10	Viruses	8	Viruses	8	Viruses	8

Annex 3 – List of standardized tests

Type	Group	Standard Org.	Method name
Ecolog.	Multiple	ISO	ISO 18311:2016 Soil quality — Method for testing effects of soil contaminants on the feeding activity of soil dwelling organisms — Bait-lamina test
Ecolog.	Multiple	OECD	Test No. 56: Guidance document on the breakdown of organic matter in litter bags
Ecolog.	Macrofauna	ISO	ISO 23611-5 Soil quality – Sampling of soil invertebrates – Part 5: Sampling and extraction of soil macro-invertebrates
Ecotox	Earthworms	ISO	ISO 11268-1:2012 Soil quality — Effects of pollutants on earthworms — Part 1: Determination of acute toxicity to Eisenia fetida/Eisenia andrei
Ecotox	Earthworms	ISO	ISO 11268-2:2023 Soil quality — Effects of pollutants on earthworms — Part 2: Determination of effects on reproduction of Eisenia fetida/Eisenia andrei and other earthworm species
Ecotox	Earthworms	ISO	ISO 11268-3:2014 Soil quality — Effects of pollutants on earthworms — Part 3: Guidance on the determination of effects in field situations
Ecotox	Earthworms	ISO	ISO 17512-1:2008 Soil quality — Avoidance test for determining the quality of soils and effects of chemicals on behaviour — Part 1: Test with earthworms (Eisenia fetida and Eisenia andrei)
Ecotox	Earthworms	OECD	Test No. 207: Earthworm, Acute Toxicity Tests
Ecotox	Earthworms	OECD	Test No. 222: Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei)
Ecotox	Earthworms	ASTM	ASTM E1676-12(2021) Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests with the Lumbricid Earthworm Eisenia Fetida and the Enchytraeid Potworm Enchytraeus albidus
Ecotox	Earthworms	Environment Canada	Biological Test Method: Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (Eisenia andrei or Dendrodrilus rubidus) Exposed to Contaminants in Soil
Ecolog.	Earthworms	ISO	ISO 23611-1:2018 Soil quality — Sampling of soil invertebrates — Part 1: Hand-sorting and extraction of earthworms
Ecotox	Enchytraeids	ISO	ISO 16387:2014 Soil quality — Effects of contaminants on Enchytraeidae (Enchytraeus sp.) — Determination of effects on reproduction
Ecotox	Enchytraeids	OECD	Test No. 220: Enchytraeid Reproduction Test
Ecolog.	Enchytraeids	ISO	ISO 23611-3:2019 Soil quality — Sampling of soil invertebrates — Part 3: Sampling and extraction of enchytraeids
Ecolog.	Microarthropods	ISO	ISO 23611-2:2006 Soil quality — Sampling of soil invertebrates — Part 2: Sampling and extraction of micro-arthropods (Collembola and Acarina)
Ecotox	Collembola	ISO	ISO 11267:2014 Soil quality — Inhibition of reproduction of Collembola (Folsomia candida) by soil contaminants
Ecotox	Collembola	ISO	ISO 17512-2:2011 Soil quality — Avoidance test for determining the quality of soils and effects of chemicals on behaviour — Part 2: Test with collembolans (Folsomia candida)
Ecotox	Collembola	OECD	Test No. 232: Collembolan Reproduction Test in Soil
Ecotox	Collembola	Environment	Biological Test Method: Test for Measuring Survival and Reproduction

		Canada	of Springtails Exposed to Contaminants in Soil
Ecotox	Mites	ISO	ISO 21285:2019 Soil quality — Inhibition of reproduction of the soil mite (Hypoaspis aculeifer) by soil contaminants
Ecotox	Mites	ISO	ISO 23266:2020 Soil quality — Test for measuring the inhibition of reproduction in oribatid mites (Oppia nitens) exposed to contaminants in soil
Ecotox	Mites	OECD	Test No. 226: Predatory mite (Hypoaspis (Geolaelaps) aculeifer) reproduction test in soil
Ecotox	Mites	Environment Canada	Biological Test Method: Test for Measuring Reproduction of Oribatid Mites Exposed to Contaminants in Soil
Ecolog.	Nematodes	ISO	ISO 23611-4:2022 Soil quality — Sampling of soil invertebrates — Part 4: Sampling, extraction and identification of soil-inhabiting nematodes
Ecotox	Nematodes	ISO	ISO 10872:2020 Water and soil quality — Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of Caenorhabditis elegans (Nematoda)
Ecotox	Nematodes	ASTM	ASTM E2172-22 Standard Guide for Conducting Laboratory Soil Toxicity Tests with the Nematode Caenorhabditis elegans
Ecolog.	Microorganisms	ISO	ISO 11063:2020 Soil quality — Direct extraction of soil DNA
Ecolog.	Microorganisms	ISO	ISO 14238:2012 Soil quality — Biological methods — Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes
Ecolog.	Microorganisms	ISO	ISO 14240-1:1997 Soil quality — Determination of soil microbial biomass — Part 1: Substrate-induced respiration method
Ecolog.	Microorganisms	ISO	ISO 14240-2:1997 Soil quality — Determination of soil microbial biomass — Part 2: Fumigation-extraction method
Ecolog.	Microorganisms	ISO	ISO 16072:2002 Soil quality — Laboratory methods for determination of microbial soil respiration
Ecolog.	Microorganisms	ISO	ISO 17601:2016 Soil quality — Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil
Ecolog.	Microorganisms	ISO	ISO 23753-1:2019 Soil quality — Determination of dehydrogenases activity in soils — Part 1: Method using triphenyltetrazolium chloride (TTC)
Ecolog.	Microorganisms	ISO	ISO 23753-2:2019 Soil quality — Determination of dehydrogenases activity in soils — Part 2: Method using iodotetrazolium chloride (INT)
Ecolog.	Microorganisms	ISO	ISO/TS 29843-1:2010 Soil quality — Determination of soil microbial diversity — Part 1: Method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis
Ecolog.	Microorganisms	ISO	ISO/TS 29843-2:2021 Soil quality — Determination of soil microbial diversity — Part 2: Method by phospholipid fatty acid analysis (PLFA) using the simple PLFA extraction method
Ecotox	Microorganisms	ISO	ISO 15685:2012 Soil quality — Determination of potential nitrification and inhibition of nitrification — Rapid test by ammonium oxidation
Ecotox.	Microorganisms	ISO	ISO 18187:2016 Soil quality — Contact test for solid samples using the dehydrogenase activity of Arthrobacter globiformis
Ecotox.	Microorganisms	ISO	ISO 20130:2018 Soil quality — Measurement of enzyme activity patterns in soil samples using colorimetric substrates in micro-well plates

Ecotox.	Microorganisms	ISO	ISO/TS 20131-1:2018 Soil quality — Easy laboratory assessments of soil denitrification, a process source of N2O emissions — Part 1: Soil denitrifying enzymes activities
Ecotox.	Microorganisms	ISO	ISO/TS 20131-2:2018 Soil quality — Easy laboratory assessments of soil denitrification, a process source of N2O emissions — Part 2: Assessment of the capacity of soils to reduce N2O
Ecotox.	Microorganisms	ISO	ISO/TS 22939:2019 Soil quality — Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates
Ecotox.	Microorganisms	OECD	Test No. 217: Soil Microorganisms: Carbon Transformation Test
Ecotox.	Microorganisms	OECD	Test No. 216: Soil Microorganisms: Nitrogen Transformation Test
Ecotox	Mycorrhiza	ISO	ISO/TS 10832:2009 Soil quality — Effects of pollutants on mycorrhizal fungi — Spore germination test
Ecolog.	Plants	ISO	ISO 21479:2019 Soil quality — Determination of the effects of pollutants on soil flora — Leaf fatty acid composition of plants used to assess soil quality
Ecotox	Plants	ISO	ISO 11269-1:2012 Soil quality — Determination of the effects of pollutants on soil flora — Part 1: Method for the measurement of inhibition of root growth
Ecotox	Plants	ISO	ISO 11269-2:2012 Soil quality — Determination of the effects of pollutants on soil flora — Part 2: Effects of contaminated soil on the emergence and early growth of higher plants
Ecotox	Plants	ISO	ISO 17126:2005 Soil quality — Determination of the effects of pollutants on soil flora — Screening test for emergence of lettuce seedlings (Lactuca sativa L.)
Ecotox	Plants	ISO	ISO 18763:2016 Soil quality — Determination of the toxic effects of pollutants on germination and early growth of higher plants
Ecotox	Plants	ISO	ISO 22030:2005 Soil quality — Biological methods — Chronic toxicity in higher plants
Ecotox	Plants	ISO	ISO 29200:2013 Soil quality — Assessment of genotoxic effects on higher plants — Vicia faba micronucleus test
Ecotox	Plants	OECD	Test No. 227: Terrestrial Plant Test: Vegetative Vigour Test
Ecotox	Plants	OECD	Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test
Ecotox	Plants	ASTM	ASTM E1197-12(2021) Standard Guide for Conducting a Terrestrial Soil-Core Microcosm Test
Ecotox	Plants	EC	Biological Test Method: Test for Measuring Emergence and Growth of Terrestrial Plants Exposed to Contaminants in Soil
Ecotox	Plants	EC	Biological Test Method: Test for Growth in Contaminated Soil Using Terrestrial Plants Native to the Boreal Region

Annex 4 - Minutes of the online expert workshop on “Bioindicators for monitoring plant protection product residues” September 2023

External participants: Anna-Sofia Hug - NABO, Céline Pelosi - INRAE, Avignon, Claire Le Bayon - University Neuchatel, Cornelis van Gestel - Vrije Universiteit Amsterdam, Elias Barmettler – Agroscope, Fabrice Martin-Laurent - INRAE, Dijon, Florian Walder – Agroscope, Franco Widmer – Agroscope, Fritz Oehl – Agroscope, José Paulo Sousa - Universidade de Coimbra, Sebastian Höss – ECOSSA, Sylvie Cotelle - Université de Lorraine, Thierry Heger - HES-Changins.

Earthworms

Questions provided to help guide the workshop:

- *Would the standard earthworm reproduction test (ISO 11268-2 - Eisenia fetida / andrei) be a good ecotoxicological indicator?*
- *Would non-standard species (e.g. A. caliginosa) be more appropriate despite higher technical demands?*
- *If the earthworm reproduction test is not feasible, would the enchytraeid reproduction test (ISO 16387) be a suitable surrogate for all oligochaetes?*
- *Can the bait-lamina test (ISO 18311) provide informative data in the sampling period of February/March for earthworms?*
- *Is earthworm sampling (ISO 23611-1) feasible in Switzerland in February/March?*
- *Is the sampling effort necessary for measuring small effect sizes (e.g. 10% effect) feasible under routine monitoring?*

Note: Not all questions may have been discussed

Experts' opinions on the 3 sampling options:

Earthworms	Indicator type	Sampling option 1: Soil samples provided	Sampling option 2: Soil sampling + site assessment	Sampling option 3: Full sampling
	Ecotoxicology	The earthworm reproduction test (ISO 11268-2) is an appropriate ecotoxicological indicator. Both the fitness (i.e. no previous exposure to PPP) and the exact identity of the earthworms (i.e. correct species) must be guaranteed when ordering earthworms from suppliers. The ISO 11268-2 has recently been updated to include potential alternative earthworm species to *E. fetida/andrei,* such as *A. caliginosa. A. caliginosa* is more sensitive than *Eisenia sp.* but is also more difficult to culture. The enchytraeus reproduction test (ISO 16387) may be used as a surrogate for the earthworm reproduction test, as there is no difference in sensitivity compared to earthworms (*E. crypticus/albidus* versus *E. fetida/andrei*). In addition, earthworms are sometimes picky about soil conditions (soil properties). This is not the case with enchytraeids, making them a good substitute. Looking for bioaccumulation of PPP in earthworms could be an indication of risk. An OECD guideline (n°317) is available to assess bioaccumulation in terrestrial oligochaetes.		
	Ecology	No recommendation	Biomass of earthworm surface casts (collected over one square meter), as well as earthworm individual biomass and juvenile to adult ratio would be relevant endpoints. The bait lamina test (ISO 18311) measuring soil organisms feeding activity would be an appropriate complement but is not exclusive to earthworms (See General remarks below).	Earthworm hand sampling according to ISO 23611-1 can be performed. Ideally, identification should be to the species level (some species are more exposed to PPP in the field than others), but this requires advanced knowledge or DNA barcoding. Earthworm monitoring, such as RMQS in France, goes to species level. In addition, abundance, earthworm individual biomass, ecological categories and juvenile to adult ratio can also be evaluated.

Enchytraeids

Questions provided to help guide the workshop:

- *Would the Enchytraeid reproduction test, with the adaptations of Castro-Ferreira et al., 2012 for Enchytraeus crypticus be a good indicator under the ecotoxicological line of evidence?*
- *Can bait-lamina provide informative data in the sampling period of February March for enchytraeids?*
- *Is enchytraeid sampling in February/March feasible and pertinent in Switzerland?*

Note: Not all questions may have been discussed

Experts' opinions on the 3 sampling options:

Enchytraeids	**Indicator type**	**Sampling option 1: Soil samples provided**	**Sampling option 2: Soil sampling + site assessment**	**Sampling option 3: Full sampling**
	Ecotoxicology	Enchytraeus reproduction test (ISO 16387) is a well suited ecotoxicological indicator. The test is very easy to perform and requires only a small amount of soil. *Enchytraeus crypticus* would be a good candidate as the test variability is much lower compared to other enchytraeid species. Enchytraeids are also proposed as a surrogate for all oligochaetes (see section on earthworms) for ecotoxicological tests.		
	Ecology	No recommendation	No recommendation. The bait lamina test (ISO 18311) is not adapted to provide information on enchytraeids in the field. Although enchytraeids also feed on bait, it is difficult to see anything when earthworms are also present. When both organisms are present, the feeding activity is dominated by earthworms rather than enchytraeids, with earthworms having a higher feeding rate than enchytraeids.	Enchytraeid sampling according to ISO 23611-3 could be performed. Total abundance is not an appropriate endpoint and there are no ecological categories as for earthworms. Identification to genus level could provide interesting information (species level would be ideal).

Microarthropods (Collembola and Mites)

Questions provided to help guide the workshop:

- *Would the Collembola reproduction test under ISO 11267 with Folsomia candida be a good ecotoxicological indicator?*
- *Can bait-lamina provide meaningful data for microarthropods in the sampling period of February/March?*
- *Is microarthropod sampling according to ISO 23611-2 in February/March feasible and pertinent in Switzerland?*
- *Is a combined functional (bait-lamina) and structural (Microarthropod sampling) approach adequate under sampling option 3?*

Note: Not all questions may have been discussed

Experts' opinions on the 3 sampling options:

Microarthropods	**Indicator type**	**Sampling option 1: Soil samples provided**	**Sampling option 2: Soil sampling + site assessment**	**Sampling option 3: Full sampling**
	Ecotoxicology	The collembola reproduction test (ISO 11267) is adequate as an ecotoxicological indicator for collembola. Regarding species: *F. candida* has similar sensitivity compared to *Folsomia fimetaria,* but *F. fimetaria* has slightly more technical requirements (e.g. more difficult to select males and females) and higher variability, making it difficult to see small effect sizes. Therefore, for routine testing, *F. candida* will be the ideal candidate, even though it is less common in the field compared to *F. fimetaria*.		
	Ecology	No recommendation. eDNA may be used in the future but still requires major development.	The bait-lamina test (ISO 18311) is not suitable for measuring microarthropod activity in the field (nor is the litter bag test). The bait lamina test is not necessarily species specific but will show activity of soil organisms in general (see general remarks on the bait lamina test below).	Microarthropod sampling could be done using ISO 23611-2, focusing on springtails, as they are easier to identify to species level compared to mites. Mites are much more difficult to identify than springtails, but the main groups could still be analyzed. For a monitoring approach, the morphotype or morphospecies analysis might be sufficient, depending on the level of precision we want to achieve. Focusing on springtails will also allow comparison with the ecotoxicological line of evidence. Metabarcoding seems promising. However, the database is not yet complete (species names may not be available).

Nematodes

Questions provided to help guide the workshop:

- *Is the standard single species C. elegans test (ISO 10872), adequate as an ecotoxicological indicator?*
- *Is nematode sampling (ISO 23611-4) in February/March feasible and pertinent in Switzerland.*
- *Are nematode community indices sufficiently sensitive to PPP residues or more sensitive to management practices?*

Note: Not all questions may have been discussed

Experts' opinions on the 3 sampling options:

Nematodes	**Indicator type**	**Sampling option 1: Soil samples provided**	**Sampling option 2: Soil sampling + site assessment**	**Sampling option 3: Full sampling**
	Ecotoxicology	The C. elegans toxicity test (ISO 10872) is well suited as an ecotoxicological indicator, although it may be a problem for soils with high clay content.		
	Ecology	The soil nematode community could be assessed according to (ISO 23611-4). Maturity and structure indices would be particularly appropriate as they are highly responsive to chemicals. Nematodes are very sensitive to fungicides (less to insecticides). Nematodes can in principle also be sampled during winter.		

Microorganisms (Bacteria, Fungi, Mycorrhiza, Protozoa)

Questions provided to help guide the workshop:

- *What standard methods would the experts recommend for ecological and ecotoxicological microorganism indicators to measure the effects of PPP residues, considering the standards listed in Annex 1?*
- *Are general microorganism indicators adequate for representing Bacteria, Fungi, Protozoa and Mycorrhiza in a combined approach (e.g. molecular approaches)?*
- *Are there well-established ecological or ecotoxicological methods for microorganism in general not listed in Appendix 1 that should be considered?*
- *Are there adequate ecological and ecotoxicological methods specific for Bacteria / Fungi / Protozoa / mycorrhiza?*
- *For mycorrhiza is the mycorrhizal spore germination test (ISO/TS 10832) with G. mosseae adequate as an ecotoxicological indicator?*

Note: Not all questions may have been discussed

Experts' opinions on the 3 sampling options:

Bacteria Fungi	Indicator type	Sampling option 1: Soil samples provided	Sampling option 2: Soil sampling + site assessment	Sampling option 3: Full sampling
	Ecotoxicology	Functional approaches could be a gain by targeting a group of organisms. EFSA recommended nitrifying groups for N cycling and AMF. However, other functional groups and enzymatic measurements need to be targeted, but this requires fresh soil. Nitrification is a good target and should be an option for the ecotoxicological line of evidence. Several ISO methods are available for a direct measurement of this endpoint. For example, the EU-funded ARISTO project (2020) for the assessment of the soil microbial toxicity of pesticides is specifically targeting nitrification with various assessment methods. Biological N fixation is sensitive to PPP contamination (also for N fixation by Mycorrhiza). Enzyme-based assays and multiple enzyme protocols could also be used. However, care should be taken in interpreting the data as factors other than PPP could influence the enzymatic response. The Ecoplate is an easy assay to measure the metabolism of microbial community and to measure changes of microbial communities according to the use of PPP (Ecoplates were unfortunately not included in the last ISO revision). The *Arthrobacter globiformis* test (ISO 18187) measuring dehydrogenase activity could also be used for testing the soil matrix in the ecotoxicological line of evidence. Soil respiration should not be recommended as the method is not very sensitive and results can vary greatly depending on the sampling period, soil moisture and other factors. Controlled laboratory conditions can slightly reduce variability. Culturomics is an innovative tool for assessing the impact of anthropogenic practices on cultivable micro-organisms. Winter is not the best season for microbes (some are not active at all). Sampling should preferably be done in spring (February/March) when temperatures are higher but before the first treatments. As with earthworms and fungi, soil water content is an important criterion (not just temperature).		
	Ecology	DNA-based tools are powerful tools for assessing the composition and diversity of microbial communities. They are well established and easy to perform, but it may be difficult to link them to pesticide exposure. The advantage is that a similar extraction can be performed for different groups of organisms (bacteria, fungi, protozoa...). However, monitoring should not be based on a single DNA extraction from a single sample, as this is too risky. Soil samples must be frozen and stored at - 40°C or - 80°C (-18°C is not adequate). It would be even better to store DNA extracts instead of soil samples. The ISO 17601 based on quantitative PCR (qPCR) for measuring abundance of selected microbial gene sequence from soil DNA extract could be used. It is is under revision for targeting functional group (among which nitrifiers) and should be published in 1 or 2 years.		

		Tea bags is a well-established and easy to use method for looking at soil quality /soil functioning, but uncertainties remain about how response relates to PPP effect. Use in winter could be difficult due to the decrease in microbial activity.		

Mycorrhiza	**Indicator type**	**Sampling option 1: Soil samples provided**	**Sampling option 2: Soil sampling + site assessment**	**Sampling option 3: Full sampling**
	Ecotoxicology	There is considerable evidence from microbial indicators that mycorrhizal fungi appear to be sensitive to PPP residues. Mycorrhiza ecotoxicity tests with soil sample using pre-symbiotic and symbiotic phases can be used: the ISO mycorrhizal ecotoxicity test on spore germination (currently under revision) is sensitive and could be implemented. An assay for the mycorrhizal symbiotic phase (root colonization) should also be available soon.		
	Ecology	Molecular approaches similar to those described for bacteria and fungi can be used.		

Protozoa	**Indicator type**	**Sampling option 1: Soil samples provided**	**Sampling option 2: Soil sampling + site assessment**	**Sampling option 3: Full sampling**
	Ecotoxicology	No recommendation		
	Ecology	Protozoa may be even more sensitive to some PPPs than bacteria and fungi and could therefore complement this group of organisms. Protozoa and algae/microalgae can be assessed using the same extraction protocol as for bacteria and fungi.		

Plants

Questions provided to help guide the workshop:

- *Would germination and seedling growth be a good ecotoxicological indicator? If yes, which standard guideline is most adequate?*
- *Different species can be selected in the guidelines, which indicator plant species should be prioritized?*
- *For the ecological line of evidence is there an appropriate indicator to consider under the three sampling options?*

Note: Not all questions may have been discussed

Experts' opinions on the 3 sampling options:

Plants	**Indicator type**	**Sampling option 1:** **Soil samples provided**	**Sampling option 2:** **Soil sampling + site assessment**	**Sampling option 3:** **Full sampling**
	Ecotoxicology	Ecotoxicity testing with cover or winter crops would be a good approach. These plant species are representative of the February/March sampling period. OECD guideline 227 provide a substantial list of species, including cover or winter crops, that might be used and for which ecotoxicological data might be available. Several endpoints may be considered for plants, in particular germination but also genotoxicity, as some pesticides are mutagens. Nutrient factors can have important influence on plant growth performance and must be considered when looking for PPP residue effects. It would be important to consider below-ground plant parts / root network (biomass, root depth, exudates, associated symbiotic microflora (AMF, bacteria, etc.)) but could be difficult to assess and would be more easily achieved under controlled conditions. Algae would also be a nice approach to assess effect of PPP residues (should be included under microorganism group).		
	Ecology	The performance of cover crops in winter may be a good indicator. Counting weeds in the field may be an indication of PPP residues.		

Ants

Questions provided to help guide the workshop:

- *Are there well-established methods for ant ecotoxicology?*
- *Are there adequate surrogates species or groups to representative the sensitivity of Ants?*
- *Is macrofauna sampling according to ISO 11268-5 adequate to represent this group in the ecological line of evidence?*

Note: Not all questions may have been discussed

Experts' opinions on the 3 sampling options:

Ants	**Indicator type**	**Sampling option 1: Soil samples provided**	**Sampling option 2: Soil sampling + site assessment**	**Sampling option 3: Full sampling**
	Ecotoxicology	No recommendation, no standard methods available.		
	Ecology	No recommendation. Some tests have been performed , but they are very complex and not yet standardized.		

General remarks

Bait lamina test (ISO 18311)

The bait lamina test should be used as a general surrogate for the assessment of soil function and not linked to any particular group of organisms. It is an interesting complement to other bioindicators in the field, is easy to implement and does not require a high level of expertise. However, the bait lamina test is temperature dependent and will show a reduced response in winter. Sensitivity to PPP still needs to be evaluated.

Long Lived Species

The issue of the time shift of effect should be considered: even if sampling is done in winter, we might see an effect that comes from the summer application, especially for slowly reproducing organisms.

Management information

For the ecological indicators it is important to have information of management and the time since changes of management occurred.