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Final report

Correctly assessing the performance and threats of microorganisms in agricultural soils – identifying meaningful endpoints under field-relevant pesticide, biocide and pharmaceutical exposure

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Abstract: Correctly assessing the performance and threats of microorganisms in agricultural soils – identifying meaningful endpoints under field-relevant pesticide, biocide and pharmaceutical exposure

The MICROSOIL project aimed to identify meaningful endpoints for assessing effects on microorganisms exposed to chemicals in agricultural soils.

In the actual risk assessment for plant protection products (PPP), the soil microbial communities and their ecosystem services are considered not to be at risk if the maximum predicted environmental concentration causes no more than a 25% effect on N-transformation (OECD 216) at least after 100 days. A literature review idendified five methods considered to be most suitable to replace or supplement the currently used OECD 216 tests: potential nitrification (ISO 15685), substrate induced respiration using MicroResp™, enzymatic activity (ISO 20130), spore germination of arbuscular mycorrhiza fungi (AMF) (ISO 10832) and the fingerprinting method ARISA. For comparison, these methods were experimentally tested in the laboratory with six test substances in three different soils. Based on the results obtained in MICROSOIL and considering the recommendations of EFSA PPR panel (2017), it is advised to enhance the first-tier risk assessment by an additional test on bacterial function (ISO 20130, enzymatic activity) and a structural test covering effects on AMF. A fingerprinting method to assess effects on the community structure is also recommended, but needs further research before implantation in the regulatory practice.

Single-species toxicity tests with microorganisms play an important role during the ERA of antibiotics (Brandt et al. 2015). While the EUCAST database provides data on minimum inhibitory concentrations (MIC) for clinical bacteria, similar information for soil bacteria is lacking. Therefore, within MICROSOIL, MICs were determined for four soil-relevant bacteria strains. For the four antibiotics tested, no systematic difference between MICs for the tested soil and clinical strains (from the EUCAST database) was found. For three of the four antibiotics tested, the soil bacteria showed a similar sensitivity than the clinical strains. Only in one case, Chlortetracyline hydrochlorine, the MIC of the soil bacterium *Acidovorax facilis* was lower than all MICs of tested clinical strains. Due to missing data for organic carbon-water partition co-efficient (K_{OC}) or soil predicted environmental concentration (PEC_{soil}), risk quotients could not be calculated except for tiamulin hydrogen fumarate. For this antibiotic, the MIC indicated a higher risk than the standard ecotoxicological tests (risk quotient > 100). Thus, current risk assessment for veterinary pharmaceuticals might not cover the development of antibiotic resistance in environmentally relevant soil bacteria.

The degradability of PPPs in soil is currently estimated in laboratory tests (OECD Test Guideline 307) with standardised soils and one maximum application rate. However, agricultural used soils are exposed to multiple chemicals. Within one growing season, several PPPs are applied which may include more than one active substance, but the occurrence of multiple residues in agricultural used soils is not reflected by the current risk assessment schemes for PPPs or other chemicals. Thus, it was analysed whether multiple applications of one substance, binary mixtures and background contamination affect the degradation time in soils using the active substances, pyraclostrobin and ethofumesate in the representative standard soils, RefeSol 02A and LUFA 2.1. Multiple applications of the same substance as well as the presence of another substance in soil may have both positive and negative effects on the degradation rate of the test substances. However, a negative impact on the degradation rate was shown for ethofumesate when applied to a soil where a second substance (here: pyraclostrobin) was already present. The results indicate that further research on multiple exposure is necessary to consider more realistic use patterns and agricultural practices.

The MICROSOIL project underlines the necessity of the European commission's mandate to update the Guidance document on terrestrial ecotoxicology (EFSA, 2025) and provides detailed recommendations how to update this guidance document.

Kurzbeschreibung: Korrekte Bewertung der Leistung und Gefährdung von Mikroorganismen in landwirtschaftlichen Böden - Ermittlung aussagekräftiger Endpunkte bei feldrelevanter Pestizid-, Biozid- und Arzneimittelexposition

Ziel des Projektes MlCROSOIL war es aussagekräftige Endpunkte für die Bewertung der Auswirkungen von Pflanzenschutzmitteln (PSM), Tierarzneimitteln und Bioziden auf Mikroorganismen in landwirtschalftlichen Böden zu identifizieren.

Bei der aktuellen Risikobewertung für Pflanzenschutzmittel (PSM) wird davon ausgegangen, dass die mikrobiellen Gemeinschaften im Boden und ihre Ökosystemleistungen nicht gefährdet sind, wenn die vorhergesagte Höchstkonzentration in der Umwelt zumindest nach 100 Tagen nicht mehr als 25 % der Stickstoff (N)-Transformation (OECD 216) beeinträchtigt. Auf Basis einer Literaturrecherche in Kombination mit einem Bewertungsschema wurden fünf Methoden identifiziert, die sich am Besten eignen könnten, um den momentan verwendeten OECD 216-Tests zu ersetzen oder zu ergänzen: potenzielle Nitrifikation (ISO 15685), substratinduzierte Atmung mit MicroRespTM, enzymatische Aktivität (ISO 20130), Sporenkeimung von arbuskulären Mykorrhizapilzen (AMF) (ISO 10832) und die Fingerprinting-Methode ARISA. Zum Vergleich der Empfindlichkeit wurden diese Methoden auf sechs Testsubstanzen in drei Böden getestet. Auf der Grundlage der MICROSOIL Ergebnisse und unter Berücksichtigung von EFSA PPR panel (2017) wird empfohlen, die Risikobewertung der ersten Stufe um einen zusätzlichen Test zur Funktion der Bakterien (ISO 20130, enzymatische Aktivität) und einen strutkurellen Test zu den Auswirkungen auf AMF zu erweitern. Eine Fingerprinting-Methode zur Bewertung der Auswirkungen auf die Struktur der Lebensgemeinschaften wird ebenfalls empfohlen, muss aber vor der Einführung in die Regulierung von Chemikalien weiter erforscht werden.

Tests zur Toxizität einzelner Spezies mit Mikroorganismen spielen bei der Risikobewertung von Antibiotika eine wichtige Rolle (Brandt et al. 2015). Während die EUCAST-Datenbank Daten zu minimalen Hemmkonzentrationen (MHK) für klinische Bakterien liefert, fehlen ähnliche Informationen für Bodenbakterien. Daher wurden im Rahmen von MICROSOIL die MHKs für vier bodenrelevante Bakterienstämme bestimmt. Für drei der vier getesteten Antibiotika wurde kein systematischer Unterschied zwischen den MHKs für die getesteten Boden- und klinischen Stämme (aus der EUCAST-Datenbank) festgestellt. Nur in einem Fall, Chlortetracylinhydrochlorin, war die MHK eines Bodenbakteriums (*Acidovorax facilis*) niedriger als alle MHKs der klinischen Stämme. Aufgrund sonst fehlender Daten zur Exposition konnte nur für Tiamulinhydrogenfumarat ein Risikoquotient (RQ) bestimmt werden. Hier wurde ein höheres Risiko der MHK als durch die ökotoxikologischen Standardtests angezeigt. Die derzeitige Risikobewertung für Tierarzneimittel deckt also möglicherweise die Entwicklung von Antibiotikaresistenzen in umweltrelevanten Bodenbakterien nicht ab.

Die Abbaubarkeit von PSM wird routinemäßig in Laborstudien mit standardisierten Böden und einer maximalen Aufwandmenge bestimmt (OECD 307). Innerhalb einer Vegetationsperiode können aber mehrere PSM aufgebracht werden, die auch mehre Wirkstoffe enthalten können. Dies wird jedoch in der aktuellen Risikobewertung von Chemikalien nicht berücksichtigt. Anhand der Testung von Pyraclostrobin und Ethofumesat bei Nutzung von repräsentativen Standardböden (RefeSol 02A und LUFA 2.1) wurde untersucht, ob Mehrfachanwendungen eines Wirkstoffes, eines binären Gemisches und auch Hintergrundkontaminationen die Abbaubarkeit im Boden beeinflussen. Mehrfache Applikation derselben Substanz sowie das Vorhandensein einer anderen Substanz im Boden hatten sowohl positive als auch negative Auswirkungen auf die Abbaugeschwindigkeit der Testsubstanzen. Eine negative Auswirkung auf die

Abbaugeschwindigkeit wurde beispielsweise für Ethofumesat nachgewiesen, wenn es auf einen Boden aufgebracht wurde, in dem bereits Pyraclostrobin vorhanden war. Die Ergebnisse deuten darauf hin, dass weitere Untersuchungen zur Mehrfachexposition erforderlich sind, um realistischere Anwendungsmuster und landwirtschaftliche Praktiken zu berücksichtigen.

Das MICROSOIL Projekt unterstreicht die Notwendigkeit des Auftrags der Europäischen Kommission, den Leitfaden zur terrestrischen Ökotoxikologie zu aktualisieren (EFSA, 2025) und gibt konkrete Empfehlungen wie dieser Leitfaden aktualisiert werden sollte.

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AMF	Arbuscular Mycorrhizal Fungi
ATCC	American Type Culture Collection
CEC	Cation Exchange Capacity
CLPP	Community Level Physiological Profiles
Dw	Dry weight
DDAC	Didecyldimethylammonium chloride
DFOP	Double First Order in Parallel (kinetic model)
DT50 / 90	Time to 50 / 90 % degradatione
ECx	Effective Concentration for x % effect
EFSA	European Food Safety Authority
EGS	Euro-GeoSurveys
ERA	Environmental Risk Assessment
ESDAC	European Soil Data Centre
FOMC	First Order Multi Compartment (kinetic model)
GAP	Good Agricultural Practice
GEMAS	Geochemical mapping of agricultural soil
HS	Hockey stick (kinetic model)
ICx	Inhibitory Concentration of x % inhibition
ISO	International Organization for Standardization
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOEC	Lowest Observed Effect Concentration
MoA	Mode of action
NOEC	No Observed Effect Concentration
OECD	Organisation for Economic Co-operation and Development
PLEL	Phospholipid ether lipids
PLFA	Phospholipid-derived fatty acids
PPP	Plant protection product
PPR panel	Panel on Plant Protection Products and their Residues
SFO	Single First Order (kinetic model)
SO	Scientific Opinion
TG	Test guideline
UBA	Umweltbundesamt (German Environment Agency)
WHC_{max}	Maximum water holding capacity
WP	Work package

Summary

Soil health is the most important driving factor for sustainable agriculture and must be maintained and promoted accordingly. Microorganisms such as bacteria or (arbuscular mycorrhizal) fungi are an essential and integral part of living soil and influence various biogeochemical cycles of major nutrients such as carbon, nitrogen, sulphur and phosphorus. They play a greater role in maintaining soil health than other biological components of soil (Sathya et al. 2016). Microorganisms are actively involved in soil formation by initiating the process of biological weathering of rocks, decomposition of organic matter and nutrient cycling (Kaviya et al. 2019). They are responsible for various biological transformations of different sources of carbon and macro- and micronutrients, which facilitate the subsequent establishment of soil-plant-microbe interactions (Sahu et al. 2017). The humus content in Central European soils is continuously decreasing (Jacobs et al. 2013). Due to multiple pressures and an inadequate environmental assessment approach with regard to the microorganisms in soils the basic protection goal of "maintaining soil biodiversity" cannot be achieved. As a result, resilience can decrease significantly and with it the ability of soil microorganisms to react to e.g. stressors such as climate change. A more differentiated risk assessment for soil microorganisms is therefore necessary, which is equally weighted towards the other assessment areas such as soil invertebrates or non-target arthropods.

The aim of the project was to identify test methods and endpoints which can be used within an environmental risk assessment for chemicals to ensure the protection of soil microorganisms and thus the important ecosystem services of soils against various pollutants and to determine their applicability in practice. Three main questions were answered:

1. Which methods are suitable for recording and describing changes in soil function and the structural composition of the microbial community in soils for regulatory assessment of chemicals like plant protection products, biocides and veterinary pharmaceuticals? (Chapter 2)
2. Second key question: Can the exposure of soil microorganisms to antibiotics/veterinary pharmaceuticals lead to the development of resistance genes and are there interactions with other chemical stressors? (Chapter 3)
3. What effect do multiple applications of active substances from plant protection products and a background pollution have on the microbial degradation of pollutants in soils? (Chapter 4)

Effects on function and structure of soil microbial communities

A literature search was conducted to compile a list with available test methods for recording the functional response of microorganisms to chemicals (e.g. enzymatic tests, Biolog© or MicroResp™, mycorrhizal fungi) considering the recommendations of EFSA PPR panel (2017). Afterwards, the suitability of chosen test methods for identifying changes in the soil microbial function and the structural composition of the soil microbial community was tested exemplarily using six different test substances and three different natural soils.

To find adequate publications in peer-reviewed journals, a **literature search** was performed using Science Direct, Google Scholar, Web of Science and Scopus as well as in an internal literature database for the identification of suitable test methods for soil microorganisms exposed to chemicals. The literature search was performed from April to October 2021. Care was taken to ensure that the methods identified are suitable for determining the influence of chemicals on soil functions as well as on structural changes in the soil microbial community. Furthermore, the methods were supposed to cover both bacteria and fungi and should also include exo- and endo-enzymes in soil. The terms of the literature search focused on three different areas: *(i)* general terms, *(ii)* direct presentation of methods/assays and *(iii)* effect

studies. The general terms were used to find relevant literature regarding the measurement of soil function and soil microbial structure and their assessment with no particular description of the presented method. From this literature, the source of the described method, which appeared to be appropriate for the testing strategy, was retrieved. In addition, terms focusing on "methods" or "assays" were used to identify literature directly presenting certain methods to determine or assess relevant soil processes. In a last step, we searched for studies presenting the effect of pesticides or other chemicals on soil microorganisms to describe the sensitivity of the methods. Evaluation parameters were defined to distinguish between different methods. The parameters proposed in the following pages are based on existing evaluations in the literature, but have been modified and supplemented to a greater or lesser extent, if necessary. Thiele-Bruhn et al. (2020) combined and slightly adapted parameters used by Pulleman et al. (2012) and Faber et al. (2013) and evaluated different methods based on a score from 1 to 5 in combination with a color code consisting of red, orange, yellow, light green and green. This approach may increase the precision of the assessment, but complexity is raised and the decision about suitability of a test method becomes more difficult. Therefore, in a first step the assessment based on a traffic light system was kept as simple as possible. For the assessment within this project five parameters were selected: (i) practicability, (ii) estimated costs, (iii) replicability/reproducibility, (iv) feasibility depending on soil type and (v) relevance for regulatory purposes. These parameters were considered essential for the assessment of methods for regulatory purposes. Accordingly, we used a simple traffic light system for each individual parameter. In this system green, yellow and red stand for reasonable, possible and not reasonable, respectively. Based on the literature search and the evaluation of different methods to assess the effect on soil functions and structure, five methods with the highest score each for aerobic heterotrophic soil microorganisms, nitrifiers, enzyme activity, AMF and structural soil diversity were elaborated.

Activity of aerobic, heterotrophic microorganisms: Substrate induced respiration using MicroResp™. The method appeared to be a promising add-on to the standard test (OECD 216) to address several nutrient cycles as also N-, P-, and S-cycles are presented. It is a simple and rapid method which can be performed within one day. In addition, the method was already recommended by the EFSA (European Food Safety Authority 2017).

Activity of nitrifying bacteria: The ammonium oxidation test (ISO 15685) appeared to be appropriate as additional parameter for nitrogen mineralisation (alternative to N-transformation test). It is a simple and rapid method which can be performed within one day. It is very sensitive as only a small microbial group with low potential for resilience is addressed. Although the environmental relevance is limited, it can be used as indicator for toxic effects.

Enzyme activity: The ISO 20130 "Soil quality — Measurement of enzyme activity patterns in soil samples using colorimetric substrates in micro-well plates" was used to assess the effect on the activity of exoenzymes. The test is comparable with the method described in ISO 22939 but cheaper since no fluorogenic substrates have to be used. Furthermore, this method offers a higher flexibility in the selection of the substrates and therefore in the considered enzymatic activities. While only a limited number of fluorogenic substrates is available, every substrate with sufficient solubility can be used in the method described in ISO 20130. Unlike methods in which the effect on only a small group of enzymes is investigated, with this method the effect on a number of different groups can be considered.

Fungal activity: The ISO 10832 "Effects of pollutants on mycorrhizal fungi — Spore germination test" was used to observe the effect on fungi since it appeared to be the most suitable test to address potential effects on this ecologically relevant group which is otherwise not covered by the proposed tests and was also already recommended by the EFSA (European Food Safety Authority 2017).

Microbial structure: ARISA was determined as a promising method which provide in-depth knowledge about effects on the microbial community structure and shifts within the community. However, these methods are expensive and the evaluation might be difficult. To better address the biodiversity of the soil microorganism community, the ARISA method might be an appropriate fingerprinting method to be used exemplary. The method was evaluated as less expensive than other methods at the time of the literature search and not so sophisticated to require a really high-level statistical evaluation.

Once the test methods were chosen, they were applied by **testing six test substances** ethofumesate, tebuconazole, propamocarb hydrochloride, pyraclostrobin, tiamulin hydrogen fumarate and didecyl-dimethylammonium chloride in the **three soils** RefeSol 02A, RefeSol 04A and Lufa 2.1 to draw conclusions on the sensitivity of the methods and the influence of different soil properties on the test sensitivity.

The choosen test soils cover the common soil characteristics of European soils very well, constituting a representative sub-sample of soils. With this, it was expected that the results of the studies within the project would be readily transferable to many different soil conditions throughout Europe.

According to the OECD TG 216, at least two concentrations must be tested. The lower concentration corresponds to the maximum amount expected to reach the soil under practical conditions (e.g. maximum PEC or application rate), and the higher concentrations should be a multiple (five- or ten-fold) of the lower concentration. In addition, care was taken to ensure that the test concentrations were comparable with those from the OECD 216/OECD 217 data provided by UBA. For DDAC, no data regarding the application rate was available and therefore based on the data of dose response tests the nominal test concentrations were chosen. While three concentrations were used for the tests in Lufa 2.1. and RefeSol 02A, for the tests with RefeSol 04A additionally a 4th test concentration was added to get better information on concentration response.

The effect of the chosen test substances on ammonium oxidizing bacteria (ISO 15685), the substrate induced respiration with the associated nutrient cycles (MicroResp™) and on the enzymatic activity (ISO 20130) was investigated in the three soils (LUFA 2.1, RefeSol 02A, RefeSol 04A). In the test with Lufa 2.1 measurements were performed at test initiation and after 14 and 28 days of incubation, while in tests with RefeSol 04A and RefeSol 02A effects were determined only after 14 and 28 days. If effects above 25% occurred or if there were peculiarities with regard to occurring effects below 25%, selected test methods were extended to 56 and/or 84 days. In addition, the effect on the structural diversity (ARISA) of the soil microorganisms was determined in soil samples taken at day 28 of the beforementioned tests for the three soils. The effect of the chosen test substances on the spore germination of *F. mosseae* was determined only in RefeSol 02A and exemplarily in RefeSol 04A, since pre-tests indicated that Lufa 2.1 is not appropriate as test soil for testing the effect at least on the AMF *F. mosseae* due to its low pH level. This might be different if other AMF species as e.g. *Rhizophagus irregularis* are used.

Key question 1 focussed on assessing the risks posed by plant protection products, biocides and veterinary pharmaceuticals for soil microorganisms with the aim to compare five additional test methods to the current approach based on OECD TG 216 (N-transformation): ISO 15685 on potential nitrification, MicroResp™ on basal and substrate induced respiration, ISO 20130 on enzymatic activity, ISO 10832 on the spore germination of AMF and the fingerprinting method ARISA on the microbial community structure. Based on six test substances and three soils the following main results were obtained:

- The N-Transformation test (OECD 216) was often less sensitive and the lowest observed effect concentration (LOEC) of alternative methods was found to be up to a factor of 100 lower.
- The potential nitrification test (ISO 15685) showed no relevant differences to the currently used N-Transformation test (OECD 216), although if in a sandy soil (LUFA 2.1) the effects were stronger than in the N-Transformation limit test (OECD 216).
- The microbial respiration test (MicroResp™) showed the lowest variability of controls and the lowest minimum detectable differences. However, it often did not provide the lowest LOEC values and therefore was therefore not the most sensitive test system.
- The enzymatic activity test (ISO 20130) most often gave the lowest LOEC and was therefore the most sensitive test method, sometimes indicating long-term effects (no recovery to deviations < 25 % within 84 days). It was often the only method for functional endpoints that gave the lowest LOEC.
- The ARISA data evaluated using ordination plots revealed often gave the same low estimated LOECs as the enzymatic activity test (ISO 20130), but was less often the only approach resulting in the lowest LOEC. However, due to the aim of testing six test items in three soils, only a limited number of test concentrations and replicates could be tested and statistical testing was not always possible.
- The Spore gemination test on Arbuscular mycorrhiza fungi (AMF) (ISO 10832), conducted with the species *Funneliformis mosseae* was tested in one natural soil. Test results were never among the methods with the lowest LOECs. The results of the project indicate that the existing guideline for spore germination testing of AMF (ISO 10832) needs to be revised if natural soils are used as test substrate. Following adaptations should be considered:
- The establishment of other AMF species (e.g. *Rhizophagus irregularis*), which could be used in the spore germination test and which may be a more sensitive species, should be considered.
- For *F. mosseae*, the maximum water holding capacity of the soil has to be reduced from 90%, as indicated in the guideline, to about 50%, if natural soils are used as test substrate. However, this value probably may need to be adjusted depending on the characteristics of the natural soil intended to be used for testing.
- Soils used for regulatory testing with a pH below 5 (e.g.; Lufa 2.1) were not suitable for testing F. mosseae. The soil pH of natural test soils should be above pH 5.
- Increasing the temperature improves the spore germination of F. mosseae. Using the upper end of the permitted range of 24 ± 2 °C according to ISO 10832 improved the spore germination in RefeSol 02A.
- Of the three natural soils tested, RefeSol 02A, the soil with the lowest sand content, the highest pH value, but a lower organic carbon content compared to e.g. RefeSol 04A or Lufa 2.1, showed the lowest effects for the five tested methods due to the application of the exemplarily used six active substances.

The framework of ecotoxicological testing of microorganisms differs in many respects from other areas in environmental risk assessment, such as soil organisms, birds and mammals, or aquatic organisms. Test systems contain intact communities of soil microorganisms and deliver

either information on their functional (as described in the above section 2.1) or on their structural state. This poses a fundamental aspect of contextualizing them in a tiered ERA approach. The ranking criterion *complexity of a test system* drops out.

From these results the following conclusions are drawn:

- Microbial respiration (MicroResp™) and potential nitrification (ISO 15685) were less sensitive than the other tested, functional endpoints. Enzymatic activity (ISO 20130) was the most sensitive test method indicating long-term effects where N-transformation (OECD 216) was not. Therefore, it is proposed to complement OECD 216 by ISO 20130 in the first step of the risk assessment to cover a broad set of soil microbial functions.
- Spore gemination of AMF (ISO 10832) was more sensitive than N-Transformation (OECD 216) but less sensitive than enzymatic activity (ISO 20130). Thus, a test with AMF is also proposed for lower tier testing. A revision of the method is required in advance of its implementation within the risk assessment framework.
- Soil type affects the test results. LUFA 2.1 was not always the soil resulting in the lowest endpoints.
- The ARISA method was the only technique tested in the project specifically addressing microbial community structure and, by extension, the biodiversity. While ARISA revealed impacts on biodiversity, the limited available data do not allow for definitive conclusions regarding the long-term implications of these changes. Therefore, its recommendation as a routine test should be further evaluated and supported by additional data, studies and agreements for evaluation of results. Decision criteria need to be set for the acceptable or unacceptable risks. Moreover, during the three-year project duration, the costs of microbiome metabarcoding and metagenomics have significantly decreased, making these techniques more feasible for routine use. Now, for slightly higher costs in relation to the costs for ARISA, these methods offer greater taxonomic and functional resolution compared to ARISA. However, the turnaround time and the lack of standardization in sequencing methods as well as test evaluation remain limitations that need to be addressed in future.

In the current risk assessment for plant protection products and veterinary pharmaceuticals the microbial community in soils and its ecosystem services are considered to be not at risk, if effects of the maximum PEC in-field on the Nitrogen transformation (OECD TG 216) above 25 % are restricted to less than 100 days. Based on the results obtained here and considering the recommendations of the EFSA PPR panel (2017) it is recommended to supplement the Tier 1 assessment by an additional test on bacterial function and a test covering effects on arbuscular mycorrhizal fungi. Due to its higher sensitivity found for the substances tested within the project, the ISO 20130 is preferred over MicroResp™. In contrast to EFSA PPR panel (2017) we do not consider additional functional tests (providing additional endpoints) to be a useful intermediate tier option because a risk for N-transformataion (OECD 216) is not refined by testing other functions. Instead, refined exposure could be tested with additional soils, e.g. sampled in the field as refinement option. At the higher tier, natural communities in field test (with known structural composition, e.g. by microbiome sequencing) can be tested. This would serve as the reference tier for calibrating or validating lower tiers. Without a comparison with a reference tier it is not clear whether the current assessment (based only on OECD TG 216) is sufficiently protective.

Antibiotic resistance

Here, the aim was to investigate the extent to which inputs of antibiotics/veterinary pharmaceuticals favour the development of antibiotic resistance in soil relevant bacteria **(WP3).** For this purpose, Minimum Inhibitory Concentrations (MIC) of active substances were determined for environmentally relevant soil bacteria.

"Single-species toxicity tests with microorganisms play an important role during the ERA of antibiotics" (Brandt et al. 2015). The advantage of these methods is that they are standardized, well defined, rapid and robust and results from different laboratories are thus more comparable than for community tests. An important consideration for all single-species tests is that even single species of bacteria may contain both antibiotic-resistant and sensitive strains and the choice of test strain is thus critical (Dias et al. 2015). We therefore proposed that proper strain selection should be included in standard guidelines for single-species toxicity testing with bacteria to be used for ERA. Hence, thorough genomic and phenotypic characterization of a broad range of standard test strains is needed in order to select sensitive strains for ecotoxicity testing.

In order to make a proper strain selection for WP3, the availability of genomic information of the strains available at IME was revised. From the 22 strains (19 species) revised, 16S rRNA (partial) sequence information and whole genome information is available for ten and nine species, respectively. Additionally, five strains (*Janibacter terrae* DSM 13953; *Arthrobacter* sp. DSM 917; *Lactobacillus plantarum* DSM 20205; *Pseudomonas fluorescens* ATCC 17571; *Pseudomonas putida* DSM 291) were sent for whole genome sequencing with the aim to determine their suitability for antibiotic resistance testing. These strains were selected for whole genome sequencing as they are relevant soil bacteria. The following test substances were chosen together with the UBA: colistin sulfate, neomycin sulfate, chlortetracyclin hydrochloride, tiamulin hydrogen fumarate, copper sulfate and TWEEN 20.

The MIC was determined by the broth microdilution method following EUCAST protocols. In the main test, 96-well flat base polystyrene microtiter plates were used. For each substance, eight concentrations and ten replicates were tested. Dilutions of the substance were prepared from the stock solutions. Three controls, used as validity criteria, were included in the plates: inoculation control (between 0.08 and 0.13 at OD625) substance control (no growth), and media control (no growth).

All tests were conducted successfully and have fulfilled the validity criteria, and MIC was determined for each bacteria/substance tested. Using soil-relevant bacteria for MIC determination is an important step in understanding the effectiveness of antimicrobial agents in environments that are ecologically relevant. Variability of MICs between the four bacterial strains tested spanned up to two orders of magnitude. No single strain was the most sensitive one but *Ac. facilis* provided the *lowest* MIC for two of the four antibiotics while *Arthrobacter* sp. was never the most sensitive strain. The minima MICs of the four antibiotics tested were at least two orders of magnitude lower that for the two other substances ($CuSO_4$ and TWEEN 20) tested. The lowest MIC found was 0.06 mg/L for chlortetracycline hydrochloride.

For the four antibiotics tested, the soil bacteria were on average not more sensitive than the clinical strains. Only in one case, Chlortetracyline hydrochlorine, a MIC from a soil bacterium (Acidovorax facilis) was lower than all the MICs of the clinical strains. Due to missing data for KOC or PECsoil, risk quotients could not be calculated except for tiamulin hydrogen fumarate. For this antibiotic, the MIC indicated a higher risk than the standard ecotoxicological tests (RQ > 100). Thus, current risk assessment for veterinary pharmaceuticals might not cover the development of antibiotic resistance in environmentally relevant soil bacteria.

Degradation performance of soil microorganisms

Here, the influence of more realistic pollution scenarios compared to the standard scenario of a common OECD degradation study on microbial degradation was investigated. The data was used to clarify whether, for example, multiple applications or background pollution must also be considered in these studies in order to adequately protect the soil microorganisms.

The tests were designed to investigate different situations of single and multiple chemical exposure at realistic concentrations and include the following steps:

1. STEP 1: Determination of the degradation performance of soil microorganisms after a single application of a test substance compared to the repeated (multiple) application of the same test substance (simulating the repeated application of a pesticide with a designated interval).
2. STEP 2: Determination of the degradation performance of the soil microbial community of a test substance in the presence of one further chemical.
3. STEP 3: Determination of the degradation performance of soil microorganisms of a test substance in an outdoor agricultural soil already containing a realistic mixture of chemicals. The outdoor soil should have comparable soil properties to one of the soils used in step 1 or 2 to relate any degradation effect to the multiple chemicals additionally present in the soil.

Soil degradation tests based on OECD 307 were carried out using the two test substances pyraclostrobin (fungicide) and ethofumesate (herbicide) in two soils RefeSol 02A (silt loam, STEP 1-2) and LUFA 2.1 (sand, STEP 1) as well as in a soil with a field under agricultural use with expected background contaminantion (STEP 3) freshly sampled at the original sampling location of RefeSol 02A. Two test concentrations for both substances were chosen and were consistent throughout the degradation tests. One test concentration level reflected the maximum application rate for the two active substances (0.667 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate) being used for single applications. The second test concentration level was half the maximum application rate and was applied twice for multiple applications (2 x 0.333 mg/kg for pyraclostrobin and 2 x 0.400 mg/kg for ethofumesate) and, hence, resulting in total in the same concentration level used for single applications. The following main results were obtained:

- Degradation times of both substances applied as single application were higher in LUFA 2.1. compared to degradation times in Refesol 02A, depending on the characteristic of the tested substance as well as used test soil. DT50-values of pyraclostrobin were about twice as high in Lufa 2.1 compared to DT50-values observed in Refesol 02A, whereas DT50-values of ethofumesate were about seven times higher in LUFA 2.1 compared to Refesol 02A. Observed differences in degradation times between different representative soils are in line with the current state of knowledge. Even if existing differences in degradation times between soils are well know, they are not sufficiently reflected in the current risk assessment scheme for microorganisms exposed to chemicals.

- Single applications of the two active substances pyraclostrobin and ethofumesat individually applied to both soils were compared to multiple applications (i.e. two successive applications) of the same substance with an interval of 21 days. The effect of multiple application on the degradation half-life did not show a uniform picture: DT50-values of pyraclostrobin were higher in both soils after the second application, whereas the DT50-values for ethofumesat were comparable in soil Refesol 02A but were lower in soil Lufa 2.1 after multiple application. These results indicated that the presence of the test substance may have a negative effect on its further degradation when applied for a second time.

- The effect of binary mixtures on the soil degradation of pyraclostrobin and ethofumesate was investigated using single and multiple applications of both substances at the same time as well as single applications of both substances individually but time-delayed into the same soil (RefeSol 02A). The results showed that the presence of a second substance (within a mixture or already present on the soil) did not have a consistent effect on degradation of the given substance in the binary mixture. However, a similar pattern could be observed for pyraclostrobin and ethofumesate. For single application, degradation times of pyraclostrobin and ethofumesat were slightly prolonged, if applied as mixture, but the difference was small so it was regarded as comparably high since a statistical evaluation was not possible. For multiple application of the mixture of both substances, degradation of both substances was slower after the first applicaiton (0 – 21d), but degradation of both substances was faster after the second application (21 – 120d). For time-delayed applications, the application of a substance after 21 days with a second substance already present in soil led to an increase of the half-life of – at least - ethofumesate.
- Obtained DT50-values for soil degradation tests using single applications of pyraclostrobin and ethofumesate individually applied to an agriculturally used soil were lower compared to the results for soil degradation tests using single applicatons of pyraclostrobin and ethofumesat in soil RefeSol 02A (STEP 1-2).

Soil degradation studies according to OECD 307 are usually carried out to determine degradation rates of active substances which are used for the estimation of the predicted environmental concentration of an intended use of chemicals like plant protection products. Usually, degradation tests are performed with a set of different soils and the respecting mean or worst-case value is used for the estimation of the predicted environmental concentration which is compared to effect values for test organisms like microorganisms within the risk assessment of chemicals.

The results of the MICROSOIL project show that multiple application of the same substance as well as the presence of another substance may have an effect on the degradation time of the tested substances: Both positive and negative effects on the degradation rate of the test substances used in this study were observed. However, a negative impact on the degradation rate was found, for instance, for Pyraclostrobin when applied for a second time during multiple application testing or for ethofumesate when applied to a soil where a second substance (here: pyraclostrobin) was already present.

The results of this project are specific for the used test substances pyrclostrobin and ethofumesate as well as for the tested soils RefeSol 02A and Lufa 2.1. Further research, reflecting real application patterns of plant protection products as well as fertilizing practices including residues of biocides, industry chemicals and pharmaceuticals is needed to determine the actual and correct degradation time of substances which are used for the estimation of the predicted environmental concentration of substances. The estimation of predicted environmental concentrations of actives substances should acknowledge the presence of a variety of soils in Europe as well as the occurrence of multiple applications and mixture residues in agricultural used soils in order to ensure a sufficient protection of the environment.

Zusammenfassung

Die Gesundheit des Bodens ist der wichtigste Faktor für eine nachhaltige Landwirtschaft und muss entsprechend erhalten und gefördert werden. Mikroorganismen wie Bakterien oder Pilze (arbuskuläre Mykorrhizapilze) sind ein wesentlicher und integraler Bestandteil des lebenden Bodens und beeinflussen verschiedene biogeochemische Kreisläufe der wichtigsten Nährstoffe wie Kohlenstoff, Stickstoff, Schwefel und Phosphor. Sie spielen eine größere Rolle bei der Erhaltung der Bodengesundheit als andere biologische Komponenten des Bodens (Sathya et al. 2016). Mikroorganismen sind aktiv an der Bodenbildung beteiligt, indem sie den Prozess der biologischen Verwitterung von Gestein, die Zersetzung organischer Stoffe und den Nährstoffkreislauf in Gang setzen (Kaviya et al. 2019). Sie sind für verschiedene biologische Transformationen unterschiedlicher Kohlenstoffquellen und Makro- und Mikronährstoffe verantwortlich, die die anschließende Etablierung von Boden-Pflanzen-Mikroben-Interaktionen erleichtern (Sahu et al. 2017). Der Humusgehalt in mitteleuropäischen Böden nimmt kontinuierlich ab (Jacobs et al. 2013). Aufgrund vielfältiger Belastungen und eines unzureichenden Umweltbewertungsansatzes in Bezug auf die Mikroorganismen in Böden kann das grundlegende Schutzziel "Erhalt der biologischen Vielfalt im Boden" nicht erreicht werden. In der Folge kann die Resilienz und damit die Fähigkeit der Bodenmikroorganismen, z. B. auf Stressoren wie den Klimawandel zu reagieren, deutlich abnehmen. Daher ist eine differenziertere Risikobewertung für Bodenmikroorganismen erforderlich, die auch die anderen Bewertungsbereiche wie wirbellose Bodenlebewesen oder Nichtziel-Arthropoden berücksichtigt.

Ziel des Projekts war es, Testmethoden und Endpunkte zu identifizieren, die im Rahmen einer Umweltverträglichkeitsprüfung zum Schutz von Bodenmikroorganismen und damit der wichtigen Ökosystemleistungen von Böden vor verschiedenen Schadstoffen eingesetzt werden können, und deren Anwendbarkeit in der Praxis zu ermitteln. Drei Schlüsselfragen wurden beantwortet:

1. Welche Methoden sind geeignet, um Veränderungen der Bodenfunktion und der strukturellen Zusammensetzung der mikrobiellen Gemeinschaft in Böden für die regulatorische Bewertung von Pflanzenschutzmitteln, Bioziden und Tierarzneimitteln zu erfassen und zu beschreiben (Kapitel 2)?
2. Zweite Schlüsselfrage: Kann die Exposition von Bodenmikroorganismen gegenüber Antibiotika/Tierarzneimitteln zur Entwicklung von Resistenzgenen führen und gibt es Wechselwirkungen mit anderen chemischen Stressoren? (Kapitel 3)
3. Wie wirken sich Mehrfachanwendungen von Wirkstoffen aus Pflanzenschutzmitteln und eine Hintergrundbelastung auf den mikrobiellen Schadstoffabbau im Boden aus? (Kapitel 4)

Auswirkungen auf Funktion und Struktur von mikrobiellen Bodengemeinschaften

In einer Literaturrecherche wurde eine Liste mit verfügbaren Testmethoden zur Erfassung der funktionellen Reaktion von Mikroorganismen auf die Wirkstoffkonzentration (z.B. enzymatische Tests, Biolog© oder MicroResp™, Mykorrhizapilze), unter Berücksichtigung der Empfehlungen des EFSA-Bodengutachtens, zusammengestellt. Anschließend wurde die Eignung ausgewählter Testmethoden zur Ermittlung von Veränderungen der mikrobiellen Bodenfunktion und der strukturellen Zusammensetzung der mikrobiellen Bodengemeinschaft exemplarisch an sechs verschiedenen Testsubstanzen getestet.

Um geeignete Veröffentlichungen in begutachteten Fachzeitschriften zu finden, wurde eine **Literaturrecherche** über Science Direct, Google Scholar, Web of Science und Scopus durchgeführtsowie in einer internen Literaturdatenbank nach geeigneten Testmethoden für Bodenmikroorganismen gesucht. Die Literaturrecherche fand von April bis Oktober 2021 statt.

Es wurde darauf geachtet, dass die identifizierten Methoden geeignet sind, den Einfluss von Chemikalien auf Bodenfunktionen sowie auf strukturelle Veränderungen der mikrobiellen Gemeinschaft im Boden zu bestimmen. Außerdem sollten die Methoden sowohl Bakterien als auch Pilze abdecken und auch Exo- und Endo-Enzyme im Boden einbeziehen. Die Begriffe der Literatursuche konzentrierten sich auf drei verschiedene Bereiche: *(i)* allgemeine Begriffe, *(ii)* direkte Darstellung von Methoden/Assays und *(iii)* Effektstudien. Die allgemeinen Begriffe wurden verwendet, um einschlägige Literatur über die Messung der Bodenfunktion und der mikrobiellen Struktur des Bodens und deren Bewertung zu finden, ohne dass eine besondere Beschreibung der vorgestellten Methode erfolgte. Aus dieser Literatur wurde die Quelle der beschriebenen Methode, die für die Teststrategie geeignet erschien, herausgesucht. Darüber hinaus wurden Begriffe mit dem Schwerpunkt "Methoden" oder "Assays" verwendet, um Literatur zu finden, in der bestimmte Methoden zur Bestimmung oder Bewertung relevanter Bodenprozesse direkt vorgestellt werden. In einem letzten Schritt wurde nach Studien gesucht, in denen die Wirkung von Pestiziden oder anderen Chemikalien auf Bodenmikroorganismen beschrieben wird, um einen Eindruck von der Empfindlichkeit der Methoden zu erhalten. Es wurden Bewertungsparameter festgelegt, um zwischen verschiedenen Methoden unterscheiden zu können. Die auf den folgenden Seiten vorgeschlagenen Parameter beruhen auf bestehenden Bewertungen in der Literatur, wurden aber, falls erforderlich, mehr oder weniger stark modifiziert und ergänzt. Thiele-Bruhn et al. (2020) kombinierte und leicht angepasste Parameter von Pulleman et al. (2012) und Faber et al. (2013) und bewerteten verschiedene Methoden anhand einer Punktzahl von 1 bis 5 in Kombination mit einem Farbcode, der aus den Farben Rot, Orange, Gelb, Hellgrün und Grün besteht. Dieser Ansatz kann die Genauigkeit der Bewertung erhöhen, aber die Komplexität steigt und die Entscheidung über die Eignung einer Prüfmethode wird schwieriger. Daher wurde in einem ersten Schritt die Bewertung auf der Grundlage eines Ampelsystems so einfach wie möglich gehalten. Für die Bewertung im Rahmen dieses Projekts wurden fünf Parameter ausgewählt: (i) Durchführbarkeit, (ii) geschätzte Kosten, (iii) Wiederholbarkeit/Reproduzierbarkeit, (iv) Durchführbarkeit je nach Bodentyp und (v) Relevanz für regulatorische Zwecke. Diese Parameter wurden als wesentlich für die Bewertung von Methoden für Regulierungszwecke angesehen. Dementsprechend haben wir für jeden einzelnen Parameter ein einfaches Ampelsystem verwendet. In diesem System stehen grün, gelb und rot für sinnvoll, möglich bzw. nicht sinnvoll. Auf der Grundlage der Literaturrecherche und der Bewertung verschiedener Methoden zur Beurteilung der Auswirkungen auf Bodenfunktionen und -struktur wurden fünf Methoden mit der jeweils höchsten Punktzahl für aerobe heterotrophe Bodenmikroorganismen, Nitrifikanten, Enzymaktivität, AMF und strukturelle Bodenvielfalt ausgearbeitet.

Aktivität von aeroben, heterotrophen Mikroorganismen: Die Substratinduzierte Atmung durch MicroResp™ erscheint eine vielversprechende Ergänzung zu den Standardtests (OECD 216) zu sein, um mehrere Nährstoffkreisläufe zu erfassen, da auch N-, P- und S-Kreisläufe dargestellt werden. Es handelt sich um eine einfache und schnelle Methode, die innerhalb eines Tages durchgeführt werden kann. Darüber hinaus wurde die Methode bereits von der EFSA empfohlen (European Food Safety Authority 2017).

Aktivität der nitrifizierenden Bakterien: Der Ammoniumoxidationstest (ISO 15685) schien als zusätzlicher Parameter für die Stickstoffmineralisierung geeignet zu sein (alternativ zum N-Transformationstest, OECD 216). Es handelt sich um eine einfache und schnelle Methode, die innerhalb eines Tages durchgeführt werden kann. Sie ist sehr empfindlich, da nur eine kleine mikrobielle Gruppe mit geringem Resilienzpotenzial angesprochen wird. Obwohl die Umweltrelevanz begrenzt ist, kann er als Indikator für toxische Wirkungen verwendet werden.

Enzymaktivität: Die ISO 20130 "Bodenqualität - Messung von Enzymaktivitätsmustern in Bodenproben unter Verwendung kolorimetrischer Substrate in Micro-Well-Platten" wurde

verwendet, um die Auswirkungen auf die Aktivität von Exoenzymen zu bewerten. Der Test ist vergleichbar mit der in ISO 22939 beschriebenen Methode, jedoch kostengünstiger, da keine fluorogenen Substrate verwendet werden müssen. Außerdem bietet diese Methode eine größere Flexibilität bei der Auswahl der Substrate und damit bei den berücksichtigten enzymatischen Aktivitäten. Während nur eine begrenzte Anzahl von fluorogenen Substraten zur Verfügung steht, kann bei der in ISO 20130 beschriebenen Methode jedes Substrat mit ausreichender Löslichkeit verwendet werden. Im Gegensatz zu Methoden, bei denen die Wirkung auf nur eine kleine Gruppe von Enzymen untersucht wird, kann bei dieser Methode die Wirkung auf eine Reihe verschiedener Gruppen berücksichtigt werden.

Pilzaktivität: Die ISO 10832 "Effects of pollutants on mycorrhizal fungi - Spore germination test" wurde zur Beobachtung der Wirkung auf arbuskuläre Mykorrhizapilze verwendet, da er als der am besten geeignete Test erschien, um potenzielle Wirkungen auf diese ökologisch relevante Gruppe zu untersuchen, die ansonsten nicht von den vorgeschlagenen Tests abgedeckt wird, und auch bereits von der EFSA empfohlen wurde (European Food Safety Authority 2017).

Mikrobielle Struktur: ARISA wurde als vielversprechende Methode eingestuft, die tiefgreifende Erkenntnisse über die Auswirkungen auf die Struktur der mikrobiellen Gemeinschaft und die Verschiebungen innerhalb der Gemeinschaft liefert. Allerdings sind diese Methoden teuer und die Auswertung könnte schwierig sein. Um die Artenvielfalt der Mikroorganismengemeinschaft im Boden besser zu erfassen, könnte die ARISA-Methode eine geeignete Fingerprinting-Methode sein, die beispielhaft eingesetzt werden sollte. Die Methode wurde zum Zeitpunkt der Literaturrecherche als weniger kostspielig als andere Methoden bewertet und ist nicht so anspruchsvoll, dass eine wirklich anspruchsvolle statistische Auswertung erforderlich wäre.

Nach der Auswahl der **Testmethoden** wurden diese angewandt, indem die **sechs Testsubstanzen** Ethofumesat, Tebuconazol, Propamocarbhydrochlorid, Pyraclostrobin, Tiamulinhydrogenfumarat und Didecyl-Dimethylammoniumchlorid in den **drei Böden** RefeSol 02A, RefeSol 04A und Lufa 2.1 getestet wurden, um Rückschlüsse auf die Empfindlichkeit der Methoden und den Einfluss verschiedener Bodeneigenschaften auf die Testempfindlichkeit zu ziehen.

Es wurde festgestellt, dass die RefeSols die allgemeinen Bodeneigenschaften der europäischen Böden sehr gut abdecken und eine repräsentative Unterprobe von Böden darstellen. Daher wurde erwartet, dass die Ergebnisse der Studien im Rahmen des Projekts ohne weiteres auf viele verschiedene Bodenbedingungen in ganz Europa übertragbar sind. LUFA 2.1 ist der Referenzboden, der üblicherweise in Studien mit behördlicher Zielsetzung verwendet wird. Daher wurde LUFA 2.1 als Testboden für das Projekt ausgewählt.

Den Vorgaben der OECD TG 216 folgend müssen mindestens zwei Konzentrationen getestet werden. Die niedrigere Konzentration entspricht der maximalen Menge, die unter praktischen Bedingungen (z. B. maximale PEC oder Ausbringungsrate) voraussichtlich den Boden erreicht und die höheren Konzentrationen sollten ein Vielfaches (das Fünf- oder Zehnfache) der niedrigeren Konzentration betragen. Außerdem wurde darauf geachtet, dass die Testkonzentrationen mit denen vom UBA zur Verfügung gestellten OECD 216/OECD 217-Daten vergleichbar sind. Für DDAC lagen keine Daten zur Ausbringungsmenge vor, daher wurden auf der Grundlage der Daten von Dosis-Wirkungs-Tests die nominalen Testkonzentrationen gewählt. Während für die Tests in Lufa 2.1. und RefeSol 02A drei Konzentrationen verwendet wurden, wurde für die Tests mit RefeSol 04A zusätzlich eine vierte Testkonzentration hinzugefügt, um bessere Informationen über die Konzentrationsreaktion zu erhalten.

In den drei Böden (LUFA 2.1, RefeSol 02A, RefeSol 04A) wurde die Wirkung der ausgewählten Testsubstanzen auf ammoniumoxidierende Bakterien (ISO 15685), die substratinduzierte Atmung mit den damit verbundenen Nährstoffkreisläufen (MicroResp™) und auf die

enzymatische Aktivität (ISO 20130) untersucht. Bei dem Test mit Lufa 2.1 wurden die Messungen zu Beginn des Tests sowie nach 14 und 28 Tagen Inkubation durchgeführt, während bei den Tests mit RefeSol 04A und RefeSol 02A die Auswirkungen erst nach 14 und 28 Tagen bestimmt wurden. Traten Effekte über 25 Prozent auf oder gab es Auffälligkeiten in Bezug auf auftretende Effekte unter 25 Prozent, wurden ausgewählte Testmethoden auf 56 bzw. 84 Tage ausgedehnt. Zusätzlich wurde die Wirkung auf die strukturelle Diversität (ARISA) der Bodenmikroorganismen in Bodenproben, die am Tag 28 der vorgenannten Tests entnommen wurden, für die drei Böden bestimmt. Die Wirkung der ausgewählten Testsubstanzen auf die Sporenkeimung von *F. mosseae* wurde nur in RefeSol 02A und exemplarisch in RefeSol 04A bestimmt, da Vorversuche darauf hindeuteten, dass Lufa 2.1 aufgrund seines niedrigen pH-Wertes als Testboden für die Prüfung der Wirkung zumindest auf den AMF *F. mosseae* nicht geeignet ist. Dies könnte anders sein, wenn andere AMF-Arten wie z. B. *Rhizophagus irregularis* verwendet werden.

Die erste Schlüsselfrage konzentrierte sich auf die Bewertung der von Pflanzenschutzmitteln, Bioziden und Tierarzneimitteln ausgehenden Risiken für Bodenmikroorganismen mit dem Ziel, fünf zusätzliche Testmethoden mit dem derzeitigen Ansatz auf der Grundlage der OECD TG 216 (N-Transformation) zu vergleichen: ISO 15685 zur potenziellen Nitrifikation, MicroResp™ zur basalen und substratinduzierten Atmung, ISO 20130 zur enzymatischen Aktivität, ISO 10832 zur Sporenkeimung von AMF und die Fingerprinting-Methode ARISA zur mikrobiellen Gemeinschaftsstruktur.

Auf der Grundlage von sechs Testsubstanzen und drei Böden wurden die folgenden Hauptergebnisse erzielt:

- Der N-Transformationstest (OECD 216) war häufig weniger empfindlich, und die niedrigste beobachtete Wirkkonzentration (LOEC) alternativer Methoden war bis zu einem Faktor 100 niedriger.
- Der Test zur potenziellen Nitrifikation (ISO 15685) zeigte keine relevanten Unterschiede zum derzeit verwendeten N-Transformationstest (OECD 216), obwohl die Auswirkungen in einem sandigen Boden (LUFA 2.1) stärker waren als im N-Transformationsgrenzentest (OECD 216).
- Der mikrobielle Respirationstest (MicroResp™) zeigte die geringste Variabilität der Kontrollen und die geringsten nachweisbaren Unterschiede. Allerdings lieferte er häufig nicht die niedrigsten LOEC-Werte und war daher nicht das empfindlichste Testsystem.
- Der Test auf enzymatische Aktivität (ISO 20130) ergab am häufigsten die niedrigste LOEC und war daher die empfindlichste Testmethode, die manchmal langfristige Auswirkungen anzeigte (keine Erholung bis zu Abweichungen < 25 % innerhalb von 84 Tagen). Bei den funktionellen Endpunkten war es oft die einzige Methode, die die niedrigste LOEC ergab.
- Die anhand von Ordinationsdiagrammen ausgewerteten ARISA-Daten ergaben häufig die gleichen niedrigen geschätzten LOECs wie der Test auf enzymatische Aktivität (ISO 20130), waren jedoch seltener der einzige Ansatz, der zu den niedrigsten LOECs führte. Aufgrund des Ziels, sechs Prüfgegenstände in drei Böden zu testen, konnte jedoch nur eine begrenzte Anzahl von Testkonzentrationen und Wiederholungen getestet werden, und eine statistische Prüfung war nicht immer möglich.
- Der Sporenkeimtest für arbuskuläre Mykorrhizapilze (ISO 10832), durchgeführt mit der Art *Funneliformis mosseae*, wurde in einem natürlichen Boden getestet. Die Testergebnisse gehörten nie zu den Methoden mit den niedrigsten LOECs. Die Ergebnisse des Projekts deuten darauf hin, dass der bestehende Leitfaden für Sporenkeimungstests von AMF (ISO

10832) überarbeitet werden muss, wenn natürliche Böden als Testsubstrat verwendet werden. Folgende Anpassungen sollten in Betracht gezogen werden:

- Die Etablierung anderer AMF-Arten (z. B. *Rhizophagus irregularis*), die im Sporenkeimungstest verwendet werden könnten und möglicherweise eine empfindlichere Art sind, sollte in Betracht gezogen werden.
- Für *F. mosseae* muss die maximale Wasserhaltekapazität des Bodens von 90 Prozent, wie in der Leitlinie angegeben, auf etwa 50 Prozent reduziert werden, wenn natürliche Böden als Testsubstrat verwendet werden. Dieser Wert muss jedoch je nach den Eigenschaften des natürlichen Bodens, der für die Tests verwendet werden soll, möglicherweise angepasst werden.
- Für vorgeschriebene Tests verwendete Böden mit einem pH-Wert unter 5 (z. B. Lufa 2.1) waren für die Untersuchung von F. mosseae nicht geeignet. Der Boden-pH-Wert natürlicher Testböden sollte über pH 5 liegen.
- Eine Erhöhung der Temperatur verbessert die Sporenkeimung von *F. mosseae*. Die Verwendung des oberen Endes des zulässigen Bereichs von 24 ± 2 °C gemäß ISO 10832 verbesserte die Sporenkeimung in RefeSol 02A.
- Von den drei getesteten natürlichen Böden zeigte RefeSol 02A, der Boden mit dem geringsten Sandgehalt, dem höchsten pH-Wert aber einem niedrigeren Gehalt an organischem Kohlenstoff im Vergleich zu z.B. RefeSol 04A oder Lufa 2.1, die geringsten Auswirkungen für die fünf getesteten Methoden aufgrund der Anwendung der beispielhaft verwendeten sechs Wirkstoffe.

Der Rahmen der ökotoxikologischen Prüfung von Mikroorganismen unterscheidet sich in vielerlei Hinsicht von anderen Bereichen der Umweltverträglichkeitsprüfung, wie Bodenorganismen, Vögel und Säugetiere oder Wasserorganismen. Testsysteme enthalten intakte Gemeinschaften von Bodenmikroorganismen und liefern entweder Informationen über deren Funktion (wie im obigen Abschnitt beschrieben 2.1) oder über ihren strukturellen Zustand. Dies ist ein grundlegender Aspekt für ihre Einordnung in einen mehrstufigen ERA-Ansatz. Das Ranking-Kriterium *Komplexität eines Testsystems* fällt weg.

Aus diesen Ergebnissen werden die folgenden vorläufigen Schlussfolgerungen gezogen:

- Die mikrobielle Atmung (MicroResp™) und die potenzielle Nitrifikation (ISO 15685) waren weniger empfindlich als die anderen getesteten funktionellen Endpunkte. Die enzymatische Aktivität (ISO 20130) war die empfindlichste Testmethode, die langfristige Auswirkungen anzeigte, während die N-Umwandlung (OECD 216) dies nicht tat. Daher wird vorgeschlagen, die OECD 216 im ersten Schritt der Risikobewertung durch die ISO 20130 zu ergänzen, um eine breite Palette von mikrobiellen Bodenfunktionen abzudecken.
- Die Sporenbildung von AMF (ISO 10832) war empfindlicher als die N-Transformation (OECD 216), aber weniger empfindlich als die enzymatische Aktivität (ISO 20130). Daher wird ein Test mit AMF auch für Tests der unteren Stufen vorgeschlagen. Eine Überarbeitung der Methode ist erforderlich, bevor sie im Rahmen der Risikobewertung eingesetzt werden kann.
- Die Art des Bodens beeinflusst die Testergebnisse. LUFA 2.1 war nicht immer der Boden, der zu den niedrigsten Endpunkten führte.
- Die ARISA-Methode war die einzige im Rahmen des Projekts getestete Technik, die sich speziell mit der Struktur der mikrobiellen Gemeinschaft und damit auch mit der

biologischen Vielfalt befasste. Während ARISA Auswirkungen auf die biologische Vielfalt aufzeigte, lassen die begrenzten verfügbaren Daten keine endgültigen Schlussfolgerungen hinsichtlich der langfristigen Auswirkungen dieser Veränderungen zu. Daher sollte seine Empfehlung als Routinetest weiter evaluiert und durch zusätzliche Daten, Studien und Vereinbarungen zur Bewertung der Ergebnisse und zur Festlegung von Entscheidungskriterien für akzeptables Risiko unterstützt werden. Darüber hinaus sind während der dreijährigen Projektlaufzeit die Kosten für das Mikrobiom-Metabarcoding und die Metagenomik erheblich gesunken, so dass diese Techniken für den Routineeinsatz besser geeignet sind. Für etwas höhere Kosten im Vergleich zu den Kosten für ARISA bieten diese Methoden nun eine größere taxonomische und funktionelle Auflösung im Vergleich zu ARISA. Die Durchlaufzeit und die fehlende Standardisierung der Sequenzierungsmethoden sowie die Testauswertung stellen jedoch nach wie vor Einschränkungen dar, die in Zukunft angegangen werden müssen.

In der aktuellen Risikobewertung für Pflanzenschutzmittel und Tierarzneimittel wird davon ausgegangen, dass die mikrobielle Gemeinschaft im Boden und ihre Ökosystemleistungen nicht gefährdet sind, wenn die Auswirkungen der maximalen PEC im Feld auf die Stickstofftransformation (OECD TG 216) über 25 % auf weniger als 100 Tage beschränkt sind. Auf der Grundlage der hier erzielten Ergebnisse und unter Berücksichtigung von EFSA PPR panel (2017) wird empfohlen, die Stufe -1-Bewertung durch einen zusätzlichen Test zur bakteriellen Funktion und einen Test zu den Auswirkungen auf arbuskuläre Mykorrhizapilze zu ergänzen. Aufgrund der höheren Empfindlichkeit, die für die im Rahmen des Projekts getesteten Stoffe festgestellt wurde, wird der ISO 20130 (Enzymaktivit) gegenüber der subtract induzierten Atmung (MicroResp™) bevorzugt. Im Gegensatz zum EFSA PPR-Gremium (2017) halten wir zusätzliche Funktionstests (die zusätzlichen Endpunkte liefern) nicht für eine sinnvolle Option für eine Zwischenstufe, da das von z. B. OECD 216 angegebene Risiko für die N-Transformation durch die Prüfung anderer Funktionen nicht besser abgeschätzt wird. Stattdessen könnte realistischere Exposition mit zusätzlichen Böden getestet werden, z. B. durch Probenahme im Feld als Verfeinerungsoption. Auf Stufe 3 können natürliche Gemeinschaften im Feldversuch (mit bekannter struktureller Zusammensetzung, z. B. durch Mikrobiom-Sequenzierung) getestet werden. Solche Studien zu Effekten auf die Funktion und Strukture der Bodenmikroorganismen im Freiland würden als Referenz für die Kalibrierung oder Validierung der niedrigeren Stufen dienen.

Antibiotikaresistenz

Hier wurde untersucht, inwieweit der Eintrag von Antibiotika/Tierarzneimitteln die Entwicklung von Antibiotikaresistenzen bei bodenrelevanten Bakterien begünstigt**.** Zu diesem Zweck wurden die Minimalen Hemmkonzentrationen (MHK) von Wirkstoffen für umweltrelevante Bodenbakterien bestimmt.

"Einzelspezies-Toxizitätstests mit Mikroorganismen spielen eine wichtige Rolle bei der ERA von Antibiotika" (Brandt et al. 2015). Der Vorteil dieser Methoden ist, dass sie standardisiert, gut definiert, schnell und robust sind und die Ergebnisse aus verschiedenen Labors daher besser vergleichbar sind als bei Gemeinschaftstests. Eine wichtige Überlegung bei allen Einzelspezies-Tests ist, dass selbst einzelne Bakterienarten sowohl antibiotikaresistente als auch empfindliche Stämme enthalten können und die Wahl des Teststamms daher entscheidend ist (Dias et al. 2015). Wir haben daher vorgeschlagen, eine angemessene Stammauswahl in die Standardrichtlinien für Toxizitätstests mit Bakterien, die für die ERA verwendet werden sollen, aufzunehmen. Daher ist eine gründliche genomische und phänotypische Charakterisierung einer breiten Palette von Standardteststämmen erforderlich, um empfindliche Stämme für Ökotoxizitätstests auszuwählen.

Um eine geeignete Stammauswahl treffen zu können, wurde die Verfügbarkeit von Genominformationen der beim IME verfügbaren Stämme überprüft. Von den 22 überprüften Stämmen (19 Arten) sind 16S rRNA (Teil-)Sequenzinformationen und vollständige Genominformationen für zehn bzw. neun Arten verfügbar. Zusätzlich wurden fünf Stämme (*Janibacter terrae* DSM 13953; *Arthrobacter* sp. DSM 917; *Lactobacillus plantarum* DSM 20205; *Pseudomonas fluorescens* ATCC 17571; *Pseudomonas putida* DSM 291) zur Ganzgenomsequenzierung eingesandt, um ihre Eignung für Antibiotikaresistenztests zu ermitteln. Diese Stämme wurden für die Ganzgenomsequenzierung ausgewählt, da es sich um relevante Bodenbakterien handelt. Folgende Testsubstanzen wurden zusammen mit dem UBA ausgewählt: Colistinsulfat, Neomycinsulfat, Chlortetracyclinhydrochlorid, Tiamulinhydrogenfumarat, Kupfersulfat und TWEEN 20.

Die MHK wurde nach dem Brühe-Mikroverdünnungsverfahren gemäß den EUCAST-Protokollen bestimmt. Für den Haupttest wurden 96-Well-Polystyrol-Mikrotiterplatten mit flachem Boden verwendet. Für jede Substanz wurden acht Konzentrationen und zehn Wiederholungen getestet. Die Verdünnungen der Substanz wurden aus den Stammlösungen hergestellt. Die Platten enthielten drei Kontrollen, die als Validitätskriterien dienten: Inokulationskontrolle (zwischen 0,08 und 0,13 bei OD625), Substanzkontrolle (kein Wachstum) und Medienkontrolle (kein Wachstum).

Alle Tests wurden erfolgreich durchgeführt und haben die Validitätskriterien erfüllt, und die MHK wurde für jede getestete Bakterie/Substanz bestimmt. Die Verwendung bodenrelevanter Bakterien für die MHK-Bestimmung ist ein wichtiger Schritt zum Verständnis der Wirksamkeit antimikrobieller Mittel in ökologisch relevanten Umgebungen. Die Variabilität der MHKs zwischen den vier getesteten Bakterienstämmen lag in einer Größenordnung von bis zu zwei Größenordnungen. Kein einzelner Stamm war der empfindlichste, aber *Ac. facilis* wies für zwei der vier Antibiotika die *niedrigste* MHK auf, während *Arthrobacter* sp. nie der empfindlichste Stamm war. [4]Die minimalen MHKs der vier getesteten Antibiotika lagen um mindestens zwei Größenordnungen unter denen der beiden anderen getesteten Substanzen (CuSO und TWEEN 20). Die niedrigste MHK wurde mit 0,06 mg/L für Chlortetracyclinhydrochlorid festgestellt.

Bei den vier getesteten Antibiotika wurde kein systematischer Unterschied zwischen den MHKs der getesteten Boden- und klinischen Stämme (aus der EUCAST-Datenbank) festgestellt. Nur in einem Fall, Chlortetracylinhydrochlorin, war die MHK eines Bodenbakteriums (*Acidovorax facilis*) niedriger als alle MHKs der klinischen Stämme. Aufgrund ansonsten fehlender Daten für die Expositionsabschätzung konnten keine Risikoquotienten berechnet werden, außer für Tiamulinhydrogenfumarat. Für dieses Antibiotikum zeigte die MHK ein höheres Risiko an als die ökotoxikologischen Standardtests (RQ > 100). Die derzeitige Risikobewertung für Tierarzneimittel erfasst also möglicherweise nicht die Entwicklung von Antibiotikaresistenzen in umweltrelevanten Bodenbakterien.

Abbauleistung von Bodenmikroorganismen

Hier wurde der Einfluss von realistischeren Belastungsszenarien, im Vergleich zum Standardszenario einer gängigen OECD-Abbaustudie, auf den mikrobiellen Abbau untersucht. Die Daten wurden genutzt, um zu klären, ob beispielsweise Mehrfachanwendungen oder Hintergrundbelastungen in diesen Studien ebenfalls berücksichtigt werden müssen, um die Bodenmikroorganismen ausreichend zu schützen.

Die Tests wurden so konzipiert, dass sie verschiedene Situationen der Einzel- und Mehrfachexposition gegenüber Chemikalien in realistischen Konzentrationen untersuchen und die folgenden Schritte umfassen:

SCHRITT 1: Bestimmung der Abbauleistung von Bodenmikroorganismen nach einmaliger Anwendung einer Testsubstanz im Vergleich zur wiederholten (mehrfachen) Anwendung derselben Testsubstanz (Simulation der wiederholten Anwendung eines Pestizids in einem bestimmten Intervall).
SCHRITT 2: Bestimmung der Abbaubarkeit einer Testsubstanz durch die mikrobielle Bodengemeinschaft in Gegenwart einer weiteren Chemikalie.
SCHRITT 3: Bestimmung der Abbauleistung von Bodenmikroorganismen für eine Testsubstanz in einem landwirtschaftlich genutzten Boden, der bereits eine realistische Mischung von Chemikalien enthält. Der Freilandboden sollte vergleichbare Bodeneigenschaften aufweisen wie einer der in Schritt 1 oder 2 verwendeten Böden, um etwaige Abbaueffekte mit den mehreren zusätzlich im Boden vorhandenen Chemikalien in Verbindung bringen zu können.

Mit den beiden Testsubstanzen Pyraclostrobin (Fungizid) und Ethofumesat (Herbizid) wurden Bodenabbauversuche gemäß OECD 307 in den beiden Böden RefeSol 02A (toniger Schluff, STEP 1-2) und LUFA 2.1 (Sand, Schritt 1) sowie in einem Boden mit einer Hintergrundkontamination (Schritt 3) durchgeführt, der an der ursprünglichen Probenahmestelle von RefeSol 02A frisch beprobt worden war. Für beide Substanzen wurden zwei nominale Testkonzentrationen gewählt, die während der gesamten Abbaubarkeitstests konstant gehalten wurden. Eine der beiden Testkonzentrationen entsprach der maximalen Ausbringungsmenge für die beiden Wirkstoffe (0,667 mg/kg für Pyraclostrobin und 0,800 mg/kg für Ethofumesat) und wurde für die Tests mit einer Chemikalie verwendet. Die zweite Testkonzentration entsprach der Hälfte der maximalen Ausbringungsmenge und wurde zweimal für Mehrfachanwendungen eingesetzt (2 x 0,333 mg/kg für Pyraclostrobin und 2 x 0,400 mg/kg für Ethofumesat), so dass sich insgesamt die gleiche Applikationsmenge ergab, die auch für einmalige Applikation verwendet wurde. Die folgenden Hauptergebnisse wurden erzielt:

- Die Abbauzeiten beider Substanzen, die einmalig als als Einzelsubstanz appliziert wurden, waren in Boden LUFA 2.1 höher als in Boden Refesol 02A. Die DT50-Werte von Pyraclostrobin waren in Lufa 2.1 etwa doppelt so hoch wie die in Refesol 02A beobachteten DT50-Werte, während die DT50-Werte von Ethofumesat in LUFA 2.1. etwa siebenmal höher waren als in Refesol 02A. Die beobachteten Unterschiede der DT50-Werte je nach Bodentyp stimmen mit dem bisherigen Kenntnisstand überein. Auch wenn diese Unterschiede bekannt sind, werden sie zurzeit während der Risikoabschätzung für Bodenmikroorganismen, die Chemikalien exponiert sind, nicht berücksichtigt.

- Die einmalige Applikation der beiden Wirkstoffe Pyraclostrobin und Ethofumesat, die als Einzelsubstanzen in die Bodenproben appliziert wurden, wurden mit der mehrmaligen Applikation (d. h. zwei aufeinanderfolgenden Applikationen) desselben Wirkstoffs im Abstand von 21 Tagen verglichen. Die Auswirkung der Mehrfachapplikation auf die Abbauhalbwertszeit zeigte kein einheitliches Bild: Die DT50-Werte von Pyraclostrobin waren in beiden Böden nach der zweiten Anwendung höher, während die DT50-Werte für Ethofumesat im Boden Refesol 02A vergleichbar hoch waren, im Boden Lufa 2.1 nach der Mehrfachanwendung jedoch niedriger. Diese Ergebnisse deuten darauf hin, dass sich eine mehrfache Applikation negativ auf den weiteren Abbau der Testsubstanz nach 21 Tagen auswirken kann.

- Die Auswirkung von Gemischen aus Pyraclostrobin und Ethofumesat auf den Bodenabbau beider Substanzen wurde anhand von einmaliger und mehrmaliger Applikation beider Substanzen zur gleichen Zeit sowie von einmaliger, aber zeitversetzter Applikation beider Substanzen in denselben Boden (RefeSol 02A) untersucht. Die Ergebnisse zeigten, dass das Vorhandensein einer zweiten Substanz (in einem Gemisch oder bereits im Boden vorliegend) keine einheitlichen Effekte auf den Abbau der jeweiligen Substanz im Gemisch

hatte. Es konnte jedoch ein ähnliches Muster für Pyraclostrobin und Ethofumesat beobachtet werden: Bei einmaliger Applikation waren die Abbauzeiten von Pyraclostrobin und Ethofumesat geringfügig länger, wenn sie als Gemisch angewendet wurden. Der Unterschied war gering, so dass er als vergleichbar hoch angesehen wurde, da eine statistische Auswertung nicht möglich war. Bei mehrmaliger Applikation des Gemisches beider Substanzen war der Abbau beider Substanzen nach der ersten Anwendung (0 - 21 Tage) langsamer, nach der zweiten Anwendung (21 - 120 Tage) jedoch schneller. Bei der zeitversetzten Anwendunge führte die Applikation einer Substanz nach 21 Tagen mit einer zweiten, bereits im Boden vorhandenen Substanz zu einer Verlängerung der Halbwertszeit von - zumindest - Ethofumesat.

- Die erhaltenen DT50-Werte für Bodenabbauversuche nach einmaliger Applikation von Pyraclostrobin und Ethofumesat als Einzelsubstanzen in einen Boden aus landwirtschaftlicher Nutzung waren niedriger als die Ergebnisse für Bodenabbauversuche in Boden RefeSol 02A (Schritt 1-2), das heißt es konnten keine negativen Effekte in dem Freilandboden nachgewiesen werden.

Bei Bodenabbauversuchen gemäß OECD 307 werden in der Regel die Abbauraten von Wirkstoffen bestimmt, welche dann für die Abschätzung der voraussichtlichen Umweltkonzentration nach einer beabsichtigten Ausbringung einer Chemikalie - wie z.B. eines Pflanzenschutzmittels - verwendet werden. In der Regel werden die Abbauversuche mit einer Reihe verschiedener Böden durchgeführt, wobei der jeweilige Mittelwert oder der ungünstigste Fall („worst case“) für die Abschätzung der voraussichtlichen Umweltkonzentration herangezogen wird. Im Rahmen der Risikobewertung von Chemikalien werden diese voraussichtlichen Umweltkonzentrationen mit den Effektwerten für die Bodenmikroorganismen verglichen.

Die Ergebnisse des MICROSOIL-Projekts zeigen, dass die mehrmalige Anwendung derselben Substanz sowie das Vorhandensein einer anderen Substanz Auswirkungen auf die Abbaurate der getesteten Substanzen haben kann: Es wurden sowohl positive als auch negative Effekte auf die Abbaurate der in dieser Studie verwendeten Testsubstanzen beobachtet. Ein negativer Effekt auf die Abbaurate wurde beispielsweise für Pyraclostrobin festgestellt, wenn es bei mehrmaliger Applikation ein zweites Mal ausgebracht wurde oder für Ethofumesat, wenn es auf einen Boden appliziert wurde, in dem bereits eine zweite Substanz (hier: Pyraclostrobin) vorhanden war.

Die Ergebnisse dieses Projekts sind spezifisch für die verwendeten Testsubstanzen Pyrclostrobin und Ethofumesat sowie für die getesteten Böden RefeSol 02A und Lufa 2.1. Es sind daher weitere Forschungsarbeiten erforderlich, die die realistischen Anwendungsscenarios von Pflanzenschutzmitteln sowie die Düngepraktiken einschließlich der Rückstände von Bioziden, Industriechemikalien und Arzneimitteln widerspiegeln, um die tatsächliche Abbaurate der Substanzen unter realistischen Bedingungen zu bestimmen und die voraussichtliche Umweltkonzentration der Stoffe korrekt abschätzen zu können. Dabei sollte berücksichtigt werden, dass es in Europa eine Vielzahl von Bodentypen gibt und dass in landwirtschaftlich genutzten Böden Mehrfachanwendungen und Mischbelastungen aus Rückständen vorkommen, damit ein ausreichender Schutz der Bodenfunktion und der Umwelt gewährleistet werden kann.

1 Introduction

Soil health is the most important driving factor for sustainable agriculture and must be maintained and promoted accordingly. Microorganisms such as bacteria or (arbuscular mycorrhizal) fungi are an essential and integral part of living soil and influence various biogeochemical cycles of major nutrients such as carbon, nitrogen, sulphur and phosphorus. They play a greater role in maintaining soil health than other biological components of soil (Sathya et al. 2016). Microorganisms are actively involved in soil formation by initiating the process of biological weathering of rocks, decomposition of organic matter and nutrient cycling (Kaviya et al. 2019). They are responsible for various biological transformations of different sources of carbon and macro- and micronutrients, which facilitate the subsequent establishment of soil-plant-microbe interactions (Sahu et al. 2017).

The humus content in Central European soils is continuously decreasing (Jacobs et al. 2013). Due to multiple pressures and an inadequate environmental assessment approach with regard to the microorganisms in soils the basic protection goal of "maintaining soil biodiversity" cannot be achieved. As a result, resilience can decrease significantly and with it the ability of soil microorganisms to react to e.g. stressors such as climate change. A more differentiated risk assessment for soil microorganisms is therefore necessary, which is equally weighted towards the other assessment areas such as soil invertebrates or non-target arthropods.

The aim of the project was to identify test methods and endpoints which can be used within an environmental risk assessment to ensure the protection of soil microorganisms and thus the important ecosystem services of soils against various pollutants and to determine their applicability in practice.

The project aimed to answer three key questions:

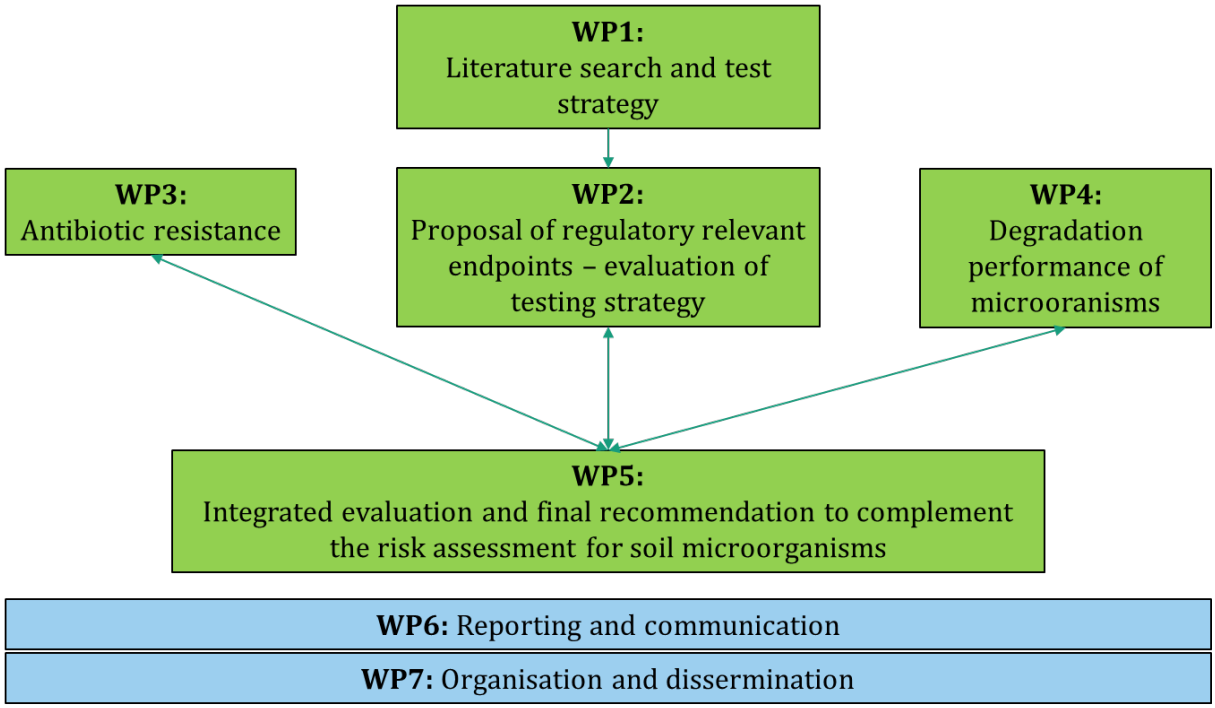
First key question: Which methods are suitable for recording and describing changes in soil function and the structural composition of the microbial community in soils for regulatory assessment of plant protection products, biocides and veterinary pharmaceuticals? A literature search was conducted to compile a list with available test methods for recording the functional response of microorganisms to the active substance concentration (e.g. enzymatic tests, Biolog© or MicroResp™, mycorrhizal fungi) considering the recommendations of the EFSA soil opinion (EFSA PPR Panel, 2017). Afterwards, the suitability of chosen test methods for identifying changes in the soil microbial function and the structural composition of the soil microbial community was tested exemplarily using six different test substances.

Second key question: Can the exposure of soil microorganisms to antibiotics/veterinary pharmaceuticals lead to the development of resistance genes and are there interactions with other chemical stressors? Here, the aim was to investigate the extent to which inputs of antibiotics/veterinary pharmaceuticals favour the development of antibiotic resistance in soils. For this purpose, minimum selective antibiotic concentrations of active substances for environmentally relevant soil bacteria were determined. The existence of specific environmental conditions for the formation or transfer of resistance was considered.

Third key question: What influence do multiple applications of active substances from plant protection products and a background pollution have on the microbial degradation of pollutants in soils? Here, the influence of more realistic pollution scenarios compared to the standard scenario of a common OECD degradation study on microbial degradation was investigated. The data was used to clarify whether, for example, multiple applications or background pollution must also be considered in these studies in order to adequately protect the soil microorganisms.

The project included 7 different work packages (Figure 1). In work package 1 a literature review was conducted, resulting in proposals for test methods, which were then analysed in work package 2. Work package 3 examined antibiotic resistance, while work package 4 focussed on the effect of multiple applications and background concentrations on the degradation performance of soil microorganisms. Finally, work package 5 summarised the data to enable an integrative analysis. Work packages 6 and 7 involved organisational work.

Figure 1: Project structure of the MICROSOIL project.



Source: own illustration, Fraunhofer IME

This report is structured into three main chapters:

- Chapter 2: Function and structure of soil microorganisms.
  Six chemicals were tested in three soils using six different methods (Table 1) to evaluate whether the current assessment based on the OECD test 216 on N-transformation should be replaced or supplemented. The focus is on ecological risk assessment for plant protection products but the results are also relevant for other regulations.

- Chapter 3: Antibiotic resistance
  Four antibiotics and two other chemicals (a disinfectant and a heavy metal, (Table 1)) were tested on antibiotic resistance building in soils using four typical soil bacteria strains in contrast to the typically tested clinically relevant strains. Thus, the background is risk assessment for human and animal health.

- Chapter 4: Degradation performance of soil microorganisms
  Effects of multiple applications, a second chemical stressor and of background contamination of agricultural soil were tested. Results may affect the calculation of Predicted Environmental Concentrations in Soil (PEC_{soil}) if they would indicate higher background levels due to slower biodegradation.

The final Chapter 5 summarizes results and provides recommendations for improving risk assessments.

Table 1: Overview on tests items and soils tested with different methods within the project.

Test item	Application	Microbial function and structure (OECD 216, ISO 15685, MicroResp, ISO 20130, ARISA) in RefeSol 02A, RefeSol 04A LUFA 2.1 Chapter 2	Mycorrhiza germination ISO 10832 in RefeSol 02A Chapter 2	Antibiotic resistance using 4 bacterial strains Chapter 3	Degradation OECD 307 (modified) in RefeSol 02A LUFA 2.1 Chapter 4
Propamocarb-hydrochloride	Fungicide	x	X	-	-
Pyraclostrobin	Fungicide	x	x	-	Alone & with Ethofumesate
Tebuconazole	Fungicide	x	x	-	x
Tiamulin hydrogen fumarate	Antibiotic	x	x	x	-
Ethofumesate	Herbicide	x	x	-	Alone & with Pyraclostrobin
Didecyl-dimethyl-ammonium chloride (DDAC)	Disinfectant	x	x	-	-
Colistin sulfate	Antibiotic	-	-	x	-
Neomycin sulfate	Antibiotic	-	-	x	-
Chlortetracyline hydrochlorid	Antibiotic	-	-	x	-
Copper sulfate	Fungicide, algicide, molluscicide	-	-	x	-
TWEEN 20 (Polysorbate 20)	Detergent	-	-	x	-

2 Effects on function and structure of soil microbial communities – literature research and exemplary testing

2.1 Available standardized and non-standardized methods

In work package (WP) 1, a literature search was conducted to identify currently available test methods to determine effects of chemicals on the structure and composition of the soil microbial community (especially bacteria and fungi) and to retrieve suitable test methods to record and describe changes in soil functions related to soil microorganisms. Based on both the results and the evaluation of the literature search, a research strategy for this project was developed.

The aim of WP1 was to evaluate at least one to a maximum of three suitable test method(s) with respect to meaningful endpoints and their suitability within the regulatory framework.

Identified test methods were evaluated according to various criteria such as feasibility in the laboratory, costs, relevance and replicability. Care was taken to avoid particularly expensive, specialised equipment for the suggested methods. Therefore, the selection process focused on the effect of chemicals on the widest possible range of enzymatic reactions (e.g. on the activity of exoenzymes and endoenzymes) but also on microbial specialists (e.g. ammonium oxidizing bacteria).

Furthermore, general statements describing effects of chemicals on microbial populations were also developed within WP1. Finally, effects of chemicals on mycorrhizal fungi, particularly on arbuscular mycorrhizal fungi, were also considered.

2.1.1 Literature search and results

To find adequate publications in peer-reviewed journals, literature was searched in various search engines, namely in Science Direct, Google Scholar, Web of Science and Scopus as well as in an internal literature database for suitable test methods for soil microorganisms. The literature search was performed from April to October 2021.

Care was taken to ensure that the methods identified are suitable for determining the influence of chemicals on soil functions as well as on structural changes in the soil microbial community. Furthermore, the methods were supposed to cover both bacteria and fungi and should also include exo- and endo-enzymes in soil.

The terms of the literature search focused on three different areas: *(i)* general terms, *(ii)* direct presentation of methods/assays and *(iii)* effect studies. The general terms were used to find relevant literature regarding the measurement of soil function and soil microbial structure and their assessment with no particular description of the presented method. From this literature, the source of the described method, which appeared to be appropriate for the testing strategy, was retrieved. In addition, terms focusing on “methods” or “assays” were used to identify literature directly presenting certain methods to determine or assess relevant soil processes. In a last step, we searched for studies presenting the effect of pesticides or other chemicals on soil microorganisms to get an idea about the sensitivity of the methods.

For the literature search a time span from 2000 to 2021 was selected. If earlier literature was described in the sources, it was retrieved separately. The first 8 to 10 pages (comprising 10 hits per page) of the search results were checked by evaluation of the title and/or abstract of the references. If the evaluation showed that the references were not relevant to the project, the search was stopped and another term was used. To observe more recent literature, the time period of the search was narrowed down to a time span from 2010 to 2021, which was sorted

chronologically from new to old. Furthermore, the search was mainly limited to biological, geological or (eco)toxicological journals.

The results of the literature search are summarized in the Appendix, Table 150. The methods found in the literature search are summarized in Table 151.

2.1.2 Parameters for the assessment of test methods

Evaluation parameters were determined to be able to distinguish between different methods. The parameters proposed in the following pages are based on existing evaluations in the literature, but have been modified and supplemented to a greater or lesser extent, if necessary. Thiele-Bruhn et al. (2020) evaluated different methods based on a score from 1 to 5 in combination with a color code consisting of red, orange, yellow, light green and green. Thiele-Bruhn et al. (2020) combined and slightly adapted parameters used by Pulleman et al. (2012) and Faber et al. (2013). This approach may increase the precision of the assessment, but complexity is raised and the decision about suitability of a test method becomes more difficult. Therefore, in a first step the assessment based on a traffic light system should be kept as simple as possible. For the assessment within this project five parameters were selected: (i) practicability, (ii) estimated costs, (iii) replicability/reproducibility, (iv) feasibility depending on soil type and (v) relevance for regulatory purposes. These parameters were considered essential for the assessment of methods for regulatory purposes.

Accordingly, we used a simple traffic light system for each individual parameter. In this system green, yellow and red stand for reasonable, possible and not reasonable, respectively. This is explained in more detail below.

2.1.2.1 Practicability

Green: Methods which require neither expensive (analytical) equipment, nor special skills.

Yellow: Methods which require either expensive (analytical) equipment or special skills.

Red: Methods which require expensive (analytical) equipment and special skills.

(Analytical) equipment: This refers mainly to standard laboratory equipment, e.g. measuring devices such as a simple photometer including a 96-well microplate reader. For non-standard equipment, a one-time investment of about 10,000€ is assumed to be a reasonable amount for the implementation of a new method. Regarding chemicals, costs of about 500€ are considered reasonable.

Special skills (i.e. for preparation of test solutions or non-standard statistical methods as multi-variant analysis): For the statistical evaluation of a test, the calculation of ECx values based on a concentration-response curve as well as the calculation of inhibitions compared to the control are assumed to be standard, while e.g. more advanced statistical methods (e.g. principal component analysis) for which special programs as well as expert knowledge is required are considered non-standard.

2.1.2.2 Possible costs for the method

Green: In total overall low costs

Yellow: In total overall moderate costs

Red: In total overall high costs

Total costs result from the consideration of all individual costs related to a certain method (i.e. method implementation, costs per sample measurement and costs per working hour of laboratory staff).

Costs per sample measurement and working hour of laboratory staff: Low costs result from personal with basic laboratory experience AND work load for test preparation (≤ 10 h) AND for determination per sampling day and calculation of results (≤ 10 h).

Moderate costs result from personal with increased laboratory experience OR work load for test preparation (≥ 10 h) OR for determination per sampling day and calculation of results (≥ 10 h).

High costs result from personal with increased laboratory experience AND work load for test preparation (≥ 10 h) AND for determination per sampling day and calculation of results (≥ 10 h).

2.1.2.3 Replicability/Reproducibility

Green: Information on laboratory comparison or ring tests are available.
Low variability based on the available data: ≤25% of the mean CV of the control replicates for the determined endpoint (e.g. expected for standardised ISO guidelines).

Yellow: Information on laboratory comparison or ring tests are available.
Acceptable variability based on the available data: 26 - 40% of the mean CV of the control replicates for the determined endpoint (e.g. expected for standardised ISO guidelines).

Red: No information on laboratory comparison or ring tests available (e.g. expected for non-standardised methods).

Criteria for variability: To set a limit, data from ring tests of the ISO standard guidelines (ISO 14240-2 (2011), ISO 17155 (2012), ISO 15685 (2012), ISO 20130 (2018) and ISO 18187 (2024)) were summarised. The mean value of the coefficient of variation and the respective standard deviation was calculated (Table 2). The coefficients of variation of the ISO 15685 ring test had a high variability and therefore, these values were not considered for possible limits.

Methods with a mean coefficient of variation below or equal to 25% were considered as methods with a low variability. Methods with a mean coefficient of variation above 25 and up to 40% were considered as methods with moderate variability.

The threshold values were set based on the data available in the ISO guidelines (see Table 2), but were slightly adapted to receive standard values (i.e. 25% were used as threshold value instead of 26% to implement threshold for low variability). In addition, 46% were considered to be quite high and therefore, the upper threshold value for moderate variability was set to be at 40% by expert judgement.

Table 2: Available coefficients of variations [%] from single tests of ring tests conducted with five ISO standard guidelines for the assessment of soil quality.

Soil microbial biomass – Fumigation extraction method (ISO 14240 -2)	**Abundance and activity of soil microflora using respiration curves (ISO 17155)**		**Potential nitrification – Rapid test by ammonium oxidation (ISO 15685)**	**Measurement of enzyme activity patterns (ISO 20130)**		**Dehydrogenase activity of *Arthrobacter globiformis* (ISO 18187)**
CV [%] of measured C concentration	CV [%] of measured CO_2 or O_2 concentration		CV [%] of measured NO_2-N/g concentration	CV [%] calculated for each activity considering all laboratories and a single soil		CV [%] calculated for inhibition of dehydrogenase activity
21	66	84	17	10	22	8
15	53	40	40	18	33	22

Soil microbial biomass – Fumigation extraction method (ISO 14240 -2)	**Abundance and activity of soil microflora using respiration curves (ISO 17155)**		**Potential nitrification – Rapid test by ammonium oxidation (ISO 15685)**	**Measurement of enzyme activity patterns (ISO 20130)**		**Dehydrogenase activity of *Arthrobacter globiformis* (ISO 18187)**
23	37	48	154	10	10	36
18	50	98	143	24	20	8
11	27	43	136	27	14	13
15	16	47		37	36	23
	10	44		13	16	22
	29	45		23	32	29
	41	65		6		
	65	36		23		
	28	32		16		
	43	65		31		
Mean CV%: 17	**Mean CV%:** 46		**Mean CV%:** 98	**Mean CV%:** 21		**Mean CV%:** 20
Overall Mean CV%: 40						
Overall Standard deviation: 34						
Overall Mean CV% excluding the data from ISO 15685: 26						
Overall Standard deviation excluding the data from ISO 15685: 14						

2.1.2.4 Feasibility depending on soil type

Green: No influence of soil properties (e.g. particle size distribution) in the test system and representative arable soil.

Yellow: Influence due to soil properties (e.g. a high clay content and an associated high soil density or sorption of the test substrate to soil) are expected or reported in the literature (e.g. forest soil, peat), however, these soil types are of no relevance for agriculturally used sites.

Red: Strong influence of soil properties (e.g. a high clay content and an associated high soil density or sorption of the test substrate to soil) and influence due to soil types are reported in the literature; use of these soils for agriculture is not excluded (e.g. loamy soil).

Some ISO guidelines point out that different soil types are not suitable for the respective test systems. For example, forest soils or soils with waterlogging are not suitable for some test methods. However, it has to be considered that e.g. very loamy soils form large agglomerates, which might induce problems at the distribution of soil in microplates. Some methods have primarily been used on forest soils or other special soils that do not correspond to the arable soils targeted in the project.

2.1.2.5 Relevance for regulatory purposes / expressiveness in a tiered approach

Green: Comprehensive assessment of the soil microbial community including mycorrhizal fungi, with a high relevance/transferability to all of the following aspects: microbial diversity, function and structure; ecological function and ecosystem services.

Yellow: Partial assessment of the soil microbial community including mycorrhizal fungi with lesser relevance/ transferability to at least one of the following points: microbial diversity, function and structure; ecological function or ecosystem services.

Red: Single species tests with a low relevance/transferability to microbial diversity, function and structure; ecological function or ecosystem services.

In accordance to EFSA PPR panel (2017)) seven ecosystem services were identified as being driven by in-soil organisms in the agricultural landscape. In six of these services, microorganisms play an important role regarding *(i)* genetic resources and biodiversity, *(ii)* the cycling of nutrients in soils, *(iii)* pest and disease control, *(iv)* natural attenuation, *(v)* soil structure formation and water retention and *(vi)* as part of the soil food web. Therefore, the main goal is the protection of the soil microbial community including mycorrhizal fungi. Test methods should reflect effects on the community, i.e. it should be possible to demonstrate the effect of a substance on the abundance, function and structure of the soil microbial community including mycorrhizal fungi. This can be expressed through the effect on different nutrient cycles, enzymatic activities or species richness.

Mycorrhizal fungi are part of the soil microbial community and depending on the used test system, the test substance and the test soil, effects on mycorrhizal fungi will be most likely. Since particularly arbuscular mycorrhizal fungi (AMF) are responsible for important ecosystem services, they are an essential microbial group of the in-soil community. As tests on AMF are conducted as single species tests, they are considered separately besides tests targeting the whole microbial in-soil community.

2.1.2.6 Handling and availability of organisms

The parameter 'handling and availability of organisms' has been used by Thiele-Bruhn et al. (2020) and Pulleman et al. (2012) to compare different test methods. This parameter may be applicable to various tests on in-soil meso- or macro-organisms, but it is not suitable to rank tests on soil microorganisms including AMF, which are available throughout the whole year. According to the standardized ISO guidelines, experimental soil can be stored refrigerated (<4°C) for up to 3 months. Therefore, its availability is also given throughout the year. As availability of AMF throughout the year is possible, care as well as expertise will be necessary to establish test on AMF. Due to the low importance of this parameter for in-soil microorganisms, 'handling and availability' was not considered further in this project.

2.1.3 Methods for the testing strategy and their evaluation

In the following, various test methods identified in the literature are listed, briefly described, and evaluated in terms of their suitability for this project. Methods for which radiolabeled macromolecules (e.g. ^{14}C, ^{3}H) are necessary were not considered as appropriate test methods and therefore were not listed below. This type of testing requires an appropriate radiation protection area, specialized know-how and equipment, which cannot be provided by most of the standard laboratories.

In addition, plate count and microbial cultures as well as direct microscopy of these cultures from soil combined with cell staining as e.g. discussed by Blagodatskaya and Kuzyakov (2013) or Brandt et al. (2015) were not considered as appropriate methods which would fit in the scope of the project. The reason for this is two-fold. On the one hand, it is impossible to extract

microorganisms quantitatively from soil. On the other hand, the number of cultivable microbes from soil using plate techniques is very small (~1%) and depends on the used medium. Therefore, according to Blagodatskaya and Kuzyakova (2013), the microbial biomass quantified via plating is strongly underestimated.

Six methodological groups were formed. These groups include methods for determining (i) the activity of aerobic and anaerobic heterotrophic microorganisms, (ii) nitrifying and denitrifying bacteria, (iii) the activity of exo- and endoenzymes, (iv) the effect on arbuscular mycorrhizal fungi, (v) the determination of functional genes and structural changes in soils and (vi) carbon cycling and sequestration.

2.1.3.1 Activity of aerobic and anaerobic heterotrophic microorganisms

2.1.3.1.1 Activity of aerobic, heterotrophic microbial biomass (comparable methods to OECD 217)

As a reference the standard test guideline OECD TG 217 (2000), which is used for the assessment of chemical effects on soil microorganisms, was evaluated based on the parameters described in chapter 2.1.2. Due to this a direct comparison of the alternative test systems is possible.

I. **OECD Test No. 217 – Soil Microorganisms: Carbon Transformation Test.** A laboratory test method designed to investigate long term potential effects of a single exposure of chemicals on carbon transformation activity of soil microorganisms.

   Endpoint: Effect on the carbon transformation activity of soil microorganisms.

   Practicability: Method requires neither expensive (analytical) equipment nor special skills. Therefore, the parameter was evaluated green.

   Costs: In total overall moderate costs (10 – 30 h per test). Therefore, the parameter was evaluated yellow.

   Replicability/reproducibility: No information on laboratory comparison or ring tests available in the guideline. However, before a method becomes an OECD guideline, it goes through an expert panel. In addition, it is the actual guideline used for environmental risk assessments (ERA). Therefore, it can be assumed that the replicability/reproducibility is good and the parameter is evaluated accordingly (green).

   Soil type: No influence of soil properties on use in the test system and representative arable soil. Therefore, the parameter was evaluated green.

   Relevance for regulatory purpose: Assessment of the soil microbial activity of aerobic microorganisms. The soil is tested directly and therefore, the environmental relevance is high. Due to low expressive power, the test is no longer used for regulatory purposes. The regulatory relevance is low, therefore the parameter was evaluated red.

The following guidelines listed below (II – V) describe ISO guidelines that are comparable to the OECD 217 test guideline concerning carbon transformation of soil microorganisms. These methods are considered as 'beyond the scope'. These ISO guidelines have been included for completeness only. However, for the final evaluation of the test strategy it will be necessary to compare results of the OECD 217 test as well as these ISO guidelines for different test substances directly.

II. **ISO 14240-1:2011 - Determination of soil microbial biomass – Part 1: substrate-induced respiration method.** A method for the determination of the active aerobic, heterotrophic microbial biomass in aerated agricultural and mineral soils.

III. **ISO 14240-2:2011 - Determination of soil microbial biomass – Part 2: fumigation-extraction method.** A method for the determination of microbial biomass of soils by measurement of total extractable organic biomass material mainly from fumigated microorganisms. It is also applicable to the estimation of microbial nitrogen and ninhydrin-reactive nitrogen in soil.

IV. **ISO 16072:2002 - Laboratory methods for determination of microbial soil respiration.** Methods for the determination of soil microbial respiration of aerobic, unsaturated soils. The methods are suitable for the determination of O_2 uptake or CO_2 release, either after addition of a substrate (substrate-induced respiration), or without substrate addition (basal respiration).

V. **ISO 17155:2020 - Determination of abundance and activity of soil microflora using respiration curves.** A test method for determining the activity of the active aerobic, heterotrophic microbial biomass in soils. This method is applicable to the monitoring of soil quality and to the evaluation of the ecotoxic potential of soils and soil materials. It is also applicable to soils that are contaminated experimentally in the field or in the laboratory (chemical testing) and for soils sampled along contamination gradients in the field.

2.1.3.1.2 Activity of aerobic, heterotrophic microbial biomass (additional to OECD 217)

1. **BIOLOG® – Garland and Mills (1991) – Classification and Characterization of Heterotrophic Microbial Communities on the Basis of Patterns of Community-Level Sole-Carbon-Source Utilization. Applied and Environmental Microbiology. 57:2351-2359 (Garland and Mills 1991).** A method based on tetrazolium dye reduction as an indicator of sole-carbon-source utilization as a rapid, community-level method to characterize and classify heterotrophic microbial communities. Direct incubation of whole environmental samples (aquatic, soil, and rhizosphere) in BIOLOG plates containing 95 separate carbon sources produced community-dependent patterns of sole carbon-source utilization.

   Endpoint: Effect on nutrient cycles or turnover of carbon, nitrogen, phosphorus and/or sulfur containing substrates.

   Practicability: Method requires neither expensive (analytical) equipment nor special skills. For the method a spectrophotometer, an incubation chamber and 96-well multiwall plates is all that is needed. The method can be easily handled without the need for special additional training. Therefore, the parameter was evaluated green.

   Costs: In total overall low costs (≤10 h per test). BIOLOG® ECO plates should be available throughout the year. Therefore, the parameter was evaluated green.

   Replicability/reproducibility: No information on laboratory comparison or ring tests available. However, for this commercially available system this is not surprising. However, there is a range of publication dealing with the BIOLOG® system and its applicability in soil science (see chapter 2.1.5). At least the quality of ECO plates should be equal throughout the whole year. No information could be obtained about a quality assurance system. However, it was decided to evaluate this parameter with yellow instead of red (=no laboratory comparison or ring test available), since it is assumed that the quality of the ECO plates should be high in this well-established system and therefore comparable throughout different tests.

Soil type: Since an eluate is produced, there is no dependence on the soil type. However, only a small proportion of the soil microorganisms will be captured by this method. Therefore, the parameter was evaluated yellow.

Relevance for regulatory purpose: Since only a fraction of the soil microbial community is assessed, the test barely represents the complete community. In addition, the test has a lower flexibility regarding the observed nutrient cycles due to the given substrates (partly unnecessary substrates) in the ECO plates. In conclusion, the method can be used to assess a limited number of metabolic transformations performed by a fraction of the soil microbial community or mycorrhizal fungi. Therefore, lower relevance/transferability to at least one of the following points: microbial diversity, function and structure; ecological function or ecosystem services. The parameter was evaluated yellow.

2. **MicroResp™ - A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil (Campbell et al. 2003).** The origin of the MicroResp™ system, which is a microplate-based respiration system with DeepWell™ plates that enables the user to analyse up to 96 soil, sediment or water samples and to test a range of carbon sources and/or replicates in a small compact space. While some sole C-source tests tend to select for fast-growing bacteria and rely on growth of organisms, MicroResp™ gives more immediate responses to these substrates and reflects activity rather than growth by measuring responses in the first 6 hours.

   Endpoint: Effect on nutrient cycles or turnover of carbon, nitrogen, phosphorus and/or sulfur containing substrates.

   Practicability: Method requires neither expensive (analytical) equipment nor special skills. For the method a spectrophotometer, an incubation chamber, substrates and 96-well multiwall plates is all that is needed. The methods can be easily handled without the need of special additional training. Therefore, the parameter was evaluated green.

   Costs: In total overall low costs (≤10 h per test). Therefore, the parameter was evaluated green.

   Replicability/reproducibility: No information on laboratory comparison or ring tests available. Substrate solutions must be prepared new each time. However, for this commercially available system this is not surprising. However, there is a range of publications dealing with the MicroResp™ system and its applicability in soil science (see chapter 2.1.5).

   Greater susceptibility to errors than with EcoPlates. Based on the IME own experiences, the use of stored plates can result in a higher standard deviation of the transformation rates. Use of fresh plates overcomes this problem. As the solutions are easy to prepare and the variations in the replicates can be reduced, it was decided to evaluate this parameter with yellow instead of red (=no laboratory comparison or ring test available).

   Soil type: Distribution in DeepWell™ plates could be difficult e.g. with soils with a high clay content. Therefore, the probability for errors might be higher. However, there is no available data and this would have to be tested. Based on IMEs own experience this will be no issue with arable soils and therefore the parameter was evaluated green.

   Relevance for regulatory purpose: Assessment of the soil microbial community. The soil is tested directly and therefore, the environmental and regulatory relevance is high. Therefore, the parameter was evaluated green.

2.1.3.1.3 Microbial activity of aerobic and anaerobic soil microorganisms

3. **Fe(III) reduction test according to "Microbial inhibition by pharmaceutical antibiotics in different soils – dose response relations determined with the Iron(III) reduction test. Environmental Toxicology and Chemistry, 24, 869 – 876" (Thiele-Bruhn 2005).** The iron(III) reduction test as described by Welp and Brümmer is a method to determine the effect on aerobic and anaerobic microorganisms. The microbial Fe(III) reduction has been found to be affected by environmentally relevant concentrations of antibiotics in soil as stated in Brandt et al. (2015).

   Endpoint: Effect on the soil microbial activity after addition of glucose, measured by the iron concentration of the solutions, which are indicative for the soil microbial activity.

   Practicability Method requires expensive (analytical) equipment, since an ICP-MS or ICP-OES will be necessary to measure Fe in the supernatant. Therefore, the parameter was evaluated yellow.

   Costs: In total overall moderate costs (10 – 30 h per test) and additional costs per sample measurement via ICP-MS or ICP-OES. Therefore, the parameter was evaluated yellow.

   Replicability/reproducibility: No information on laboratory comparison or ring tests available. Therefore, the parameter was evaluated red.

   Soil type: No influence of soil properties on use in the test system and representative arable soil. Therefore, the parameter was evaluated green.

   Relevance for regulatory purpose: Assessment of the soil microbial activity of aerobic and anaerobic microorganisms. The soil is tested directly and therefore, the environmental and regulatory relevance is high. Therefore, the parameter was evaluated green.

2.1.3.2 Nitrifying and denitrifying bacteria

2.1.3.2.1 Nitrifying and denitrifying bacteria (comparable method to OECD 216)

As a reference the standard test guideline OECD TG 216 (2000), which is used for the assessment of chemical effects on soil microorganisms, was evaluated based on the parameters described in chapter 2.1.2. Due to this a direct comparison of the alternative test systems is possible.

i. **OECD Test No. 216 – Soil Microorganisms: Nitrogen Transformation Test.** A laboratory test method designed to investigate the long-term effects of chemicals, after a single exposure, on nitrogen transformation activity of soil microorganisms.

   Endpoint: Effect on the nitrogen transformation activity of soil microorganisms.

   Practicability: Method requires neither expensive (analytical) equipment nor special skills.

   Costs: In total overall moderate costs (10 – 30 h per test).

   Replicability/reproducibility: No information on laboratory comparison or ring tests available. However, before a method becomes an OECD guideline, it goes through an expert panel. In addition, it is the actual guideline used for environmental risk assessments. Therefore, it can be assumed that the replicability/reproducibility is good and the parameter is evaluated accordingly (green).

Soil type: No influence of soil properties on use in the test system and representative arable soil.

Relevance for regulatory purpose: Assessment of the soil microbial activity of aerobic microorganisms. The soil is tested directly, however, there are doubts about the significance of the results due to the fact that the determined parameters are sum parameters and therefore, the environmental and regulatory relevance is considered moderate.

The guideline listed below (ii) describes an ISO guideline that is comparable to the OECD 216 test guideline concerning nitrogen transformation of soil microorganisms. The guideline has been included for completeness only. This method is considered 'beyond the scope' of the project. However, for the final evaluation of the test strategy it will be necessary to compare results of the OECD 216 test as well as these ISO guidelines for different test substances directly.

ii. **ISO 14238:2012 - Determination of N mineralization and nitrification in soils and the influence of chemicals on these processes.** Laboratory procedures for measuring the mineralization and nitrification of nitrogen by the soil microbiota.

2.1.3.2.2 Nitrifying and denitrifying bacteria (additional to OECD 216)

4. **ISO 15685:2012 - Determination of potential nitrification and inhibition of nitrification – rapid test by ammonium oxidation.** A rapid method for the determination of the potential rate of ammonium oxidation and inhibition of nitrification in soils. This method is suitable for all soils containing a population of nitrifying microorganisms. It can be used as a rapid screening test for monitoring soil quality and quality of wastes, and is suitable for testing the effects of cultivation methods, chemical substances [except volatiles i.e. H > 1 (Henry's constant)], extracts of biosolids and pollution in soils.

   Endpoint: Effect on ammonium-oxidizing bacteria (AOB). Step of the transformation of ammonium to nitrite during nitrification.

   Practicability: Method requires neither expensive (analytical) equipment nor special skills. For the method a spectrophotometer, an incubation chamber, substrates and 96-well multiwall plates is all that is needed. The methods can be easily handled without the need for special additional training. Therefore, the parameter was evaluated green.

   Costs: In total overall low costs (≤ 10 h per test). Therefore, the parameter was evaluated green.

   Replicability/reproducibility: Information on laboratory comparison or ring tests available. Acceptable variability based on the available coefficients of variation (26 - 40%). Note: For this evaluation, only the reference soil and soil No. 1 showed coefficients of variation within the recommended range of 200 ng N/g to 800 ng N/g (dw/h) in the ring test. In the ring test, also contaminated soils were tested which showed a very low ammonium oxidation activity. The high coefficients of variation of the contaminated soils are linked to very low values (close to zero). For this reason, only the coefficient of variation of the reference soil and soil No.1 were used for the evaluation, which ranged between 17 and 40% (mean 28.5%). The parameter was evaluated yellow.

   Soil type: Since soil is tested directly after addition of a test solution, there is no dependence on the soil type. The pH should not be below 5, but it was assumed that test soils usually have a pH of above 5 and therefore this should be no issue. Therefore, the parameter was evaluated green.

Relevance for regulatory purpose: Partial assessment of the soil microbial community (the ammonium oxidizing bacteria) with lesser relevance/transferability to microbial diversity, function and structure and to ecological function or ecosystem services. A small group of nitrifiers is considered, which can be very sensitive. In conclusion, the relevance for regulatory purposes is moderate (yellow).

5. **ISO 20131-1:2018 – Easy laboratory assessments of soil denitrification, a process source of N_2O emissions – Part 1: Soil denitrifying enzyme activities.** The guidance specifies a laboratory test for characterizing the denitrifying enzyme activities in soils. It globally characterizes the transformation of nitrate to nitrous oxide and dinitrogen.

   Endpoint: Assessment of the effect on denitrifying enzyme activities in soils.

   Practicability: Method requires either expensive (analytical) equipment or special skills. Gas chromatographs (GC) with ECD and thermal conductivity detector (TCD) detector and substrates/chemicals are necessary. Therefore, the parameter was evaluated yellow.

   Costs: In total overall high costs. Due to additional GC analysis costs might be high since personal with increased laboratory experience is required (≥ 10 h) and because determination per sampling day and calculation of results is time-consuming (≥ 10 h). Therefore, the parameter was evaluated red.

   Replicability/reproducibility: Information on laboratory comparison or ring tests available. Low variability based on the available data: ≤25%. Therefore, the parameter was evaluated green.

   Soil type: As the method is performed on sieved soils, it is quite easy to be done and can be used for a wide range of soils. The parameter was evaluated green.

   Relevance for regulatory purpose: Denitrifying enzyme activities are considered to be representative for the denitrifying enzyme pool present in the soil sample at the time of sample collection. Denitrification is the main process releasing nitrogen to the atmosphere. This process corresponds to the reduction of nitrate to nitrite and then to the gaseous forms nitric oxide, nitrous oxide and dinitrogen.

   In conclusion this method only partially assesses the soil microbial community and has a moderate relevance/transferability to microbial diversity, function and structure and to ecological function or ecosystem services. In conclusion, the parameter was evaluated yellow.

6. **ISO 20131-2:2018 - Easy laboratory assessments of soil denitrification, a process source of N_2O emissions – Part 2: Assessment of the capacity of soils to reduce N_2O.** A laboratory test for characterizing the ability (or inability) of soils to reduce the greenhouse gas N_2O to N_2.

   Endpoint: Assessment of the effect on the ability (or inability) of soils to reduce the greenhouse gas N_2O to N_2

   Practicability: Method requires either expensive (analytical) equipment or special skills. Gas chromatographs (GC) with ECD and thermal conductivity detector (TCD) detector and substrates/chemicals are necessary. Therefore, the parameter was evaluated yellow.

   Costs: In total overall high costs. Due to additional GC analysis costs might be high since personal with increased laboratory experience is required (≥ 10 h) and because

determination per sampling day and calculation of results is time-consuming (≥ 10 h). The parameter was evaluated red.

Replicability/reproducibility: Information on laboratory comparison or ring tests available. Low variability based on the available data: ≤25%. The parameter was evaluated green.

Soil type: The method can be performed on all types of soils sampled all over the year except in very exceptional and extreme conditions of dryness. Results obtained are stable over time for situations that do not receive neither organic nor lime amendments. Experiments are performed with sieved soil samples. The parameter was evaluated green.

Relevance for regulatory purpose: Denitrifying enzyme activities is considered to be representative for the denitrifying enzyme pool present in the soil sample at the time of sample collection. Denitrification is the main process releasing nitrogen to the atmosphere. This process corresponds to the reduction of nitrate to nitrite and then to the gaseous forms nitric oxide, nitrous oxide and dinitrogen.

In conclusion this method only partially assesses the soil microbial community and has a moderate relevance/transferability to microbial diversity, function and structure and to ecological function or ecosystem services. Therefore, the parameter was evaluated yellow.

2.1.3.3 Microbial enzymatic activities

7. **ISO 20130:2018 - Measurement of enzyme activity patterns in soil samples using colorimetric substrates in micro-well plates.** A method for the simultaneous measurement of several hydrolase activities (arylamidase, arylsulfatase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-glucosaminidase, acid, alkaline and global phosphatases, urease) in soil samples, using colorimetric substrates. Enzyme activities of soil vary seasonally and depend on its chemical, physical and biological characteristics. This method can be applied either to detect harmful effects on soil enzyme activities derived from toxic substances or other anthropogenic agents in contaminated soils against a control soil, or to test chemicals.

   Endpoint: Influence on nutrient cycles or turnover of carbon, nitrogen, phosphorus and/or sulfur containing substrates.

   Practicability: Method requires neither expensive (analytical) equipment nor special skills. A spectrophotometer, an incubation chamber, substrates and 96-well multiwall plates is all that is needed. The methods can be easily handled without the need for special additional training. Therefore, the parameter was evaluated green.

   Costs: In total overall moderate costs (10 – 30 h per test). The parameter was evaluated yellow.

   Replicability/reproducibility: Information on laboratory comparison or ring tests available. Low variability based on the available data: ≤25%. Therefore, the parameter was evaluated green.

   Soil type: No influence of soil properties on use in the test system and representative arable soil. The parameter was evaluated green.

   Relevance for regulatory purpose: Assessment of the soil microbial community with a high significance/ transferability to microbial diversity, function and structure and to ecological function and ecosystem services. Due to the fact that different enzyme

activities can be assessed by using different substrates a large part of the microbial community is captured. Therefore, the relevance of this method is high. In conclusion, the parameter was evaluated green.

8. **ISO 22939:2019 - Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates.** A method for the simultaneous measurement of several enzyme activities (arylsulfatase, α −glucosidase, β -glucosidase, cellubisidase, β -xylosidase, phosphodiesterase (PDE), chitinase, phosphomonoesterase (PME), leucine-aminopeptidase, Alanine-aminopeptidase) using fluorigenic substrates in soil samples. Enzyme activities of soil vary seasonally and depend on the chemical, physical and biological characteristics of soil. The application of this method for the detection of harmful effects of toxic chemicals or other anthropogenic impacts relies on on the simultaneous comparison of enzyme activities in both a control soil and the test soil, or on exposure tests with chemicals or treatments.

   Endpoint: Influence on nutrient cycles or turnover of carbon, nitrogen, phosphorus and/or sulfur containing substrates.

   Practicability: Method requires neither expensive (analytical) equipment nor special skills. However, it should be considered that apart from other test systems there is only a narrower spectrum of different fluorogenic substrates which can be used. For the method a spectrophotometer, an incubation chamber and 96-well multiwall plates is all that is needed. The methods can be easily handled without the need for special additional training. Therefore, the parameter was evaluated green.

   Costs: In total overall high costs. The method is time-consuming and fluorogenic substrates are expensive. Therefore, the method was evaluated red.

   Replicability/reproducibility: Information on laboratory comparison or ring tests available. Low variability based on the available data: ≤25%. The parameter was evaluated green.

   Soil type: No influence of soil properties on use in the test system and representative arable soil. Therefore, the parameter was evaluated green.

   Relevance for regulatory purpose: Assessment of the soil microbial community with a high significance/transferability to microbial diversity, function and structure and to ecological function and ecosystem services. Using different substrates, various exoenzyme activities can be studied (Hoppe 1983, Hoppe et al. 1988). Most organic substances in the environment are macromolecular and have to be broken down by extracellular substrate decomposition before taken up into the microbial cell. The stability of these enzymes is affected by physico-chemical soil properties (Allison 2006). Nevertheless, the relevance is high, as a wide spectrum of enzymatic activities is considered at the same time and the parameter was evaluated green.

9. **ISO 18187:2016 - Contact test for solid samples using the dehydrogenase activity of *Arthrobacter globiformis*.** A rapid method for assessing solid samples in an aerobic suspension, by determining the inhibition of dehydrogenase activity of *Arthrobacter globiformis* using the redox dye resazurin. The test yields a result within 6 hours and can therefore be used for screening potentially contaminated material.

   Endpoint: Determination of the inhibition of the dehydrogenase activity of *Arthrobacter globiformis* using the redox dye resazurin.

   Practicability: Method requires neither expensive (analytical) equipment nor special skills and therefore the parameter was evaluated green.

Costs: In total overall low costs (≤ 10 h per test) and therefore the parameter was evaluated green.

Replicability/reproducibility: Information on laboratory comparison or ring tests available. Low variability based on the available data: ≤25%. The parameter was evaluated green.

Soil type: No influence of soil properties on use in the test system and representative arable soil. The parameter was evaluated green.

Relevance for regulatory purpose: Single species tests with a low significance/ transferability to microbial diversity, function and structure and to ecological function or ecosystem services. Therefore, low relevance only and the parameter was evaluated red.

10. **ISO 23753-1:2019 - Determination of dehydrogenase activity in soils. Part 1: Method using triphenyltetrazolium chloride (TTC).** A method for determining the dehydrogenase activity in soil using 2,3,5-triphenyltetrazolium chloride (TTC).

    Endpoint: Inhibition of dehydrogenase enzyme activity in soil with 2,3,5-triphenyltetrazolium chloride (TTC).

    Practicability: Method requires neither expensive analytical equipment nor special skills and therefore the parameter was evaluated green.

    Costs: In total overall moderate costs (10 – 30 h per test). The parameter was evaluated yellow.

    Replicability/reproducibility: No information on laboratory comparison or ring tests available. Therefore, the parameter was evaluated red.

    Soil type: No influence of soil properties on use in the test system and representative arable soil. The method is not applicable for determining the dehydrogenase activity in the upper layers (L, F, H horizons) of forest humus forms with low microbial activity or in soils showing reducing properties (e.g. waterlogged soils). In conclusion, the parameter was evaluated green.

    Relevance for regulatory purpose: Assessment of the soil microbial community with a high relevance/ transferability to microbial diversity, function and structure and to ecological function or ecosystem services. Among all enzymes in the soil environment, dehydrogenases are one of the most important, and are used as an indicator of overall soil microbial activity (Quilchano and Marañón 2002). Therefore, high regulatory relevance and the parameter was evaluated green.

11. **ISO 23753-2:2019 - Determination of dehydrogenase activity in soils. Part 2: Method using iodotetrazolium chloride (INT).** A method for determining soil dehydrogenase activity using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT). As the INT reduction is less sensitive to O_2, the method is more reproducible than the TTC-method described in ISO 23753-1.

    Endpoint: Inhibition of dehydrogenase enzyme activity in soil with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT).

    Practicability: Method requires neither expensive (analytical) equipment nor special skills. Therefore, the parameter was evaluated green.

    Costs: In total overall moderate costs (10 – 30 h per test). Therefore, the parameter was evaluated yellow.

Replicability/reproducibility: No information on laboratory comparison or ring tests available. Therefore, the parameter was evaluated red.

Soil type: No influence of soil properties on use in the test system and representative arable soil. The method is not applicable for determining the dehydrogenase activity in the upper layers (L, F, H horizons) of forest humus forms with low microbial activity, or in soils showing reducing properties (e.g. waterlogged soils). Therefore, the parameter was evaluated green.

Relevance for regulatory purpose: Assessment of the soil microbial community with a high relevance/transferability to microbial diversity, function and structure and to ecological function or ecosystem services. Among all enzymes in the soil environment, dehydrogenases are one of the most important, and are used as an indicator of overall soil microbial activity (Quilchano and Marañón 2002). Therefore, high regulatory relevance and the parameter was evaluated green.

12. **Urease activity according to "Assay for soil urease activity. Plant and Soil 45, 301 – 305" (May and Douglas 1976).** A precise and simple procedure to determine the soil urease activity.

    Endpoint: Inhibition of urease activity.

    Practicability: Method requires neither expensive (analytical) equipment nor special skills. Therefore, the parameter was evaluated green.

    Costs: In total overall low costs (≤ 10 h per test). Therefore, the parameter was evaluated green.

    Replicability/reproducibility: No information on laboratory comparison or ring tests available (e.g. expected for non-standardized methods). Therefore, the parameter was evaluated red.

    Soil type: No influence of soil properties on use in the test system and representative arable soil. Therefore, the parameter was evaluated green.

    Relevance for regulatory purpose: Partial assessment of the soil microbial community with a lesser relevance/ transferability to microbial diversity, function and structure and to ecological function or ecosystem services. Of the total bacterial population 17 – 30% can hydrolyze urea and therefore, by measuring urease activity, only a portion (~1/4) of the microbial community is considered (Lloyd and Sheaffe 1973, Fisher et al. 2017). In conclusion, the parameter was evaluated yellow.

13. **(ABTS assay) Phenol oxidase according to "ABTS assay of phenol oxidase activity in soil. Journal of Microbiological Methods, 71, 319 – 324 (Floch et al. 2007)".** The ABTS assay is a simple method to assay phenol oxidase activity directly in bulk samples by spectrophotometric test using 2,2′-azinobis-(-3 ethylbenzothiazoline-6-sulfononic acid) diammonium salt (ABTS) as the substrate.

    Endpoint: Inhibition of phenol oxidase activity. The phenol oxidases are involved in the degradation of many recalcitrant aromatic compounds and may be sensitive to pollutants (Floch et al. 2007).

    Practicability: Method requires either expensive (analytical) equipment or special skills. Based on the descriptions in the literature, a relatively complex preparation of various test solutions is necessary. Therefore, the parameter was evaluated yellow.

    Costs: In total overall moderate costs (10 – 30 h per test). Therefore, the parameter was evaluated yellow.

Replicability/reproducibility: No information on laboratory comparison or ring tests available. Therefore, the parameter was evaluated red.

Soil type: Limitations due to several soil properties (e.g. a high clay content and an associated high soil density or sorption of the test substrate to soil) is reported (Floch et al. 2007). Therefore, the parameter was evaluated red.

Relevance for regulatory purpose: Partial assessment of the soil microbial community with a lesser relevance/transferability to microbial diversity, function and structure and to ecological function or ecosystem services. Therefore, the relevance is classified as moderate (yellow).

Soil enzymes play an important role in the geochemical processes in soil. Soil enzymes are constantly synthesized, accumulated, inactivated and/or decomposed, hence they are central for e.g. the nutrient cycling (Makoi and Ndakidemi 2008). Activities of soil enzymes undergo complex biochemical processes affecting among others the enzyme stability. Therefore, all soils contain a group of enzymes that determine soil metabolic processes (McLaren 1975, Makoi and Ndakidemi 2008), depending on the soils physical, chemical, microbiological and biochemical properties. Phenol oxidases and peroxidases for example are less stable in the environment than extracellular hydrolases, especially when associated with organic particles (Sinsabaugh 2010). Therefore, it is an uncertainty factor, whether the activity in the collected soil is sufficiently present for robust statements. This applies especially for soils which had been stored.

For methods determining the soil enzymatic activities, it is difficult to assess if the result of a performed method reflects effects on the complete soil microbial community or only a part of the community. While other methods (ISO 20130, 22939) examine the effect on a number of soil enzymes as part of the method, the method presented here examines phenol oxidases or peroxidases alone.

Finally, it was decided that for this reason the method was evaluated as "partial assessment of soil microbial community" and the relevance is classified as moderate (yellow).

14. **Peroxidase activity according to "Measuring phenol oxidase and peroxidase activities with pyrogallol, L-DOPA, and ABTS: Effect of assay conditions and soil type. Soil Biology and Biochemistry 67, 183 – 191 (Bach et al. 2013)".** The assay on phenol and peroxidase activity is a method to determine the enzymatic activity in soil samples by combining soil suspensions with the respective test substrate and measurements of absorbance. It is recommended to perform the assays at both the soil pH and a reference pH (e.g., pH 5.0) to determine the effect of assay pH on oxidase activity. These recommendations should contribute to greater comparability of oxidase potential activities across studies.

    Endpoint: Inhibition of phenol oxidase and peroxidases mediated biogeochemical processes in soil.

    Practicability: Method requires neither expensive (analytical) equipment nor special skills. Therefore, the parameter was evaluated green.

    Costs: In total overall moderate costs (10 – 30 h per test). Therefore, the parameter was evaluated yellow.

    Replicability/reproducibility: No information on laboratory comparison or ring tests available. Therefore, the parameter was evaluated red.

    Soil type: No influence of soil properties on use in the test system and representative arable soil. Therefore, the parameter was evaluated green.

Relevance for regulatory purpose: Partial assessment of the soil microbial community with a lesser relevance/ transferability to microbial diversity, function and structure and to ecological function or ecosystem services.

Soil enzymes play an important role in the geochemical processes in soil. Soil enzymes are constantly synthesized, accumulated, inactivated and/or decomposed, hence they are central for e.g. the nutrient cycling (Makoi and Ndakidemi 2008). Activities of soil enzymes undergo complex biochemical processes affecting among others the enzyme stability. Therefore, all soils contain a group of enzymes that determine soil metabolic processes (McLaren 1975, Makoi and Ndakidemi 2008) depending on the soils physical, chemical, microbiological and biochemical properties. Phenol oxidases and peroxidases are less stable in the environment than extracellular hydrolases, especially when associated with organic particles (Sinsabaugh 2010). Therefore, even if the enzymes mediate important biogeochemical processes, it might be difficult to receive reliable results and the transferability might be hampered.

For methods determining the soil enzymatic activities, it is difficult to assess if the result of a performed method reflects effects on the complete soil microbial community or only a part of the community. While other methods (ISO 20130, 22939) examine the effect on a number of soil enzymes as part of the method, the method presented here examines phenol oxidases or peroxidases alone.

Finally, it was decided that for this reason the method was evaluated as "partial assessment of soil microbial community" and the relevance is classified as moderate (yellow).

15. **Fluorescein diacetate assay according to "Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. Soil Biology and Biochemistry 33, 943 – 951 (Adam and Duncan 2001)".** An accurate and simple method for measuring total microbial activity in a range of environmental samples including soils. Colorless fluorescein diacetate is hydrolyzed by both free and membrane bound enzymes, releasing a colored end product fluorescein which can be measured by spectrophotometry.

    Endpoint: Inhibition of enzyme activity in soil.

    Practicability: Method requires neither expensive (analytical) equipment nor special skills. Therefore, the parameter was evaluated green.

    Costs: In total overall low costs (≤ 10 h per test). The parameter was evaluated green.

    Replicability/reproducibility: No information on laboratory comparison or ring tests available. Therefore, the parameter was evaluated red.

    Soil type: Limitations due to some soil properties were reported, however, due to the adaptations presented in the reference, suitable for a wide range of soils as arable soils. Therefore, the parameter was evaluated green.

    Relevance for regulatory purpose: Partial assessment of the soil microbial community with a lesser relevance/transferability to microbial diversity, function and structure and to ecological function or ecosystem services. The study is described as an indicator for total microbial community, however according to Hund et al. (1988) impacts on fungi were more obvious than on bacteria. The study showed that fungicides have a higher effect in this test than herbicides or bactericides. This was not the case for other activities, e.g. microbial respiration. This suggests that the method is predominantly detecting fungal activities and the relevance is classified as moderate (yellow).

2.1.3.4 Soil fungal community

16. ISO/TS 10832:2009 - Effects of pollutants on mycorrhizal fungi – Spore germination test. A method to evaluate the effects of pollutants on spore germination of arbuscular mycorrhizal fungus *Funneliformis mosseae* (formerly known as *Glomus mosseae*). This direct acute toxicity bioassay allows the evaluation of potential effects of pollutants and contaminated soils on beneficial soil microorganisms important for plant growth within the concept of sustainable agriculture.

Remark: Arbuscular mycorrhizal fungi (AMF) are a distinct fungal lineage (Glomeromycetes) which provide important ecosystem services. Therefore, the spore germination test with *Funneliformis mosseae* is considered to be representative for arbuscular mycorrhizal fungi in general, even though this test relies on a single species only. Tests with mycorrhizal fungi have thus far not been part of the canonical assessment strategies for PPPs, biocides and antibiotics. Therefore, they are listed within this report in addition to tests concerning the remaining soil microbial community.

The publications by Mallmann et al. (2018) and Hannula et al. (2016) are considered to provide additional information on alternative AMF species or primer sets for functional genes of the fungal community and were not evaluated.

a. Mallmann et al. (2018): Placing arbuscular mycorrhizal fungi on the risk assessment test battery of plant protection products (PPPs). Ecotoxicology. 27, 809 – 818. This study aimed to contribute to improve the ISO Protocol (ISO 10832: 2009) by assessing the feasibility of using other arbuscular mycorrhizal fungi (AMF) species under different test conditions. Overall, results showed that AMF species *Gigaspora albida* and *Rhizophagus clarus* (selected out of five AMF species) are suitable to be used in spore germination tests using the ISO protocol (14 days incubation with sand or artificial soil as substrate) to test PPPs.

b. Hannula and van Veen (2016): Primer sets developed for functional genes reveal shifts in functionality of fungal community in soils. Frontiers in Microbiology. 7:1897. The sequencing of genomes of various species of fungi (http://1000.fungalgenomes.org/) has enabled us to investigate the presence and types of functional genes and subsequently design probes to target as many fungal groups as possible.

Endpoint: Inhibition of spore-germination of *Funneliformis mosseae* or additional arbuscular mycorrhiza species.

Practicability: Method requires neither expensive (analytical) equipment (i.e. measurement devices), nor special skills. Because of the standardization, test performance should be reasonably easy to learn. *Funneliformis mosseae* can be obtained, information on this is also provided in the guideline. Therefore, the parameter was evaluated green.

Costs: In total overall moderate costs, especially since the test will last for 14 days and because in the first instance the cultivation of the test organisms might be more time consuming until e.g. a stable culture can be established.

Replicability/reproducibility: No information on laboratory comparison or ring tests available, but according to UBA which has experience with this test system through another research project, the replicability/reproducibility is sufficient. Therefore, the parameter was evaluated yellow.

Soil type: No influence of soil properties on use in the test system and representative arable soil. Therefore, the parameter was evaluated green.

Relevance for regulatory purpose: Even though the spore germination test is a single species test, it is considered to represent the whole group of arbuscular mycorrhizal fungi. Therefore, the assessment of arbuscular mycorrhizal fungi has a high significance/transferability to ecological function and ecosystem services in soil. This is the more true as there is no other known test system which provides information on the effect of AMF. However, as the successful germination of the fungal spore may not reflect the successful establishment of a functional mycorrhizal symbiosis the regulatory relevance is questionable and the parameter was evaluated yellow.

17. **Enzyme activity of fungi according "Laccase activity in soils: Considerations for the measurement of enzyme activity. Chemosphere 88, 1154 – 1160 (Eichlerová et al. 2012)".** Laccases from *Trametes versicolor* and *Pyricularia sp.* and tyrosinase from *Agaricus bisporus* were used. The following compounds were used as substrates for enzyme activity measurements: 2,20-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,6-dimethoxyphenol (DMP), L-3,4-dihydroxyphenylalanine (DOPA), 3,30-dimethylaminobenzoic acid (DMAB), 3-methyl-2-benzothiazolinone hydrazone (MBTH), 2-methoxyphenol (guaiacol), 4-hydroxy-3,5-dimethoxybenzaldehyde [(1E)-(4-hydroxy-3,5-dimethoxyphenyl)methylene]hydrazone (syringaldazine), 4-methylbenzene-1,2-diol (4-methylcatechol) and Ltyrosine.

    Endpoint: Inhibition of the enzyme (laccase) activity of fungi in soil.

    Practicability: Method requires neither expensive (analytical) equipment nor special skills. Therefore, the parameter was evaluated green.

    Costs: In total overall moderate costs (10 – 30 h per test). The parameter was evaluated yellow.

    Replicability/reproducibility: No information on laboratory comparison or ring tests available. The parameter was evaluated red.

    Soil type: Since an eluate is produced, there is no dependence on the soil type. However, only a small proportion of the soil microorganisms will be captured by this method. Therefore, the parameter was evaluated yellow.

    Relevance for regulatory purpose: Partial assessment of the soil microbial community with a lesser relevance/transferability to microbial diversity, function and structure and to ecological function or ecosystem services.

    Soil enzymes play an important role in the geochemical processes in soil. Soil enzymes are constantly synthesized, accumulated, inactivated and/or decomposed, hence play an important role in e.g. the nutrient cycling (Makoi and Ndakidemi 2008). Activities of soil enzymes undergo complex biochemical processes affecting among others the enzyme stability. Therefore, all soils contain a group of enzymes that determine soil metabolic processes ((McLaren 1975, Makoi and Ndakidemi 2008)) depending on the soils physical, chemical, microbiological and biochemical properties.. Laccases are a group of phenol oxidases in soil and therefore also less stable in the environment than extracellular hydrolases, especially when associated with organic particles (Sinsabaugh 2010). Therefore, even if the enzymes mediate important biogeochemical processes, it might be difficult to receive reliable results and the transferability might be hampered.

Finally, it was decided that for this reason the method was evaluated as "partial assessment of soil microbial community" and the relevance is classified as moderate (yellow).

2.1.3.5 Functional genes and structural changes

2.1.3.5.1 Functional genes assessed by real time qPCR

18. **ISO 17601:2018 - Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil.** The guidance specifies the crucial steps of a quantitative real-time polymerase chain reaction (qPCR) method to measure the abundance of selected microbial gene sequences from soil DNA extract which provides an estimation of selected microbial groups.

    Note: This ISO guideline provides only the general method. In addition, functional genes have to be chosen to allow the assessment of substance related effects on the microbial function or structure. Additionally, an ISO guideline for the extraction of DNA from soil (ISO 11063; a) is available as additional guidance. Both guidelines may serve as a basis for studies using qPCR (see i – iii). Several studies in which microbial functional genes were investigated are described below.

    - **ISO 11063:2020 - Method to directly extract DNA from soil samples.** A method for direct extraction of DNA from soil samples to analyse the global structure and the abundance of soil bacterial communities using PCR-based technologies. This method is mainly dedicated to agricultural and forest soils and is likely not suitable for soils rich in organic matter (e.g. peat soils) and soils heavily polluted with organic pollutants or heavy metals.

    i. **Levy-Booth et al. (2014): Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. Soil Biology and Biochemistry 75, 11 – 25.** A method to quantify the abundance of nitrifying microorganisms. The majority of N entering ecosystems is biologically-derived from fixation of atmospheric N_2. Molecular studies of N-fixation use the nitrogenase reductase (nifH) marker gene, and can be used to link N-fixation to other N- and C-cycling processes. Ammonia-oxidizing bacteria (AOB) and archaea (AOA) oxidize ammonia (NH_3) to NO_3^- as the first step of nitrification and are studied using the ammonium monooxygenase (amoA) marker.

    ii. **Norton et al. (2002): Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. Archives of Microbiology. 177, 139 – 149.** A method to quantify the abundance of nitrifying microorganisms. Autotrophic ammonia-oxidizing bacteria use the essential enzyme ammonia monooxygenase (AMO) to transform ammonia to hydroxylamine. The amo operon consists of at least three genes, amoC, amoA, and amoB; amoA encodes the subunit containing the putative enzyme active site. The use of the amo genes as functional markers for ammonia-oxidizing bacteria in environmental applications requires knowledge of the diversity of the amo operon on several levels.

    iii. **Bergkemper et al. (2016): Novel oligonucleotide primers reveal a high diversity of microbes which drive phosphorous turnover in soil. Journal of Microbiological Methods 125, 91 – 97.** A method to quantify the abundance of microorganisms responsible for phosphorous transformation. In this study seven oligonucleotide primers are presented which target genes coding for microbial

acid and alkaline phosphatases (phoN, phoD), phytases (appA), phosphonatases (phnX) as well as the quinoprotein glucose dehydrogenase (gcd) and different P transporters (pitA, pstS).

Endpoint: Inhibition of microorganisms or groups of microorganisms within the hierarchy of taxonomic levels from phylum to species level by measurements of microbial functional genes.

Practicability: Method requires expensive (analytical) equipment and special skills. Therefore, the parameter was evaluated red.

Costs: In total overall high costs. Therefore, the parameter was evaluated red.

Replicability/reproducibility: No information on laboratory comparison or ring tests available and therefore, the parameter was evaluated red.

Soil type: No influence of soil properties on use in the test system and representative arable soil. The parameter was evaluated green.

Relevance for regulatory purpose: Assessment of the soil microbial community including mycorrhizal fungi. Brandt et al. (2015) mentioned that nucleic acid–based protocols could serve as amendments to existing standard laboratory protocols such as the OECD 216 and OECD 217 tests in soil in order to monitor community shifts during long-term toxicity tests. Therefore, the regulatory relevance is classified as high (green).

2.1.3.5.2 Structural profile of microbial community

19. **ISO 29843-1:2010 - Determination of soil microbial diversity. Part 1: Method by PLFA analysis and PLEL analysis.** An extended method for the extraction and determination of both phospholipid fatty acids (PLFA) and phospholipid ether lipids (PLEL) from soils.
ISO 29843-2:2011 - Determination of soil microbial diversity. Part 2: Method by PLFA analysis using the "simple PLFA extraction method". A simple method for the extraction of only phospholipid fatty acids (PLFA) from soils.

    Endpoint: Phospholipids are essential components of membranes of all living cells, and their fatty acids (PLFA: phospholipid fatty acids) or ether-linked isoprenoid side chains (PLEL: phospholipid ether lipids) enable taxonomic differentiation within complex microbial communities. Determination of the total PLFA and PLEL contents provides a quantitative measure of the viable biomass of soil (bacteria, fungi, and archaea).

    Practicability: Method requires expensive (analytical) equipment and special skills. The analysis and evaluation of a PLFA requires experience and especially statistical know-how. Therefore, the parameter was evaluated red.

    Costs: In total overall high costs. A gas chromatograph coupled with either a mass spectrometer detector or a flame ionization detector is needed. Therefore, the parameter was evaluated red.

    Replicability/reproducibility: No information on laboratory comparison or ring tests available. The parameter was evaluated red.

    Soil type: No influence of soil properties on use in the test system and representative arable soil. The parameter was evaluated green.

    Relevance for regulatory purpose: Partial assessment of the soil microbial community. Brandt et al. (2015) indicated that many widely used taxonomic signature PLFA markers are unspecific, and changes in profiles cannot be linked to specific ecosystem services. The relevance is therefore moderate (yellow).

20. **Denaturing gradient gel electrophoresis (DGGE) according to "New and Current Microbiological Tools for Ecosystem Ecologists: Towards a Goal of Linking Structure to Function. The American Midland Naturalist. 160:140 – 159 (Drenovsky et al. 2008)".** Denaturing gradient gel electrophoresis (DGGE) is a molecular technique for rapid fingerprint analysis of microbial community composition, diversity, and dynamics (Green et al. 2010). The method is rapid and affordable, allowing multiple samples to be processed simultaneously.

    Endpoint: Shifts of the microbial community composition

    Practicability: Method requires either expensive (analytical) equipment or special skills. The technique itself is inexpensive but requires a lot of technical expertise for the gel preparation, which results also in longer preparation times. The results provide a band pattern, representing the community of the sample, in which each band belong to an operational taxonomic unit (OUT). The analysis of the results can be qualitative or semi quantitative. For a semi quantitative analysis, a photo documentation equipment is required as well as a software to analyze the size of the bands obtained. Samples can be compared based on the differences/similarities of their community profiles by using multidimensional scaling statistical analysis. In conclusion, the parameter was evaluated yellow.

    Costs: In total overall high costs. The parameter was evaluated red.

    Replicability/reproducibility: No information on laboratory comparison or ring tests available. Based on IMEs own experience the method has some drawbacks regarding the reproducibility due to the evaluation based on different gels and the software used for the evaluation. Therefore, the parameter was evaluated red.

    Soil type: No influence of soil properties on use in the test system and representative arable soil. The parameter was evaluated green.

    Relevance for regulatory purpose: Assessment of a soil microbial community. In accordance to Drenovsky et al. (2008) the method provides a high resolution microbial community characterization compared to lipid-based approaches. Therefore, the regulatory relevance is classified as high (green).

21. **Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis according to "New and Current Microbiological Tools for Ecosystem Ecologists: Towards a Goal of Linking Structure to Function. The American Midland Naturalist. 160:140 – 159 (Drenovsky et al. 2008)".** Terminal Restriction Fragment Length Polymorphism analysis (T-RFLP) is a method for rapid profiling of mixed populations of a homologous amplicon (i.e., diverse sequences of a single gene). It combines restriction fragment analysis of a PCR-amplified gene marker with automated sequencing gel technology. One primer used in PCR amplification of the marker gene is labeled at the 5′ terminus with a fluorescent dye, in order that the terminal restriction fragments (T-RFs) of the digested amplicon can be detected and quantified (Liesack and Dunfield 2004).

    Endpoint: Shifts of the microbial community composition

    Practicability: Method requires expensive (analytical) equipment and special skills. Drenovsky et al. (2008) mention that the method needs optimization of conditions and standardization of data. The method requires the use of a capillary electrophoresis system, which is not common to have in a standard laboratory. In addition, the use of restriction enzymes to digest the amplified DNA (16S rRNA or other gene targeting functional groups) is required. The main drawbacks of RFLPs are the requirement of using several endonucleases (restriction enzymes) for enhancing the resolution and

specificity of the terminal fragments. The restriction pattern among samples provides an indication on community changes among samples. Samples can be compared based on the differences/similarities of their community profiles by using multidimensional scaling statistical analysis. In conclusion, the parameter was evaluated red.

Costs: In total overall high costs, therefore, the parameter was evaluated red.

Replicability/reproducibility: No information on laboratory comparison or ring tests available. The parameter was evaluated red.

Soil type: No influence of soil properties on use in the test system and representative arable soil. Therefore, the parameter was evaluated green.

Relevance for regulatory purpose: Assessment of the soil microbial community. According to Drenovsky et al. (2008) the method provides a resolution on the genus level, high relevance (green).

22. **Automated rRNA intergenic spacer analysis (ARISA) according "Characterisation of Bacterial and Fungal Soil Communities by Automated Ribosomal Intergenic Spacer Analysis Fingerprints: Biological and Methodological Variability. Applied and Envionmental Microbiology, 67, 4479 – 4487 (Ranjard et al. 2001)".** Automated rRNA intergenic spacer analysis (ARISA) is a rapid and effective community analysis technique that can be used in conjunction with more accurate but labor-intensive methods (e.g., 16S rRNA gene cloning and sequencing) when fine-scale spatial and temporal resolution is needed (Fisher and Triplett 1999).

    Endpoint: Change of bacterial and fungal community composition

    Practicability: Method requires either expensive (analytical) equipment or special skills. Similar to T-RFLP this method requires the use of a capillary electrophoresis system. The technique is quite simple as it does not require any additional step after the PCR amplification and purification (in contrast to T-RFLP which requires the use of endonucleases). Results are provided as an electropherogram in which each peak represents an OTU. The analysis of the results can be qualitative or semi quantitative. Samples can be compared based on the differences/similarities of their community profiles by using multidimensional scaling statistical analysis. In conclusion, the parameter was evaluated yellow.

    Costs: In total overall moderate costs (10 – 30 h per test) and therefore, the parameter was evaluated yellow.

    Replicability/reproducibility: No information on laboratory comparison or ring tests available. Based on IMEs own experience the method shows a good reproducibility. Therefore, the parameter was evaluated comparable with ring-test information with a variability of 26 – 40% ending in a final evaluation yellow.

    Soil type: No influence of soil properties on use in the test system and representative arable soil. Therefore, the parameter was evaluated green.

    Relevance for regulatory purpose: Assessment of the soil microbial community (bacterial and fungal). ARISA is a rapid and effective community analysis technique if low scale resolution is sufficient. It can be used in conjunction with more accurate but labor-intensive methods (e.g., 16S rRNA gene cloning and sequencing) when fine-scale spatial and temporal resolution is needed (Fisher and Triplett 1999). It is considered of high relevance for regulatory purposes (green).

2.1.3.6 Carbon cycling and sequestration

23. **OECD Series on Testing and Assessment No. 56, Guidance document on the breakdown of organic matter in litterbags (2006).** A method designed to assess the effects of chemicals in general, and pesticides in particular on the breakdown of organic matter in soil. The test can be used to address concerns regarding the breakdown of plant litter material, particularly when exposed to persistent compounds in agricultural and horticultural soils. After appropriate modifications, this method can be used to assess the effects of other chemicals (e.g. biocides) as well as for the assessment of soil quality at contaminated sites.

    Endpoint: Inhibition of the functional process of organic matter breakdown

    Practicability: Method requires neither expensive (analytical) equipment nor special skills. The parameter was evaluated green.

    Costs: In total overall high costs. Even though there may be only a few steps during the test, an incubator or some laboratory space will still be occupied for a very long period of time. In this case, this must also be considered in the costs incurred. Therefore, the parameter was evaluated red.

    Replicability/reproducibility: No information on laboratory comparison or ring tests available. The guidance document mentions that due to a lack of time and money no validation exercise (ring-test) was performed, but is considered to be an important research need. In addition, it is mentioned that 15 data set of already performed studies was available in which all of the described validity criteria were met. Since no official ring test data are available, but an estimate can be made from existing other data, this parameter is set comparable to acceptable ring test data with a variability of 26 to 40%. Finally, the parameter was evaluated yellow.

    Soil type: The test was developed for field studies but it should be possible to transfer the method into the laboratory. Since there are still uncertainties regarding this assumption, the parameter was evaluated yellow.

    Relevance for regulatory purpose: Assessment of the soil microbial community or mycorrhizal fungi, with a moderate significance/transferability to microbial diversity, function and structure and to ecological function and ecosystem services. However, based on UBAs experience and consensus within international experts the current test protocol does not show a high sensitivity in the suggested time frame of one year after application. Therefore, the relevance for regulatory purposes is low (red).

2.1.4 Evaluation of test methods for a testing strategy

The evaluations of the methods provided above are summarized in Table 3 using a traffic light system for each of the five evaluation parameters used, i.e. practicability, costs, replicability/reproducibility, influence of soil type and relevance.

The traffic light system with green (well suited), yellow (suited) and red (barely suited) was used to evaluate the individual test methods. Furthermore, for the purpose of decision making a score based on individual numerical values was introduced. The individual criteria were each assigned a score from 1 (red) over 2 (yellow) to 3 (green) and coloured accordingly. Various approaches are possible for the evaluation. First, all parameters are considered equally. Second, the parameters are differently prioritised. Parameters which are considered to be more important regarding the identification of suitable methods for regulation, are given more weight. To limit the probability of a wrong conclusion, both approaches were considered.

Approach 1: Equal consideration of the parameters

The sum of all scores was calculated resulting in a total score. The total score was then divided by the number of parameters considered, resulting in a score between 1 and 3. Due to this, a better comparability should be achieved. Nevertheless, both values are given in the following tables. Based on the achieved score a first evaluation was possible and methods, which appeared to be appropriate for the testing strategy were highlighted (Table 3).

Approach 2: Different weighting of the parameters

Different weighting strategies were applied. The results are presented in Table 4.

As 1st modification a weighting factor was implemented. Since the parameter's practicability, costs and relevance were considered to be more important than the parameters influence of soil type and replicability/reproducibility they received a weighting factor of 2. The parameters practicability and costs were weighted higher due to economic reasons. Smaller laboratories who are not necessarily equipped with e.g. expensive measurement devices can still easily implement the test methods without losing the economic connectivity. Although the parameter replicability/reproducibility of a method is also very important a weighting of this parameter was excluded from the modification, as the evaluation strongly depends on whether a ring test result is available or not. This should not be further enhanced by an additional higher weighting. There is no standardized approach which methods become subject of a ring test, the selection is not always based on technical reasons and the selection can be arbitrary. Due to these new methods with a high potential for the focus of this project could be sorted out at an early stage.

The individual scores of the methods (score from 1 to 3) were multiplied with the weighting factor for the specific parameters (e.g. practicability). Again, the total score was then divided by the number of parameters considered, resulting in a score between 1 and 3 (Table 4).

For the parameter replicability/reproducibility, it must be considered that methods can have a very high accuracy and precision even without ring test results and without standardization. However, it is difficult to obtain an accurate assessment only on the basis of literature data. Therefore, in additional modifications, the impact of the parameter replicability/reproducibility was modified. In consultation with German Environment Agency (Umweltbundesamt (UBA), web conference, September 10, 2021) it was decided that in absence of results from a ring test, this parameter should be scored as 0 (2nd modification). Afterwards, the total score was divided by the number of parameters considered, resulting in a score between 1 and 3. If no ring test results were available, the number of parameters was set to 4. If results from ring tests were available, the number of considered parameters was 5.

Furthermore, scores without weighting and without the consideration of the parameter reproducibility (3rd modification) were calculated as were scores with weighting but without the parameter reproducibility (4th modification).

The results of the respective modifications of the evaluation are presented in Table 4.

Independent of the chosen evaluation (with or without modifications), there is a clear tendency highlighting certain methods, which are appropriate for the testing strategy. Care was taken to ensure that the test strategy consists of a balanced mixture of tests, covering responses of bacteria and fungi as well as function and structure of the microbial community in the soil. Moreover, tests evaluating enzymatic reactions as well as alternative methods such as determination of species richness by PCR based approaches (e.g. ARISA-PCR) were selected. Based on the traffic light system, the evaluations presented in Table 3 and Table 4 and the final score - which was achieved for each test method within the 6 assigned groups (i.e. activity of aerobic, heterotrophic microbial biomass; nitrifying and denitrifying bacteria etc.) - following test systems were highlighted to be most appropriate:

- Activity of aerobic, heterotrophic microorganisms: MicroResp™
- Activity of nitrifying and denitrifying bacteria: ISO 15685
- Enzymatic activity: ISO 20130
- Fungal community: ISO 10832
- Functional genes and structural profile: ARISA
- Carbon cycling and sequestration: OECD 56

While for the majority of assigned groups the results are clear, the result for the determination of enzymatic activities does not clearly identify one test. The ISO 20130 was identified independently from the chosen evaluation method (see Table 3 and Table 4). Therefore, the ISO 20130 is listed above and is considered as most appropriate test method for the evaluation of the methodological group 'enzymatic activity'. However, also the ISO 23753-1/2 was identified with the modified evaluation, if weighting was performed and the parameter replicability/reproducibility was not considered. In one case, if no weighting was performed and the parameter replicability/reproducibility was not considered for evaluation, also the methods for determination of urease activity and fluorescein diacetate were identified. As the resulting score was comparable to the score of ISO 20130 in individual cases, these tests are not listed in the testing strategy at the moment. However, both methods are established at IME and could be included, if necessary (e.g., by insensitive response of ISO 20130).

Table 3: Assessment of possible methods considered to be included in the testing strategy.
Bold and blue: The method with the highest score in each of the assigned groups was highlighted.

No.	Method	Endpoint	Practicability	Costs	Replicability/ reproducibility	Influence of soil type	Relevance	Final score [1]
Activity of aerobic (and anaerobic), heterotrophic microbial biomass								
Ref	*OECD 217*	*Carbon transformation*	***3***	***2***	3	3	*1*	*2.40 (12)*
1	Biolog®	Nutrient cycles or turnover of C-, N-, P- and/or S-containing substrates	**3**	**3**	**2**	**2**	**2**	2.40 (12)
2	**MicroResp™**	Nutrient cycles or turnover of C-, N-, P- and/or S-containing substrates	**3**	**3**	**2**	**3**	**3**	**2.80 (14)**
3	Fe(III) reduction test	Soil microbial activity	**2**	**2**	**1**	**3**	**3**	2.20 (11)
Nitrifying and denitrifying bacteria								
Ref	*OECD 216*	*Nitrogen transformation*	***3***	***2***	***3***	***3***	***2***	*2.60 (13)*
4	**ISO 15685**	Ammonium-oxidizing bacteria (AOB); Transformation of NH_4 to NH_2	**3**	**3**	**2**	**3**	**2**	**2.60 (13)**

No.	Method	Endpoint	Practicability	Costs	Replicability/ reproducibility	Influence of soil type	Relevance	Final score [1]
5	ISO 20131-1	Denitrifying enzyme activities	2	1	3	3	2	2.20 (11)
6	ISO 20131-2	Reduction of the greenhouse gas N_2O into N_2 by soil microorganisms	2	1	3	3	2	2.20 (11)
Enzymatic activity								
7	**ISO 20130**	Nutrient cycles or turnover of C-, N-, P- and/or S-containing substrates	**3**	**2**	**3**	**3**	**3**	**2.80 (14)**
8	ISO 22939	Nutrient cycles or turnover of C-, N-, P- and/or S-containing substrates	3	1	3	3	3	2.60 (13)
9	ISO 18187	Dehydrogenase activity	3	3	3	3	1	2.60 (13)
10	ISO 23753-1	Dehydrogenase activity	3	2	1	3	3	2.40 (12)
11	ISO 23753-2	Dehydrogenase activity	3	2	1	3	3	2.40 (12)
12	Urease	Urease activity	3	3	1	3	2	2.40 (12)

No.	Method	Endpoint	Practicability	Costs	Replicability/ reproducibility	Influence of soil type	Relevance	Final score [1]
13	ABTS	Phenol oxidase activity	2	2	1	1	2	1.60 (8)
14	Peroxidase	Peroxidase activity	3	2	1	3	2	2.20 (11)
15	Fluorescein diacetate (FDA)	Total microbial activity; measured by both free and membrane bound enzymes	3	3	1	3	2	2.40 (12)
Fungal community								
16	**ISO 10832**	Spore-germination of *F. mosseae* or additional AMF species	**3**	**2**	**2**	**3**	**2**	**2.40 (12)**
17	Laccase	Laccase (enzyme from fungi) activity	3	2	1	2	2	2.00 (10)
Functional genes and structural profile								
18	ISO 17601	Shifts of the microbial community composition	1	1	1	3	3	1.80 (9)
19	ISO 29843-1/2	Total PLFA and PLEL contents as quantitative measure of the viable soil biomass	1	1	1	3	2	1.60 (8)

No.	Method	Endpoint	Practicability	Costs	Replicability/ reproducibility	Influence of soil type	Relevance	Final score [1]
20	DGGE	Shifts of the microbial community composition	2	1	1	3	3	2.00 (10)
21	T-RFLP	Shifts of the microbial community composition	1	1	1	3	3	1.80 (9)
22	**ARISA**	Shifts of the microbial community composition	**2**	**2**	**2**	**3**	**3**	**2.40 (12)**
Carbon cycling and sequestration								
23	**OECD 56**	**Organic matter breakdown**	**3**	**1**	**2**	**2**	**1**	**1.80 (9)**

Bold values: For each of the 6 assigned groups representing the overall microbial biomass, specialized groups, enzymes, functional genes or fungi, the method with the highest score was highlighted.
[1]Final score: The presented value is the total score divided by the number of parameters, while the value in brackets is the overall total score.

Table 4: Possible modification of the assessment of methods for the testing strategy and the resulting score. The presented value is the total score divided by the number of parameters, while the value in brackets is the overall total score. Bold and blue: The method with the highest score in each of the assigned groups was highlighted.

No.	Method	Endpoint	Final score without weighting (as presented in Table 3)	1st modification (Final score considering weighting factors)[1]	2nd modification (Final score without weighting and neglecting reproducibility if no ring tests were available)[2]	3rd modifications (Final score without weighting and neglecting reproducibility for all methods)	4th modification (Final score with weighting and neglecting reproducibility for all methods)
Activity of aerobic (and anaerobic), heterotrophic microbial biomass							
Ref	*OECD 217*	*Carbon transformation*	2.40 (12)	2.29 (16)	2.40 (9)	2.25 (9)	2.17 (13)
1	Biolog®	Nutrient cycles or turnover of C-, N-, P- and/or S-containing substrates	2.40 (12)	2.43 (17)	2.40 (12)	2.50 (10)	2.50 (15)
2	**MicroResp™**	**Nutrient cycles or turnover of C-, N-, P- and/or S-containing substrates**	**2.80 (14)**	**2.86 (20)**	**2.80 (14)**	**3.00 (12)**	**3.00 (18)**
3	Fe(III) reduction test	Soil microbial activity	2.20 (11)	2.29 (16)	2.50 (10)	2.50 (10)	2.50 (15)
Nitrifying and denitrifying bacteria							
Ref	*OECD 216*	*Nitrogen transformation*	2.60 (13)	2.57 (18)	2.60 (10)	2.50 (10)	2.50 (15)
4	**ISO 15685**	**Ammonium-oxidizing bacteria (AOB); Transformation of NH_4 to NH_2**	**2.60 (13)**	**2.57 (18)**	**2.60 (13)**	**2.75 (11)**	**2.67 (16)**

No.	Method	Endpoint	Final score without weighting (as presented in Table 3)	1st modification (Final score considering weighting factors)[1]	2nd modification (Final score without weighting and neglecting reproducibility if no ring tests were available)[2]	3rd modifications (Final score without weighting and neglecting reproducibility for all methods)	4th modification (Final score with weighting and neglecting reproducibility for all methods)
5	ISO 20131-1	Denitrifying enzyme activities	2.20 (11)	2.14 (15)	2.20 (11)	2.00 (8)	2.00 (12)
6	ISO 20131-2	Reduction of the greenhouse gas N_2O into N_2 by soil microorganisms	2.20 (11)	2.14 (15)	2.20 (11)	2.00 (8)	2.00 (12)
Enzymatic activity							
7	**ISO 20130**	**Nutrient cycles or turnover of C-, N-, P- and/or S-containing substrates**	2.80 (14)	**2.86 (20)**	**2.80 (14)**	**2.75 (11)**	**2.83 (17)**
8	ISO 22939	Nutrient cycles or turnover of C-, N-, P- and/or S-containing substrates	2.60 (13)	2.71 (19)	2.60 (13)	2.50 (10)	2.67 (16)
9	ISO 18187	Dehydrogenase activity	2.60 (13)	2.43 (17)	2.60 (13)	2.50 (10)	2.33 (14)
10	**ISO 23753-1**	**Dehydrogenase activity**	2.40 (12)	2.57 (18)	2.75 (11)	**2.75 (11)**	**2.83 (17)**
11	**ISO 23753-2**	**Dehydrogenase activity**	2.40 (12)	2.57 (18)	2.75 (11)	**2.75 (11)**	**2.83 (17)**
12	Urease	Urease activity	2.40 (12)	2.43 (17)	2.75 (11)	**2.75 (11)**	2.67 (16)
13	ABTS	Phenol oxidase activity	1.60 (8)	1.71 (12)	1.75 (7)	1.75 (7)	1.83 (11)
14	Peroxidase	Peroxidase activity	2.20 (11)	2.29 (16)	2.50 (10)	2.50 (10)	2.50 (15)

No.	Method	Endpoint	Final score without weighting (as presented in Table 3)	1st modification (Final score considering weighting factors)[1]	2nd modification (Final score without weighting and neglecting reproducibility if no ring tests were available)[2]	3rd modifications (Final score without weighting and neglecting reproducibility for all methods)	4th modification (Final score with weighting and neglecting reproducibility for all methods)
15	**Fluorescein diacetate (FDA)**	**Total microbial activity; measured by both free and membrane bound enzymes**	2.40 (12)	2.43 (17)	2.75 (11)	**2.75 (11)**	2.67 (16)
Fungal community							
16	**ISO 10832**	**Spore-germination of *F. mosseae* or additional AMF species**	**2.40 (12)**	**2.43 (17)**	**2.40 (12)**	**2.50 (10)**	**2.50 (15)**
17	**Laccase**	**Laccase (enzyme from fungi) activity**	2.00 (10)	2.14 (15)	2.25 (9)	2.25 (9)	2.33 (14)
Functional genes and structural profile							
18	ISO 17601	Shifts of the microbial community composition	1.80 (9)	1.86 (13)	2.00 (8)	2.00 (8)	2.00 (12)
19	ISO 29843-1/2	Total PLFA and PLEL contents as quantitative measure of the viable soil biomass	1.60 (8)	1.57 (11)	1.75 (7)	1.75 (7)	1.67 (10)
20	DGGE	Shifts of the microbial community composition	2.00 (10)	2.14 (15)	2.25 (9)	2.25 (9)	2.33 (14)
21	T-RFLP	Shifts of the microbial community composition	1.80 (9)	1.86 (13)	2.00 (8)	2.00 (8)	2.00 (12)

No.	Method	Endpoint	Final score without weighting (as presented in Table 3)	1st modification (Final score considering weighting factors)[1]	2nd modification (Final score without weighting and neglecting reproducibility if no ring tests were available)[2]	3rd modifications (Final score without weighting and neglecting reproducibility for all methods)	4th modification (Final score with weighting and neglecting reproducibility for all methods)
22	**ARISA**	**Shifts of the microbial community composition**	2.40 (12)	**2.43 (17)**	**2.40 (12)**	**2.50 (10)**	**2.50 (15)**
Carbon cycling and sequestration							
23	**OECD 56**	**Organic matter breakdown**	**1.80 (9)**	**1.86 (13)**	**1.80 (9)**	**1.75 (7)**	**1.83 (11)**

[1] The parameters practicability, costs and relevance were considered more important (factor = 2) than the parameters influence of soil type and replicability (factor 1).[2] For methods, for which no ring tests were available, the parameter replicability was not considered in the calculation of the total score.

2.1.5 Supporting literature for the evaluation of the test methods

Literature was reviewed for further advantages, disadvantages, and general recommendations. These recommendations were also considered to finally determine the test strategy.

Imfeld and Vuilleumier (2012) propose a systematic combination of culture based and culture independent methods for a more in-depth assessment of environmental risk. The main drawback of cultivation-dependent methods is that only a minor fraction of the diversity of soil bacteria can be easily grown in the laboratory, and is thus accessible to experimental investigation.

2.1.5.1 Reviews

Next to articles dealing with the application of a limited set of selected test methods (e.g., Thiele-Bruhn (2005), Floch et al. (2007)), several reviews on available test methods were identified. These reviews were used as the basis for the evaluation of the reviewed test methods. While most of the identified reviews mainly summarize available standardized ISO test methods, which could be used in addition to the available OECD guidelines as OECD 216 or 217, a few also include non-standardized methods of which some may be standardized in the near future (Imfeld and Vuilleumier (2012), Blagodatskaya and Kuzyakov (2013), Brandt et al. (2015), Römbke et al. (2018) and Thiele-Bruhn et al. (2020)).

Thiele-Bruhn et al. (2020) have gathered all available, already validated and published ISO standards for determining potential effects on microbial biomass and/or activities for soil quality. Moreover, they also listed potential new methods for the ISO standardization process and assessment. These potentially new methods were evaluated according to a list of criteria for the selection of indicators for microbial functional indicators, based on Faber et al. (2013) and Pulleman et al. (2012), with slight modifications by the authors. Criteria such as practicability, cost efficiency, policy relevance, sensitivity, selectivity, reproducibility, use as an indicator, handling and availability of organisms, fit for use as an indicator, experience and data evaluation were used for the evaluation of all the piled-up methods. The criteria of Thiele-Bruhn et al. (2020) can be used for the evaluation of available test methods as well as for the preparation of a testing strategy within this project.

In 2018, Römbke et al. (2018) a critical review on standard methods for the assessment of structural and functional diversity of soil organisms is published. Standard methods of sampling, identifying, determining and assessing soil organisms currently applied or proposed were described and evaluated by using well-accepted criteria such as ecological relevance; practicability of usage in terms of resources, time, and costs; and the level of standardization. The presented methods addressed the structure and the functions of soil organisms, with a special focus on new molecular methods based on nucleic acid extraction and further analyses by polymerase chain reaction (PCR) based approaches for microorganisms (Römbke et al. 2018). The presented list of available ISO methods allows the estimation of a range of parameters, describing abundance, structure, and activity of soil microorganisms. The results are in line with the list of Thiele-Bruhn et al. (2020).

Brandt et al. (2015) reviewed approaches for elucidating ecotoxicological effects of antibiotics on microorganisms for an environmental risk assessment and concluded that microbial community-based tests should be used to complement single-species tests to offer more targeted protection of key ecosystem services. The ecotoxicological tests should not only assess microbial community function, but also microbial diversity ('species' richness) and antibiotic susceptibility. Several standardized ISO guidelines (soil quality tests) with relevance for ecotoxicological testing of microorganisms were listed, but also non-standardized approaches for the investigation of effects to microorganisms in the environment were provided and

evaluated for their suitability for a test strategy. For a detailed evaluation, the substance sulphadiazine was used to compare the sensitivity of different tests on target groups such as bacteria, fungi, anaerobic Fe(III)-reducing bacteria, cultivable bacteria etc. In addition, a conceptual framework for understanding effects of antibiotics on microbial communities was provided. The authors highlight, that the major limitation of single-species tests – which are planned in work package 3 (antibiotic resistance) – is the lack of representativity of the high complexity of the microbial communities occurring in soil. Therefore, single-species tests should be complemented by tests targeting intact microbial communities.

Imfeld and Vuilleumier (2012) reviewed methods to measure and interpret the effects of pesticides exposure on the soil microbial microflora and ongoing advances in microbial molecular ecology. They indicate biomass, microcalorimetry, [^{3}H]-leucine or [^{3}H]-thymidine incorporation, substrate-induced respiration and soil enzymes (dehydrogenase, phosphatase, urease) as physiological measurements and community-level physiological profiling (CLPP) or community-level catabolic profiles (CLCPs) as fingerprinting methods next to PCR-based DNA profiling and lipid analysis (FAME, PFLA) and comment on their possible application and advantages or shortcomings.

The focus of the review paper by Blagodatskaya and Kuzyakov (2013) is on active microorganisms. According to the authors, microbial functioning refers to microbial activity because only active microorganisms drive biogeochemical processes. However, only 0.1 – 2% of the total microbial biomass in soil are active, while the part of potentially active microorganisms (ready to start utilization of available substrates within a few hours) is much higher comprising between 10 to 40% of the total microbial biomass. Therefore, the focus was to list methods to evaluate the majority of active and potentially active microorganisms. Methods such as plate counts and microbial cultures, direct microscopy combined with cell staining, ATP, PLFA, DNA and RNA content, microarray analyses, PCR-based approaches, stable isotope probing, soil proteomics, enzyme activities and various approaches based on respiration and substrate utilization were evaluated.

2.1.5.2 Activity, aerobic heterotrophic microbial biomass

Two test systems are commercially available which can be used to determine community level physiological profiles (CLPP) in soils, the BIOLOG® system and the MicroResp™ system.

In 1991, Garland and Mills (1991) introduced the BIOLOG® system, a redox technology based on tetrazolium dye reduction acting as an indicator of sole-carbon-source utilization. The system was evaluated as a rapid, community-level method to characterize and classify heterotrophic microbial communities by the manufacturer. The BIOLOG® plates (namely ECO plates) contain 95 separate carbon sources which produce community-dependent patterns of sole-carbon-source utilization. Meanwhile, various microplates such as the ECO plate or plates for the determination of filamentous fungi are commercially available from BIOLOG. The ECO plate is certainly the microplate that seems most suitable for this project as it allows measurements of the metabolism of 31 carbon sources per assay, with each assay panel in triplicate per plate. The evaluation is based on a simple colorimetric readout, readable with any microplate reader.

The MicroResp™ system is also a microplate-based respiration system that enables the user to analyse up to 96 soil, sediment or water samples and test a range of carbon sources and/or replicates in a small compact space. While some sole C-source tests tend to select for fast-growing bacteria and rely on growth of organisms, MicroResp™ gives more immediate responses to these substrates and reflects activity rather than growth by measuring responses in the first 6 hours.

In a review article from Chapman et al. (2007) various methods among others BIOLOG® and MicroResp™ were compared to outline the advantages and applications of the MicroResp™ system. According to Chapman et al. (2007), the original method for CLPP determination was based on the BIOLOG® plate with its range of 95 carbon substrates. However, the main point of criticisms is that BIOLOG® relies upon the growth of an extracted microbial population, which may not represent the true functioning of the whole soil. Briefly, for the BIOLOG® system, soil extracts must be prepared, which are added to the microplates together with available substrates. The MicroResp™ system has been developed to overcome the deficiencies in both methods. The system has been applied to a range of media including mineral and organic soils, sediments and litter. In addition, Chapman et al. (2007) have adapted the method to the testing of a wide range of carbon substrates such as hydrocarbons, terpenes and pesticides. A wide range of possible C-, N-, P- and S-containing substrates (n > 100) that can be used for the test system is now available.

2.1.5.3 Nitrifying and denitrifying bacteria

The potential ammonium oxidation test in accordance to ISO 15685 was mentioned in several reviews as an alternative test method to the nitrogen transformation test (OECD 216) ((Brandt et al. 2015), (Römbke et al. 2018), (Thiele-Bruhn et al. 2020)). The intent of this method is to measure the ammonia oxidation potential, which provides an indication of the size of the ammonia oxidizing bacterial population.

2.1.5.4 Enzymatic activity

Imfeld and Vuilleumier (2012) stated that results from studies on the effect of pesticides on several soil enzymes have to be evaluated carefully since the response and/or recovery of bacteria to pesticides exposure is fast and depends on dose and exposure frequency. Imfeld and Vuilleumier (2012) outlined for the cultivation dependent methods that testing several relatively specific enzymatic activities or a combination of them need to be used to characterize the response of soil bacterial communities to pesticides. This approach can be followed e.g. by using the methods of ISO guideline 20130 for the measurement of several important hydrolase activities such as arylamidase, arylsulfatase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-glucosaminidase, acid, alkaline and global phosphatases and urease. Microplate-scale fluorometric or colorimetric assays offer a fast throughput approach for studying the response of multiple enzyme activities involved in main soil biochemical processes (Giacometti et al. 2014) and increase the precision of the measurements, enabling sensitive analysis of complex experiments with sufficient replicates (Joergensen and Emmerling 2006).

For several selected pesticides, Imfeld and Vuilleumier (2012) showed the observed effects were highly variable depending on soil characteristics, the type of pesticide and the observed enzyme activity. For the fungicide propiconazole, the insecticide profenofos and the herbicide pretilachlor a decreased activity of phosphatase, urease and dehydrogenase was observed, while for the fungicide mancozeb an enhanced activity of alkaline phosphatase, protease and amidase but a decreased activity of urease and asparaginase was found.

Riah et al. (2014) reviewed effects of pesticides on the activity of soil enzymes in the terrestrial environment. In 61% of the analyzed studies, dehydrogenase was inhibited while mainly no effects on aryl-sulfatase occurred. In more than 50% cellulose degradation was stimulated. In this review, a large survey on effects of various fungicides, insecticides and herbicides on different enzyme activities is presented, considering respective pesticide doses and soil pH.

2.1.5.5 Fungal community

Arbuscular mycorrhizal fungi (AMF) are considered as a key group in soil systems since they occur widely in terrestrial ecosystems and provide important ecosystem services (Mallmann et al. 2018). Currently, the ISO 10832 describes a method to evaluate effects of pollutants on spore germination of the AMF species *Funneliformis mosseae.* However, there are new publications describing *Gigaspora albida* and *Rhizophagus clarus* as possible test organisms (Mallmann et al. 2018). Currently, AMFs are not considered in ERA despite their importance in various processes in soils and their interactions with plants. However, EFSA PPR panel (2017) identified AMF as possible test species for the ERA of plant protection products. At the moment, the development of a corresponding guideline is conducted that will produce results suitable for an ERA. For this reason, studies on the effect of PPP, biocides and pharmaceuticals on AMF will also be carried out in the current project and, if sensitive, will be part of the testing strategy.

2.1.5.6 Functional genes and structural profile

Buyer and Drinkwater (1997) compared the Biolog® system with results obtained via fatty acid analysis (PLFA) of soil microbial communities and found that results were quite comparable, but certain shifts in the microbial community could be found by fatty acid analysis but not within the Biolog® system (substrate utilization) suggesting that there have been shifts in the microbial composition which did not affect the microbial function. Based on this result, the question can be derived, how far-reaching and specific a test method has to be to achieve a protective risk assessment for soil microorganisms. PLFAs produce very specific results and, from a research point of view, useful results. However, changes in structure do not necessarily mean an impact on function and a less specific test method which often is less expensive and easier to conduct appears to be more appropriate.

Using quantitative real-time PCR (qPCR) will enable the possibility to observe effects on several functional endpoints which might be more sensitive compared to other endpoints ((Römbke et al. 2018), (Thiele-Bruhn et al. 2020)). Here, a wide variety of functional genes or single marker gene sequences which are indicative for specific transformations processes or soil functions as *nifh*, *amoA* and *amoB* or *nirS/nirK* are already used for quantification of nitrogen fixing microbes, nitrifiers and denitrifiers (Thiele-Bruhn et al. 2020). As outlined by Brandt et al. (2015) in most of the reviewed studies fingerprinting techniques as DGGE or ARISA were used, in which mainly the 16S rRNA gene was targeted. These methods may be also used to determine functional genes/single marker gene sequences indicated above e.g. for nitrifies/denitrifiers. However, these methods require extensive and complex multivariate statistical procedures. They are therefore rated very low in terms of practicability and cost-effectiveness in the available reviews, e.g. by Thiele-Bruhn et al. (2020), and cannot be performed by every standard laboratory. Sequencing functional or taxonomic gene markers is actually replacing the above-mentioned fingerprinting methods such as DGGE or ARISA and it is possible to measure the species richness, which could be a good indicator for effects of pollutants on the microbial community (Brandt et al. 2015). However, this method is also costly and lacks the necessary practicality.

Thiele-Bruhn et al. (2020) lists a number of different advantages and disadvantages of qPCR assay to quantify gene sequence numbers as proxies for microbial functions. The advantages mentioned here are that the methods are standardized, sensitive, selective and reproducible and can be performed in high-throughput. Once purchased and after thorough familiarization of the personnel with the method, the costs are reduced relatively quickly. On the other hand, the method always depends on the soil DNA extracts, primer pairs sometimes cannot be amplified properly and no distinction is made between active, dormant and dead microorganisms. Here,

one would have to resort to RNA analyses, which in turn bring with them the problem that changes occur very dynamically and changes can occur accordingly during sample processing.

Methods such as DGGE, ARISA or T-RFLP might be relevant approaches but they are not applicable for an ERA due to certain difficulties. However, it was concluded that nucleic acid based protocols are to be used as an amendment to already existing standardized protocols (OECD 216/217) in order to monitor community shifts during long-term toxicity tests (Brandt et al. 2015).

2.1.5.7 Carbon cycling and sequestration

The OECD 56 "Guidance Document on the Breakdown of Organic Matter in Litter Bags" test is a long-term test with a test duration of several months and is conducted as field experiment. Nevertheless, it has been listed here, because in principle one could also think about a modified application in the laboratory. However, due to the low score and the fact that the test is actually intended for the field and shows low relevance for regulatory purposes (information given by UBA), it was not included for the test strategy.

2.1.6 Conclusion for the project's test strategy and recommendations of the statistical analysis of test methods

2.1.6.1 Practical implementation

In most of the reviews and research papers it was outlined that not only the properties of the test item governs the effect on the soil microorganisms but also the soil properties (Dick et al. 2000). Therefore, it appears of high relevance to observe the effect of chemicals with several test methods for a variety of test soils as intended in the actual project.

Based on the evaluation of different methods to assess the effect on soil functions and structure, five methods with the highest score each for aerobic heterotrophic soil microorganisms, nitrifiers, enzyme activity, AMF and structural soil diversity were elaborated.

Activity of aerobic, heterotrophic microorganisms: MicroResp™ including basal respiration and glucose for standard substrate-induced respiration, which allows to determine the metabolic quotient $Q_{R,}$ as additional information. The method could be a promising add-on to standard tests (OECD 216, 217) to address several nutrient cycles as also N-, P-, and S-cycles are presented. It is a simple and rapid method which can be performed within one day. In addition, the method was already recommended by the EFSA PPR Panel (2017). Therefore, this could be a good indicator for effects not only on the carbon or nitrogen transformation but also on phosphorus or sulphur transformation. Creamer et al. (2016) and Hund-Rinke et al. (2019) recommended several substrates for this test method and these should be used in the actual project.

Activity of nitrifying bacteria: The ammonium oxidation test (ISO 15685) can be used as additional parameter for nitrogen mineralisation (alternative to N-transformation test). The test was also included in the various reviews stated above. It is a simple and rapid method which can be performed within one day. It is very sensitive as only a small microbial group with low potential for resilience is addressed. Although the environmental relevance is limited (score 2) it can be used as indicator for toxic effects.

Enzyme activity: The ISO 20130 "Soil quality — Measurement of enzyme activity patterns in soil samples using colorimetric substrates in micro-well plates" should be used to assess the effect on the activity of exoenzymes. The test is comparable with the method described in ISO 22939 but cheaper since no fluorogenic substrates have to be used. Furthermore, this method offers a higher flexibility in the selection of the substrates and therefore in the

considered enzymatic activities. While only a limited number of fluorogenic substrates is available, every substrate with sufficient solubility can be used in the method described in ISO 20130. Unlike methods in which the effect on only a small group of enzymes is investigated, with this method the effect on a number of different groups can be considered.

Fungal activity: The ISO10832 "Effects of pollutants on mycorrhizal fungi — Spore germination test" (or an adaptation from Sousa et al.; 2nd UBA project) will be used to observe the effect on fungi since it appears to be the most suited test to address potential effects on this ecologically relevant group which is otherwise not covered by the proposed tests.

Microbial structure: qPCR, DGGE, ARISA, PLFA and PLEL are probably promising methods which will bring real in-depth knowledge about effects on the microbial community structure and shifts within the community. However, these methods are expensive and the evaluation might be difficult. In a tiered approach, it does not seem realistic that these methods can be used in a lower tier. They would rather be useful in a higher tier assessment. To better address the biodiversity of the soil microorganism community, the ARISA method might be an appropriate fingerprinting method to be used exemplary. The method is less expensive than the other methods evaluated and not so sophisticated to require a really high-level statistical evaluation.

2.1.6.2 Statistical analysis

The systematic literature search in Section 2.1 showed that several methods for describing the effects of contaminants on soil microorganisms were developed as international standards or adapted from well-established approaches described in the scientific literature. The methods were evaluated regarding practicability, expected costs, reproducibility, and influence of different soil types. This evaluation deduced the overall relevance and a final score for defining a test strategy. The selected methods were practically implemented and tested for six test items and three soils (section 2.4). Statistical aspects of the different methods are discussed in the following section.

2.1.6.2.1 Data evaluation following the guidelines for Nitrogen Transformation (OECD 216) as well as Carbon Transformation (OECD 217)

For the practical implementation, three methods (ISO 15685 – Ammonium-oxidizing bacteria, MicroResp™ (substrate-induced respiration), ISO 20130 Enzymatic activity) were already applied in tests designed following the Nitrogen Transformation (OECD TG 216 (2000)) and Carbon Transformation (OECD TG 217 (2000)) test guidelines[1]. Therefore, soil samples were treated with the test item, and the measurements were made at specific intervals over at least 28 days. Regarding the test design and data evaluation, OECD 216 and 217 are equivalent to the statistically relevant design parameters and can be summarised as follows. Here, OECD TG 216 was used as an example because it poses the single default method to assess toxic effects on soil microorganisms and, thus, represents the current state:

- A minimum of three replicates for both treated and untreated soils is recommended

[1] Note, that the ISO 15685 – Ammonium oxidizing bacteria - is a description to assess soil quality with respect to nitrification and thus, has another focus than OECD test guidelines. Nevertheless, it includes a short section 8.2 "Testing the effects of chemical substances". It is recommended to use twice as many control replicates as treatment replicates (without suggesting specific numbers) and five test concentrations in a geometric series. No recommendations for statistical evaluation are made. Only a graphical representation of the inhibition of nitrification against the test concentration and an indication of ID25 and ID50 in the diagram is recommended. It is not described how the test substances should be applied and when the nitrification should be measured.

- *For agrochemicals*,
  - a minimum of two test concentrations is recommended (the maximum PEC and fivefold of maximum PEC), and
  - the data is analysed on days 0, 7, 14, and 28. If a prolonged test is required, further measurements should be made at 14-day intervals after day 28.
- *For non-agrochemicals*,
  - at least five test concentrations are required, and
  - the data is analysed at the start of the experiment (on day 0) and the end of the exposure period (after 28 days). An intermediate measurement, e.g., on day 7, may be added if necessary.

- The quantities of nitrate formed are expressed in mg nitrate-N/kg soil dry weight/day.
- The validity criterion is that the variation between replicate control samples should be less than ± 15%'. It is not explicitly defined which measure of variations should be calculated. Usually, the coefficient of variation (standard deviation/arithmetic mean *100) is used.
- The quantity of nitrate formed (alternatively, the carbon dioxide released or oxygen consumed, respectively) in each replicate soil sample should be recorded, and the mean values of all replicates should be provided in tabular form. The mean nitrate formation rate in each treatment is compared with that in the control, and the per cent deviation from the control treatment is calculated.
- Nitrogen transformation rates should be evaluated using appropriate and generally acceptable statistical methods (e.g., F-test, 5 % significance level). However, if, on the 28th day, differences between treated and untreated soils are equal to or greater than 25 %, measurements are continued to a maximum duration of 100 days. When results from tests with agrochemicals are evaluated, and the difference in the rates of nitrate formation between the lower treatment (i.e. the maximum predicted concentration) and control is equal to or less than 25 % at any sampling time after day 28, the product can be evaluated as having no long-term influence on nitrogen transformation in soils.
- As requested for non-agrochemicals, tests with multiple concentrations are analysed using a regression model to calculate EC_x values for inhibition (i.e. EC_{50}, EC_{25} and EC_{10}). The %-inhibition values for each test concentration are calculated. These percentages are plotted against concentration, and statistical procedures are then used to calculate the EC_x values. Confidence limits (probability 95 %) for the calculated EC_x are also determined using standard procedures (OECD TG 216). References relate to probit analysis for ECx calculation.

In most cases in this project, the tests on microbial function described in chapter 2.3 were conducted with three test concentrations plus control with three replicates each. In a few cases, four test concentrations were used. Thus, the design fulfilled the minimum requirement in OECD TG 216 for the number of replicates but tested more treatment levels than requested for agrochemicals but less as required for non-agrochemicals to allow a calculation of EC_x. Thus, the number of test concentrations usually used did not allow for a reliable calculation of EC_x-values with confidence intervals.

The trigger for acceptable or not acceptable effects of plant protection products on soil microorganisms is the effect magnitude (25 %) and not a statistical significance between control

and treatment. Therefore, the effect of the expected maximum PEC is considered for the decision. The rate above the PEC is only used as a positive control, not as margin of safety.

The OECD guidelines do not explain the rationale behind setting the 25 % criterion to decide on long-term effects. It also is also unusual that an OECD test guideline includes guidance for the risk assessment, e.g., on specific criteria to decide on the absence of long-term effects or different data requirements for agrochemicals and other chemicals.

An evaluation via regression analysis to calculate EC_x values is preferable for a more flexible risk assessment. The assessment aims to avoid long-term effects on microbial function in-crop (no deviation from control larger than 25 % after 100 days). The option of testing only two concentrations for agrochemicals is explained by addressing only the recommended application rates or predicted maximum concentrations in agricultural practice. However, if the test item affects the microbial activity at environmentally realistic concentrations, a regression approach would allow the calculation of different threshold concentrations. These could vary depending on the specific protection goals defined by authorities responsible for risk management, e.g. safe concentrations could be deduced from different effect sizes (EC_{10}, EC_{20}). Therefore, as a recommendation, at least five test concentrations should be tested. If fewer concentrations are tested in a limit-test design, the number of replicates should be increased to allow a more robust statistical analysis of significant differences to controls if this is required.

Further steps will examine if the reported expectable variability (refer to Table 2) between replicates would allow for exemplary power analysis for the difference between control and treatment group microbial activities. In this context, data derived from ring test results would be helpful. However, not all of the methods proposed were properly ring-tested.

It remains unclear why the guidelines suggest an F-test (they probably mean an ANOVA F-test that is conducted in a limit-test setting only) and not a multiple Dunnett or Williams test or alternatives for non-parametric data (for general guidance on statistics, see (OECD GD 54 2006), which would indicate which treatments are significantly different to the control if more than one treatment level has been tested. However, it would make sense to set an acceptable magnitude of effect if the variability in the tests is so low that deviations considered not ecologically relevant are significant.

The OECD test guidelines for nitrogen (OECD TG 216) and carbon (OECD TG 217) transformation describe procedures to measure one single endpoint per study, either nitrification or respiration rate. In contrast, using MicroResp (substrate-induced respiration) and ISO 20130 (enzyme activity) provides multiple endpoints, i.e. inhibition of respiration rates for different substrates and of various enzymes, which may represent effects on various parts of the microbial community. Thus, since the three methods can be applied in the same experiment, they offer an assessment of functional diversity, which could be evaluated similarly to multivariate techniques like structural community composition (see below, ARISA).

For the statistical evaluation, the number of replicates is defined by the soil sample replicates prepared at the start of the test (e.g. three replicates for each test concentration and the control). Replicates of subsamples, e.g. on microtiter plates for the measurements, should not be used for the statistical evaluation but aggregated as mean values. Aggregating across wells of the microtiter plates is valid if no edge effects or significant differences between replicate measurements could occur, which is corrected by more complicated statistical models (e.g. linear mixed models). Regarding the number of replicates for these subsamples, we refer to the recommendations related to the different methods.

2.1.6.2.2 The spore germination test for AMF

The *DIN ISO/TS 10832 on the effect of pollutants on mycorrhizal fungi – spore germination* test is laid down in a separate test guideline. The statistical test design and data evaluation are described as follows:

- At least five concentrations in a geometric series, applying a spacing factor smaller or equal to two, plus a control group should be used.
- Six replicates per concentration, 30 spores per replicate.
- Germination rate is the number of germinated spores related to the number of retrieved spores. Calculation of mean germination rate and standard deviation per concentration level.
- Calculation of inhibition of germination in the treatment groups concerning control germination.
- Calculation of dose-response function. Specific methods for calculating the IC_x (50 % inhibitory concentration) are not proposed. Probit analysis is possible.
- Derivation of the (IC_{50}) with a 95 % confidence interval
- For statistical comparison to control germination, per cent values should be transformed by arcsine-square root since this is supposed to be a variance stabilising step for proportional data that is usually binomially distributed and could then be used as quantitative data.
- The validity criterion is set to the mean number of retrieved spores (must be > 25) and the control germination rate (>= 75 %).

Since the primary endpoint is the IC_{50}, spending more resources on the number of test concentrations than replicates would be better. Six replicates support hypothesis testing (NOEC / LOEC) calculation, which meant statistical analysis based on arcsine (i.e., arcsine-square-root-) transformed values. However, tests for %-values also do not need transformation (Chi^2-test).

2.1.6.2.3 Concluding evaluation of statistical test designs

Table 5 provides information on topics considered important for the statistical evaluation of each of the five focused test methods used to test microbial function and structured within this project. The compilation holds instructions and recommendations from the respective OECD and ISO guidelines or method descriptions that mainly give practical provisions and often no assistance for statistical evaluation. Whenever no indication was given in the guideline, it refers to the standards of the analysis and design from guideline OECD TG 216.

References to assessment endpoints and specific protection goals are not the subject matter in this section, but the issues of the use and position of the test methods in future risk assessment schemes will be broached in a later section 2.6.

Table 5: Compilation of methodological characteristics of the proposed ecotoxicological tests to assess the effects of contaminants on soil microbial communities. In cases where no provision is made in the respective guideline, the recommendation is taken from the OECD216 or own expertise in this project.

Parameter	Nitrification - Ammonium oxidation, ISO 15685	Respiration - Microbial activity, MicroResp	Enzyme activity, ISO 20130	Microbial community structure, ARISA	Germination – AMF, ISO 10832
Test objective	Determination of inhibitory doses (ID_x) for the inhibition of the transformation of NH4 to NO2- by ammonium-oxidising bacteria (nitrification)	Metabolically fingerprinting soil microbial communities - no direct relation to testing of chemical contaminants	Determination of adverse effects of toxic substances on hydrolase-activities in soils	Characterization of bacterial (B-ARISA) and fungal (F-ARISA) communities from different soil types - no direct relation to testing of chemical contaminants	Determination of inhibitory concentration of toxic substances on spore germination of mycorrhiza-fungus *Glomus mosseae*
Functional group assessed	Nitrifying bacteria	Microbial community			Arbuscular mycorrhizal fungi (AMF) e.g.; *Funneliformis mosseae*
Measurement parameter	NO_2-formation	Transformation to oxidised products of C-, N-, P-, and S-containing substrates. Oxidised products formation (D-(+)-Glucose, L-Cysteine hydrochloride, L-Malic acid, y-Amino butyric acid, N-Acetyl glucosamine, Citric acid, L-Alanine)	Oxidised product formation as extinction caused by para-Nitrophenol, ß-Naphthylamine, Ammonium-chloride as reaction products	Number of individual Operational Taxonomic Unit – OTU Peak area as a surrogate for abundance per OTU	Proportion of number of germinated spores to total spore number
Measurement unit	ng NO_2-N/g dw soil/h	µg CO_2-C | PO_4-P | SO_2-S | NO_2-N/g dw soil/h	nmol/minute/g dw soil	Number	%
Test duration [d]	not defined	not defined	not defined	not defined	14
Test design	Could be combined with one or a few of the other methods, e.g. similar to the design proposed in OECD test guidelines 216, 217				Test on its own
Measurement dates	*Following OECD 216:* 0, 7, 14, 28, for PPP prolonged if effect >25%			*In this project:* 28	14

Parameter	Nitrification - Ammonium oxidation, ISO 15685	Respiration - Microbial activity, MicroResp	Enzyme activity, ISO 20130	Microbial community structure, ARISA	Germination – AMF, ISO 10832
[d after the start of the experiment]					
Statistical design	*Following OECD 216:* Limit (agrochemicals); Regression design (non-agrochemicals)				Regression design
Number treatments	≥5 concentration levels + control group	*Following OECD 216:* ≥2 concentration levels + control group (agrochemicals); (≥5 concentration levels + control group (non-agrochemicals))			≥5 concentration levels + control group
Statistical endpoint	*Following OECD 216:* % deviation from control (agrochemicals); ECx (non-agrochemicals) for inhibition			NOEC / LOEC for diversity indices or ordination sample scores	ECx (Inhibition concentration IC_x)
Statistical - model	*Recommendation:* Limit test: normal distribution, t-distribution ECx-design: depends on the shape of the response and the mode of action; e.g., 3-parameter logistic, simple exponential, 4-parameter logistic,probit.			Univariate für diversity indices, multivariate for OTUs	ECx-design: depends on the shape of the response and the mode of action; e.g., 3-parameter logistic, simple exponential, 4-parameter logistic, probit

Analysing the five test methods for chemical structural and functional effects on microorganisms evaluated in practice in this project has yielded insights and conclusions on the designs of the studies conducted in the present work and their data analysis. It has been considered possible that several methods measuring community functions (i.e. nitrification, respiration and enzyme activity) could be practically implemented within the same test design, e.g., a test similar to OECD 216 / 217. Combining more than one test method would improve the cost-effectiveness of the experiments (fewer efforts in pre-processing soil samples) and increase the comparability between them because methodological variability due to several pre-processing steps would be circumvented. Samples to analyse community structure should preferably be taken using the same testing design. The analysis of these community data should not be considered as a refinement step but as a tier 1 endpoint on its own or as an analysis to link observed effects on a function to changes in community structure. The test on spore germination of arbuscular mycorrhizal fungi is considered a test on its own with a specific design.

A regression design allowing calculation of EC_x values is preferred over a NOEC design, i.e. testing at least five concentration levels with three true replicates each. Compromise designs that allow for both deducing EC_x and NOEC would need more replicates within each treatment group, depending on the desired power of the test design.

2.2 Representative soil characteristics across Europe

The range of characteristics of natural soils that trigger the ecotoxicological profiles of chemicals was considered before the practical test implementation and verification of the sensitivity of

individual methods. In what follows, the representativeness of the chosen reference soils (LUFA 2.1, RefeSol 02A and RefeSol 04A) for soils across the European Union was investigated.

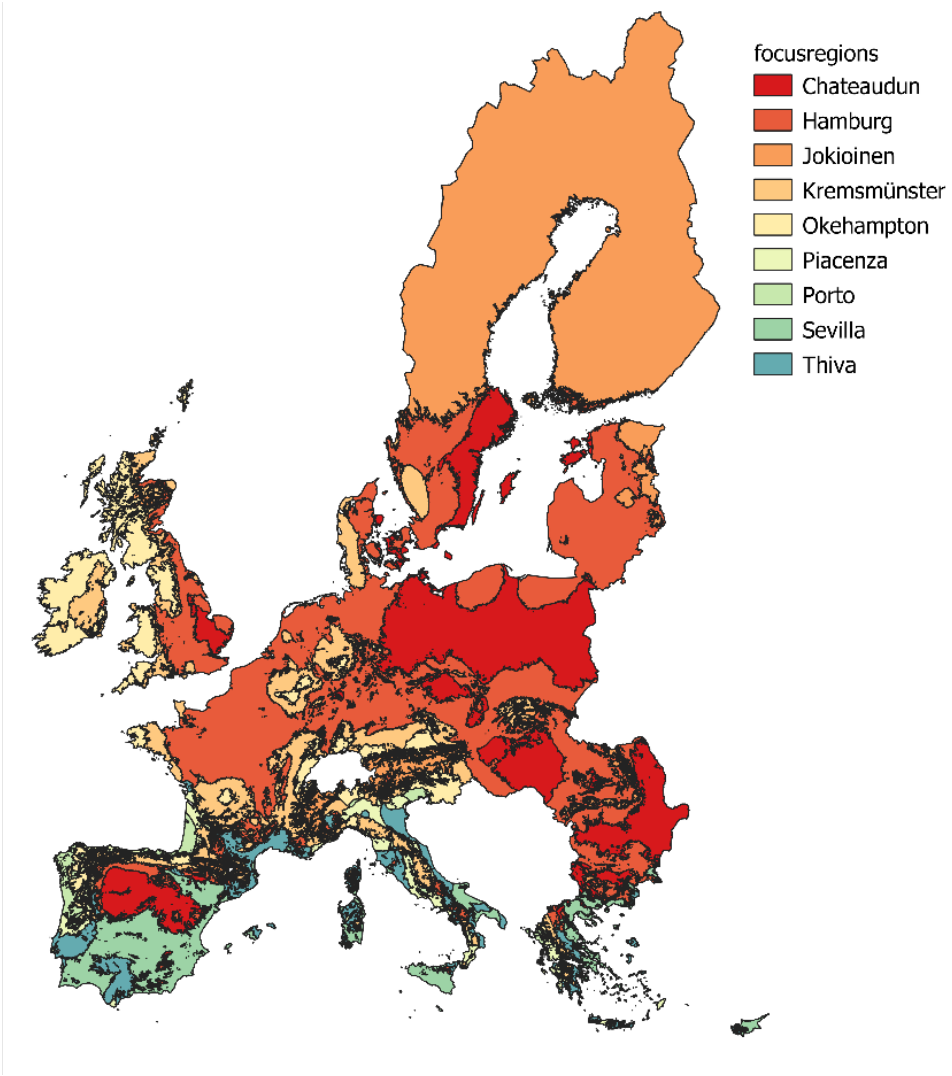
Various EU institutions or initiatives have compiled data describing European soil properties. A first overview was visualized in the *Soil Atlas of Europe* (European Commission 2005), but more detailed data are available via the Joint Research Center (JRC, Ispra, Italy). Another example is the outcome of the GEMAS (Geochemical Mapping of Agricultural Soil) project, which was organised by the Geochemistry Expert Group of Euro-GeoSurveys (EGS) and Eurometaux (Reimann et al. 2018).

On request, the European Soil Data Centre (ESDAC) – European Commission provided a large dataset on soil characteristics across the EU28 (including GB) that was queried from EFSA-JRC (Hiederer 2012) based on grid cells of 1 square kilometre size. The EFSA Spatial Data Version 1.0 database was publicly available and contained 52 spatial data layers. It was downloaded in raster format as an ASCII-text file (number of raster cells > 4.2 million) and filtered to arable soils exclusively because of the low likelihood of exposure to chemicals on non-agricultural land. The database was primarily designed to feed the regulatory fate models from the FOCUS working groups and the FATE and ECOREGION EFSA PPR working groups (Gardi et al. 2010). Five key variables are stored in a 1 x 1 km grid all over Europe and used to analyze and demonstrate the distribution of different soil properties.

- Topsoil Organic Matter content EU28 (converted to organic carbon)
- Topsoil pH EU28
- Topsoil Bulk Density EU28
- Topsoil Texture Class EU28
- Topsoil Water Content at Field Capacity

The raw data were imported into an R-environment from all raster cells, followed by computing frequencies of the levels of categorical variables (e.g., texture classes) or cumulative density functions for continuous variables (e.g. organic matter content, Figure 3). Raster data were visualized by drawing classified maps (Figure 2) according to EFSA Focus working group exposure scenarios.

Figure 2: Regions according to FOCUS scenarios across EU28.



Source: own illustration darwin statistics

2.2.1 RefeSol and LUFA 2.1 soils

IME RefeSols and LUFA soils are soils recognized by the German Environment Agency (Umweltbundesamt UBA) for application in test procedures according to the German Federal Soil Protection Act/Ordinance. On behalf of the UBA, the different RefeSol soil types were selected and tested by the Fraunhofer Institute for Molecular Biology and Applied Ecology IME (Fraunhofer IME) in cooperation with the Department of Physical Geography and Landscape Ecology, Hanover University. The focus was on the RefeSol soils 02A and 04A since they were proposed to perform ecotoxicological tests to describe the effects of chemicals on soil microorganism community structures and functions (results in chapter 2.4). Refesol 01A, originally proposed to be included in effect testing, was dismissed by the expert support of the project sponsor and replaced by LUFA 2.1 soil within this project. For the properties of the different soil types, see Table 6 and Table 7.

Table 6: Soil type and properties of RefeSols (A= arable land, G=grassland) and LUFA 2.1. https://www.refesol.de/english/analysedaten.shtml, accessed 2021-06-16.

RefeSol	soil type	properties
01A	Dystric Cambisol	sandy loam, medium acidic, very light humic
02A	Stagnic Luvisol	silt loam, sub-acidic, light humic
03G	Eutric Cambisol	silt loam, medium acidic, medium humic
04A	Gley-Podsol	loamy sand, medium acidic, medium humic
05G	Gleyic Fluvisol	loam, strongly acidic, strongly humic
06A	Cambic Rendzina	silty clay, very sub-acidic, medium humic
Lufa 2.1	not known	sand, strongly acidic, very light humic

WP2 relies on RefeSol soils 02A and 04A and LUFA 2.1 soil. The Refesol soils 01-A, 03-G, 05-G, and 06-A are included in the table as they were originally proposed for WP2.

Table 7: Analysis Data (Average AV), https://www.refesol.de/english/analysedaten.shtml, accessed 2021-06-16. Blue cells: RefeSols foreseen for testing in the MICROSOIL project.

RefeSol	Sand DIN [%]	Sand USDA [%]	Silt DIN [%]	Silt USDA [%]	Clay DIN [%]	Clay USDA [%]	pH_{CaCl2} AV [%]	Corg AV	CEC_{eff} [mmolc/kg]	WHC [g/kg]	Ntotal AV [g/kg]
01A	73.1	74.0	17.2	17.7	6.1	5.70	5.61	0.93	11.6	293	0.97
02A	2.3	4.1	82.0	80.1	15.7	15.8	6.54	1.04	81.2	471	1.20
03G	17.7	19.9	57.5	55.7	24.8	24.4	5.97	3.01	115.2	734	3.65
04A	79.7	81.2	14.9	12.2	5.40	6.50	5.11	3.04	82.4	346	1.76
05G	21.9	33.0	59.0	46.7	19.1	20.3	5.37	1.92	89.2	666	2.51
06A	11.5	12.1	41.3	40.9	47.2	47.0	7.39	2.55	271.8	699	2.86
LUFA 2.1	87.1	88.2	9.6	8.4	3.5	3.5	4.6	0.55	2.9	29.5	0.6

WP2 relies on RefeSol soils 02A and 04A and LUFA 2.1 soil. The Refesol soils 01-A, 03-G, 05-G, and 06-A are included as they were originally proposed for WP2.

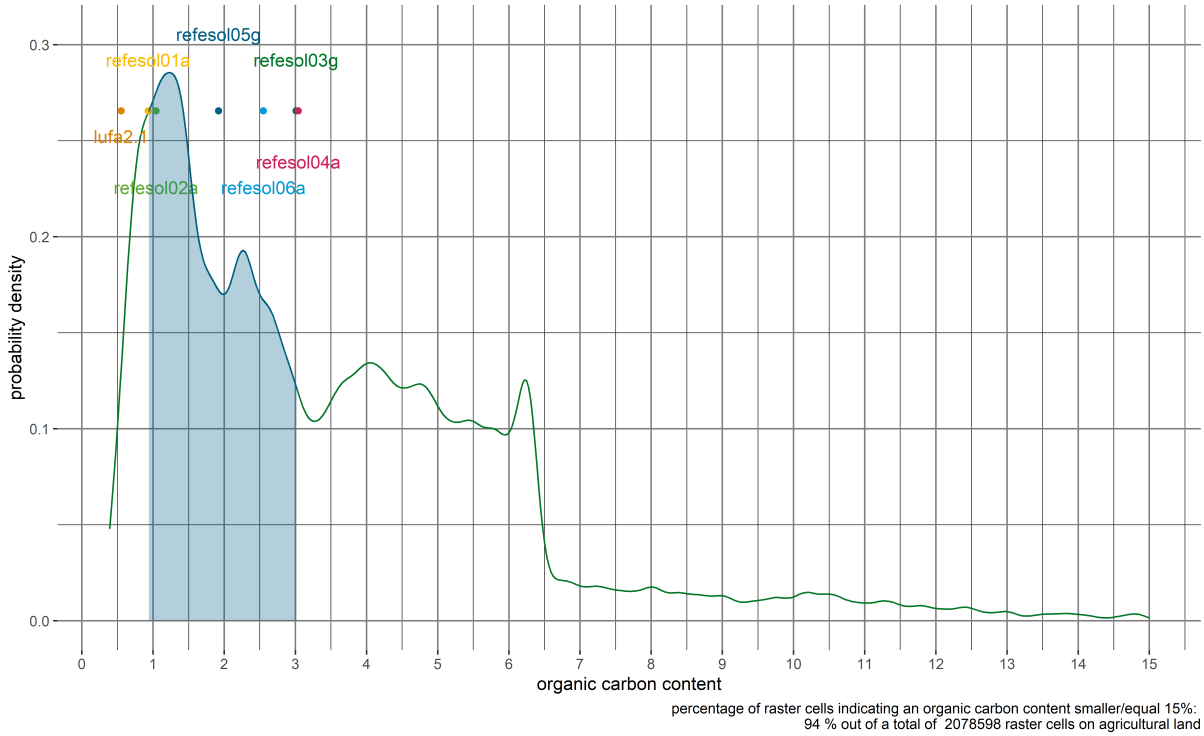
2.2.2 Comparison between the range of soil characteristics across Europe and standard natural soils

From Figure 3 and Figure 4, intervals of characteristic soil values under identifiable peaks of probability could be deduced and used for decision-making on the most relevant soils tested in our experiments. Figure 5 shows the absolute frequencies. For the present analyses, all data was filtered for agricultural soils first. Cation exchange capacity (CEC) is not implemented in the EFSA Spatial Dataset 1.0. The *kernel density* figures below indicate the most probable ranges of values for organic carbon contents and pH values. The area under the curve sums up to one, and the absolute values from the y-axis are of minor importance. Concrete probabilities must be computed from the density model for given ranges of values (e.g., the minimum and maximum organic carbon content of a set of RefeSols). Soil textures are given as categorical data and thus shown by their absolute frequencies per class. Analyses are based on about two Million raster cells. Figure 3 shows that those low values between 0.5 % and 6 % organic carbon content are

much more likely than any other higher range values due to the steep decrease of the density curve.

Figure 3: Organic carbon contents of agricultural soils in the EFSA Spatial Dataset 1.0 database combined with OC values from RefeSol soils and LUFA 2.1.

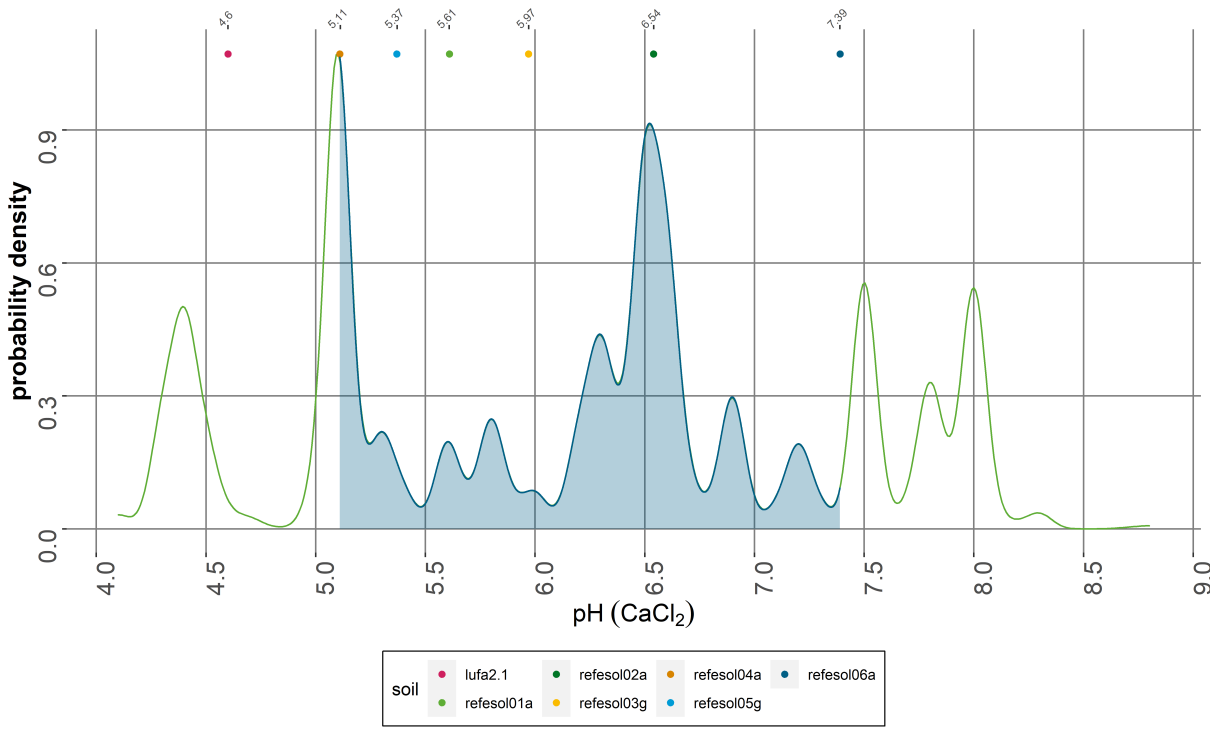
EFSA SPATIAL DATASET 1.0 values were generically converted by a factor of 0.58 from organic matter data because the maps of EFSA SPATIAL DATASET 1.0 (Hiederer 2012) were constructed vice versa with the factor of 1.72 from OC data, combined with organic carbon content from representative European soils.



Blue-shaded area: range of OC-values obtained from RefeSols. The probability of observing an OC-value in the range of the RefeSols throughout Europe is 40 %. Source: Own illustration, darwin statistics

Figure 4 shows that European agricultural soils most often exhibit pH values acidic around pH 5 and neutral around pH 7.

Figure 4: pH-values of European agricultural soils from the EFSA Spatial Dataset 1.0 database combined with pH-values from RefeSol soils and LUFA 2.1.

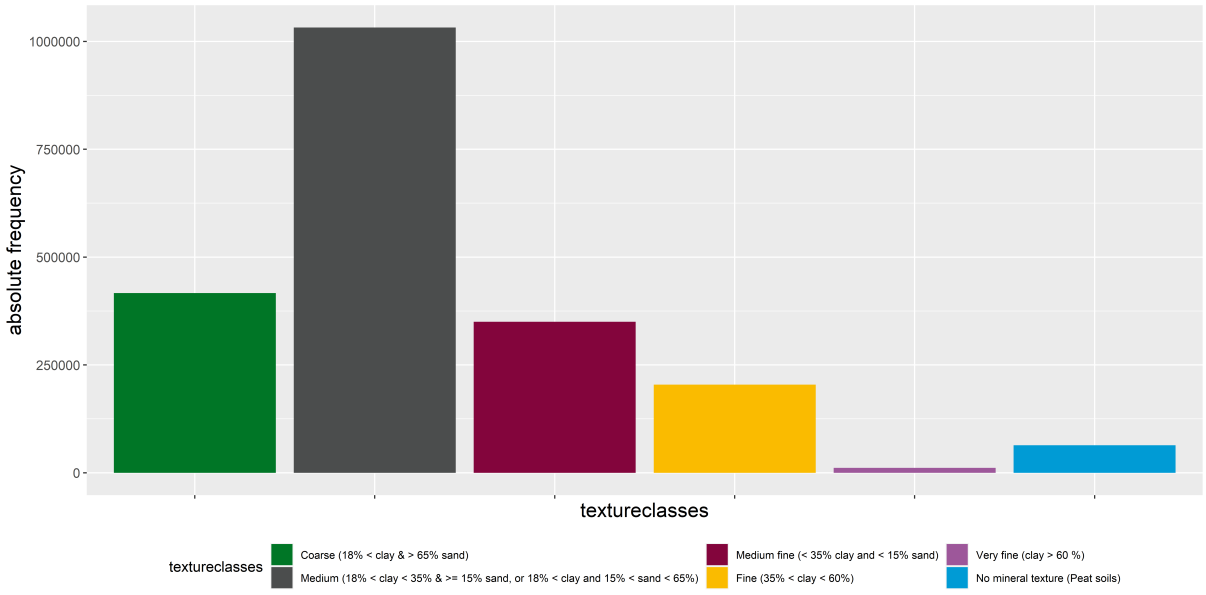


Blue-shaded area: range of pH values obtained from RefeSols. The probability of observing a pH value in the range of the RefeSols throughout Europe is 56 %.

Source: Own illustration, darwin statistics

The textures of RefeSols 01-06 (summarized in Table 8) were classified accordingly to the EFSA SPATIAL DATASET 1.0.

Figure 5: EFSA SPATIAL DATASET 1.0 texture class data



Source: Own illustration, darwin statistics

Table 8: Texture classes of RefeSol and LUFA soils according to EFSA SPATIAL DATASET 1.0 texture class data.

RefeSol Identification	texture class
01A	Coarse
02A	Medium fine
03G	Medium
04A	Coarse
05G	Medium
06A	Fine
Lufa 2.1.	Coarse

WP2 relies on RefeSol soils 02A and 04A and LUFA 2.1 soil. The Refesol soils 01A, 03G, 05G, and 06A are included in the table as they were originally proposed for WP2.

2.2.3 Representativity of RefeSol and LUFA soils in Europe

Four RefeSol soils were initially considered for laboratory studies within the project. The analysis of the organic carbon values of European agriculturally used soils showed that the probability of encountering soils within the range of the RefeSols between 0.93 and 3.04 % C_{org} is approx. 40 %. The range of pH values of RefeSols between 5.11 and 7.39 occur with 56 % probability, as derived from the EFSA soil dataset. By far, the most frequent textures, *coarse*, *medium*, *medium fine*, and *fine,* are well covered by the RefeSols 01-04.

It was concluded that the RefeSols cover the common soil characteristics of European soils very well, constituting a representative sub-sample of soils. With this, it was expected that the results of the studies within the project would be readily transferable to many different soil conditions throughout Europe.

However, it was finally decided that even if the RefeSol soils cover the common soil characteristics of Europe very well, LUFA 2.1 is the reference soil commonly used in studies with a regulatory purpose and will be very likely focused in upcoming research activities of the contracting body, the German Federal Environment Agency. Therefore, RefeSol 01A soil was replaced by LUFA 2.1 soil.

2.3 Material and methods

The proposed test strategy included five methods to determine the effect of one herbicide, three fungicides, one biocide, and one veterinary pharmaceutical on soil microorganism communities' function and structure. The compilation of the selected tests was intended to provide a picture as accurate as possible of the effect on soil microorganisms in terms of structure and function of the microbial community (bacteria and fungi).

2.3.1 Test substances

The UBA provided a list of candidate test substances before a web conference in September 2021. After a discussion at the web conference, the UBA made a final decision on the test substances. On October 15, 2021, the UBA provided a detailed list of information (e.g. Predicted Environmental Concentration (PEC) based on intended application rate(s), data on fate and effect studies) for the six chosen test substances ethofumesate, tebuconazole, propamocarb hydrochloride, pyraclostrobin, tiamulin hydrogen fumarate and didecyl-dimethylammonium

chloride. An overview of the purchased substances, their amounts, the costs and physico-chemical properties are given in Table 9, Table 10, and Table 11.

Table 9: General information on the purchased test substances.

Test substance	Category	CAS number	Producer	Culture/ application	Amount [mg]	Costs [Eur]
Propamocarb-hydrochloride	Fungicide	25606-41-1	LGC Standards	Salad, ornamental plants, potato	100	58.9
Pyraclostrobin	Fungicide	175013-18-0	Merck	Grain, potato	300	186.3
Tebuconazole	Fungicide	107534-96-3	LGC Standards	Grain	1000	235.6
Tiamulin hydrogen fumarate	Antibiotic	55297-96-6	Merck	Manure	500	146
Ethofumesate	Herbicide	26225-79-6	LGC Standards	Turnip	1000	125.4
Didecyl-dimethylammonium chloride	Quaternary ammonium compound	7173-51-5	Honeywell	Sewage sludge	1000	258.48

Table 10: Available information on the fate of the test substances to assess the suitability of the methods chosen for the test strategy.

Test substance	Soil relevant application rate [g a.s./ha]	PECsoil[1] (initial max) [mg/kg]	Log K_{ow}	Solubility in water [g/L]	DT50/90 in soil [d]
Propamocarb-hydrochloride	1350 – 3032	1.69 – 3.88	-1.3	1005	DT50 = 22.3; DT90 < 100
Pyraclostrobin	13.6 – 250	0.013 – 0.49	3.99	1.95	DT50 = 41.9; DT90 > 100
Tebuconazole	150	0.185	3.7	0.032	DT50 = 39.9[2]; DT90 > 100
Tiamulin hydrogen fumarate	No information; no agricultural use	1.1	4.5	0.000000524	DT50 = 15.6; DT90 = 59.8
Ethofumesate	799.2	1.04	2.7	0.05	DT50 = 21.6; DT90 = < 100
Didecyl-dimethylammonium chloride	No information	No information	2.59	0.39	DT50 = 29.9; DT90 = < 78.7

[1] PECsoil values were calculated with the EU approach assuming a soil distribution of the substance within the upper 5 cm.

[2] These DT50 and DT90 values are results of a field study.

Table 11: Available information regarding the toxicity of the chosen test substances based on OECD 216 data (Data provided by UBA).

Test item	Test soil	Test concentration	Test duration [d]	Inhibition on N-transformation (OECD TG 216) [%]
PPP containing Propamocarb-hydrochloride	Loamy to clayey sand (BBA type 2.3)	216.6 kg a.s./ha equal to 288.8 mg a.s./kg dw soil	14	42.8
			28	-36.3
			56	-42.3
			90	-31.4
	Sandy loam	216.6 kg a.s./ha equal to 288.8 mg a.s./kg dw soil	14	-24.7
			28	-33.2
			56	-30.5
			90	-15.8
PPP containing Pyraclostrobin	Loamy sand	1.8 and 18 kg product/ha equal to 2.4 and 24 mg product/kg dw soil (1x and 10x application rate)	14	2.4 mg/kg: -52.7 24 mg/kg: -76.0
			28	2.4 mg/kg: -21.0 24 mg/kg: -37.3
			56	2.4 mg/kg: 5.9 24 mg/kg: -4.4
	Loamy silt	1.8 and 18 kg product/ha equal to 2.4 and 24 mg product/kg dw soil (1x and 10x application rate)	14	2.4 mg/kg: 1.7 24 mg/kg: -6.1
			28	2.4 mg/kg: 1.2 24 mg/kg: 3.7
			56	n.d.
PPP containing Tebuconazole	Silty sand	0.625 and 6.25 kg a.s./ha equal to 0.83 and 8.33 mg a.s./kg dw soil (1x and 10x application rate)	7	0.83 mg/kg: 26.3 8.33 mg/kg: -26.3
			14	0.83 mg/kg: -11.8 8.33 mg/kg: -53.2
			28	0.83 mg/kg: -8.6 8.33 mg/kg: -9.7
Tiamulin hydrogen fumarate	Sandy loam	0.36 and 3.6 mg a.s./kg dw soil (1x and 10x application rate)	28	0.36 mg/kg: n.d. 3.6 mg/kg: 102
			90	0.36 mg/kg: 15.0 3.6 mg/kg: n.d.
Didecyl-dimethylammonium chloride	Sandy loam	No information available. Data was generated in a dose-response study with three	5	10 and 100 mg/kg: No information available. 1000 mg/kg: 24.8

Test item	Test soil	Test concentration	Test duration [d]	Inhibition on N-transformation (OECD TG 216) [%]
		nominal concentrations and a spacing of 10 between them.	28	10 and 100 mg/kg: No information available. 1000 mg/kg: <25 EC50 = >1000 mg a.s./kg
	Low humic acid		5	10, 100 and 1000 mg/kg: No information available.
			28	10, 100 and 1000 mg/kg: No information available. EC50 = >1000 mg a.s./kg
	Sandy loam	No information available. Data was generated in a dose-response study with eight nominal concentrations ranging from 50 to 3200 mg a.s./kg dw soil.	28	EC50 = 135.6 mg a.s./kg

Note 1: Negative effect values present a stimulation compared to the control treatment, while positive effect values indicate an inhibition compared to the control.

Note 2: Effect values for propamocarb hydrochloride and tebuconazole were determined before the release of the official OECD 216 test guideline based on the BBA guidance VI 1-1, which in general is comparable to the OECD test guideline 216 regarding the test performance and evaluation.

Table 12: Available information regarding the toxicity of the chosen test substances based on OECD 217 data.

Test item	Test soil	Test concentration	Test duration [d]	Inhibition on C-transformation (OECD TG 217) [%]
Ethofumesate	Light sandy loam	1.4 and 7.0 kg a.s./ha equal to 1.9 and 9.3 mg a.s./kg dw soil (1x and 5x application rate)	7	1.9 mg/kg: -0.84 9.3 mg/kg: -22.6
			14	1.9 mg/kg: -0.01 9.3 mg/kg: -16.5
			28	1.9 mg/kg: 1.83 9.3 mg/kg: -14.6
		1.4 and 7.0 kg a.s./ha equal to	7	1.9 mg/kg: -72.4 9.3 mg/kg: -35.5

Test item	Test soil	Test concentration	Test duration [d]	Inhibition on C-transformation (OECD TG 217) [%]
	Heavier clay loam	1.9 and 9.3 mg a.s./kg dw soil (1x and 5x application rate)	14 21 28	1.9 mg/kg: -33.2 9.3 mg/kg: -16.9 1.9 mg/kg: -41.1 9.3 mg/kg: -21.9 1.9 mg/kg: -33.6 9.3 mg/kg: -19.8

Note 1: Negative effect values present a stimulation compared to the control treatment, while positive effect values indicate an inhibition compared to the control.
Note 2: Effect values were determined before the release of the official OECD 217 test guideline based on the BBA guidance type VI, which is generally comparable to the OECD test guideline 217 regarding the test performance and evaluation.

2.3.2 Test soils

Table 13 summarizes the most important physico-chemical properties of the test soils Lufa 2.1, RefeSol 04A and RefeSol 02A.

Table 13: Analysis Data of the RefeSol and Lufa soils used in the project.

ID	Soil type	Attributes	Sand [%]	Silt [%]	Clay [%]	pHCaCl2	Corg [%]	WHC [g/kg]	Ntotal [g/kg]
LUFA 2.1	-	sand	87.5	8.7	3.9	4.7	<0.1	314	0.6
02A	Stagnic Luvisol	silt loam, sub-acid, light humic	2.30	82.0	15.7	6.5	1.04	471	1.2
04A	Gley-Podsol	loamy sand, medium acid, medium humic	79.7	14.9	5.40	5.1	3.04	346	1.8

2.3.3 Application of test items into soil

The test soil was adjusted to around 20 % of the maximum water holding capacity (WHC_{max}) for storage at 4°C. Three days prior to the test initiation, the soil was pre-incubated at 20°C. The water content was calculated gravimetrically (ISO 11465) with the Halogen Moisture Analyzer HC 103 (Mettler Toledo, Germany).

Each test substance was applied via a carrier soil (air-dried test soil) into the test soil. For this, 10 g of carrier soil, corresponding to 1% of the total amount of soil per treatment, was air-dried for at least 24 hours.

For each control and treatment, three separate replicates were prepared. Acetonic stock solutions were prepared to contain appropriate amounts of the test substances to achieve maximum solubility. The same amount of acetone was used in all treatments, including the control. Appropriate volumes of the acetonic stock solution were added to the carrier soil. The solvent was allowed to evaporate for 1 hour, and the carrier soil containing the test substance was added to the test soil. The test soil (1 kg) was spread out on a tray, and the carrier soil was spread on top of the test soil. The soil was then carefully turned over with a trowel to receive a homogeneous mixture. Deionised water was added in several steps to achieve a final water-holding capacity of 45%. After the test substance was applied, the test soil was incubated in an incubation chamber (Memmert Inkubationsschrank IPS 749, Memmert GmbH & Co KG, Schwabach) at 21°C in the dark. The mass in the test vessels was measured every two weeks. Evaporated water was replaced with deionized water.

2.3.4 Test concentrations

The nominal test concentrations used in the tests with the six model test substances for the test soils (Lufa 2.1., RefeSol 04A and Refesol 02A) are summarized in Table 14.

According to the OECD TG 216 and 217, at least two concentrations must be tested. The lower concentration corresponds to the maximum amount expected to reach the soil under practical conditions (e.g. maximum PEC or application rate), and the higher concentrations should be a multiple (five- or ten-fold) of the lower concentration. In addition, care was taken to ensure that the test concentrations were comparable with those from the OECD 216/OECD 217 data provided by UBA.

For the testing of ethofumesate, tebuconazole, pyraclostrobin, propamocarb hydrochloride, tiamulin hydrogen fumarate and DDAC we investigated three test concentrations. We considered the test concentrations on which the results of the UBA data were based which were 1x and 5x or 1x and 10x the maximum PEC or application rate. With the third concentration the test range of 1x, 5x and 10x was completed. Tiamulin hydrogen fumarate was tested slightly higher (20x PEC or application rate) in addition to concentrations reflecting the application rate and 10x the application rate, since the provided data showed only stimulations at the used test concentrations. For DDAC no data regarding the application rate was available and therefore based on the data of dose response tests the nominal test concentrations were chosen as outlined in Table 14.

While three concentrations were used for the tests in Lufa 2.1. and RefeSol 02A, for the tests with RefeSol 04A additionally a 4th test concentration was added to get better information on concentration response. Note that in preference of testing a large number of combinations of test items, soils and methods, none of the tests was conducted with the minimal design recommended for regression analysis (at least five test concentrations).

Table 14: Test concentrations used for the studies (OECD 216/OECD 217) provided by UBA and chosen for the tests conducted in this project.

Test substance	Test concentrations used in OECD 216/OECD 217 (data provided by UBA)	Test concentrations used in this project1 [mg a.s./kg dw soil]	
		in tests with Lufa 2.1. and RefeSol 02A	in tests with RefeSol 04A
Propamocarb-hydrochloride	288.8 mg a.s./kg dw soil (100x application rate)	3.0, 15 and 30 (1x, 5x and 10x application rate)	3, 15, 30 and 75
Pyraclostrobin	2.4 and 24 mg product/kg dw soil (1x and 10x application rate)	3.0, 15 and 30 (1x, 5x and 10x application rate)	3.0, 15, 30 and 75
Tebuconazol	0.83 and 8.33 mg a.s./kg dw soil (1x and 10x application rate)	1.0, 5.0 and 10 (1x, 5x and 10x application rate)	1.0, 5.0, 10 and 50
Tiamulin hydrogen fumarate	0.36 and 3.6 mg a.s./kg dw soil (1x and 10x application rate)	0.36, 3.6 and 7.2 (1x, 10x and 20x application rate)	0.36, 3.6, 7.2 and 14.4
Ethofumesat	1.9 and 9.3 mg a.s./kg dw soil (1x and 5x application rate)	2.0, 10 and 20 (1x, 5x and 10x application rate)	2.0, 10, 20 and 100

Test substance	Test concentrations used in OECD 216/OECD 217 (data provided by UBA)	Test concentrations used in this project1 [mg a.s./kg dw soil]	
		in tests with Lufa 2.1. and RefeSol 02A	in tests with RefeSol 04A
Didecyl-dimethylammonium chloride	Data was generated in a dose-response study with three nominal concentrations and a spacing of 10. Data was generated in a dose-response study with eight nominal concentrations ranging from 50 to 3200 mg a.s./kg dw soil.	3.0, 30 and 300	

[1] For ARISA and ISO 10832 only the lowest and highest test concentration was used and the ISO 10832 could be performed only in RefeSol 02A but not in Lufa 2.1 and RefeSol 04A.

2.3.5 Test methods and performance

The effect of the chosen test substances on ammonium oxidizing bacteria (ISO 15685), the substrate induced respiration with the associated nutrient cycles (MicroResp™) and on the enzymatic activity (ISO 20130) was investigated in the three soils (LUFA 2.1, RefeSol 02A, RefeSol 04A). In the test with Lufa 2.1 measurements were performed at test initiation and after 14 and 28 days of incubation, while in tests with RefeSol 04A and RefeSol 02A effects were determined only after 14 and 28 days. If effects above 25% occurred or if there were peculiarities with regard to occurring effects below 25%, selected test methods were extended to 56 or 84 days. Since it takes around 24 hours to carry out one experiment, they were performed one after another over three days in a consistent test sequence. Thus, data for D0 show results for exposure over 1- 3 days after application.

In addition, the effect on the structural diversity of the soil microorganisms was determined in soil samples taken at day 28 of the beforementioned tests for the three soils.

The effect of the chosen test substances on the spore germination of *F. mosseae* was determined only in RefeSol 02A and exemplarily in RefeSol 04A, since pre-tests indicated that Lufa 2.1 is not appropriate as test soil for testing the effect at least on the AMF *F. mosseae* due to its low pH level. This might be different if other AMF species as e.g. *Rhizophagus irregularis* are used. The methods are summarized in Table 15.

Confidential data from regulatory studies were available for the six chosen test substances. However, for the active substances tebuconazole, pyraclostrobin and propamocarb hydrochloride the test described in the study reports were performed with formulations including the active substances, which were chosen within the MICROSOIL project. Therefore, it was not clear whether the UBA data could be directly compared with the data generated in the project. For this reason, it was decided that an OECD 216 test in a limit test design had to be performed for all soils and test substances. Measurements were performed on day 14 and 28.

Table 15: Information on the test systems used in work package 2.

Test system	Test details	Endpoint	Unit
ISO 15685	-	Nitrification	ng NO_2-N/g dw soil/h
MicroResp™	Deionized water	Basal respiration (CO_2 production rate)	µg CO_2-C/g dw soil/h
	D-(+)-Glucose	Substrate induced respiration (CO_2 production rate); Carbon cycle	
	L-Cysteine hydrochloride	Substrate induced respiration (CO_2 production rate); Sulfur cycle	
	L-Malic acid	Substrate induced respiration (CO_2 production rate); Carbon cycle	
	y-Amino butyric acid	Substrate induced respiration (CO_2 production rate); Nitrogen cycle	
	N-Acetyl glucosamine	Substrate induced respiration (CO_2 production rate); Nitrogen cycle	
	Citric acid	Substrate induced respiration (CO_2 production rate); Carbon cycle	
	L-Alanine	Substrate induced respiration (CO_2 production rate); Nitrogen cycle	
ISO 20130	Phosphatase ß-Glucosidase Arylamidase Urease Arylsulfatase	Enzyme activity/hydrolase activities	nmol/minute/g dw soil
ISO 10832	*Funneliformis mosseae*	Spore germination after 14 days	Percentage of germinated spores
ARISA-PCR	PCR amplification	Inhibition of bacterial and fungi based on changes in the community composition	Species richness (OTU number)

2.3.5.1 ISO 15685

The first method of the test sequence performed was the ISO 15685:2012. This rapid test aims to determine the potential nitrification and inhibition of nitrification in soils. For each replicate of the three treatments and the control one replicate was prepared. Briefly 25 g dw soil were mixed with 100 mL of a solution containing diammonium sulfate. The test vessels were placed in an incubator (Infors HT Multitron Pro) and were incubated at 25°C while shaking at 150 revolutions per minute (rpm). After two- and six-hours subsamples of 5 mL were taken from each replicate and mixed with 5 mL M KCl. The samples were centrifuged with 10.000 g for 5 minutes. A colour reagent was added to the supernatant and absorbance was measured using an Epoch microplate spectrophotometer (BioTek, Germany) at 530 nm. Based on the measured absorbance and a calibration curve the potential nitrification rates were calculated following the instructions of the test guideline.

2.3.5.2 MicroResp™

The MicroResp™ system was carried out using deionized water for estimating basal respiration. D-(+)-Glucose, L-Cysteine hydrochloride, L-Malic acid, y-Amino butyric acid, N-Acetyl glucosamine, Citric acid and L-Alanine were used as additional carbon source determining substrate-induced respiration. Briefly, around 11 g of test soil, depending on its water content, was added to a 96-well deepwell plate. 25 µL of substrate was added into each well. The deepwell plate was covered with a seal. A 96-well microplate containing agar mixed with cresol red as colour reagent was placed on top and the complete system was fixed with a clamp. Incubation took place at 25°C in the Thermo Scientific™ Heraeus BK 6160 incubator. Prior to incubation and after 6 hours incubation, the absorbance was measured at 570 nm using an Epoch microplate spectrophotometer (BioTek, Germany) and the quantity of evolved CO_2 was calculated following the instructions as stated in the manual.

2.3.5.3 ISO 20130

The measurement of enzyme activity in accordance to ISO 20130 was determined using colorimetric substrates reflecting the hydrolase activity. Samples of 4 g dw were taken directly from the replicates and were suspended with 25 mL deionized water or buffer solution (arylamidase). A horizontal shaker (Laboshake LS 500, Gerhardt, Germany) was used for 10 minutes to homogenize the samples. Subsamples were transferred into 96-well microtiter plates before adding enzyme specific substrates. The plates were placed in an incubator (Infors HT Multitron Pro). The reaction was stopped after respective reaction times with specific reagents. The investigated enzyme activity of phosphatase, ß-glucosidase, and arylsulfatase was measured through the degradation product Para-Nitrophenol, arylamidase by ß-naphthylamine and urease by ammonium chloride.

2.3.5.4 ISO 10832

For the performance of a spore germination test with *Funneliformis mosseae* in accordance to ISO 10832, it was necessary to establish a culture at the Fraunhofer IME, to implement the test system itself and to investigate the suitability of the test design as outlined in the ISO 10832 for artificial soil for natural soils as the RefeSol 02A, 04A and Lufa 2.1.

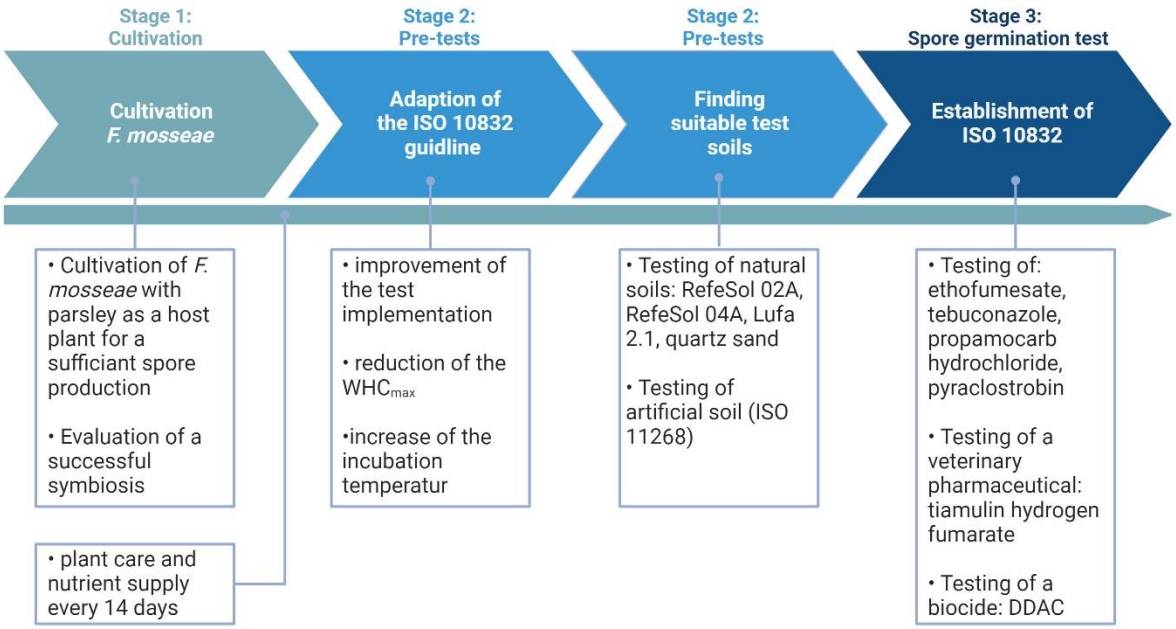
The guideline mentions “Sporocarps of the fungus *Funnelisformis mosseae*, formerly known as *Glomus mosseae*, manufactured and distributed by BioRize, are an example of a suitable commercial product. “ However, BioRize could not be found as a supplier for spores or sporocarps. Therefore, the International Bank for Glomeromycota (IBG, https://www.i-beg.eu; Dijon, France) was contacted for spores. Technicians of the Fraunhofer IME were send for a training to learn how to handle spores, how to cultivate them and how to receive sufficient

spores for testing. In this training, it became apparent during the discussion with the respective experts that the IBG would not be able to send sufficient spore materials for more than one test and that the recommendations of the ISO 10832 using 180 spores per concentration and control leading to a total of 1,260 spores for a single test will be impossible to achieve, which had to be considered for testing in the scope of the MICROSOIL project.

In order to establish the germination test, a number of tests had to be carried out in advance. In the following chapter all used methods from parsley cultivation, spore harvest, test setup and evaluation are listed. For a better overview, the rough procedure of the test is shown in Figure 6.

First a successful cultivation of *F. mosseae* with parsley as a host plant for a sufficient spore production was established. After validation of the symbiosis and spore production first pre-tests could be performed. To improve the germination rate, the WHC_{max} and temperature were adapted. In a following pre-test the use of natural and artificial soils was evaluated. Hereafter, a suitable soil was chosen and the spore germination tests with selected substances were conducted.

Figure 6: Overview of the procedure to establish the ISO 10832.



Source: Own illustration, Fraunhofer IME

2.3.5.4.1 Cultivation

In a first step, a culture of the arbuscular mycorrhizal fungi *Funneliformis mosseae*, received from the IBG, was established at Fraunhofer IME. As a symbiotic plant partner parsley (single cut 3, *Petroselium crispum*, Kiepenkerl GmbH) was used. Parsley seeds were grown in cotton wool and afterwards incubated with the AMF to initiate symbiosis.

Parsley seeds were soaked for one hour in a 3 % hydrogen peroxide (H_2O_2) solution while stirring to sterilize the seeds and washed afterwards under running deionized water. Soaking the seeds during sterilization has the advantage that the seeds can swell and thus germination can be promoted. In 6 jars (WECK, round-edged, 290 mL) the soaked seeds were evenly distributed on cotton wool (ISANA 100 % cotton) and moistened with deionized water. To ensure a humid climate and prevent desiccation, an inverted glass was placed on top to mimic greenhouse conditions. The three-week-old parsley plants were carefully removed from the

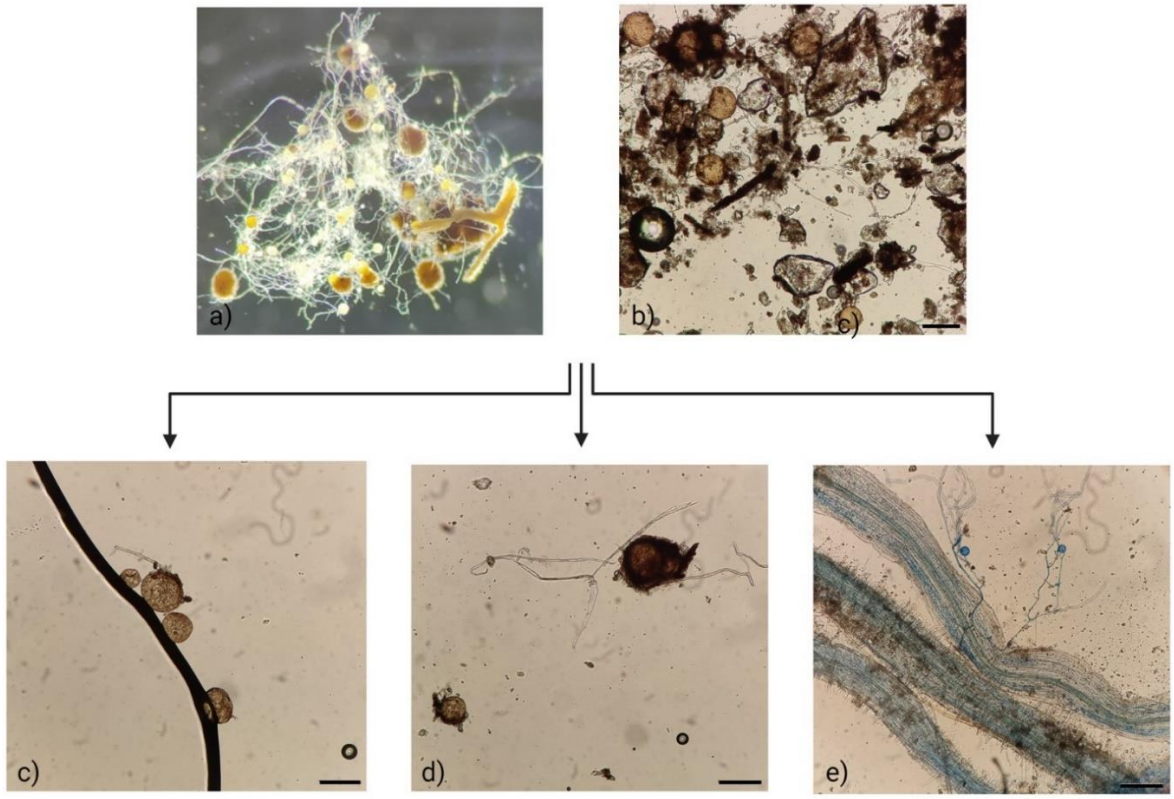
cotton wool. As the young plants had only developed one long root, the plants with the largest root were selected. Twenty plants were selected for planting. To prevent waterlogging and the soil from falling out of the holes in the pot (9x9x10 cm), a layer of coarse gravel was placed at the bottom of the pot. The selected plants were planted in well-watered, autoclaved aquarium gravel (Orbit, natural gravel, no. 4566, 1-2 mm) with 10 % RefeSol 02A soil and inoculated with spores. For inoculation, a spoonful of soil interspersed with hyphae and spores of *F. mosseae* was placed in a small hole and the plants were placed directly on it. Afterwards, more selected spores or the hyphal material were placed directly on the plant root. The fact that spores were distributed both above and below the root has the advantage that the root was completely surrounded by fungal material and possible colonization was enhanced.

Parsley plants were watered daily with tap water. Light was provided for the first months permanently and afterwards 16/8 by a lamp (Neusius Pflanzenlampe; LED type: 120x3w single chip), especially designed regarding the wavelength to improve the growth of terrestrial plants. The culture of parsley and *F. mosseae* has been cultivated at around 25 °C to create greenhouse conditions for optimal growth and to meet the incubation temperature needed for the spore germination test. Furthermore, a study showed that plants can suppress their mycorrhizal colonization if the nutrient supply is sufficient (Bücking and Kafle 2015). Therefore, it is beneficial to cultivate plants and fungi in soil with minimal nutrients. Every two weeks all plants older than one month were supplemented with 3 drops Long Ashton solution for nutrition.

2.3.5.4.2 Spore harvest and germination analysis

Up to 50 g of soil containing root material was taken and sieved through three different sieves (800 µm, 500 µm and 65 µm). The sieves containing the soil, sorted by size and placed on top of each other, were washed under running deionized water. The remaining material on each sieve was rinsed into separate glass dishes and examined for spores under a binocular. Spores were carefully transferred with a 100 µL pipette to another vial for storage or used to inoculate new plants. Spores were stored at 4 °C in deionized water for a maximum of three days as recommend in the ISO 10832. If spores were used for the spore germination test, remaining hyphae were cut off with a cannula. Figure 7 shows the different spore types and spores in a net of hyphae which had to be removed before testing.

Figure 7 **Spores in a hyphae net and different spore types. a) shows a net of hyphae with spores at different stages of proliferation in a 4x magnification; b) mature spores with left over after a 500 µm sieving; c) mature spores which are used for testing; d) juvenile spore in the left side and sporocarp in the right side; e) juvenile spore after root staining attached onto parsley roots. Black bar equals 200 µm.**



Source: Own illustration, Fraunhofer IME

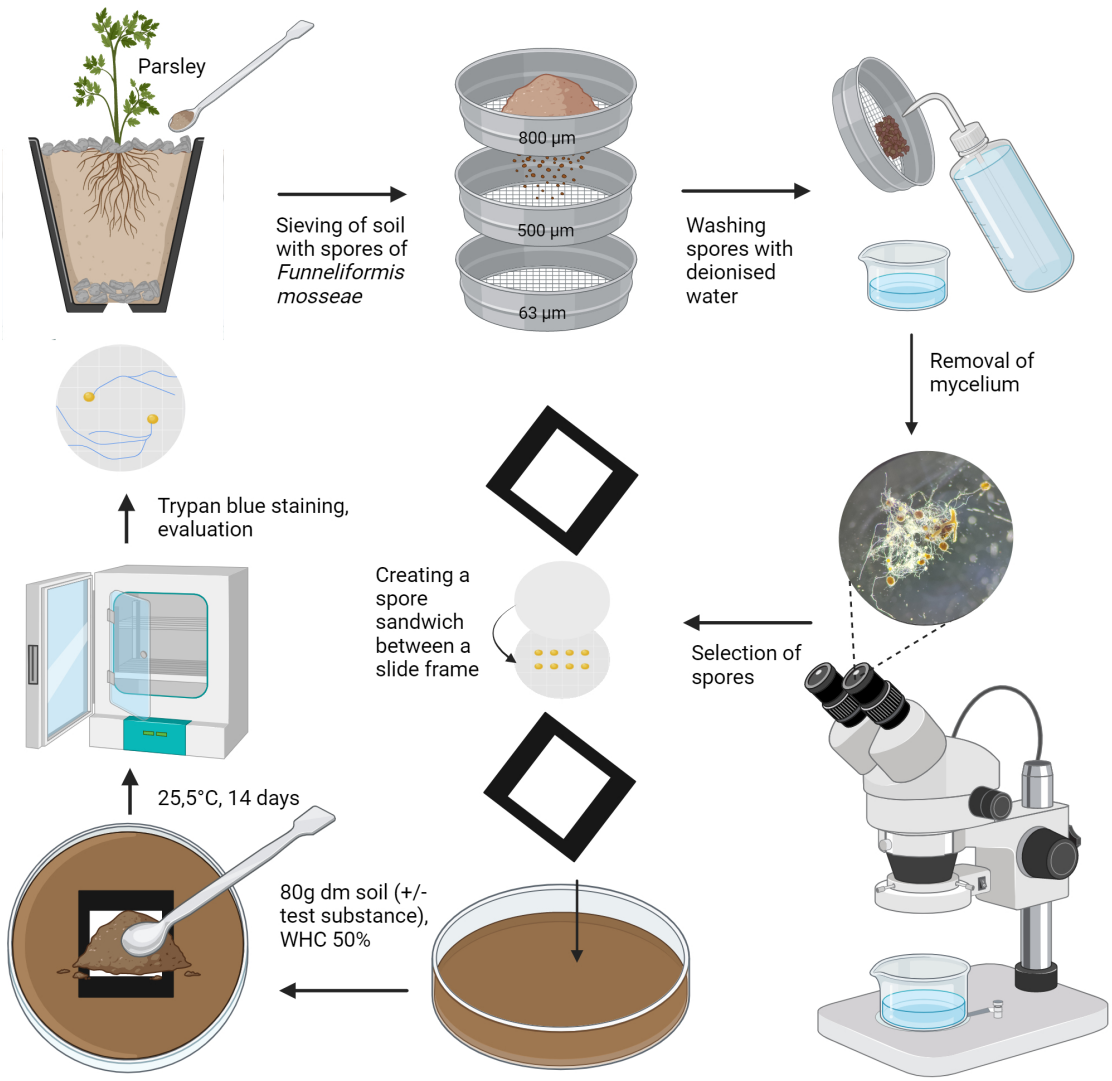
To evaluate if *F. mosseae* formed a symbiosis with parsley, a root staining was performed. For root staining, long roots were carefully separated from the plant with a spoon. Roots were washed with tap water and placed in a small glass vial with 10 mL of 10 % potassium hydroxide and boiled at 90 °C for about one hour. The incubation time depended on the degree of lignification of the root. Thin roots only need a shorter incubation time, while the incubation time for larger roots was longer. After washing with tap water, the roots were dried on a paper towel and were incubated for 5 min at 90 °C in 10 mL staining solution. Then roots were incubated for 25 min at room temperature in 8 % acetic acid solution. After washing the roots again in tap water, they were transferred directly to pure glycerol and analyzed via microscopy or stored at 4 °C for approximately 6 month or until colored out. The microscopic examination was carried out within three days.

2.3.5.4.3 Spore germination test in accordance to ISO 10832

Suitable spores (yellow, bright, no internal inclusions, intact shell) were selected under the binocular microscope and freed from adherent mycelium. These selected spores were kept in deionized water at 4 °C for a maximum of three days until testing. On the day of testing, 30 spores were placed on a filter (nitrocellulose membrane filter, diameter 47 mm, porosity 0.45 µm, white, 3 mm grid) using a 100 µL pipette. One spore at a time was placed in a square on the filter. Another with deionized water wetted filter was carefully placed on top of the wet filter with the spores. The resulting spore sandwich was placed in a slide frame (Alif Kunststofftechnik GmbH, hinged frame without glass, 5x5 cm/24x36 mm). A sterile petri dish (plastic, diameter 9

cm) was filled with 40 g dm soil with a maximum water holding capacity of 50 %, the spore sandwich was placed on top and covered with the remaining 40 g dm soil. The Petri dish was sealed with a Parafilm and incubated in the dark at 25.5 °C for 14 days. For a more detailed figure of the test setup see Figure 8. In total 6 replicates of a control and 2 concentrations per test substances were prepared.

Figure 8: Schematic setup of the DIN ISO TS 10832:2009.



Source: Own illustration created with BioRender.com, Fraunhofer IME

After 14 days of incubation, the spore sandwiches were carefully removed from the petri dish and rinsed with deionized water, if necessary. It is important to keep the spore sandwiches horizontal at all times to avoid spore loss. The sandwiches were then incubated in 0.05 % trypan blue solution for 15 minutes to visualize hyphal growth/ germination. After rinsing and drying, the sandwiches were carefully opened and observed under a binocular microscope. The total number of spores recovered and the number of spores that germinated were recorded. According to the guideline a spore was considered germinated only if the hypha was at least five times larger than the spore diameter. The number of spores recovered and the number of spores germinated were expressed as a percentage, compared with the control sample, and a standard deviation was calculated. Following the ISO 10832, the validity criteria of the test are fulfilled if the average number of spores recovered is greater than or equal to 25 and the germination rate of the control sample is greater than 75 %.

2.3.5.4.4 Adaptation of test design to natural soils

To investigate if natural soil is suitable for testing, some pre-tests were performed. During the pre-tests test conditions like water holding capacity, temperature and pH of the ISO 10832 were slightly adjusted.

2.3.5.4.4.1 Pre-test 1

The temperature was at 24 °C and incubation was performed in the dark as recommended in the ISO 10832. Tested soils were artificial soil and natural soil RefeSol 02A. The pH value of the artificial soil was set to 6.3 with $CaCO_3$, while the pH of the natural soil was not adjusted. As there were not many suitable spores, sporocarps, i.e. spores enclosed by solid mycelium, were also tested in this pre-test. Spores from a 4-month-old parsley plant (culture 2.1) was used. The two soils were tested without substance (control treatment) and with 7 mg a.s./kg dw soil benlate (reference material) to differentiate between the sensitivity of the spore germination in dependence of the used test soil. The concentrations of 1 to 10 mg a.s./kg dw soil were chosen based on the recommendations of the ISO 10832.

After the hyphae staining, most of the spores were close to the edge of the filter and some of them were lost. It was not possible to recover all used spores. Therefore, not enough spores were found to fulfill the validity criteria of the ISO 10832 (see Table 16). Spores close to the edge of the filter were identified as a major problem in the handling of the spore-soil sandwiches and therefore adapted in the following tests. Furthermore, with the exception of one sporocarp, no germination was found after 14 days exposure.

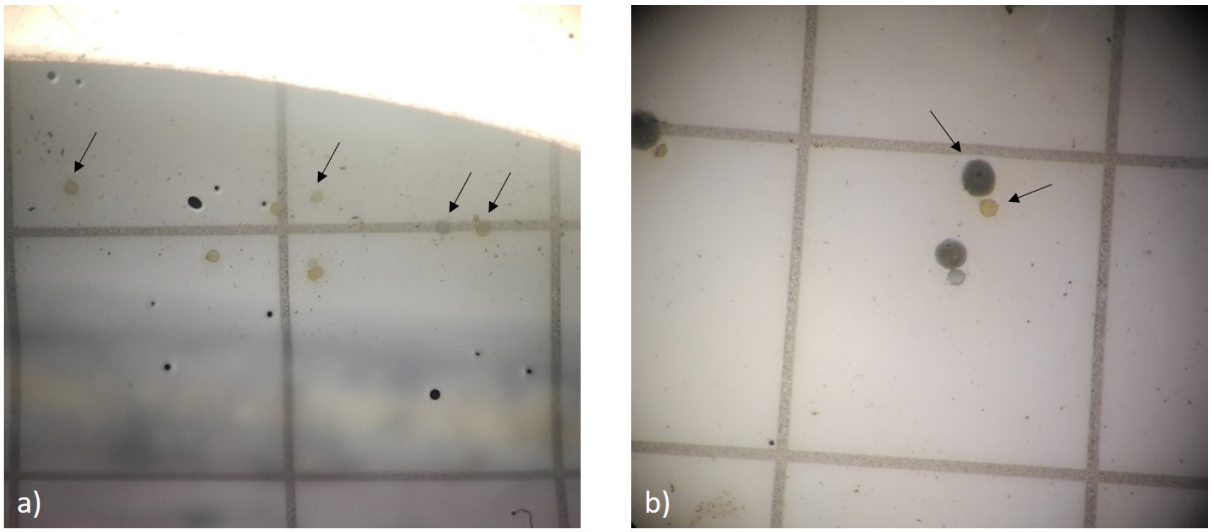
Table 16: Results of the pre-test 1 after 14 days of incubation at 24 °C with a WHC_{max} of 90 %. Spores from culture 2.1 were used. m: mature spore; s: sporocarb; - means no germination could be detected.

Soil	Number of spores used	Number of spores recovered	Recovery rate [%]	Spores germinated > 5 times the diameter	Spores germinated ≤ 5 times the diameter
Artificial soil, control	30 [m]	25	83.3	-	-
Artificial soil, control	30 [m]	25	83.3	-	-
Artificial soil, control	37 [m,s]	27	73.0	-	1
RefeSol 02A, control	30 [m]	24	80.0	-	-
RefeSol 02A, control	30 [m]	25	83.3	-	-
Artificial soil, benlate (7 mg a.s./kg dw soil)	30 [m]	26	86.7	-	-
Artificial soil, benlate (7 mg a.s./kg dw soil)	30 [m]	21	70.0	-	-
	30 [m]	21	70.0	-	-

Soil	Number of spores used	Number of spores recovered	Recovery rate [%]	Spores germinated > 5 times the diameter	Spores germinated ≤ 5 times the diameter
Artificial soil, benlate (7mg a.s./kg dw soil)[1]	20 [s]	19	95.0	-	-

[1] At one replicate (artificial soil benlate (7 mg a.s./kg dw soil), the filter was divided into two parts of which the 1st part contained mature spores and the 2nd part contained spores of sporocarbs. The treatment was chosen since soil was already prepared and no further test soil was available, however, during preparation of the spores it occurred that there were still sporocarbs available to be used within the test.

Figure 9 **Pre-test 1: Spores after 14 days of incubation and staining on a filter with a 3.1 mm grid. a) most of the spores were located near the edge of the filter b) yellow, mature spores and sporocarps.**



Source: Own illustration, Fraunhofer IME

To check whether spores were still germinating after a longer incubation period, the spore sandwiches were put back into the soil after staining and counting and incubated at 24 °C for another 7 days.

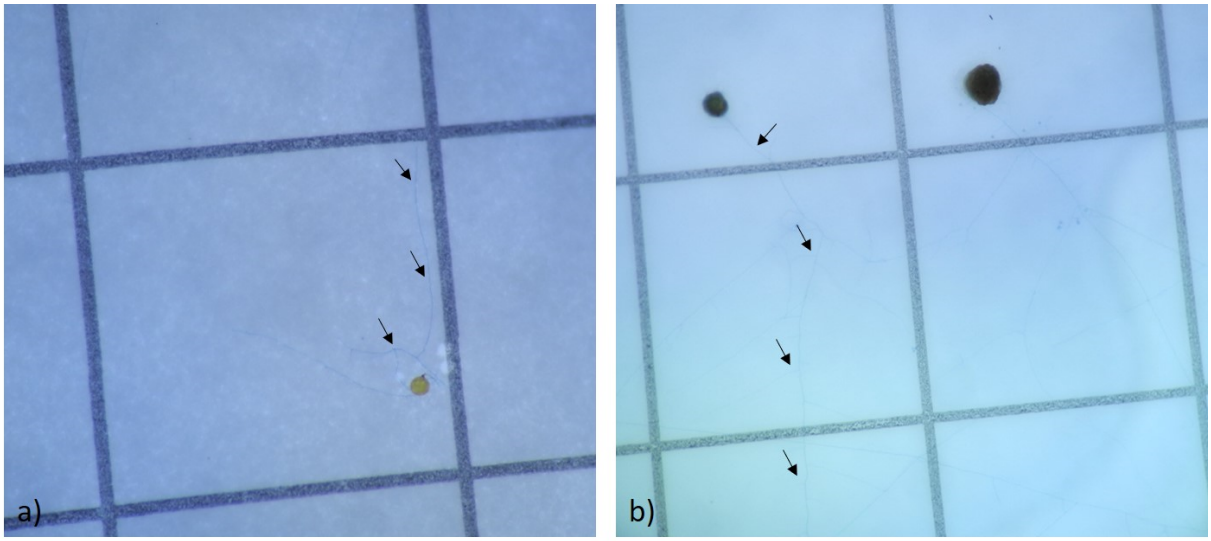
After 21 days of incubation at 24 °C, a total of 14 spores germinated (see Table 17). Only 4 of them had hyphae longer than five times the spore diameter and counted as valid. If benlate affected the spore germination it could not be assessed, since the overall germination rate was low.

Table 17: **Results of the spore germination test 1 after 21 days of incubation at 24 °C. – means no germination could be detected.**

Soil	Spores germinated and > 5 times the diameter	Spores germinated and ≤ 5 times the diameter
Artificial soil, control	-	-
Artificial soil, control	10	3

Soil	Spores germinated and > 5 times the diameter	Spores germinated and ≤ 5 times the diameter
Artificial soil, control	-	-
RefeSol 02A, control	-	1
RefeSol 02A, control	-	-
Artificial soil, benlate 7 mg a.s./kg dw soil	-	-
Artificial soil, benlate 7 mg a.s./kg dw soil	-	-
Artificial soil, benlate 7 mg a.s./kg dw soil	-	-

Figure 10 **Pre-test 1: Spores after 21 days of incubation and staining on a filter with a 3.1 mm grid. a) germinated spore and b) germinated sporocarp after 20 days of incubation at 24 °C.**



Source: Own illustration, Fraunhofer IME

The pH values were measured with 0.01 M $CaCO_3$ for natural soil and with 1 M KCl for artificial soil as recommended in the respective guideline, to check if the pH value had been changed over the time. The artificial soil had an average pH value of 6.31 and the RefeSol 02A soil had an average pH value of 7.03.

2.3.5.4.4.2 Pre-test 2

The second pre-test was performed for 14 days at 24 °C in the dark. As there was no germination in pre-test 1, the substrate was changed in the pre-test 2. Quartz sand and aquarium gravel with 10 % (w/w) of a natural soil were used to determine, if these soils are more suitable for the testing in terms of the total spore germination. For this test mature and juvenile spores and sporocarps of two 5-month-old parsley (culture 1.2 and 2.2) plants were used.

In this test the recovery rate of the spores was > 85 % except of one time. However, the germination of the spores was still limited and the germination rate of 75 % stated as validity criteria in the ISO 10832 was not reached at any of the tested treatments. Spores and sporocarps

germinated in either quartz sand and aquarium gravel including 10 % natural RefeSol 02A (see Table 18). The highest germination rate was achieved in a treatment with quartz sand with 48.3 %. Since many of the juvenile spores got damaged during incubation they were not used for further testing. The spores from the culture 2.1 had a higher germination rate than the spores of the one-month older culture 1.2 under the same conditions. It was also noticeable that the soils looked very wet overall

Table 18: Results of the pre-test 2 after 14 days of incubation at 24 °C and with a WHC_{max} of 90%. Spores from culture 2.1 and 2.2 were used m: mature spore; s: sporocarb; j: juvenile spore; - means no germination could be detected.

Soil and culture	Number of spores used	Number of spores recovered	Recovery rate [%]	Spores germinated > 5 times the diameter	Spores germinated ≤ 5 times the diameter	Germination rate [%]
Sand; 1.2	30^m	26	86.7	-	3	0
	20^s	20	100	-	2	0
Sand; 2.2[1]	30^m	29	96.7	14	3	48.3
	20^s	19	95.0	6	9	31.6
Sand; 1.1/2.2	20^j	19	95.0	7	-	36.8
Aquarium gravel + 10% RefeSol 02A; 1.2[1]	30^m	23	76.7	-	-	0
	15^s	15	100	1	1	6.7
Aquarium gravel + 10% RefeSol 02A; 1.2	20^m	17	85.0	-	1	0
Aquarium gravel + 10% RefeSol 02A; 2.2[1]	30^m	26	86.7	3	2	11.5
	20^s	19	95.0	8	1	42.1

[1] The filter was divided into two parts of which the 1st part contained mature spores and the 2nd part contained spores of sporocarbs. The treatment was chosen since soil was already prepared and no further test soil was available, however, during preparation of the spores it occurred that there were still sporocarbs available to be used within the test.

2.3.5.4.4.3 Pre-test 3

In order to test whether an increased temperature or different soil types leads to an improved germination rate, the different soils (artificial soil, Lufa 2.1, RefeSol 02A, RefeSol 04A, and quartz sand) were incubated at 25 °C for 14 days. Since other soils with lower peat content should be used, in addition to the previously used soil the natural soils Lufa 2.1 and RefeSol 04A were studied. Based on the observations of the previous pre-tests, it became apparent that the WHC_{max} of 90 %, as stated in the ISO 10832 for artificial soil, was not appropriate for natural soil. Therefore, the WHC_{max} was set to 75 % or 50 % in the 3rd pre-test.

3 g $CaCO_3$ were added to 1 kg dry mass artificial soil one week before the start of the test to increase the pH value to 7.3. Spores from a 6-month-old parsely culture were used.

Due to the high number of spores, sandwiches with 30 spores could be prepared for all approaches but not for the last approach with quartz sand and a WHC_{max} of 75 %, here a

sandwich with only 20 spores was prepared to get an idea about the influence on the spore germination.

In general, the germination rate was already higher. No germination was found in the artificial soil (WHC_{max} of 90 %) and the Lufa 2.1 soil (WHC_{max} of 75 %), whereas the RefeSol 04A (WHC_{max} of 50 %) and quartz sand (WHC_{max} of 75 %) showed a good germination rate with 65 %, indicating that a lower WHC_{max} improves the general germination rate. In case of quartz sand, a reduction of the WHC_{max} of 15 % resulted in an increased germination rate of 55 %. Reducing the WHC_{max} content of RefeSol 04A by 25 % also increased the germination rate to 65 %. Concluding, higher WHC_{max} negatively affects the germination rate.

One of the prepared replicates showed a higher evaporation and therefore loss of water which led to a much lower WHC_{max} in one of the two RefeSol 02A soils after 14 days incubation time. However, the significantly drier soil led to an increased germination rate compared to the wetter soil as can be seen in Table 19, again indicating the influence of the WHC_{max} on the germination rate of *F. mosseae* in natural soil.

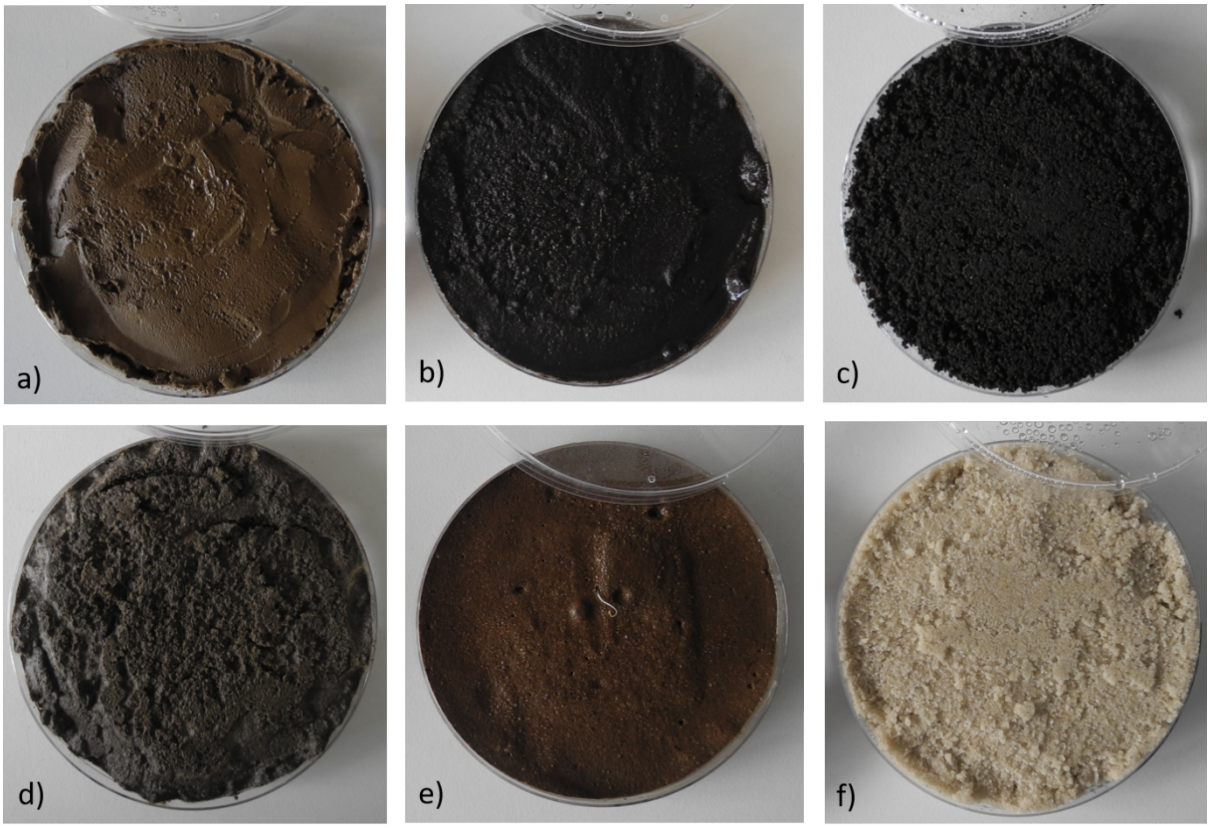
It is also worth mentioning that in the RefeSol 02A soil with the higher evaporation as well as in a test with quartz sand, more than 10 spores germinated to some length, but not over the required length of five times the spore diameter.

Table 19: Results of the pre-test 3 after 14 days of incubation at 25 °C and with different WHCs. - no germination could be detected. * RefeSol 02A soil was dried out due to a broken parafilm and lost most of the moisture during the incubation.

Soil and culture	WHC_{max} [%]	Number of spores used	Number of spores recovered	Recovery rate [%]	Spores germinated > 5 times the diameter	Spores germinated ≤ 5 times the diameter	Germination rate [%]
RefeSol 02A	75	30	26	86.7	8	1	30.8
	75*	30	30	100	13	12	43.3
RefeSol 04A	75	30	29	96.7	-	-	0
	75	30	29	96.7	-	-	0
	50	20	20	100	13	3	65.0
Artificial soil	90	30	25	83.3	-	-	0
	90	30	25	83.3	-	1	0
Lufa 2.1	75	30	27	90.0	-	-	0
	75	30	16	53.3	-	2	0
Quartz sand	90	30	30	100	13	7	43.3
	90	30	48	96.0	18	13	37.5
	75	20	20	100	13	1	65.0

At the end of the test, clear differences in the moisture content of the soil were observed. Figure 11 shows examples of RefeSol 02A, RefeSol 04A, artificial soil, Lufa 2.1 and quartz sand and after the incubation.

Figure 11 **Pre-test 3: Different soils after 14 days of incubation at 25 °C. a) RefeSol 02A WHC_{max} 75 %; b) RefeSol 04A WHC_{max} 75 %; c) RefeSol 04A WHC_{max} 50 %; d) artificial soil WHC_{max} 90 % e) Lufa 2.1 WHC_{max} 75 %; f) quartz sand WHC_{max} 75 %.**



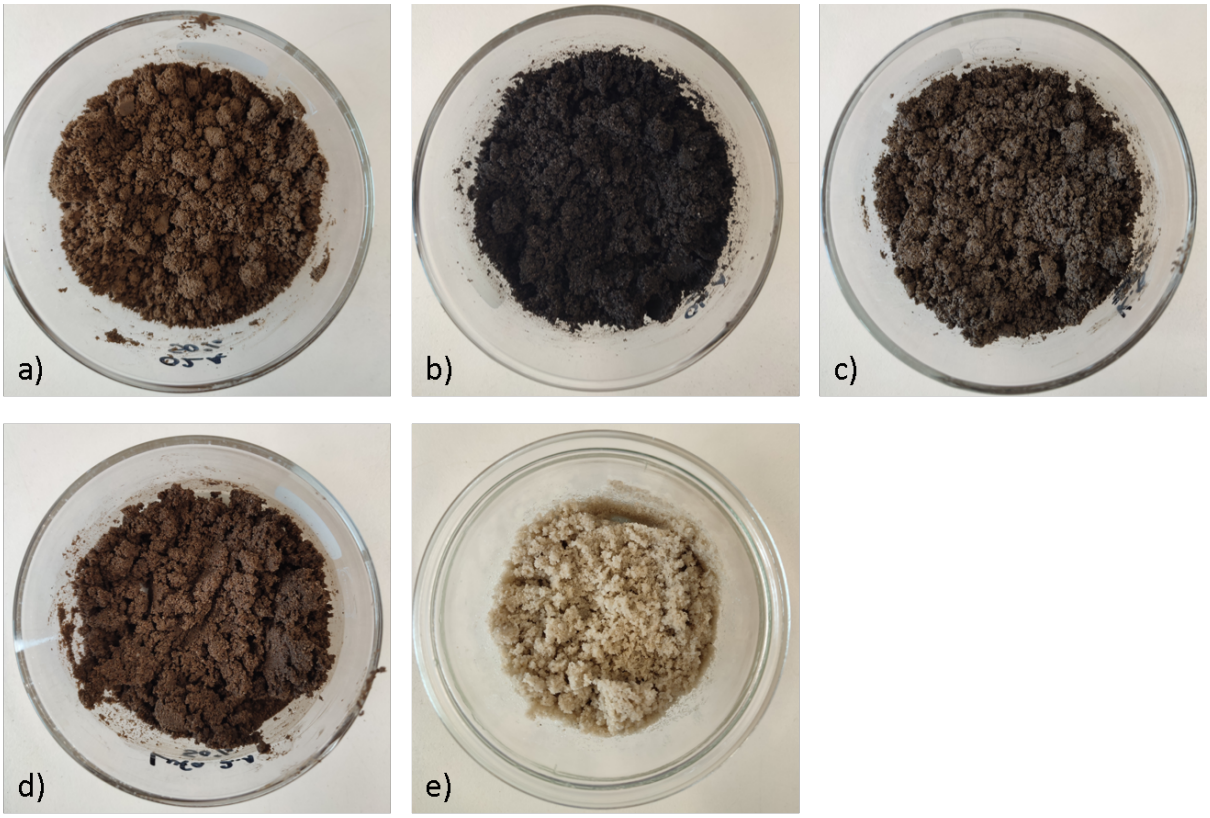
Source: Own illustration, Fraunhofer IME

2.3.5.4.4.4 Pre-test 4

Based on the results of the previous pre-test it became obvious that a lower WHC_{max} produced a better germination rate of *F. mossea*e in natural soil. Therefore, the WHC_{max} was reduced to 50 % to check whether a lower WHC_{max} can improve the germination rate. The WHC_{max} was chosen based on the visual appearance of the respective soil. As in pre-test 3, RefeSol 02A, RefeSol 04A, artificial soil, Lufa 2.1 and quartz sand were tested (see Figure 13). The temperature was set to 25 °C and triplicates with 30 spores per sandwich were tested.

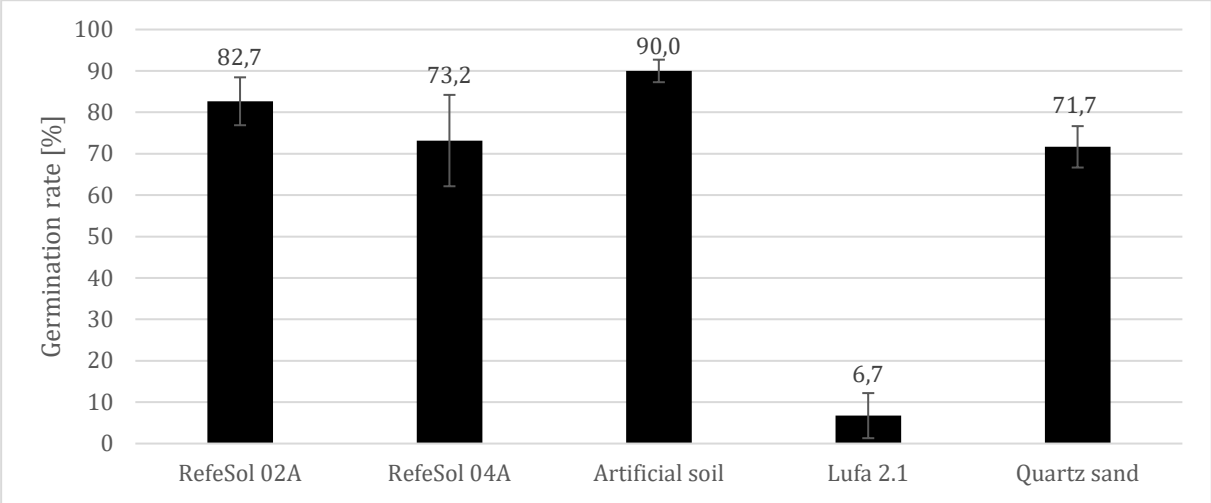
A germination of the spores was observed in every of the tested soils. Figure 12 shows that the artificial soil (WHC_{max} of 50 %) had the highest germination rate of up to 93.3 %, while Lufa 2.1 showed the lowest germination rate with an average of 6.7 %.

Figure 12 **Different soils used in the pre-test 4. a) RefeSol 02A with WHC_{max} 50 % b) RefeSol 04A with WHC_{max} 50 % c) artificial soil with WHC_{max} 50 % d) Lufa 2.1 with WHC_{max} 50 % e) quartz sand with WHC_{max} 60 %.**



Source: Own illustration, Fraunhofer IME

Figure 13: **Results of the pre-test 4 after 14 days of incubation at 25 °C and with different soils at a WHC_{max} of 50% (RefeSol 02A, RefeSol 04A, artificial soil and Lufa 2.1) and 60% (quartz sand).**



Source: Own illustration, Fraunhofer IME

2.3.5.4.4.5 Remarks on RefeSol 04A

After the various preliminary tests, definitive tests on the effect of the 6 test substances on the spore germination of *F. mosseae* have been performed in the two natural soils RefeSol 02A and

RefeSol 04A, which appeared to be most suitable, following the recommendations of the ISO 10832 with slight adaptations of the incubation conditions (see above). While the tests with RefeSol 02A fulfilled the validity criteria and are presented in chapter 2.3.6, the tests with RefeSol 04A did not fulfill the validity criteria in tests with the 6 test substances, even if the results of preliminary test 4 indicated that testing with this soil would be possible as well. In two separate experiments the spore germination rate in the control (n = 6) was below 15% (6.33% and 13.6%, respectively) at the same exposure conditions as in preliminary test 4 (see results Table 159 to Table 161). Further tests with adapted WHC_{max} (45 – 60%) at 25 °C were conducted, but the spore germination could not be improved. Therefore, further tests are necessary to determine the difference between spore germinations at the same incubation conditions.

2.3.5.4.4.6 Discussion on preliminary results for ISO 10832

The preliminary tests showed that there are various problems strictly following the ISO guideline 10832, if tests should be performed with natural soil instead of artificial substrate. Since even the spore germination tests with artificial soil did not fulfill the validity criteria when strictly following the instructions of the guideline, there is a clear indication that the guideline needs a thorough revision. Some recommendations are listed below:

- Based on the actual work described above it becomes obvious that at least for testing the effect of substances on the spore germination of *F. mosseae* in natural soil, the maximum water holding capacity has to be reduced from 90%, as indicated in the guideline, to around 50%. However, this value may need to be adapted depending on the properties (WHC_{max} or texture) of the natural soil to be used for testing.
- In addition, soil which is used for regulatory testing with soil invertebartes like Lufa 2.1, which has a pH value slightly below 5, appeared to be not appropriate for tests with *F. mosseae*. Here, it is important that the soil pH of a natural soil used should be above pH 5. Of course, the pH of a soil as Lufa 2.1. could be adapted using e.g. lime to achieve a pH slightly above 5 (measured in 0.01 M $CaCl_2$). For the present project this was not done to avoid an influence on the soil physico-chemical properties and its microbial community and therefore to allow a direct comparability of all results obtained within the project.
- The results also indicate that an increase of the temperature improves the spore germination of *F. mosseae*. To use the upper end of the permitted range of 24 ± 2 °C improved the spore germination in RefeSol 02A.
- It is very time-consuming to collect the required number of AMF spores in a specified time period in order to be able to test six concentrations and a control with six replicates each. There are methods such as separating the spores using a sugar gradient, but these did not work here. However, there are currently efforts to use other species such as *Rhizophagus irregularis* or other AMF species as test organisms within the framework of ISO 10832. The method for separating the spores may work better with these spores. If yes, it will be more likely that enough spores can be collected to test enough concentrations to determine an IC50 as outlined in the ISO 10832.

2.3.5.5 ARISA

Automated rRNA intergenic spacer analysis (ARISA) was used to characterize bacterial (B-ARISA) and fungal (F-ARISA) communities from different soil types. The method is based on PCR amplifying the 16S-23S intergenic spacer region from the bacterial rRNA operon and the 5.8S rRNA gene (ITS1-5.8S-ITS2) from the fungal rRNA operon. The amplified intergenic region displays fragments of significant heterogeneity in length and nucleotide sequence representing different OTUs (operational taxonomic units), e.g. species present in the sample.

For the performance of ARISA, samples were taken in the different tests at test termination and/or day 28. DNA and RNA of the soil samples were extracted using commercial kits, to analyse the total and living communities, respectively. The DNA and RNA of the samples from the experiments with LUFA 2.1, RefeSol 02A and RefeSol 04A soils were extracted, simultaneously, using the beads-based MagMax Microbiome Kit (ThermoFisher, Germany). Extraction were conducted using the KingFisher™ Apex purification system (ThermoFisher, Germany) according to the instructions of the kit provider. The concentration of the extracts (ng/µl) and quality (260/280 ratio) was measured using a NanoDrop. The RefeSol 02A soil, presented unsatisfactory extraction yield (< than 5 ng/ µl) and quality (< 1.7) with the MagMax Microbiome Kit, therefore extractions were repeated using the column-based DNeasy Power Kit Pro, and the RNA with the RNeasy Power Kit Pro, for DNA and RNA respectively. Results for DNA and RNA extractions can be seen in Table 20 and Table 21 respectively.

Before PCR, all samples were normalized to a concentration of 20 ng/µl, and in the case of the RNA samples, 5 µl were transcribed to cDNA using the Quantitec Reverse Transcription Kit (Qiagen, Germany). Universal fluorescence-labeled primers were used for the PCRs, and fragments of between 200 and 1,200 bp were resolved on denaturing polyacrylamide gels by use of an automated sequencer with laser detection (Eurofins).

Arisa Bact Primer

F: 5'-FAM GTC GTA ACA AGG TAG CCG TA-3'; R: 5'- GCC AAG GCA TCC ACC-3'

Arisa Fungi Primer

F: 5'-FAM-GTT TCC GTA GGT GAA CCT GC-3'; R: 5'-ATA TGC TTA AGT TCA GCG GGT-3'

Reaction mixtures (40 µl) for PCR contained 8 µl SOLIS Taq Mastermix, 1 µl primers (10 pmol/µl), 29 µl DNase/RNase free water and 2 µl normalized DNA/ cDNA. Amplification was performed in a MWG PCR Cycler with an initial denaturation at 95°C for 12 min, followed by 30 /35 (DNA/cDNA) cycles of 95°C for 20 s, 55°C for 60 s, 72°C for 60 s, with a final extension at 72°C for 5 min. PCR products were purified utilizing the peq GOLD Cycle Kit (VWR, Germany) prior to submitting to capillary electrophoresis on a 80-cm-capillary ABI Prism 3130xl genetic analyzer (Eurofins, Germany). The preprocessing and analysis of the data generated is described in section 2.3.6.3.

Table 20: DNA extraction yield (µg DNA/g soil) and purity (260/280 ratio) at day 28.

		Lufa 2.1			04A			02A		
Test substance		**Conc. [ng/µl]**	**Yield [µg/g soil]**	**Purity [260/280]**	**Conc. [ng/µl]**	**Yield [µg/g soil]**	**Purity [260/280]**	**Conc. [ng/µl]**	**Yield [µg/g soil]**	**Purity [260/280]**
Tiamulin hydrogen fumarate	control	22.8	5.01	1.68	52.0	11.39	1.58	82.7	17.50	1.88
	conc 1	22.0	5.24	1.62	37.9	8.32	1.65	79.8	17.34	1.88
	conc 3	21.0	4.75	1.72	44.8	9.65	1.61	86.9	18.20	1.88
Tebuconazol	control	29.8	7.19	1.73	79.7	16.97	1.49	88.5	18.33	1.88
	conc 1	27.1	6.55	1.59	123.5	21.03	1.45	91.0	19.50	1.89
	conc 3	52.3	11.73	1.51	119.8	25.26	1.49	88.2	19.47	1.90
DDAC	control	39.9	4.26	1.49	83.3	17.94	1.55	106.8	22.20	1.89
	conc 1	36.9	8.80	1.45	39.2	8.64	1.65	98.1	20.06	1.89
	conc 3	22.3	5.21	1.59	122.0	27.94	1.47	94.5	19.82	1.88
Pyraclostrobin	control	18.4	5.20	1.70	130.4	29.00	1.47	109.1	23.10	1.89
	conc 1	30.5	5.97	1.61	136.0	28.49	1.48	99.1	20.18	1.89
	conc 3	29.2	5.97	1.72	114.1	26.09	1.46	109.0	22.36	1.85
Propamocarb hydrochloride	control	23.8	5.20	1.71	45.6	9.79	1.51	112.0	23.47	1.91
	conc 1	28.0	5.97	1.73	41.5	8.86	1.56	105.0	22.29	1.91
	conc 3	34.8	7.25	1.64	51.6	11.31	1.49	87.3	18.73	1.92
Ethofumesat	control	24.0	5.39	1.60	60.0	12.99	1.49	108.3	22.41	1.91

		Lufa 2.1			04A			02A		
	conc 1	23.1	5.12	1.64	50.8	12.10	1.53	108.2	21.99	1.90
	conc 3	28.3	6.29	1.66	54.7	11.75	1.52	98.8	21.01	1.90

Table 21: RNA extraction yield (µg RNA/g soil) and purity (260/280 ratio) of samples at day 28.

		Lufa 2.1			04A			02A		
Test substance		**Conc. [ng/µl]**	**Yield [µg/g soil]**	**Purity [260/280]**	**Conc. [ng/µl]**	**Yield [µg/g soil]**	**Purity [260/280]**	**Conc. [ng/µl]**	**Yield [µg/g soil]**	**Purity [260/280]**
Tiamulin hydrogen fumarate	control	17.7	3.90	2.02	38.2	8.15	1.57	16.9	1.01	2.11
	conc 1	17.3	4.13	1.89	30.3	6.67	1.63	30.9	1.66	2.12
	conc 3	16.8	3.80	2.04	40.0	8.08	1.58	15.9	1.02	2.12
Tebuconazol	control	23.6	5.70	1.91	62.8	15.47	1.46	21.5	1.18	2.01
	conc 1	21.8	5.27	1.88	94.2	19.82	1.44	22.5	1.16	2.21
	conc 3	41.0	9.19	1.58	99.6	21.30	1.47	19.6	1.07	2.18
DDAC	control	31.3	6.51	1.61	67.9	14.47	1.55	21.9	1.22	2.18
	conc 1	30.4	7.27	1.60	31.7	7.00	1.62	22.4	1.19	2.23
	conc 3	18.3	4.29	1.83	104.8	25.56	1.46	25.0	1.29	2.16
Pyraclostrobin	control	15.4	3.57	1.95	100.1	21.26	1.47	22.3	1.15	2.31
	conc 1	24.6	5.80	1.81	111.3	26.25	1.47	29.7	1.52	2.30
	conc 3	23.9	4.88	1.98	89.1	21.29	1.45	33.6	1.74	2.23

		Lufa 2.1			04A			02A		
Propamocarb hydrochloride	control	20.3	4.42	1.93	37.4	8.50	1.48	28.0	1.45	2.26
	conc 1	22.1	4.71	2.02	32.1	6.68	1.53	21.5	1.11	2.29
	conc 3	28.6	5.96	1.82	41.9	8.89	1.47	23.1	1.19	2.16
Ethofumesat	control	17.0	3.82	1.77	50.0	11.00	1.47	23.8	1.23	1.97
	conc 1	18.9	4.19	1.92	41.1	10.01	1.49	24.3	1.26	2.41
	conc 3	24.8	5.54	1.84	45.4	10.11	1.48	21.8	1.12	2.40

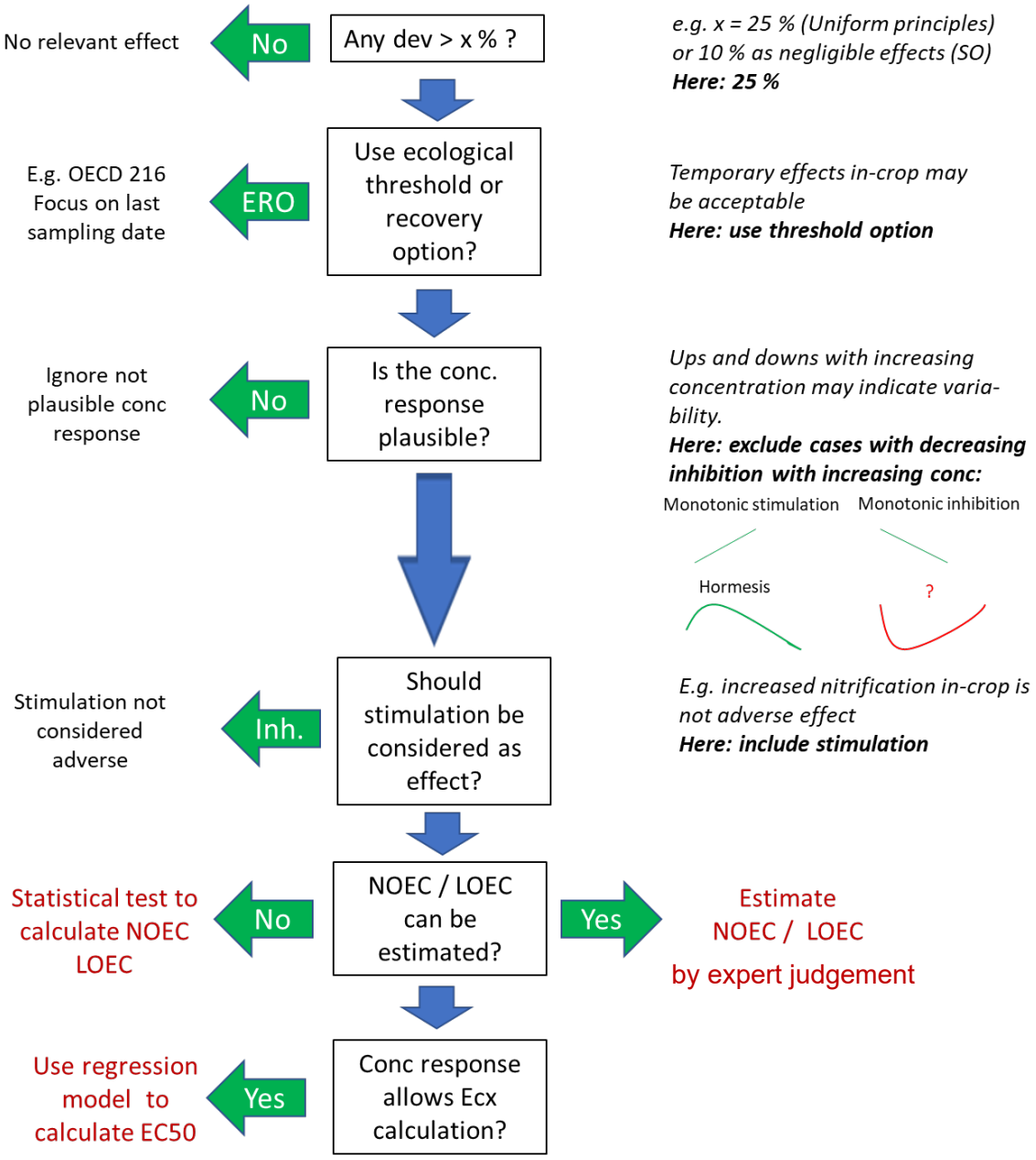
2.3.6 Test evaluation

A large number of data sets resulting from the combination of studies for six test items, up to three soils, up to twenty-four measurement endpoints and up to five sampling dates were produced within the present work and served the evaluation of treatment-related effects on bacteria and (arbuscular mycorrhiza) fungi.

2.3.6.1 Microbial function (OECD 216, ISO 15685, ISO 20130 and MicroResp™)

Figures and statistics are not shown for each possible combination within this report, and data sets were filtered for more detailed analysis and endpoint derivation. The main purpose was to compare the results between different test methods and to show which methods are best suited to serve as reliable information in a new protective risk assessment approach for soil microorganisms.

Figure 14: Decision tree for evaluating the tests on microbial function in this project.



Source: Own illustration, Fraunhofer IME

First, following the current Uniform Principles (European Commission 2011), any deviation from controls below 25 % was not further considered. However, a lower threshold level could be used in future risk assessments. For example, the SO (EFSA PPR panel, 2017) suggested using 10 % to separate negligible (‘*no increases in the frequency of effects between exposed and unexposed groups’*) from small effects (up to 35%).

The current risk assessment for soil microorganisms is related to the in-crop situation, and temporary effects are accepted: *'no authorisation shall be granted if the nitrogen or carbon mineralisation processes in laboratory studies are affected by more than 25 % after 100 days'* (Uniform Principles). Under this Ecological Recovery Option, temporary effects are ignored, and a risk is only indicated, if deviations above 25 % are observed at the end of the test. However, the Ecological Threshold Option (only negligible effects) may be used for an off-crop or off-field situation. Thus, an assessment endpoint can either be based on the data of the sampling date with the strongest effect, respectively, the lowest effect concentration (ETO) or just based on the last sampling (ERO).

In this project, only endpoints with effects ≥ 25 % on day 28 were considered in the selection of figures to be presented in this project. However, the figures for the endpoints selected show the results over the course of the test. If, for one test, more than one endpoint showed an effect ≥ 25% on day 28, the endpoint with the clearest effects was selected for plotting per test item and soil.

Some concentration-response curves are difficult to explain by common ecotoxicological mechanistic understanding, and thus, a threshold concentration is also difficult to derive. The usual expectation for an ecotoxicological test is a monotonic inhibition increase with increasing exposure level (except a stress reaction is measured). For example, stimulation with increasing exposure is also possible by trade-offs between life-cycle traits or indirect effects. In the case of bacterial function tests, this could be increased activity due to metabolization of the test item or reduced competition with a more sensitive organism group. Stimulation at a low concentration followed by inhibition at a higher concentration is also plausible (hormesis in the sense of (Forbes 2000)). For the filtering, only concentration response patterns where inhibition was stronger at lower than at higher concentration were not considered to indicate plausible effects of the treatment. In this way, setting a LOEC to a low concentration was avoided when there was no effect at higher concentrations.

According to the terrestrial guidance document (SANCO 2002), any deviation from control has to be considered (increase and decrease of activity) and the critical level is ±25 % deviation form the control. On the other hand, OECD 216 recommends, at least for non-pesticides, the calculation of EC_x values which are defined as ‚*concentration of the test substance in soil that results in a x percent inhibition of nitrogen transformation to nitrate*‘. In-crop, increased activity of microorganisms in the soil, e.g. increased nitrification may be considered positive for soil fertility, thus, whether stimulation should be considered an adverse or not was considered here as a further option of the risk management.

Thus, this scheme results in the following option to derive a relevant assessment endpoint:

ETO - S&I: consider the lowest effect concentration of different sampling dates of both inhibition and stimulation ≥ 25%

ETO – I: consider the lowest effect concentration only for inhibition of different sampling dates ≥ 25%

ERO – S&I: as ETO-S&I but consider only long-term effects (here if ≥ 25 % on day 84 or any other last sampling date)

ERO-I: as ETO-I but consider only long-term inhibition (here ≥ 25 % on day 84 or any other last sampling date)

Due to the many effects, statistical tests were not always conducted to calculate a NOEC and LOEC. Instead, if the NOEC seemed clear from the difference in the mean and the standard deviation, the NOEC and LOEC were estimated[2]. In other cases, the NOEC/LOEC was determined by statistical testing following the recommendation of the OECD GD 54 (2006) for metric data. Thus, preliminary tests on normal distribution and variance homogeneity were conducted, and depending on the outcome, a trend test was conducted to select the appropriate test to derive the NOEC.

When the means per treatment level indicated a concentration response for inhibition, non-linear regression (3-parameter logistic regression) was used to calculate EC_{50}.

The software ToxRat Professional 3.3 was used for these statistical tests.

2.3.6.2 ISO 10832

The germination test was conducted only with the Refesol 02A. Thus, the results of all six tests are presented below.

Germination rates were evaluated for significant differences to controls following the recommendation of the OECD Guidance 54 for metric data after arc-sine transformation of the percentage values. Thus, preliminary tests on normal distribution and variance homogeneity were conducted, and depending on the outcome, a trend test was conducted to select the appropriate test to derive the NOEC. The software ToxRat Professional 3.3 was used for these statistical tests.

2.3.6.3 ARISA

GeneMapper output tables were converted to sample-by-binned-OTU tables using custom R binning scripts based on the publication from (Ramette 2009). The binning algorithm was implemented in the *R* programming language (Version R: 4.1.1). A detailed description is attached to this report in the appendix E.1.

It has to be kept in mind that the derivation of Operational Taxonomic Units (OTU) as proxies of true species of bacteria and fungi presupposes that an explicit distinction could be only made within each of the experimental batches. A comparison of the community composition between batches was not considered valid without sequencing each of the single bands of a chromatographic gel. These methodological prerequisites mean all analyses should be done batch-wise or filtered to a unique combination of test item, soil and chemical because results are not directly comparable otherwise.

Without consideration of a possible position of ARISA-study results in an adapted ERA of soil microorganisms, the results were analysed for multivariate patterns using Correspondence Analysis (CA).

For the data analysis, we had to cope with the limitations posed by a low number of replicates of two chosen for this test, as it allowed the screening of the complete test setup while adhering to budgetary constraints.

2 We are aware that this should not be done for tests used for regulatory risk assessments but for the purpose of comparing the different tests, soils and assessment option within this project we consider this a pragmatic approach. Note that within this project a compromise between testing different combinations of methods, test items and soils and the effort spent on each test had to be made. Thus, in regulatory praxis often more test concentraitons and / or replicates shouold be used to proivide more suitable data sets for statistical evaluation.

The analysis of ARISA data differs substantially from the transformation and inhibition data since the basis of describing the effects of toxic substances are the multivariate patterns of operational taxonomic units (equivalently handled to the classical *species* definition). With this, the research questions and validity criteria differ.

In the community analysis here, c*hanges in soil communities due to the effects of six exemplary test substances* were visualised, and visually observed differences between community structures assigned to different treatment levels were detected. The correspondence analysis (CA) was chosen in the first instance as an example for interpretation and post-analysis from the various multivariate pattern recognition methods described in the (ecological) literature.

2.3.6.3.1 Diversity indices

Alpha diversity indexes, measure the diversity within a single sample, accounting for its richness (number of species/OTUs) and evenness (distribution of abundances across species/OTUs). The combination of both, richness and eveness, is summed up in the Shannon index by considering the proportion of each OTU and how evenly is distributed the community in a sample. These tree indices were calculated using the *diversity* function of the *vegan* package (R.) for each of the four data sets (bacterial / fungal DNA / RNA). Is is important to consider that summarizing a community structure into a single number, e.g. a diversity index, enables, in principle, the use of univariate methods for significance testing, however this comes inevitably with a loss of information. The lack of replication or test concentrations cannot be solved by converting multivariate data into univariate data.

2.3.6.3.2 Community structural composition

Relative abundance plots of the ARISA data were employed to illustrate the proportion of different taxa within the samples, represented by OTUs. These plots facilitate the comparison of community composition across multiple samples and highlight the contribution of specific OTUs to overall diversity. However, in samples with a large number of OTUs, the visualization and interpretation can become challenging, making alpha diversity indexes a more appropriate metric in such cases. In this project, 100% stacked bar plots were generated in Excel using the mean OTU values per sample, which may mask the data variability. Deviations in stacked bar plots were visually evaluated, differences between control and treatments were used to derive the ‘lowest observed effect concentration’.

2.3.6.3.3 Pattern recognition

Correspondence Analysis (CA) is a multivariate statistical method used to explore relationships between categorical variables in a dataset. When applied to ARISA data, the categorical variables are the operational taxonomic units (OTU´s) and the CA helps to visualize and interpret patterns of variation in bacterial community composition across different samples.

In this project, CA analysis were carried out to reduce the complexity of the patterns within a comparable group of samples (built from the combinations of test items, day of sampling on day 28, organism group, and genetic material) and facilitate interpretation (Hill 1973). In addition, the representations contain overlays of the treatment levels (usually controls and two treatments were tested) for visual interpretation. The following CA plots (in section 2.4) show the two dimensions (plotted on x- y-ordination axes with the highest explanatory power for the respective data sets' total variance). The number of tested combinations sums up to 72 analyses: six test substances per two organism groups times two genomic substances times three soils. Samples with similar community structures plot close together in a CA ordination, and axes represent theoretical environmental gradients, interpreted as exposure gradients causing toxic effects of the test substances.

A statistical analysis, e.g. testing on significant effect of the treatment or differences in the structure of the treated community compared to the control could not be conducted to the low number of samples (3 concentrations with 2 replicates each). Deviations between control and treatments were visually evaluated.

2.4 Test results

The following chapter includes the presentation of the test results with the six chosen test substances ethofumesate, tebuconazole, pyraclostrobin, propamocarb hydrochloride, tiamulin hydrogen fumarate and didecyl-dimethylammonium chloride in three soils and five test systems described in chapter 2.3.4.

As outlined in chapter 2.3.5.4, the spore germination test with the AMF *F. mosseae* could not be performed with Lufa 2.1 and RefeSol 04A. Therefore, results for this test system are presented for RefeSol 02A only. The raw data on the spores introduced and the determined spore germination for the tests with the 6 test substances are presented in Table 152 to Table 158 in the Annex.

The results of the OECD 216 limit test with the six substances in the three chosen soils (Lufa 2.1, RefeSol 02A, RefeSol 04A) are presented in the respective chapter of each test substance. The highest test concentration from the tests described in chapter 2.3.4. was used. In general, the observed results for the plant protection products differ from those of studies prepared for regulatory purposes, presented in chapter 2.3.1 (Table 11 and Table 12). However, it has to be noted that these studies were performed with formulations containing the active substance in the 90s, while the actual tests were performed with the sole active substance. It can, therefore, not be ruled out that formulation additives also caused effects with the formulations tested at that time.

2.4.1 Ethofumesate

2.4.1.1 Standard test results

Ethofumesate affected the C-transformation (UBA data mentions CO_2) of 15 to 23% between days 7 and 28 in a test with a light sandy loam. In a test with a heavier clay loam, effects were stronger, with 72% at the lower test concentration (1x application rate) and 35% at the higher test concentration (5x application rate) on day 7. Afterwards, the deviation from the control decreased slightly until day 14 to 33% and 17%, respectively, and remained stable until day 28 (Table 12). Within the experimental work of this project, the herbicide ethofumesate affected the nitrogen transformation by more than 25% compared to the control only on day 14 in the test soil RefeSol 02A with stimulation of 33%, while there was no observable effect after 28 days exposure (Table 28). No relevant effect was observed in Lufa 2.1 (Table 22) and RefeSol 04A (Table 25).

2.4.1.2 Lufa 2.1.

The observed inhibitions of the AOB (ISO 15685), the substrate-induced respiration (MicroResp™) and the enzymatic activity (ISO 20130) due to ethofumesate are presented in Table 22.

2.4.1.2.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

The highest inhibition of the AOB (ISO 15685) of 24% was found at test initiation, again at the lowest test concentration. Since no data on the effects of ethofumesate at test initiation was available in the UBA data, a direct comparison was not possible. Afterwards, the AOB was not inhibited on days 14 and 28; therefore, the ISO 15685 was not performed on days 56 and 84.

In the tests with LUFA 2.1, a sandy soil, which should be more comparable with the light sandy loam, the MicroResp™ system on days 0, 14 and 28 showed, compared to the control treatment, no effect above 25% on the SIR using eight different substrates. Therefore, the MicroResp™ system was not further used on days 56 and 84.

Using the ISO 20130, at test initiation in all ethofumesate treatments, the arylsulfatase activity was stimulated by 55 to 63% compared to the control. After 14 days, the arylsulfatase activity decreased by 10 to 22%. On day 14, the activity of the arylamidase increased without any concentration dependency by 133 to 192%. The urease activity was inhibited by 21 to 23%. At day 28, a concentration-dependent effect on the arylsulfatase was found, inhibited by 11 to 87%. This inhibition could not be confirmed by measurements at day 56. Here, the inhibition decreased to 11% up to a stimulation of 31%. However, on day 84, at the highest concentration of 20 mg a.s./kg dw soil, an inhibition of 40% was found. Neither of the other observed enzyme activities showed any adverse effect due to the ethofumesate on day 84.

Table 22: Effects of ethofumesate with application rates of 2, 10 and 20 mg a.s./kg soil dw in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Lufa 2.1. as test soil.

Test day	Test system	Test details	Inhibition [%]		
			2 mg/kg	10 mg/kg	20 mg/kg
Test initiation D0	ISO 15685	-	24	9	12
	MicroResp™	Deionized water	-4	2	4
		D-(+)-Glucose	2	7	3
		L-Cysteine hydrochloride	-5	-1	-6
		L-Malic acid	16	15	3
		y-Amino butyric acid	-3	14	13
		N-Acetyl glucosamine	-2	1	-2
		Citric acid	4	6	4
		L-Alanine	-4	0	0
	ISO 20130	Phosphatase	-4	13	8
		ß-Glucosidase		11	13
		Arylamidase	-18	-17	-17
		Urease	n. d.	n. d.	n. d.
		Arylsulfatase	-63	-61	-55
D14	ISO 15685	-	7	-6	9
	MicroResp™	Deionized water	5	-5	3
		D-(+)-Glucose	-9	10	17
		L-Cysteine hydrochloride	1	-5	3

Test day	Test system	Test details	Inhibition [%]		
			2 mg/kg	10 mg/kg	20 mg/kg
	ISO 20130	L-Malic acid	-8	-3	13
		y-Amino butyric acid	2	9	17
		N-Acetyl glucosamine	-1	-3	10
		Citric acid	-11	-15	5
		L-Alanine	4	7	14
		Phosphatase	1	14	23
		ß-Glucosidase	-9	13	41
		Arylamidase	-179	-133	-192
		Urease	21	23	21
		Arylsulfatase	-22	-19	-10
D28	ISO 15685	-	2	2	2
	MicroResp™	Deionized water	0	1	0
		D-(+)-Glucose	4	0	-4
		L-Cysteine hydrochloride	0	-17	-5
		L-Malic acid	9	19	15
		y-Amino butyric acid	1	-1	-5
		N-Acetyl glucosamine	-12	-8	-12
		Citric acid	8	7	4
		L-Alanine	2	2	2
	ISO 20130	Phosphatase	5	7	6
		ß-Glucosidase	-5	-3	-10
		Arylamidase	-5	-10	8
		Urease	4	12	7
		Arylsulfatase	11	39	87
D56	ISO 20130	Phosphatase	n. d.	n. d.	n. d.
		ß-Glucosidase	n. d.	n. d.	n. d.
		Arylamidase	18	12	11
		Urease	n. d.	n. d.	n. d.
		Arylsulfatase	-12	11	-31
D84	ISO 20130	Phosphatase*	0	-1	-9

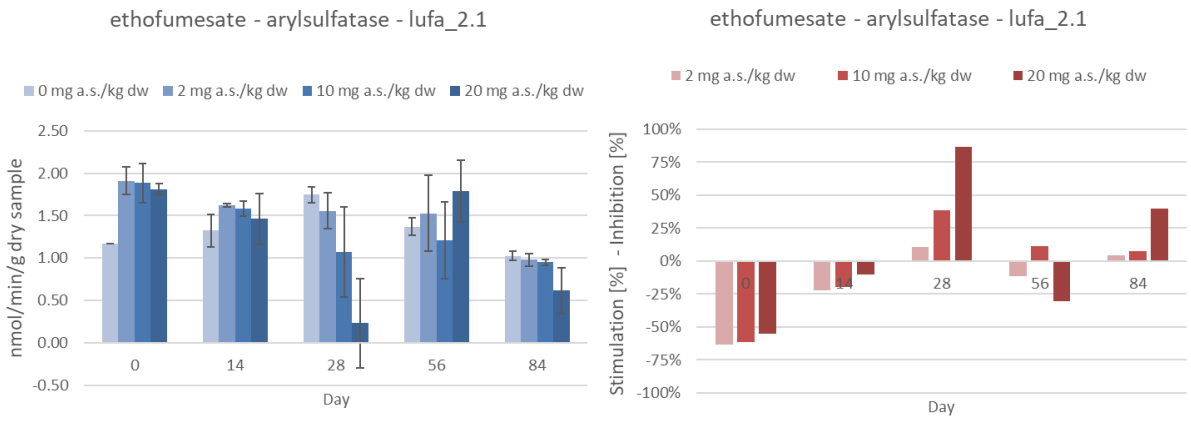
Test day	Test system	Test details	Inhibition [%]		
			2 mg/kg	10 mg/kg	20 mg/kg
		ß-Glucosidase*	-1	5	-18
		Arylamidase*	-8	15	-6
		Urease*	8	5	6
		Arylsulfatase	4	7	40
D14	*OECD 216 (reference)*	*N-Transformation*	-	*9*	-
D28			-	*-6*	-

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

*Note: Even if, after 56 days, no effect above 25% was observed, the ISO 20130 was performed with all substrates to increase the technician's training in the relevant method.

Results of the ISO 20130 regarding the effect of ethofumesate on the exoenzymatic activity were chosen exemplarily to demonstrate certain effects on the soil microorganisms and their function. On day 28, arylsulfatase showed a clear concentration-related inhibition with an estimated LOEC of 10 mg a.s./kg dw soil (Figure 15). Here, the calculation of an EC10 and an EC_{50} would be possible.

Figure 15: Exemplary results of the exoenzymatic activity (left figure) of arylsulfatase (ISO 20130) and corresponding deviation (right figure) from the treatment with ethofumesate in application rates of 2, 10 and 20 mg a.s./kg soil dw compared to the control in the test with Lufa 2.1.



Source: Own illustration, Fraunhofer IME

2.4.1.2.2 Soil microbial community structure (ARISA)

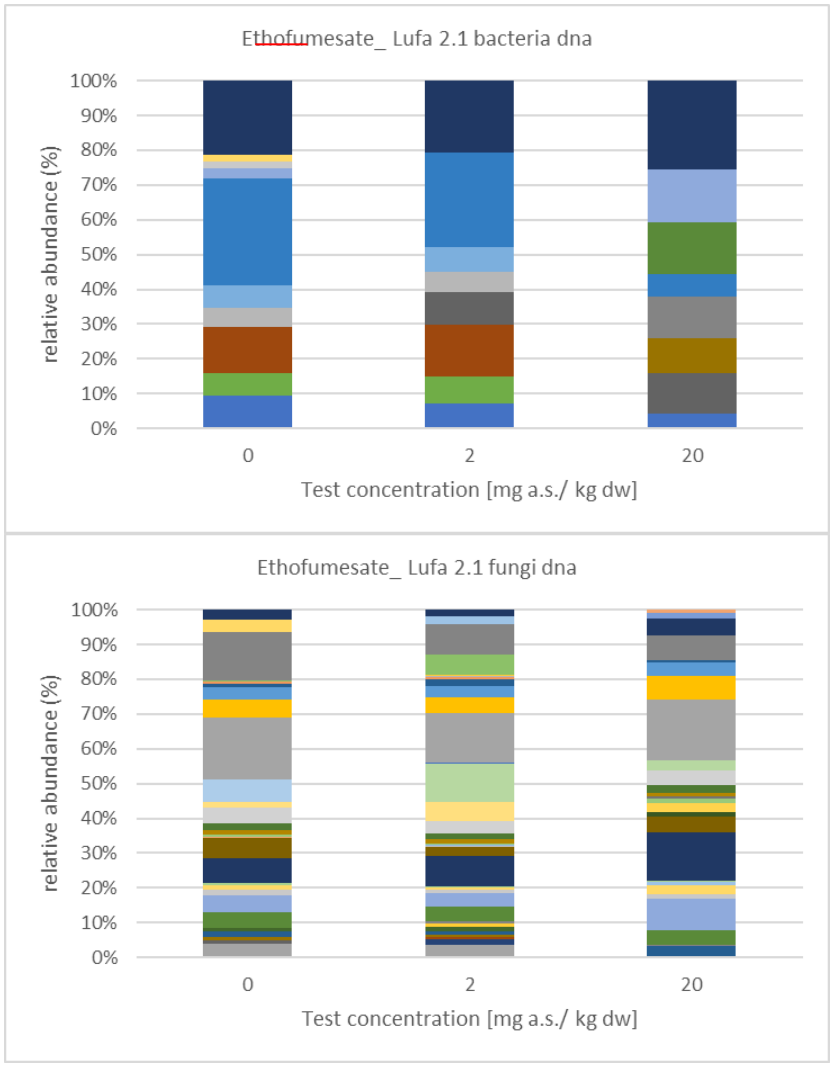
The evaluation of ARISA data revealed shifts in both bacterial and fungal community composition, as illustrated in the relative abundance plot (Figure 16) and the CA (Correspondence Analysis) plot (Figure 18). These changes were evident at both the DNA and RNA levels, particularly at a concentration of 20 mg ethofumesate/kg dry weight soil, indicating a clear response of the microbial communities to this treatment.

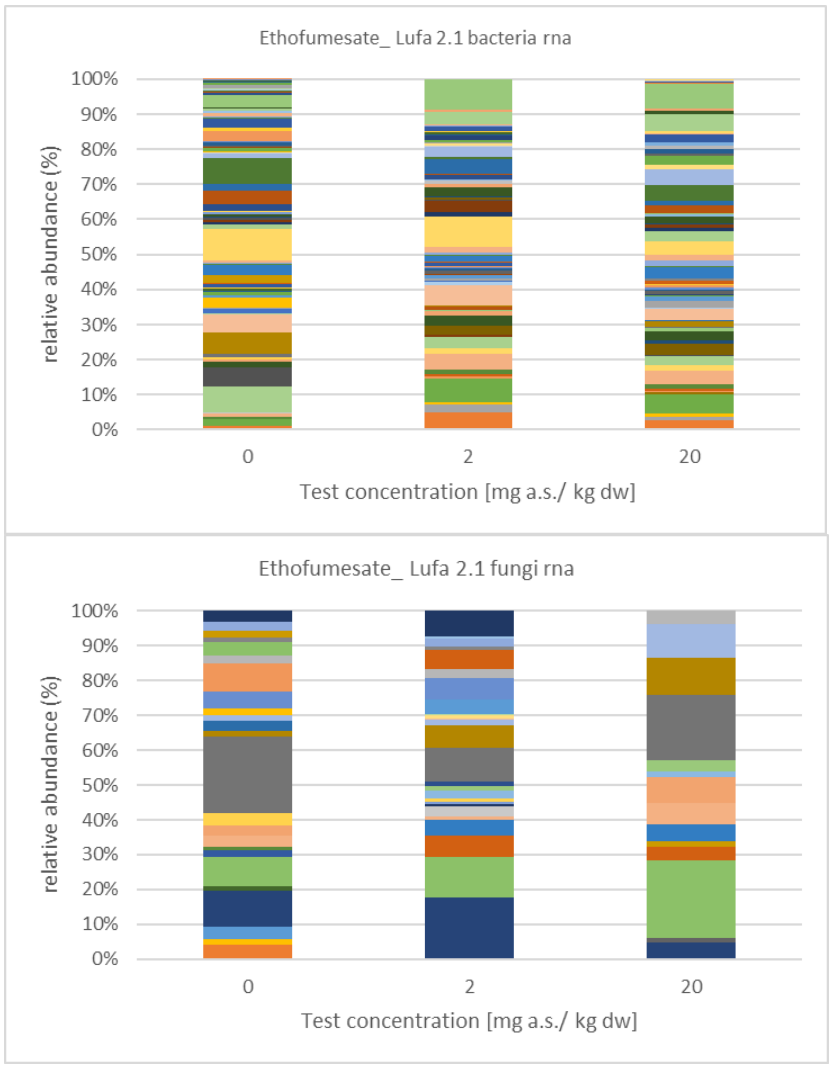
In the relative abundance plot (Figure 16), the shifts in microbial composition are visualized as changes in the proportion of specific OTUs, reflecting altered taxa prevalence under the influence of ethofumesate. Similarly, the CA analysis (Figure 17) highlights these compositional shifts by clustering samples according to their community structure, with noticeable separation

of treated samples from the controls. Despite dispersion within replicates, the overall trend shows that the treated samples exhibit distinct microbial profiles compared to the control.

The slight decrease in the Shannon diversity index (Table 23) appears to be more closely related to a reduction in OTUs richness (or the number of observed taxa) caused by the ethofumesate treatment, rather than a decline in the even distribution of OTUs. This decline suggests a loss of biodiversity within the communities, which is further supported by the clustering pattern in the CA plot.

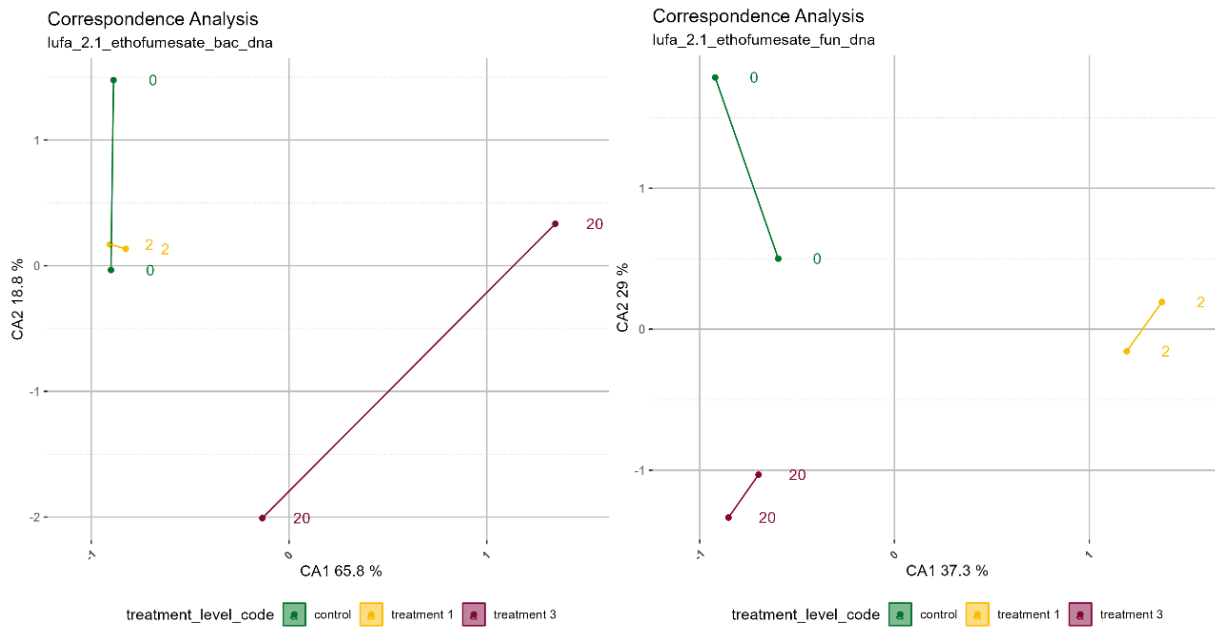
Figure 16: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 2 and 20 mg ethofumesate/kg soil dw in Lufa 2.1. after 28 days.

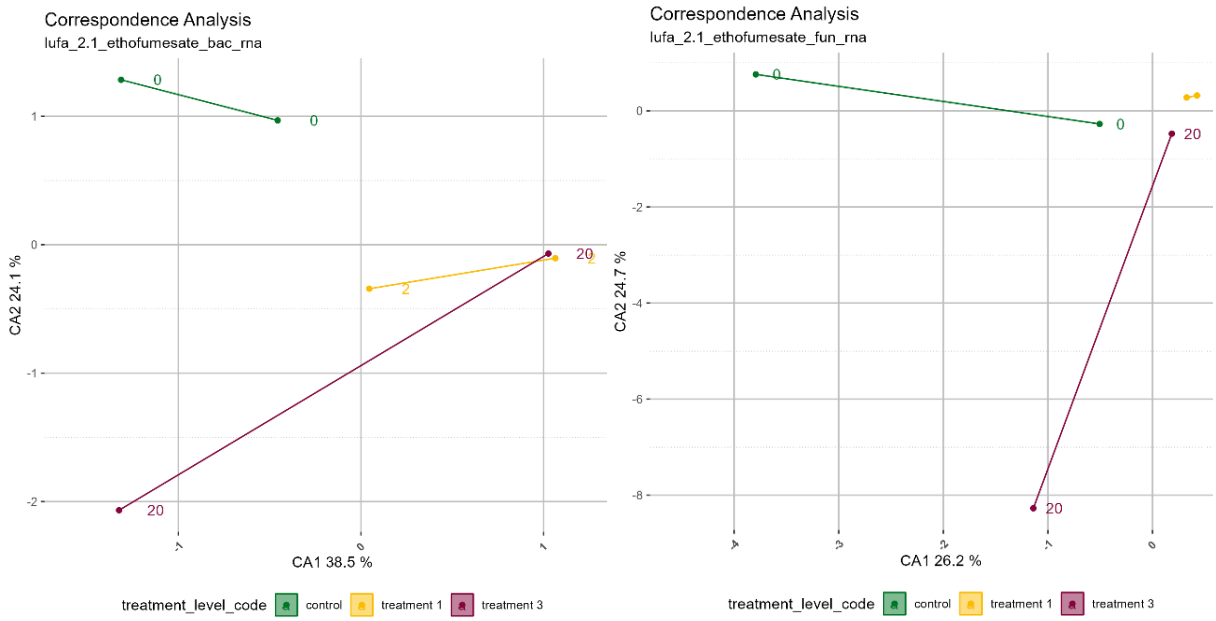




Source: Own illustration, Fraunhofer IME

Figure 17: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to 2 and 20 mg ethofumesate / kg soil dw in Lufa 2.1.





Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), bacteria RNA (bottom left) and fungi RNA (bottom right).

Table 23: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 2 mg/kg and 20 mg/kg ethofumesate in Lufa 2.1 at day 28. Values represent the average of 2 replicates

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	7.5	0.9	1.8
	2.0 mg/kg	7.5	0.9	1.9
	20.0 mg/kg	6.0	0.9	1.6
DNA fungi	Control	24.0	0.9	2.8
	2.0 mg/kg	29.0	0.9	3.0
	20.0 mg/kg	24.0	0.9	2.8
RNA bacteria	Control	49.5	0.9	3.4
	2.0 mg/kg	48.5	0.9	3.4
	20.0 mg/kg	43.0	0.9	3.4
RNA fungi	Control	16.0	0.9	2.5
	2.0 mg/kg	18.0	0.9	2.5
	20.0 mg/kg	8.5	0.9	1.8

2.4.1.2.3 Integrated evaluation

Summary

Based on the results, after approx. 100 days of exposure, at the highest test concentration of 20 mg/kg, the exoenzymatic activity of the arylsulfatase (ISO 20130) was still inhibited by more than 25% with 40% difference compared to the control, indicating a chronic effect on the soil enzyme. The two other test methods (MicroResp™, ISO 15685) appeared less sensitive than the ISO 20130 for the observed herbicide ethofumesate, tested in Lufa 2.1.

The effect of ethofumesate on the spore germination of *F. mosseae* in accordance with ISO 10832 could not be determined due to the poor spore germination of the AMF in LUFA 2.1.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to ethofumesate exposure, with the most pronounced effects seen at the highest test concentration of 20 mg/kg.

Based on the results, Table 24 summarizes the main results per test system.

Table 24: (Estimated) LOECs for effects of ethofumesate in Lufa 2.1 soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	>10	>20	>20	10	20	20
D28-I	>10	>20	>20	10	20	20
ETO-SI	>10	>20	>10	2	20	20
ETO-I	>10	>20	>10	10	20	20
ERO-SI	-	>20	>10	20	-	-
ERO-I	-	>20	>10	20	-	-

2.4.1.3 Refesol 04A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to ethofumesate are presented in Table 25.

2.4.1.3.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

After 14 days of incubation, the AOB (ISO 15685) activity was stimulated at all test concentrations without concentration dependency. The highest stimulation was found at 10 and 20 mg a.s./kg dw soil with 31 and 28%, respectively. No effect of more than 25% on the activity of the AOB was found after 28 days of exposure; therefore, ISO 15685 was not performed on days 56 and 84.

In the tests with RefeSol 04A, the data neither on day 14 nor day 28 of the MicroResp™ system showed more than 25% effect on the basal respiration and the SIR at any of the chosen test concentrations for the used substrates. Therefore, the MicroResp™ system was not further used on days 56 and 84.

The enzyme activity determined with the ISO 20130 appeared to be the most sensitive compared to the other two test systems. Ethofumesate increased the activity of the arylsulfatase and ß-glucosidase by more than 25%. Again, no concentration dependency could be observed. After 28 days of exposure, the activity of the phosphatase, arylsulfatase, urease and ß-glucosidase was inhibited by more than 25% at least by one of the chosen test concentrations. However, the inhibition occurred mainly at the lowest test concentration of 2 mg a.s./kg dw soil. The urease was most affected, but there was no clear pattern with inhibitions of 57, 120 and 30% at 2, 20 and 100 mg a.s./kg dw soil and a stimulation of 27% at 10 mg a.s./kg dw soil. Based on the results, it was decided that the test would be prolonged until day 56. Here, the arylsulfatase activity was inhibited by 45% at 10 mg a.s./kg dw soil, while the urease activity was inhibited by 43% at 2 mg a.s./kg dw soil and stimulated by 43% at 100 mg a.s./kg dw soil. After 84 days of exposure, the effect pattern again was different. No effect above 25% could be observed for ß-glucosidase and arylsulfatase, while the urease was inhibited by more than 25% at a test concentration of 20 mg a.s./kg dw soil, a concentration which was not affected at test termination and after 56 days, but after 28 days of exposure.

Table 25: Effects of ethofumesate with application rates of 2, 10, 20 and 100 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Refesol 04A as test soil.

Test day	Test system	Test details	Inhibition [%]			
			2 mg/kg	10 mg/kg	20 mg/kg	100 mg/kg
D14	ISO 15685	-	-21	-31	-28	-13
	MicroResp™	Deionized water	-8	-8	-7	-12
		D-(+)-Glucose	5	4	-6	-9
		L-Cysteine hydrochloride	9	6	4	-1
		L-Malic acid	11	8	9	2
		y-Amino butyric acid	13	11	11	5
		N-Acetyl glucosamine	15	14	15	9
		Citric acid	20	20	18	11
		L-Alanine	18	15	15	11
	ISO 20130	Phosphatase	20	9	-8	-19
		ß-Glucosidase	-15	-64	-36	-48
		Arylsulfatase	-22	-23	-64	-9
		Arylamidase	-23	2	-12	-4
		Urease	-14	-15	-1	-13

Test day	Test system	Test details	Inhibition [%]			
			2 mg/kg	10 mg/kg	20 mg/kg	100 mg/kg
D28	ISO 15685	-	14	-7	9	10
	MicroResp™	Deionized water	-10	-10	0	-3
		D-(+)-Glucose	-11	-16	2	0
		L-Cysteine hydrochloride	-11	-11	0	0
		L-Malic acid	-9	-8	2	0
		y-Amino butyric acid	-8	-10	1	-4
		N-Acetyl glucosamine	-5	-7	2	0
		Citric acid	-4	-5	1	0
		L-Alanine	-6	-9	0	-2
	ISO 20130	Phosphatase	20	48	3	12
		ß-Glucosidase	30	11	-7	-13
		Arylsulfatase	27	1	0	7
		Arylamidase	10	-5	8	-16
		Urease	57	-27	120	30
D56	ISO 20130	Phosphatase	18	19	16	-20
		ß-Glucosidase	23	6	13	-33
		Arylsulfatase	6	45	23	-11
		Arylamidase	n.d.	n.d.	n.d.	n.d.
		Urease	43	-20	-11	-43
D84	ISO 20130	Phosphatase	n.d.	n.d.	n.d.	n.d.
		ß-Glucosidase	6	-9	-22	-31
		Arylsulfatase	-2	-4	-8	-19
		Arylamidase	n.d.	n.d.	n.d.	n.d.
		Urease	20	8	52	16
D14	*OECD 216 (reference)*	*N-Transformation*	-	-10	-	-
D28			-	21	-	-

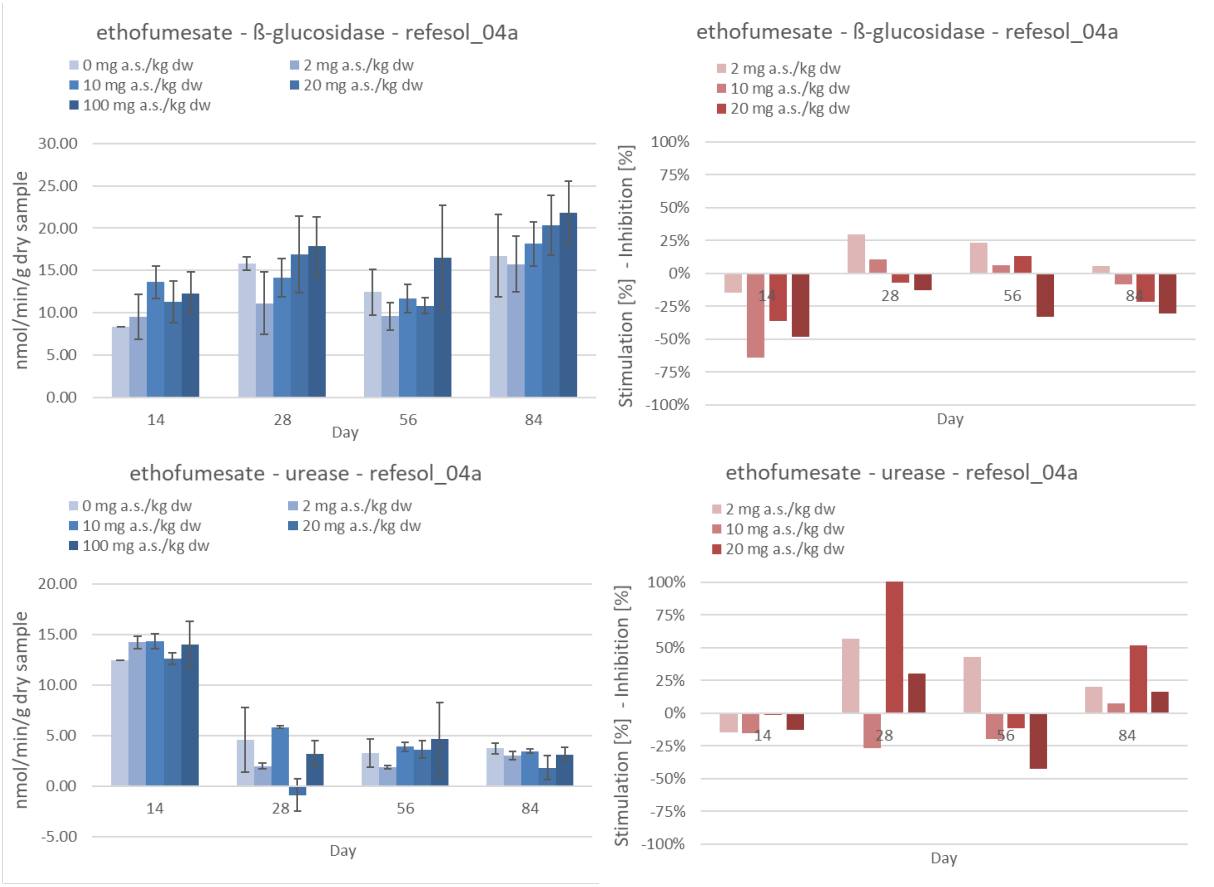
Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

For example, some effects are shown in Figure 20 to obtain a better picture of certain effects. The concentration-response of ß-glucosidase at day 28 is difficult to explain because the largest inhibition was found at 2 mg a.s./kg dw soil, decreasing with increasing exposure. Thus, the

LOEC für inhibition is estimated to be > 100 mg/kg. The lowest estimated LOEC is at 2 mg a.s./kg dw soil for stimulation on day 14. On day 84, stimulation at 100 mg a.s./kg dw soil was above 25%. Significance was not tested, but the LOEC was estimated to be 100 mg a.s./kg dw soil.

Urease was inhibited sometimes above 25 %, but this was never related to a plausible concentration-response relation. Thus, no clear effects were found.

Figure 18: Exemplary results for effects of ethofumesate on the exoenzymatic activity (ISO 20130) of ß-glucosidase (upper figure) and urease (lower figure) at application rates of 2, 10, 20 and 100 mg/kg dw soil in RefeSol 04A.



Source: Own illustration, Fraunhofer IME

2.4.1.3.2 Soil microbial community structure (ARISA)

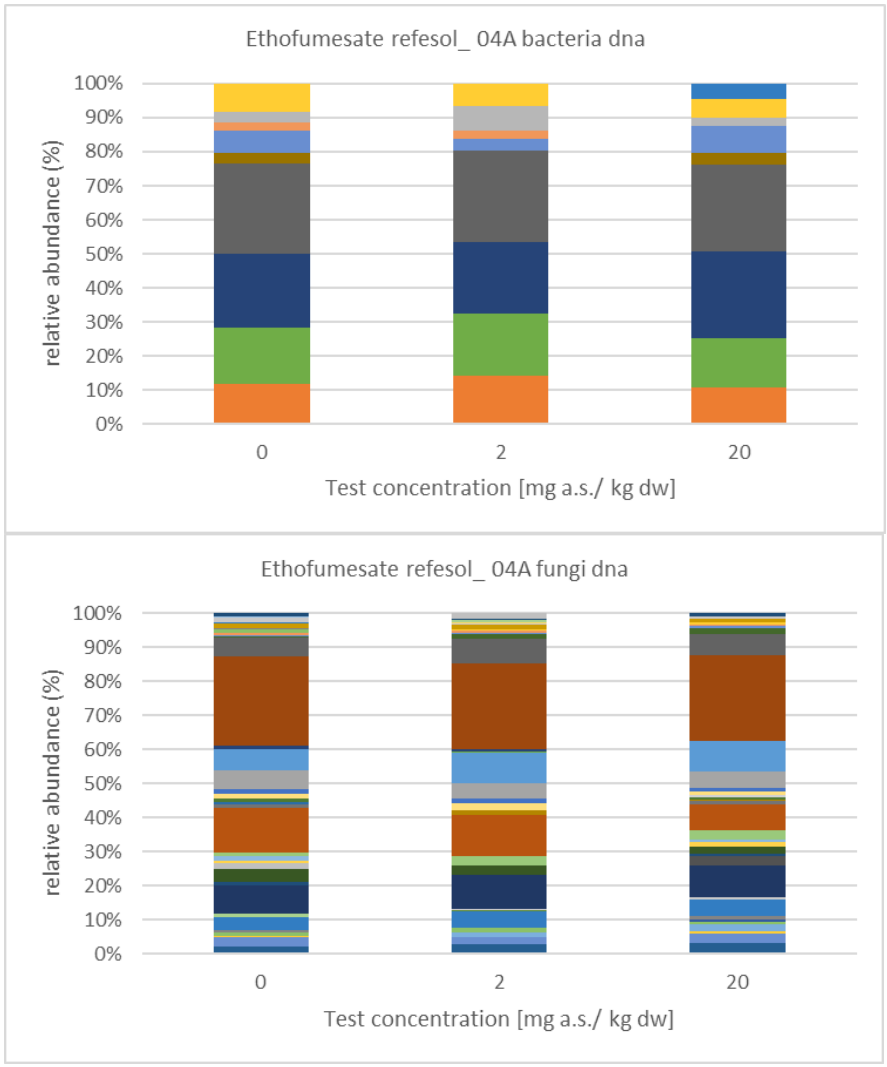
The evaluation of ARISA data revealed shifts in the bacterial community, as illustrated in the relative abundance plot (Figure 19) and the CA (Correspondence Analysis) plot (Figure 20). These changes were evident at both the DNA and RNA levels, for the concentration of 20 mg ethofumesate/kg dry weight soil, indicating a clear response of the microbial communities to this treatment. No changes were observed, at the DNA level, for the fungal community composition (Figure 19 and Figure 20). Information as well as figures regarding fungal diversity and community composition based on RNA data could not be retrieved due to data inconsistency evtl. produced by the low RNA concentration obtained for the 04A soil samples.

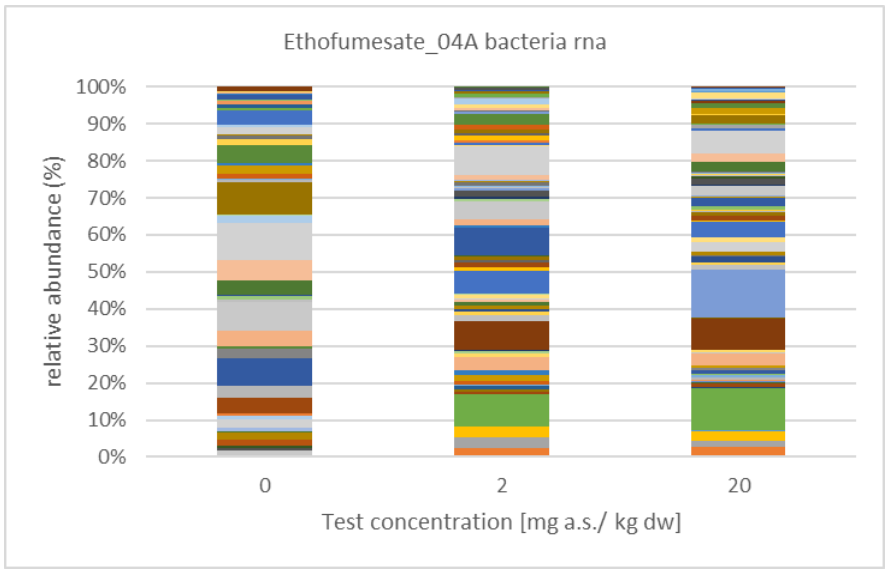
In the bacterial relative abundance plot (Figure 19), interpreting community shifts is challenging due to the high number of OTUs in the samples, complicating the analysis. However, Shannon diversity and richness indices suggest a slight increase in OTU numbers at the concentration of 20 mg ethofumesate/kg dry soil, indicating a bacterial community response to this treatment. Despite some variability within replicates, the overall trend shows that treated samples display distinct microbial profiles compared to the control. For the fungal community profile, however,

the data variability between replicates is so large that no clear trends can be observed from the analysis conducted.

While the Shannon index showed no difference in diversity levels across the treatments (Table 26), the CA results highlighted that the samples have different compositions. This implies that although the samples are similarly diverse regarding taxa richness and evenness, they might differ in the specific taxa present.

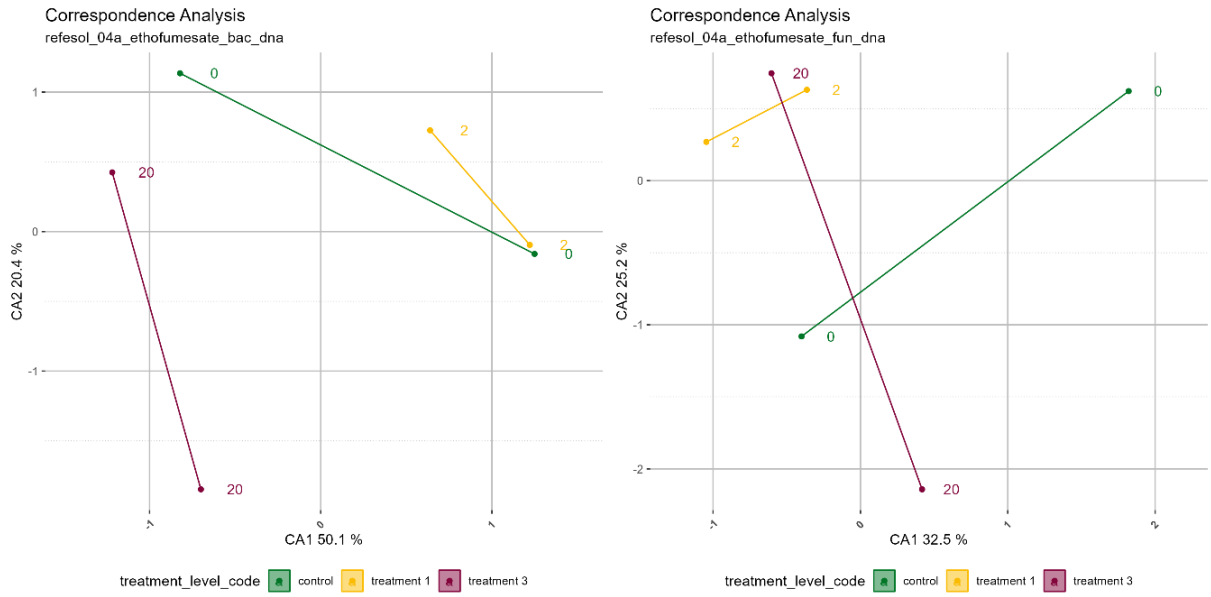
Figure 19: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 2 and 20 mg ethofumesate/kg soil dw in RefeSol 04A after 28 days.

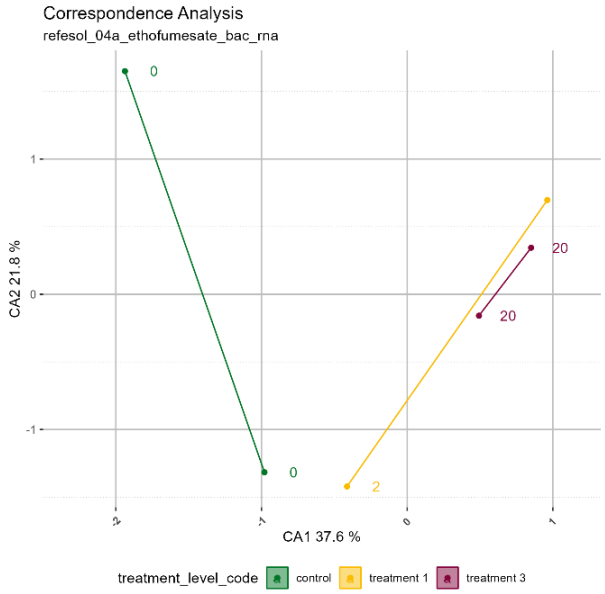




Source: Own illustration, Fraunhofer IME

Figure 20: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to ethofumesate concentrations of 2 and 20 mg/kg dw soil in RefeSol 04A.





Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), and bacteria RNA (bottom left).

Table 26: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 2 mg/kg and 20 mg/kg ethofumesate in Refesol 04A at day 28. Values represent the average of 2 replicates.

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	7.5	0.9	1.9
	2 mg/kg	7.0	0.9	1.8
	20 mg/kg	8.0	0.9	1.9
DNA fungi	Control	26.5	0.8	2.7
	2 mg/kg	24.5	0.8	2.6
	20 mg/kg	26.5	0.8	2.7
RNA bacteria	Control	31.5	0.9	3.1
	2 mg/kg	41.5	0.9	3.3
	20 mg/kg	42.0	0.9	3.2

2.4.1.3.3 Integrated evaluation

Summary

The concentration-response of glucosidase on day 28 is difficult to explain because inhibition is largest at 2 mg a.s./kg dw soil, decreasing with increasing exposure. Thus, the LOEC for inhibition is estimated to be >100 mg a.s./kg dw soil. The lowest estimated LOEC is 2 mg a.s./kg dw soil for stimulation on day 14. On day 84, stimulation at 100 mg a.s./kg dw soil was above 25%. Significance was not tested, but the LOEC was estimated to be 100 mg a.s./kg dw soil.

Based on the results with ethofumesate in RefeSol 04A, after approx. 100 days exposure, the exoenzymatic activity of the urease (ISO 20130) was still inhibited by more than 25% (52% at 20 mg a.s./kg dw soil) and the ß-glucosidase by 31% (stimulation) at 100 mg a.s./kg dw soil indicating a chronic effect on the soil enzyme. The two other test methods (MicroResp™, ISO 15685) appeared less sensitive than the ISO 20130 for the herbicide ethofumesate.

The effect of ethofumesate on the spore germination of *F. mosseae* in accordance to ISO 10832 could not be determined due to the poor spore germination of the AMF in RefeSol 04A.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial communities respond to ethofumesate exposure, with the observed effects at the lowest test concentration of 2 mg/kg. Conclusions regarding fungal communities are possible to be drawn for this soil.

Based on the results, Table 27 summarizes the main results per test system.

Table 27: LOECs for effects of ethofumesate in RefeSol 04A.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	>10	>100	>100	2	2	>20
D28-I	>10	>100	>100	2	2	>20
ETO-SI	>10	10	>100	2	2	>20
ETO-I	>10	>100	>100	2	2	>20
ERO-SI	-	>100	>100	20	-	
ERO-I	-	>100	>10	20	-	

2.4.1.4 Refesol 02A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to ethofumesate are presented in Table 28.

2.4.1.4.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

After 28 days of incubation, compared to the control, the AOB (ISO 15685) activity was not affected by more than 25% at the three chosen test concentrations. Therefore, the ISO 15685 was not performed on days 56 and 84.

In the tests with RefeSol 02A, stimulation of the SIR of more than 25% compared to the control was found for most of the used substrates except y-Amino butyric acid, N-acetyl-glucosamine and the basal respiration after 28 days of exposure. Therefore, the test was prolonged to 56 days. Only a significant difference from the control was found for citric acid. However, the test

was prolonged to observe the reliability of the data. After 84 days of exposure, again, the SIR was stimulated by more than 25% compared to the control for L-cysteine hydrochloride, L-malic acid, y-Amino butyric acid, N-acetyl glucosamine, citric acid and L-alanine. In most cases, the stimulation did not follow a concentration dependency.

The enzyme activity of the arylsulfatase, ß-glucosidase and the urease, determined with the ISO 20130, was not affected by more than 25% after 28 days of exposure. For the phosphatase, a complete inhibition was observed after 28 days of exposure, which decreased over time and after 84 days, no effect above 25% could be observed anymore. For the arylamidase, an effect was observed with the strongest effect (40% inhibition) at the lowest test concentration after 28 days of exposure. The inhibition changed afterwards, with an inhibition ranging from 19 to 26% at the different test concentrations after 56 days and finally a slight concentration-response relationship after 84 days with inhibitions compared to the control of 17%, 25% and 28% at the three test concentrations, respectively.

Table 28: Effects of ethofumesate with application rates of 2, 10 and 20 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using RefeSol 02A as test soil.

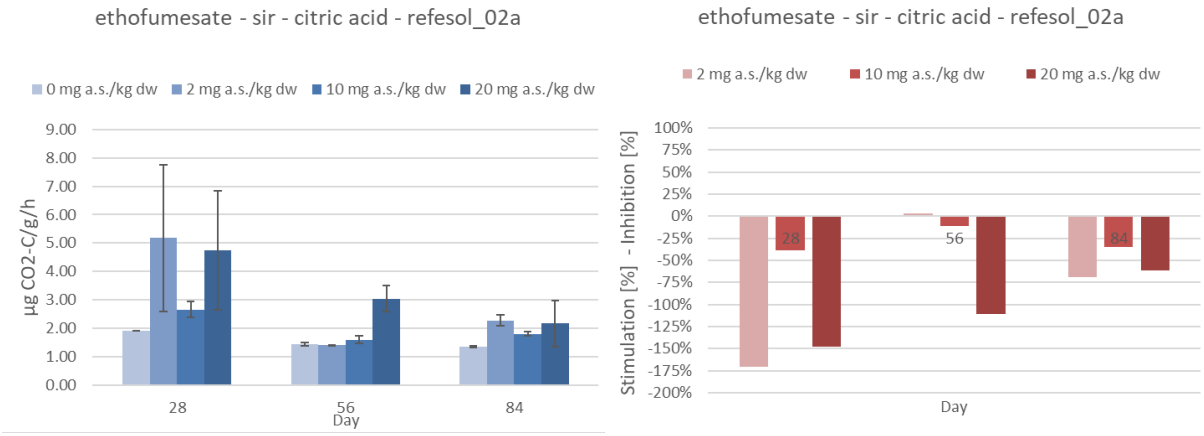
Test day	Test system	Test details	Inhibition [%]		
			2 mg/kg	10 mg/kg	20 mg/kg
D28	ISO 15685	-	-4	-1	7
	MicroResp™	Deionized water	-3	-5	3
		D-(+)-Glucose	-30	-8	-18
		L-Cysteine hydrochloride	-21	-8	-16
		L-Malic acid	-87	-7	-74
		y-Amino butyric acid	0	-8	10
		N-Acetyl glucosamine	-16	-12	-5
		Citric acid	-171	-39	-148
		L-Alanine	-38	-35	-28
	ISO 20130	Phosphatase	100	100	100
		ß-Glucosidase	14	0	3
		Arylsulfatase	13	-4	4
		Arylamidase	40	-3	19
		Urease	-11	5	5
D56	MicroResp™	Deionized water	1	-5	-2
		D-(+)-Glucose	2	-7	-2

Test day	Test system	Test details	Inhibition [%]		
			2 mg/kg	10 mg/kg	20 mg/kg
		L-Cysteine hydrochloride	2	-8	-5
		L-Malic acid	6	-7	-2
		y-Amino butyric acid	4	-9	-2
		N-Acetyl glucosamine	5	-8	-6
		Citric acid	3	-11	-111
		L-Alanine	4	-11	-16
	ISO 20130	Phosphatase	28	30	34
		ß-Glucosidase	2	-1	-6
		Arylamidases	26	19	21
D84	MicroResp™	Deionized water	-3	2	6
		D-(+)-Glucose	-13	-5	-16
		L-Cysteine hydrochloride	-21	-14	-47
		L-Malic acid	-32	-19	-170
		y-Amino butyric acid	-34	-22	-5
		N-Acetyl glucosamine	-41	-26	-9
		Citric acid	-69	-35	-61
		L-Alanine	-46	-26	4
	ISO 20130	Phosphatase	-1	21	15
		ß-Glucosidase	2	8	12
		Arylamidase	17	25	28
D14	*OECD 216 (reference)*	*N-Transformation*	-	-33	-
D28			-	*-12*	-

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

In contrast to the other two soils tested for effects of ethofumesate, the MicroResp™ reacted in RefeSol 02A, but only with stimulations. The strongest stimulation was observed for citric acid. A clear stimulation was already found at 2 mg/kg on day 28 and day 84 (estimated LOEC of 0 2 mg a.s./kg dw soil). However, the patterns related to exposure level and time were not consistent (Figure 21).

Figure 21: Exemplary results for effects of ethofumesate on microbial function at application rates of 2, 10 and 20 mg/kg dw soil in RefeSol 02A. MicroResp™ – substrate-induced respiration with the substrate citric acid and its corresponding effects.

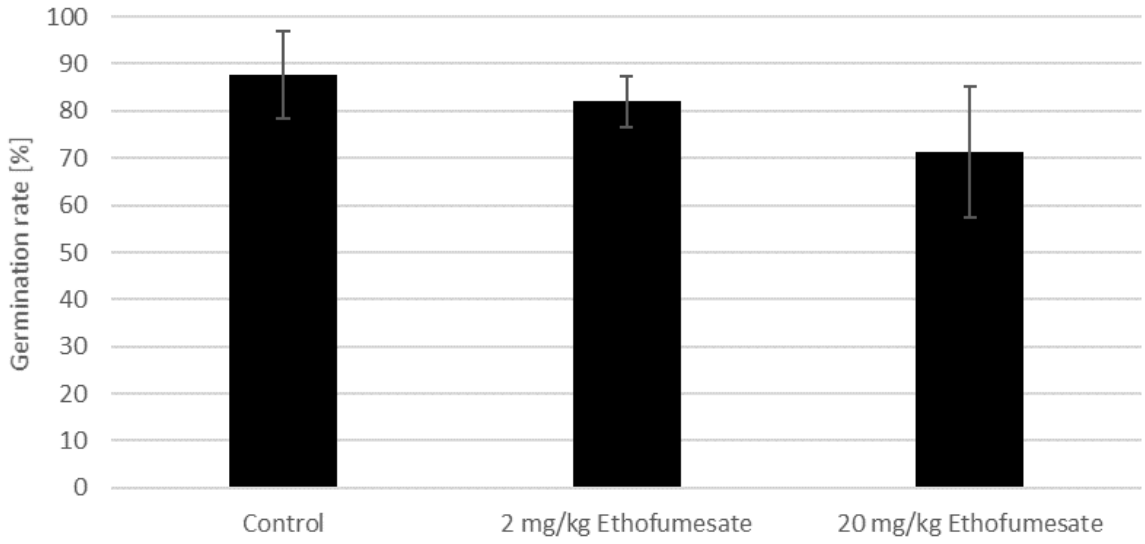


Source: Own illustration, Fraunhofer IME

2.4.1.4.2 Arbuscular mycorrhizal fungi (ISO 10832)

Nominal concentrations of 2 mg a.s./kg dw soil and 20 mg a.s./kg dw soil were tested for inhibition of germination of *F. mosseae.* At 2 mg a.s./kg dw soil, a non-significant inhibition of 7.9% was found, whereas at the highest concentration of 20 mg a.s./kg dw soil, a statistically significant inhibition of 17.8 % was observed (Figure 22). Therefore, the LOEC was determined to be at 20.0 mg a.s./kg dw soil.

Figure 22: Results of the spore germination test with ethofumesate concentrations of 2 and 20 mg/kg dw soil in RefeSol 02A after 14 days of incubation.



Source: Own illustration, Fraunhofer IME

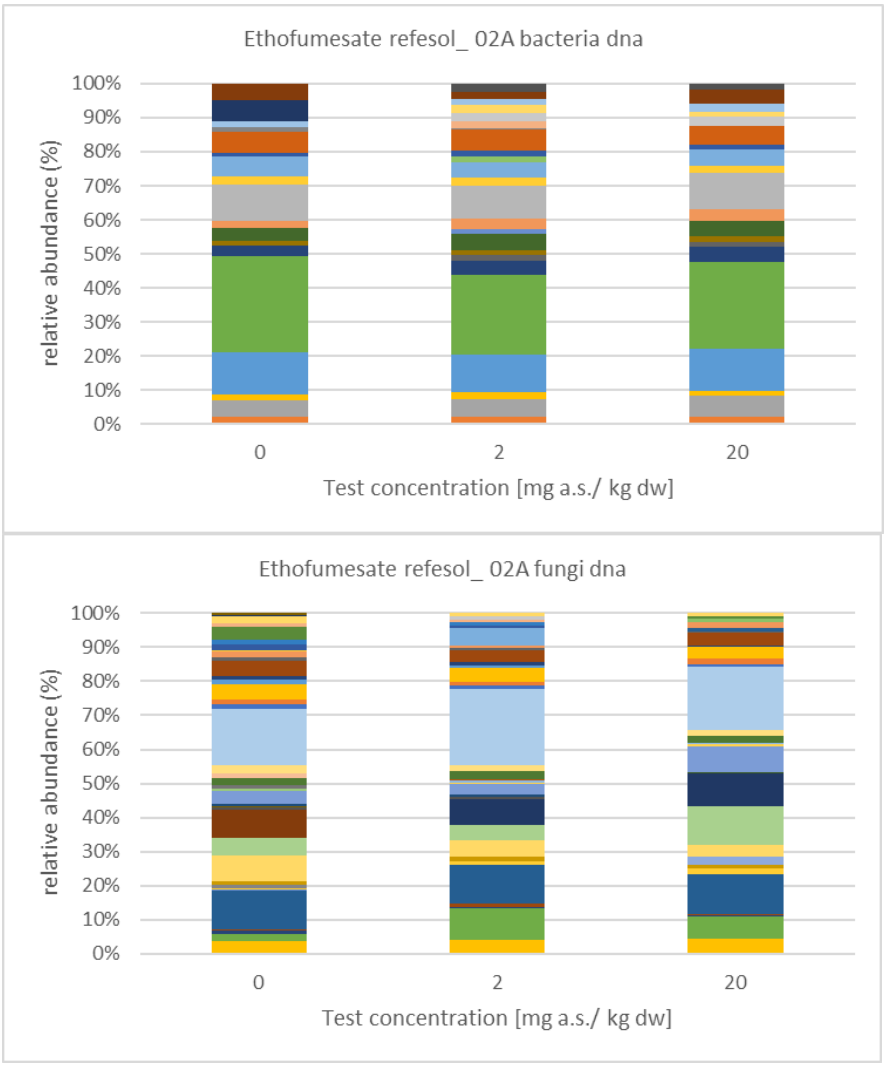
2.4.1.4.3 Soil microbial community structure (ARISA)

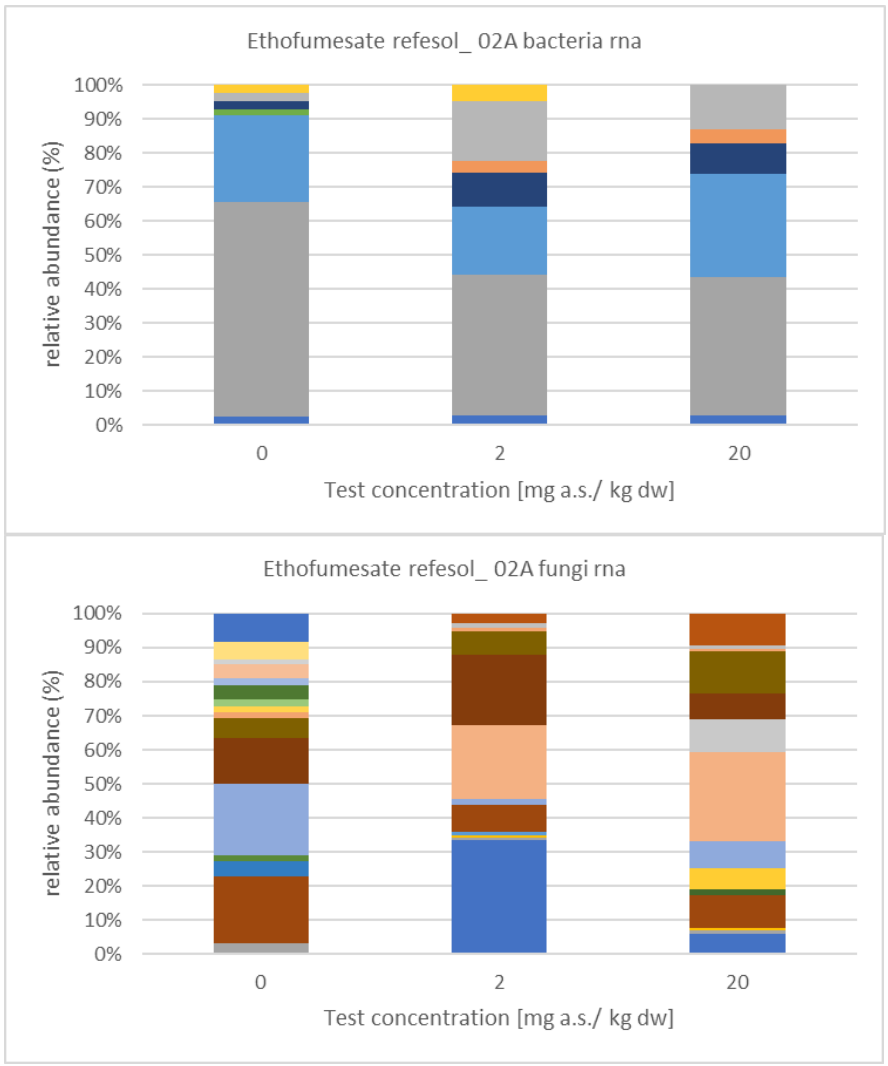
The evaluation of ARISA data revealed shifts in both bacterial and fungal community composition, as illustrated in the relative abundance plot (Figure 23) and the CA (Correspondence Analysis) plot (Figure 24). These changes were evident with the CA analysis at both the DNA and RNA levels, at a concentration of 2 and 20 mg ethofumesate/kg dry weight soil, indicating a clear response of the microbial communities to this treatment.

In the relative abundance plot (Figure 23), the shifts in microbial composition are visualized as changes in the proportion of specific OTUs, reflecting altered taxa prevalence under the influence of ethofumesate. Differences between control and treated soils were better noticiable at RNA level (lower richness compared to the DNA). With the CA analysis (Figure 24) these compositional shifts were observed for fungal and bacterial communities at both, DNA and RNA level, with noticeable separation of treated samples from the controls. Despite dispersion within replicates, the overall trend shows that the treated samples exhibit distinct microbial profiles compared to the control.

The changes observed in the Shannon diversity index (Table 29) appears to be related to both, sample richness and evenness.

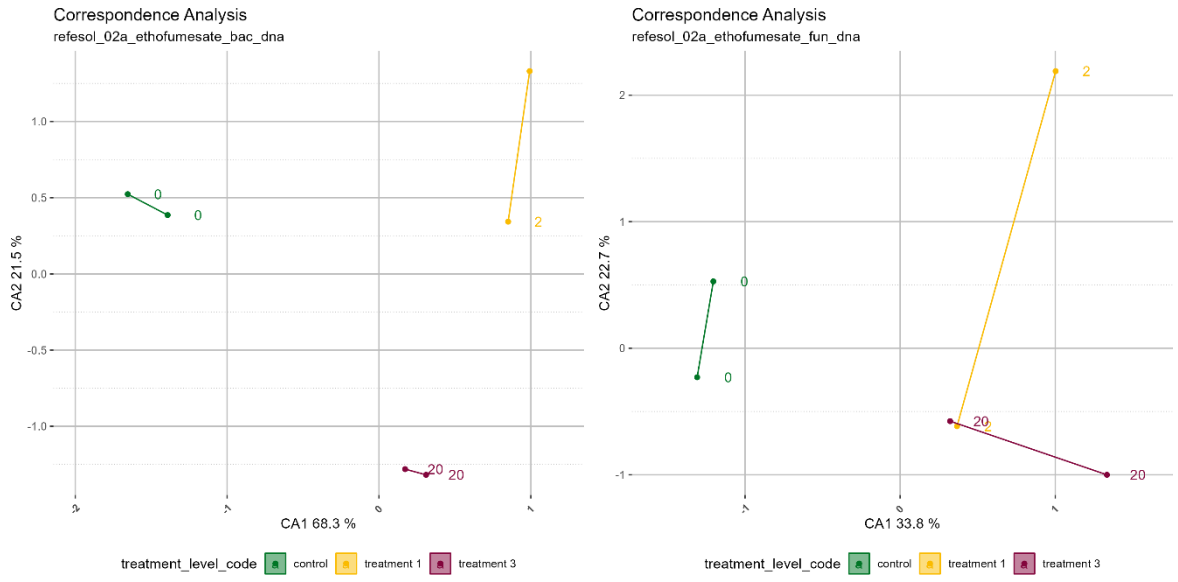
Figure 23: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 2 and 20 mg ethofumesate/kg soil dw in RefeSol 02A after 28 days.

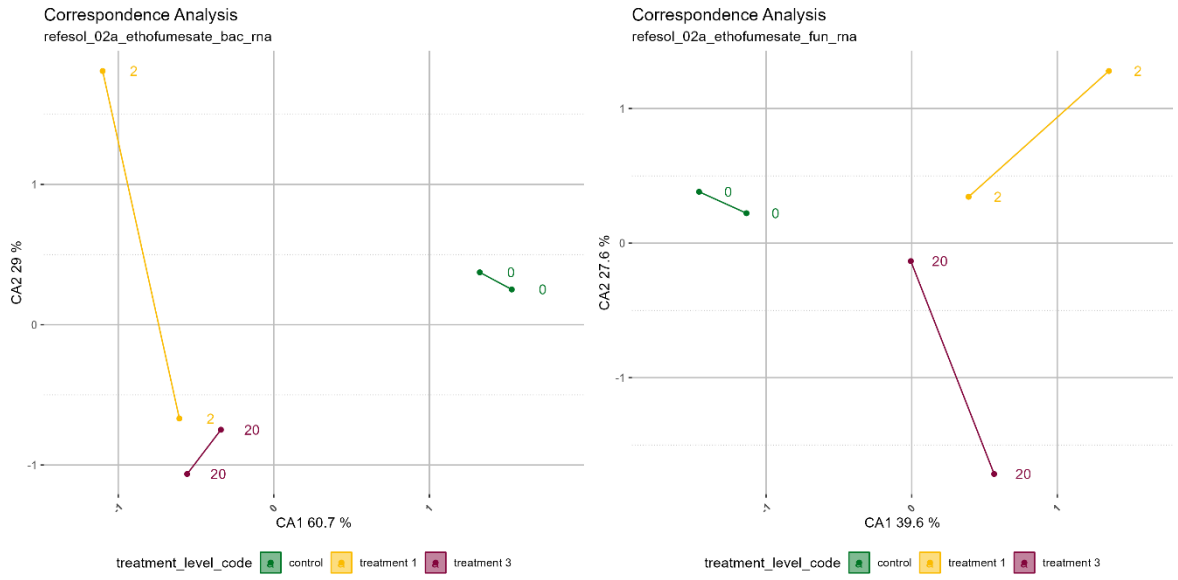




Source: Own illustration, Fraunhofer IME

Figure 24: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to ethofumesate concentrations of 2 and 20 mg/kg dw soil in RefeSol 02A.





Source: Own illustration, Fraunhofer IME

Table 29: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 2 mg/kg and 20 mg/kg ethofumesate in Refesol 02A at day 28. Values represent the average of 2 replicates.

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	18.0	0.8	2.4
	2 mg/kg	23.0	0.9	2.7
	20 mg/kg	19.5	0.9	2.6
DNA fungi	Control	31.0	0.9	3.0
	2 mg/kg	25.0	0.8	2.7
	20 mg/kg	23.0	0.8	2.6
RNA bacteria	Control	14.0	0.7	1.8
	2 mg/kg	13.0	0.9	2.2
	20 mg/kg	12.0	0.9	2.1
RNA fungi	Control	13.5	0.9	2.2
	2 mg/kg	9.0	0.8	1.7
	20 mg/kg	10.0	0.9	1.9

2.4.1.4.4 Integrated evaluation

Summary

Based on the results with ethofumesate in RefeSol 02A, the ISO 15685 appeared to be a less sensitive test method than the ISO 20130. The results of the MicroResp™ system indicate a strong

variability and high uncertainty of the results if the 25% threshold, as outlined in the OECD 216, is suitable for the test method.

The spore germination (ISO 10832) test showed a statistically significant effect at the highest test concentration, indicating a concentration-response relationship. Further tests are recommended to determine if this can be confirmed and if it is possible to receive, for example, effect concentrations. The effect on the AMF was lower than that observed on the exoenzymes; however, the results highlight the relevance of testing fungi next to soil microorganisms in more complex soils, such as RefeSol 02A.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to ethofumesate exposure. Effects were observed effects at the test concentration of 2 and 20 mg/kg.

Based on the results Table 30 summarizes the main results per test system.

Table 30: LOECs for effects of ethofumesate in Lufa RefeSol 02A.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ISO 10832	ARISA CA	ARISA Alpha diversity
D28-SI	>10	>20	2	2	20	2	2
D28-I	>10	>20	>20	>20	20	2	2
ETO-SI	10	>20	2	2	20	2	2
ETO-I	-	>20	>20	2	20	2	2
ERO-SI	-	>20	2	10	-	-	-
ERO-I	>10	>20	>20	10	-	-	-

[a]: Please note that the test duration for the spore germination test with F. mosseae (ISO 10832) was 14 days, and therefore, the values presented are the D14-I and D14-SI values.

2.4.2 Tebuconazole

2.4.2.1 Standard test results

For tebuconazole, the OECD 216 data is limited. An inhibition of 47% was found at a concentration of 8.3 mg a.s./kg dw soil after 14 days of exposure (Table 31). After 7 and 28 days of exposure at 0.83 and 8.3 mg a.s./kg dw soil, no inhibition >25% occurred. In the OECD 216 limit test, no effects above 25% (stimulation or inhibition) were found after 14 and 28 days of exposure in the three chosen soils, as indicated in the tables below.

2.4.2.2 Lufa 2.1.

The observed inhibitions of the ammonium oxidizing bacteria (ISO 15685), the substrate-induced respiration (MicroResp™) and the enzymatic activity due to tebuconazole are presented in Table 31.

2.4.2.2.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

The AOB (ISO 15685) was affected by tebuconazole at the chosen test concentrations since, at test initiation, the activity of the AOB was increased from 25 to 45%. However, there was no concentration dependency. After 14 and 28 days, the stimulation was above 25% only at a concentration of 5 mg a.s./kg dw soil. Due to the unclear pattern (effect only at mid-concentration) and low effect in the other test systems, the test was not prolonged here.

At test initiation, no effects above 25% on the SIR (MicroResp™) were determined, while after 14 days of exposure, respiration was inhibited by more than 25% using the substrate citric acid. Here, stimulation of the SIR induced by citric acid was found at the two highest test concentrations and remained above 25% until day 28, at least at 5 mg a.s./kg dw soil. Due to the unclear pattern (effect only at mid-concentration) and low effects in the other test systems, the test was not prolonged.

The enzyme activity was inhibited mainly at test initiation. Here, at 10 mg a.s./kg dw soil, the ß-glucosidase and the arylsulfatase were inhibited by 28% and 52%, respectively. On days 14 and 28, no comparable or higher effect on the different enzyme activities was found. Nevertheless, effects on the enzyme activities were also observed after 56 days since the effect on arylsulfatase could not be determined on day 28 due to the delayed received chemical. The enzyme activity using ß-glucosidase as substrate was inhibited by 29% and 22% at 5 and 10 mg a.s./kg dw soil after 56 days, but after 84-day exposure, no effect above 25% could be observed. The results for the enzyme activity of the arylsulfatase, however, showed effects, stimulation of 28% at 5 mg a.s./kg dw soil and inhibition of 37% at 10 mg a.s./kg dw soil after 56 days exposure, which still could be observed after prolongation of the exposure period. After 84 days of exposure, the arylsulfatase was affected by more than 25% at all chosen concentrations.

Table 31: Effects of tebuconazole results with application rates of 1, 5 and 10 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Lufa 2.1. as test soil.

Test day	Test system	Test details	Inhibition [%]		
			1 mg/kg	5 mg/kg	10 mg/kg
Test initiation D0	ISO 15685 MicroResp™	-	-45	-25	-31
		Deionized water	3	3	-5
		D-(+)-Glucose	5	6	-5
		L-Cysteine hydrochloride	4	4	-7
		L-Malic acid	1	1	-8
		y-Amino butyric acid	2	19	11
		N-Acetyl glucosamine	0	0	-9
		Citric acid	-9	-7	-14
		L-Alanine	5	7	0
	ISO 20130	Phosphatase	20	-7	8
		ß-Glucosidase	9	-4	28
		Arylamidase	4	-10	-4

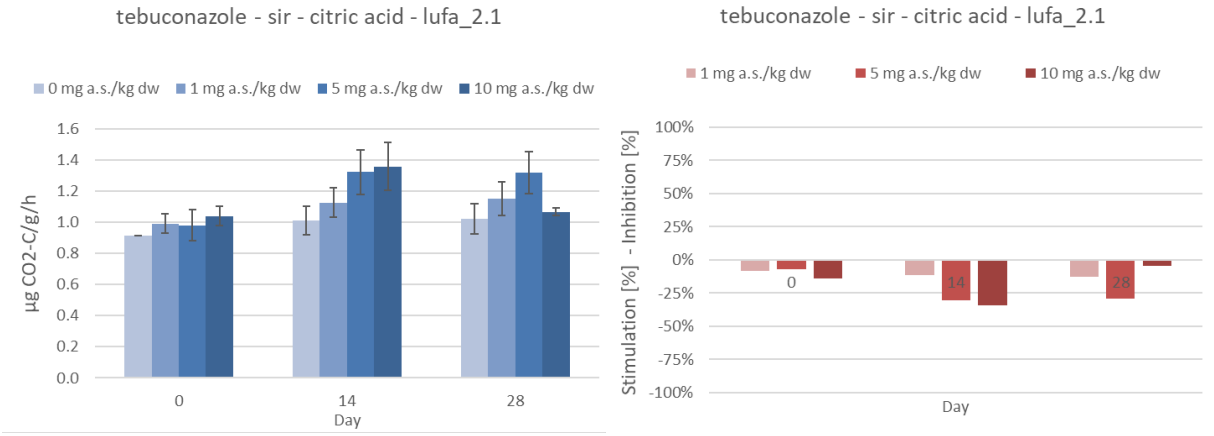
Test day	Test system	Test details	Inhibition [%]		
			1 mg/kg	5 mg/kg	10 mg/kg
		Urease	-1	2	-6
		Arylsulfatase	14	2	52
D14	ISO 15685	-	2	-31	-13
	MicroResp™	Deionized water	-7	-6	-4
		D-(+)-Glucose	1	-5	-5
		L-Cysteine hydrochloride	-5	-10	-6
		L-Malic acid	4	-1	-5
		y-Amino butyric acid	8	10	14
		N-Acetyl glucosamine	-3	-13	-13
		Citric acid	-11	-31	-34
		L-Alanine	-5	-12	-11
	ISO 20130	Phosphatase	5	16	13
		ß-Glucosidase	-16	16	-1
		Arylamidase	-20	-21	-10
		Urease	7	7	12
		Arylsulfatase	n. d.	n. d.	n. d.
D28	ISO 15685	-	-3	-45	-19
	MicroResp™	Deionized water	-3	-6	-3
		D-(+)-Glucose	-6	-10	1
		L-Cysteine hydrochloride	-1	-7	2
		L-Malic acid	-2	-12	-2
		y-Amino butyric acid	1	0	15
		N-Acetyl glucosamine	-3	-12	2
		Citric acid	-13	-29	-4
		L-Alanine	-3	-12	2
	ISO 20130	Phosphatase	0	3	1
		ß-Glucosidase	16	-9	14
		Arylamidase	-9	-21	1
		Urease	9	10	10
		Arylsulfatase	n. d.	n. d.	n. d.
D56	ISO 20130	Phosphatase	7	17	2
		ß-Glucosidase	15	29	22
		Arylamidase	16	9	10
		Urease	21	21	20
		Arylsulfatase	-9	-28	-37
D84	ISO 20130	Phosphatase	7	-7	-14
		ß-Glucosidase	13	-11	5
		Arylamidase	13	16	-8
		Urease	-3	-2	-8
		Arylsulfatase	-42	-50	-60

Test day	Test system	Test details	Inhibition [%]		
			1 mg/kg	5 mg/kg	10 mg/kg
D14	*OECD 216 (reference)*	*N-Transformation*	*-*	*-*	*5*
D28			*-*	*-*	*-3*

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

Some of the results for tebuconazole are presented in more detail in Figure 25. No effects were found in the OECD 216, while ISO 15685 revealed stimulation of nitrification on day 14 at all test concentrations, while on day 28, only the medium test concentration (5 mg/kg dw soil) resulted in a stimulation> 25%. In the MicroResp™ assay, only the use of citric acid was stimulated by more than 25% in 5 mg/kg dw soil and 10 mg/kg dw soil on day 14 and only in 5 mg/kg dw soil on day 28.

Figure 25: Substrate induced respiration (left) and corresponding inhibition (right) due to tebuconazole for arylsulfatase (ISO 20130) at application rates of 1, 5 and 10 mg/kg dw soil at day 28 in the test with Lufa 2.1.



Source: Own illustration, Fraunhofer IME

2.4.2.2.2 Soil microbial community structure (ARISA)

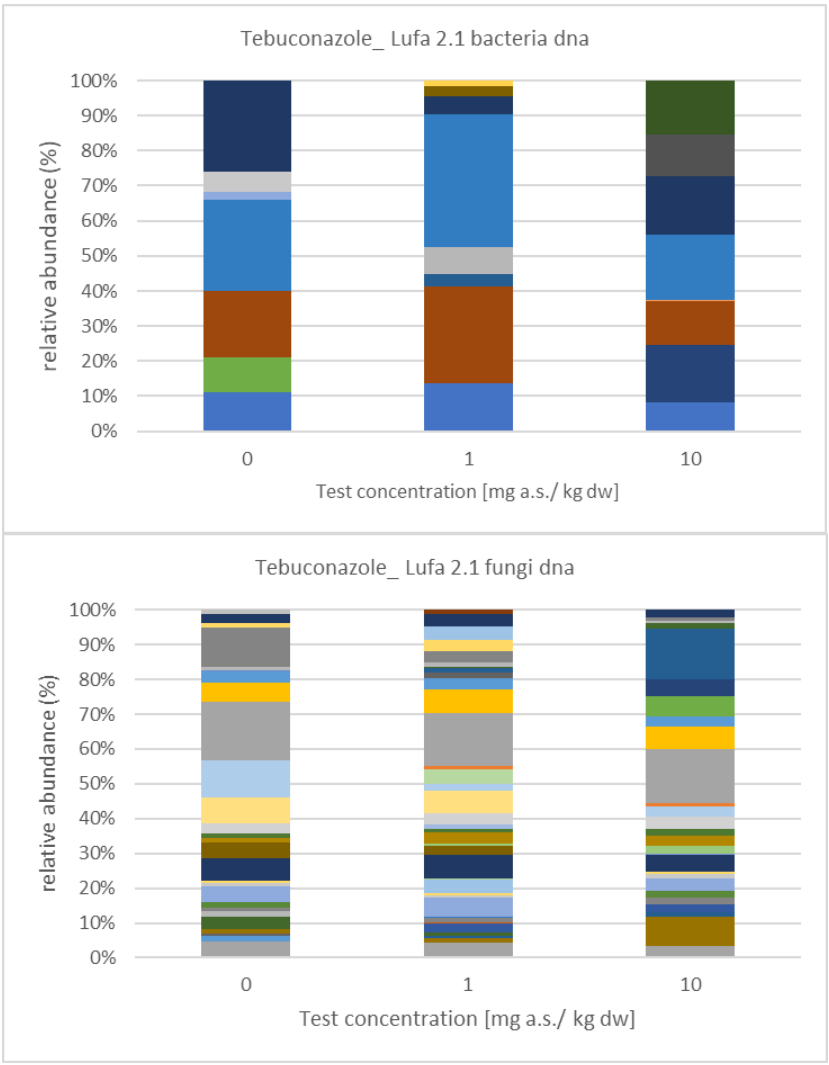
The evaluation of ARISA data revealed shifts in both bacterial and fungal community composition, as illustrated in the relative abundance plot (Figure 26) and showing very distinctive clusters in the CA plot (Figure 27), with noticeable separation of treated samples from the controls. These changes were evident at both the DNA and RNA levels, at a concentration of 1 and 10 mg tebuconazole/kg dry weight soil, indicating a clear response of the microbial communities to this substance. Despite dispersion within replicates, the overall trend shows that the treated samples exhibit distinct microbial profiles compared to the control.

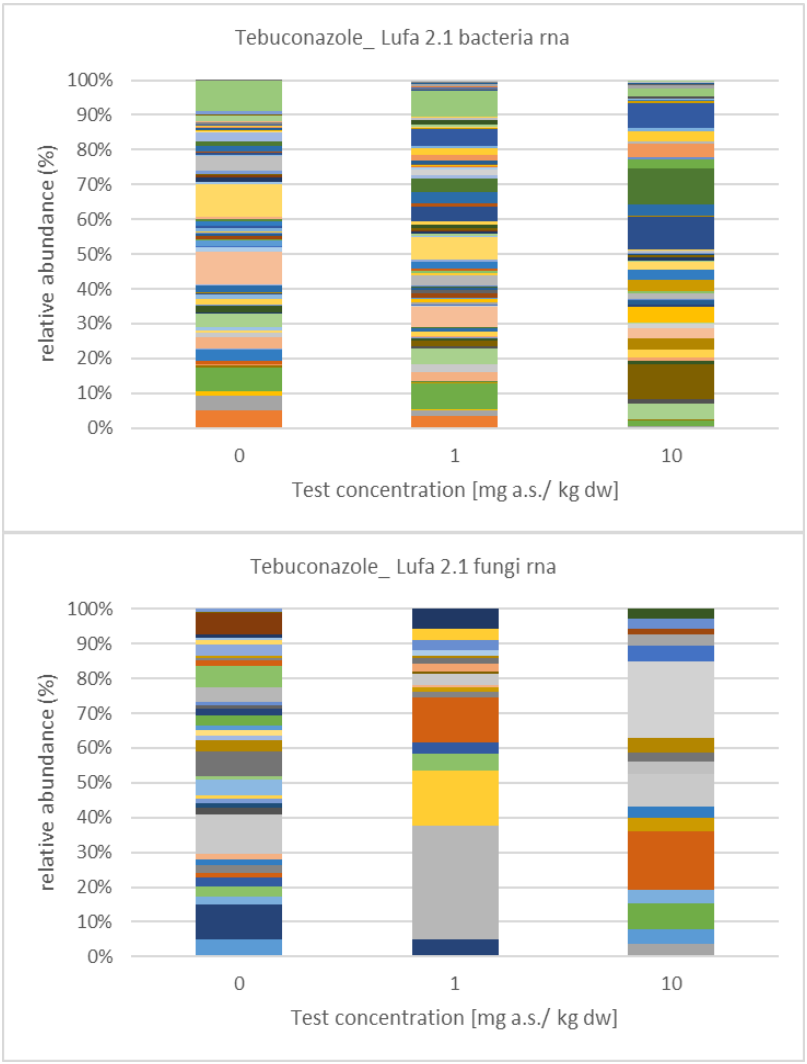
In the relative abundance plot (Figure 27), it is possible to visualize that the shifts in the bacterial communities, at DNA level, change in composition (different OTUs present) without altering necessarily the Shannon index (considering richness and evenness) (Table 32). At RNA level, the bacterial communities profile changes more dramatically in their richness, however not in the evenness generating this way only a small decrease in the Shannon index at both concentrations tested (Table 32).

At the level of fungal DNA, the analysis of the relative abundances is more complex due to the higher number of OTUs observed, and because the Shannon index do not vary significantly, it is difficult to interpret of changes occur by changes in composition, richness or evenness. However, with the CA analysis is clear that the communities are shifted by the application of Tebuconazole

at both concentrations tested. At fungal RNA level, effects are clearly observed at a both concentrations by presenting different relative abundances profiles with a decrease in the number of OTUs observed, and in the Shannon index (most likely as consequence of the biodiversity loss).

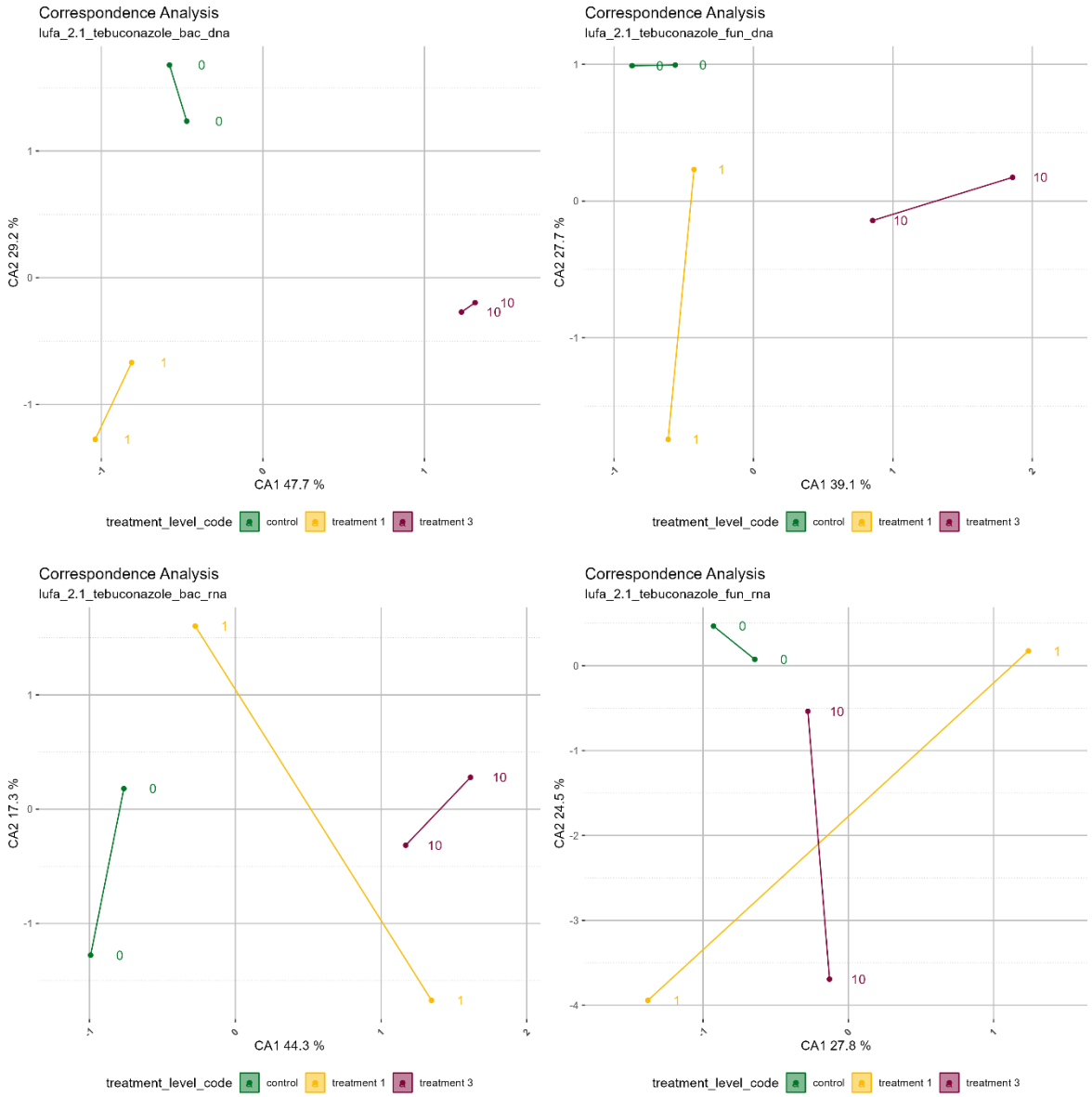
Figure 26: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 1 and 10 mg tebuconazole/kg soil dw in Lufa 2.1. after 28 days.





Source: Own illustration, Fraunhofer IME

Figure 27: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to tebuconazole concentrations of 1 and 10 mg/kg dw soil in Lufa 2.1.



Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), bacteria RNA (bottom left), and fungi RNA (bottom right).

Table 32: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 1 mg/kg and 10 mg/kg tebuconazole in Lufa 2.1 at day 28. Values represent the average of 2 replicates.

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	6.0	0.9	1.7
	1 mg/kg	6.5	0.8	1.6
	10 mg/kg	7.5	1.0	1.9
DNA fungi	Control	22.0	0.9	2.8

	1 mg/kg	27.0	0.9	3.0
	10 mg/kg	24.5	0.9	2.8
RNA bacteria	Control	53.0	0.9	3.4
	1 mg/kg	43.5	0.9	3.4
	10 mg/kg	37.0	0.9	3.2
RNA fungi	Control	22.5	0.9	2.7
	1 mg/kg	9.0	0.9	1.7
	10 mg/kg	10.0	0.9	2.1

2.4.2.2.3 Integrated evaluation

Summary

Based on the results, after approximately 100 days of exposure at the three chosen test concentrations, only the exoenzymatic activity of the arylsulfatase (ISO 20130) was still inhibited by more than 25%, indicating a chronic effect on the soil enzyme. The two other test methods (MicroResp™, ISO 15685) appeared less sensitive than the ISO 20130 for the observed fungicide tebuconazole. However, the results also indicate that even if no effects of more than 25% are observed after 28 days, this does not mean that the test substance might not influence the soil microorganisms after a longer incubation period.

The effect of tebuconazole on the spore germination of *F. mosseae* in accordance with ISO 10832 could not be determined due to the poor spore germination of the AMF in LUFA 2.1.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to tebuconazole exposure, indicating effects in the bacterial and fungal communities at concentrations of 1 and 10 mg a.s./kg dw soil.

Table 33: LOECs for effects of tebuconazole in Lufa 2.1 soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	>10	5	5	>10	1	1
D28-I	>10	>10	>10	>10	1	1
ETO-SI	>10	5	5	1	1	1
ETO-I	>10	>10	>10	5	1	1

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
ERO-SI	-	>10	>10	1	-	-
ERO-I	-	>10	>10	>10	-	-

2.4.2.3 Refesol 04A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to tebuconazole are presented in Table 34.

2.4.2.3.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

The activity of the AOB (ISO 15685) was not affected by tebuconazole by more than 25% deviation from the control level after 14 and 28 days. The ISO 15685 was not performed during the prolonged exposure period.

In the tests with RefeSol 04A, the MicroResp™ system showed an effect of more than 25% neither at day 14 nor day 28 on basal respiration and most of the substrates used for the SIR at the chosen test concentrations. However, after 28 days of exposure, a stimulation of 28% occurred at a test concentration of 20 mg a.s./kg dw soil for the substrate malic acid. Therefore, after 56 days of exposure within the MicroResp™ system, only malic acid was used as a substrate, but no effect above 25% was found, and therefore the test system was not used after 84 days of exposure.

The enzyme activity determined with the ISO 20130 appeared to be the most sensitive compared to the other two test systems. After 14 days of exposure, tebuconazole increased the activity of the urease (48%) and ß-glucosidase (31%) by more than 25% compared to the control. The effect was found at the highest test concentration of 50 mg a.s./kg dw soil.

After 28 days of exposure, the activity of phosphatase, arylsulfatase, and ß-glucosidase was affected by more than 25%, at least in one of the chosen test concentrations. Most affected was the arylsulfatase with a stimulation of 70% at the highest test concentration of 50 mg a.s./kg dw soil. The phosphatase was inhibited by 31% at the lowest and highest test concentrations, with a difference from the control of 14 and 10%, respectively. Therefore, there was no clear concentration dependency. The ß-glucosidase activity was inhibited by 28% at the lowest test concentration of 1.0 mg a.s./kg dw soil. Therefore, the effect on the three enzymes was determined after 56 days of exposure. Arylsulfatase and ß-glucosidase were still affected by more than 25%, while phosphatase was not. However, after a further 28 days of exposure, the arylsulfatase and arylamidase activities were not affected by more than 25%.

Table 34: Effects of tebuconazole with application rates of 1, 5, 10 and 20 mg/kg dw soil in three test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Refesol 04A as test soil.

Test day	Test system	Test details	Inhibition [%]			
			1 mg/kg	5.0 mg/kg	10 mg/kg	50 mg/kg
D14	ISO 15685	-	-15	-12	12	-1

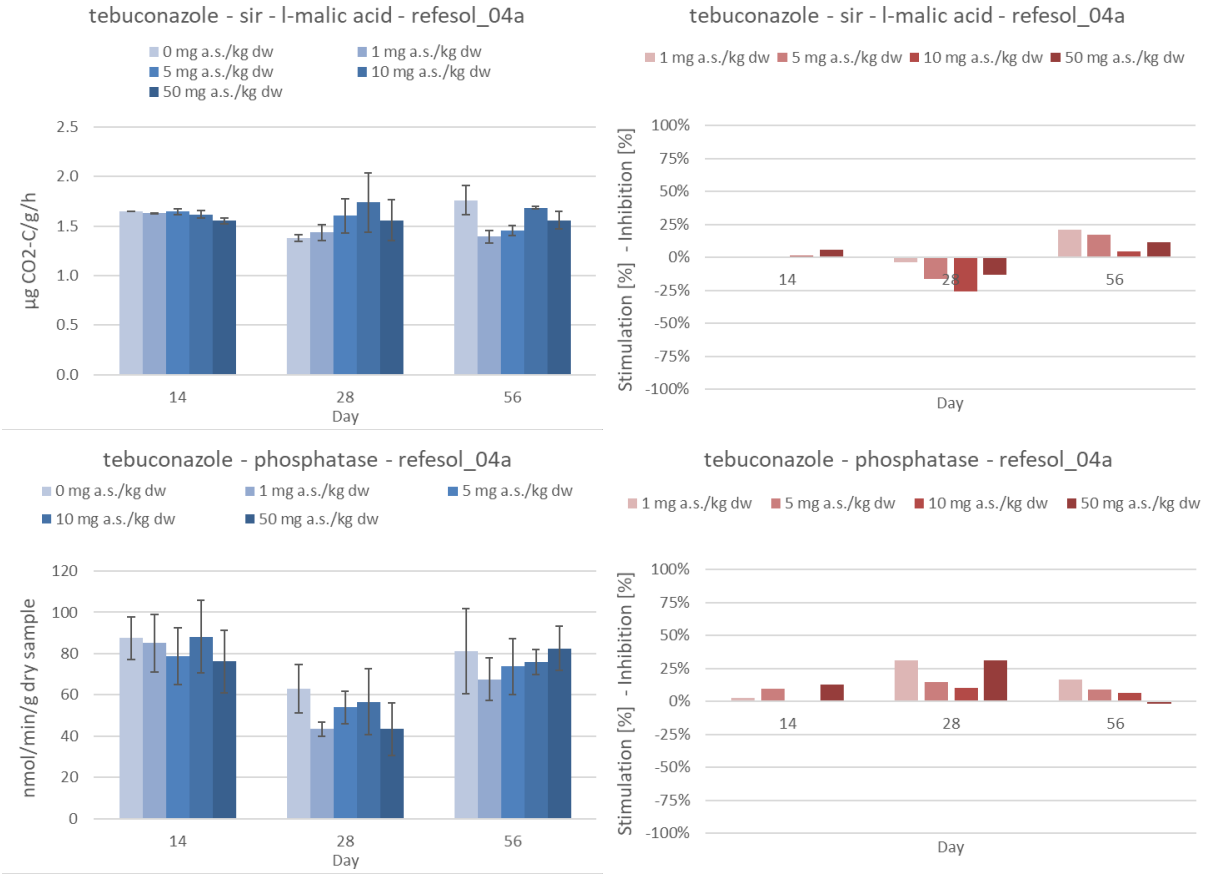
Test day	Test system	Test details	Inhibition [%]			
			1 mg/kg	5.0 mg/kg	10 mg/kg	50 mg/kg
	MicroResp™	Deionized water	4	1	-2	0
		D-(+)-Glucose	5	2	1	4
		L-Cysteine hydrochloride	4	2	4	7
		L-Malic acid	1	0	2	6
		y-Amino butyric acid	-4	-6	1	4
		N-Acetyl glucosamine	-4	-8	0	4
		Citric acid	-11	-10	-1	4
		L-Alanine	-5	-6	1	6
	ISO 20130	Phosphatase	3	10	-1	13
		ß-Glucosidase	1	3	3	-31
		Arylsulfatase	5	-9	-21	-10
		Arylamidase	9	-5	-4	-10
		Urease	-17	15	-20	-48
D28	ISO 15685	-	-4	0	9	1
	MicroResp™	Deionized water	-5	-4	-8	-5
		D-(+)-Glucose	-3	-15	0	0
		L-Cysteine hydrochloride	-6	-13	-6	-3
		L-Malic acid	-4	-16	-26	-13
		y-Amino butyric acid	3	-1	-10	-3
		N-Acetyl glucosamine	-1	-8	-17	-7
		Citric acid	2	0	-15	-6
		L-Alanine	4	-1	-4	2
	ISO 20130	Phosphatase	31	14	10	31
		ß-Glucosidase	28	9	7	-3
		Arylsulfatase	11	0	5	-70
		Arylamidase	-3	0	9	-12
		Urease	1	3	15	17

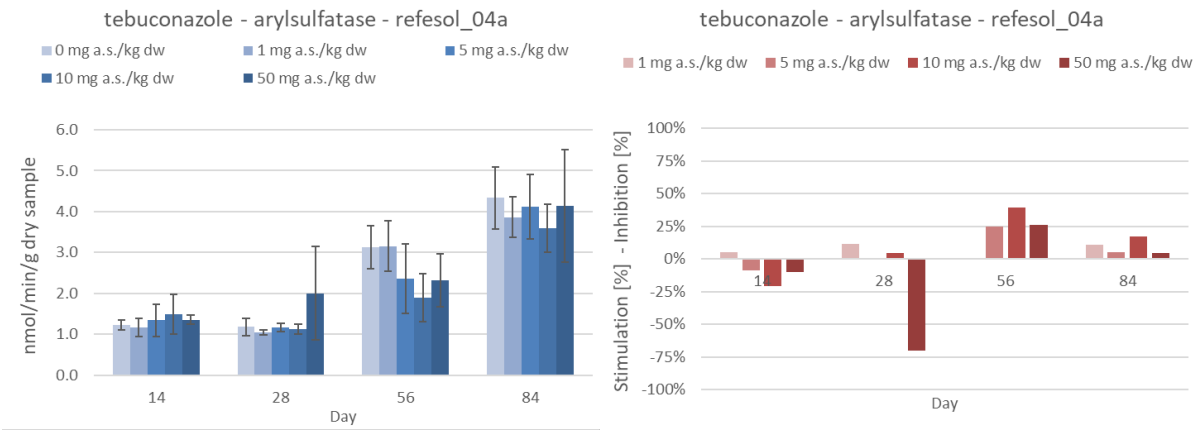
Test day	Test system	Test details	Inhibition [%]			
			1 mg/kg	5.0 mg/kg	10 mg/kg	50 mg/kg
D56	MicroResp™	L-Malic acid	21	17	4	11
	ISO 20130	Phosphatase	17	9	6	-2
		ß-Glucosidase	-6	-40	37	4
		Arylsulfatase	-1	25	40	26
D84	ISO 20130	ß-Glucosidase	8	12	-7	-7
		Arylsulfatase	11	5	17	4
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	-14	-
D28			-	-	4	-

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

As an example, representative results of test methods with observed effects are shown in Figure 28 to obtain a detailed picture of the development of the observed effects.

Figure 28: Example results for effects of tebuconazole on microbial function at application rates of 1, 5, 10 and 50 mg/kg dw soil. Substrate-induced respiration (MicroResp™; upper figure) and exoenzymatic activity (ISO 20130; mid and lowest figure).





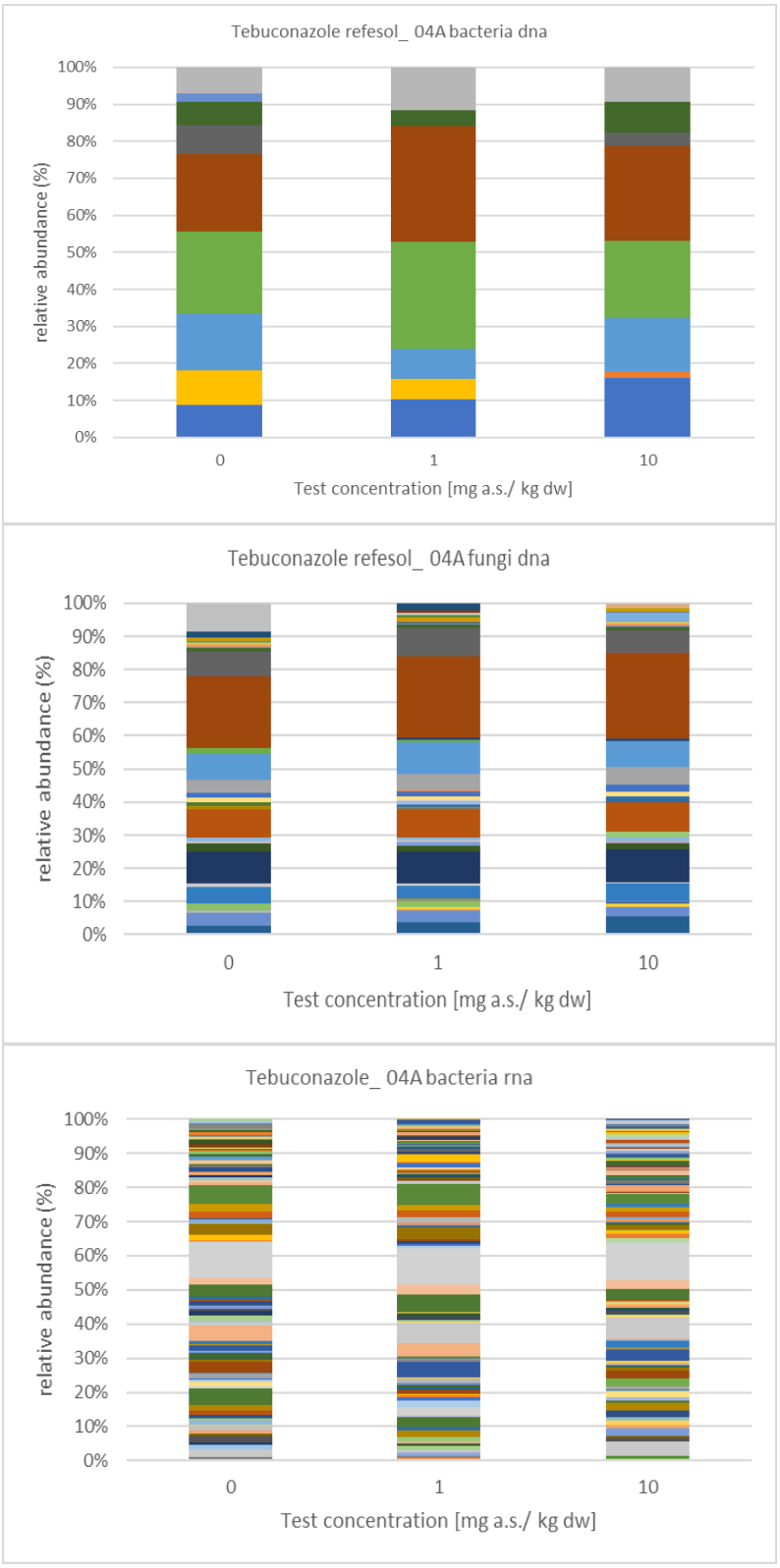
Source: Own illustration, Fraunhofer IME

2.4.2.3.2 Soil microbial community structure (ARISA)

The evaluation of ARISA data showed discrete changes in the bacterial and fungal community composition which can be easily observed in the CA analysis (Figure 30). The changes were observed at DNA and RNA level, in particular at a concentration of 1 and 10 mg tebuconazole/kg dw soil.

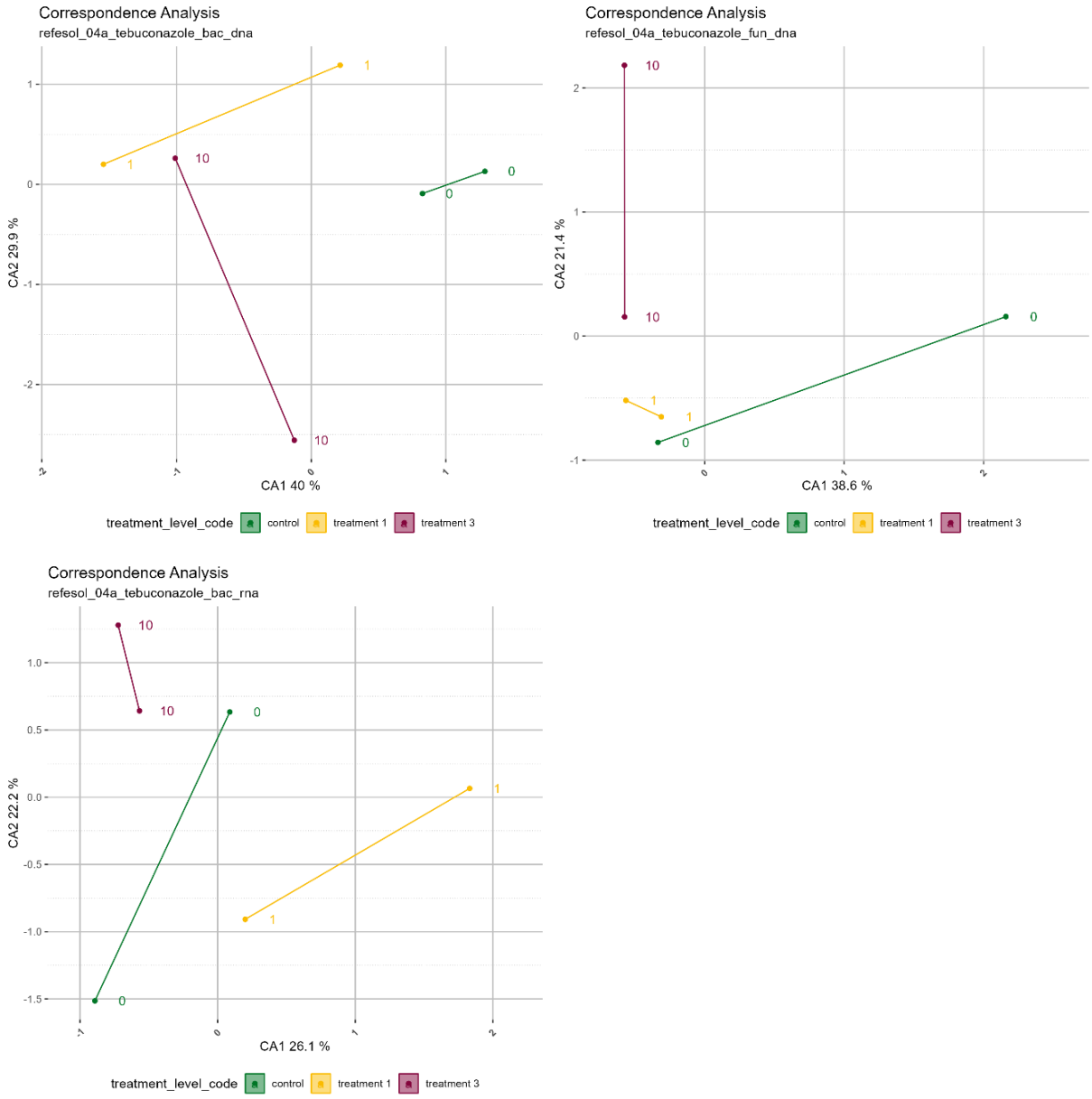
The relative abundance plots (Figure 29), since they contain a high number of OTUs, make visual evaluation difficult. In addition, the Shannon index does not show large variations, except in the case of bacterial DNA where a slight decrease is observed (Table 35). Information regarding diversity and community composition based on RNA data could not be retrieved due to data inconsistency due to the low RNA concentration obtained for the 04A soil samples.

Figure 29: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 1 and 10 mg tebuconazole/kg soil dw in RefeSol 04A after 28 days.



Source: Own illustration, Fraunhofer IME

Figure 30: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to tebuconazole concentrations of 1 and 10 mg/kg dw soil in RefeSol 04A.



Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), and bacteria RNA (bottom left).

Table 35: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 1 mg/kg and 10 mg/kg tebuconazole in Refesol 04A at day 28. Values represent the average of 2 replicates

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	8.5	0.9	2.0
	1 mg/kg	5.5	0.9	1.6
	10 mg/kg	7.0	0.9	1.8
DNA fungi	Control	24.5	0.8	2.7

	1 mg/kg	31.5	0.8	2.8
	10 mg/kg	25.5	0.8	2.6
RNA bacteria	Control	52.0	0.9	3.7
	1 mg/kg	56.0	0.9	3.6
	10 mg/kg	63.5	0.9	3.8

2.4.2.3.3 Integrated evaluation

Summary

Based on the results for tebuconazole in RefeSol 04A, after approximately 100 days of exposure, at the four chosen test concentrations, none of the used test methods (MicroResp™, ISO 15685, ISO 20130) and the thereby observed endpoints were still inhibited by more than 25%. A 4th test concentration was added, but no dose-response relationship could be observed with the applied test methods over time.

The effect of tebuconazole on the spore germination of *F. mosseae* in accordance with ISO 10832 could not be determined due to the poor spore germination of the AMF in RefeSol 04A. The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to tebuconazole exposure. While the Shannon index showed no difference in diversity levels across the treatments, the CA results highlighted that the samples have different compositions. This implies that although the samples are similarly regarding OTU richness and evenness, they might differ in the specific taxa present.

Based on the results, Table 36 summarizes the main results per test system.

Table 36: LOECs for effects of tebuconazole in RefeSol 04A soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	>50	>50	10	1	1	1
D28-I	>50	>50	>50	1	1	1
ETO-SI	>50	>50	10	1	1	1
ETO-I	>50	>50	>50	1	1	1
ERO-SI	-	>50	>50	>50	-	-
ERO-I	-	>50	>50	>50	-	-

2.4.2.4 Refesol 02A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to ethofumesate are presented in Table 37.

2.4.2.4.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

After 28 days of incubation, compared to the control, the AOB (ISO 15685) activity was not affected by more than 25% at the three chosen test concentrations. Therefore, the ISO 15685 was not performed on days 56 and 84.

In the tests with RefeSol 02A, stimulation of the SIR of more than 25% compared to the control was found for most of the used substrates except y-Amino butyric acid, N-acetyl-glucosamine and the basal respiration after 28 days of exposure. Therefore, the test was prolonged to 56 days. No further effect above 25% could be observed, and the test was terminated.

The enzyme activity of the phosphatase and the urease, determined with the ISO 20130, was not affected by more than 25% after 28 days of exposure. The strongest effect after 28 days was observed for the urease activity, with strong stimulations ranging from 56% to 132% compared to the control. The ß-glucosidase activity was stimulated by more than 25% only at the highest test concentration of 10 mg a.s./kg dw soil, while the arylamidase activity was inhibited by 38% and 44% at 5 and 10 mg a.s./kg dw soil, respectively. Therefore, the ISO 20130 was also performed after 56 days of exposure. The ß-glucosidase activity was inhibited by around 40% at all test concentrations, and a comparable effect could be observed for the arylsulfatase (inhibition between 25% and 39%), while the arylamidase was not affected anymore. After 84 days of exposure, only the ß-glucosidase was still affected by more than 25% compared to the control (5 mg a.s./kg dw soil: 30% stimulation), indicating a long-term effect on this exoenzyme activity.

Table 37: Effects of tebuconazole with application rates of 1, 5 and 10 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using RefeSol 02A as test soil.

Test date	Test system	Test details	Inhibition [%]		
			1 mg/kg	5 mg/kg	10 mg/kg
D28	ISO 15685	-	12	5	7
	MicroResp™	Deionized water	-4	-8	6
		D-(+)-Glucose	-36	-39	10
		L-Cysteine hydrochloride	-37	-30	12
		L-Malic acid	-111	-95	14
		y-Amino butyric acid	8	-4	15
		N-Acetyl glucosamine	-6	-15	12
		Citric acid	-285	-121	9
		L-Alanine	-28	-32	2
	ISO 20130	Phosphatase	-14	-7	5

Test date	Test system	Test details	Inhibition [%]		
			1 mg/kg	5 mg/kg	10 mg/kg
		ß-Glucosidase	-23	-20	-58
		Arylsulfatase	-132	-56	-84
		Arylamidase	-23	38	44
		Urease	16	9	-1
D56	MicroResp™	Deionized water	-3	14	7
		D-(+)-Glucose	-8	14	6
		L-Cysteine hydrochloride	-10	12	6
		L-Malic acid	-12	13	5
		y-Amino butyric acid	-12	10	6
		N-Acetyl glucosamine	-12	12	7
		Citric acid	-12	18	6
		L-Alanine	-12	15	6
	ISO 20130	ß-Glucosidase	45	43	44
		Arylsulfatase	39	25	30
		Arylamidase	-15	13	-4
D84	ISO 20130	ß-Glucosidase	4	-30	-13
		Arylsulfatase	21	4	14
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	*-10*
D28			-	-	*-1*

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

Results of MicroResp™ and ISO 20130 regarding the effect of tebuconazole on substrate-induced respiration and the exoenzymatic activity were chosen exemplarily to demonstrate certain effects on the soil microorganisms and their function.

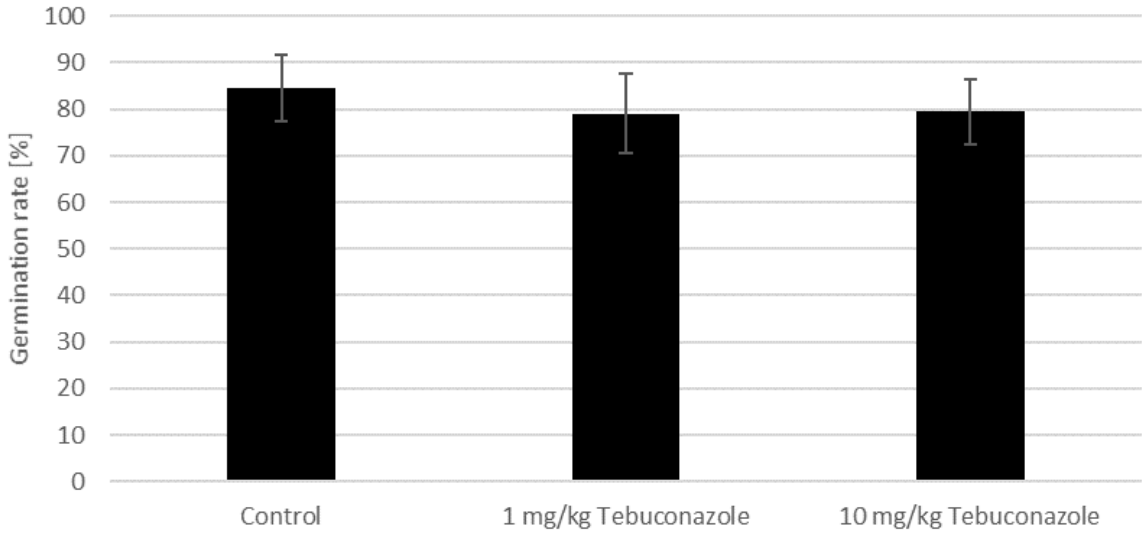
Figure 31: Exemplary results for effects of tebuconazole on the microbial function in RefeSol 02A at application rates of 1, 5 and 10 mg/kg dw soil. SIR with citric acid (MicroResp™, upper figure) and exoenzymatic activity (ISO 20130) of arylsulfatase (mid figure) and arylamidase (lower figure).

Source: Own illustration, Fraunhofer IME

2.4.2.4.2 Arbuscular mycorrhizal fungi (ISO 10832)

For the test with the fungicide tebuconazole, besides the control, two concentrations of 1 mg a.s./kg dw soil (PEC) and 10 mg a.s./kg dw soil (10x PEC) were tested. Due to the high recovery rate (see Table 153) the test fulfilled the validity criteria. The two chosen test concentrations did not affect the spore germination after 14 days of exposure (Figure 32). There was no statistically significant difference to the control at the two test concentrations (PEC: inhibition of 6.3%; 10x PEC: inhibition of 5.9%). Therefore, the determined NOEC appeared to be higher than or equal to the highest test concentration of 10 mg a.s./kg dw soil.

Figure 32: Results of the spore germination test with tebuconazole at concentrations of 1 and 10 mg/kg dw soil in RefeSol 02A after 14 days of incubation.



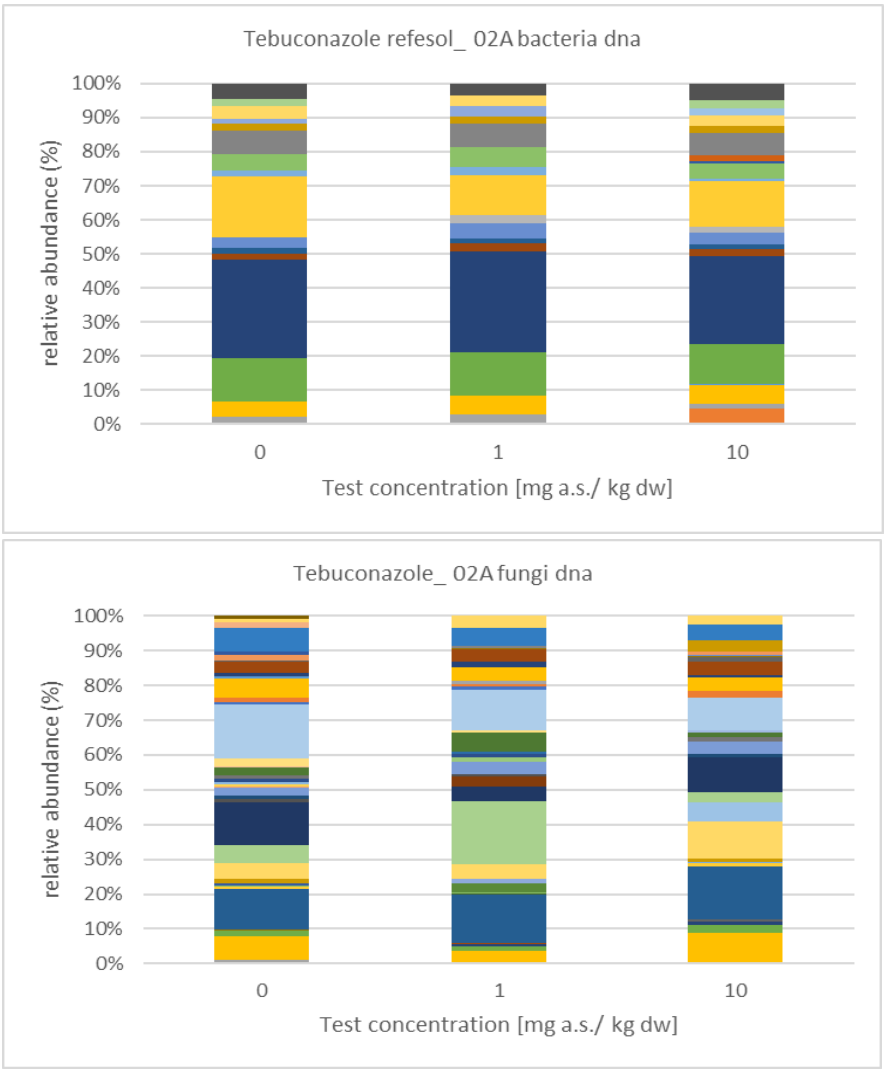
Source: Own illustration, Fraunhofer IME

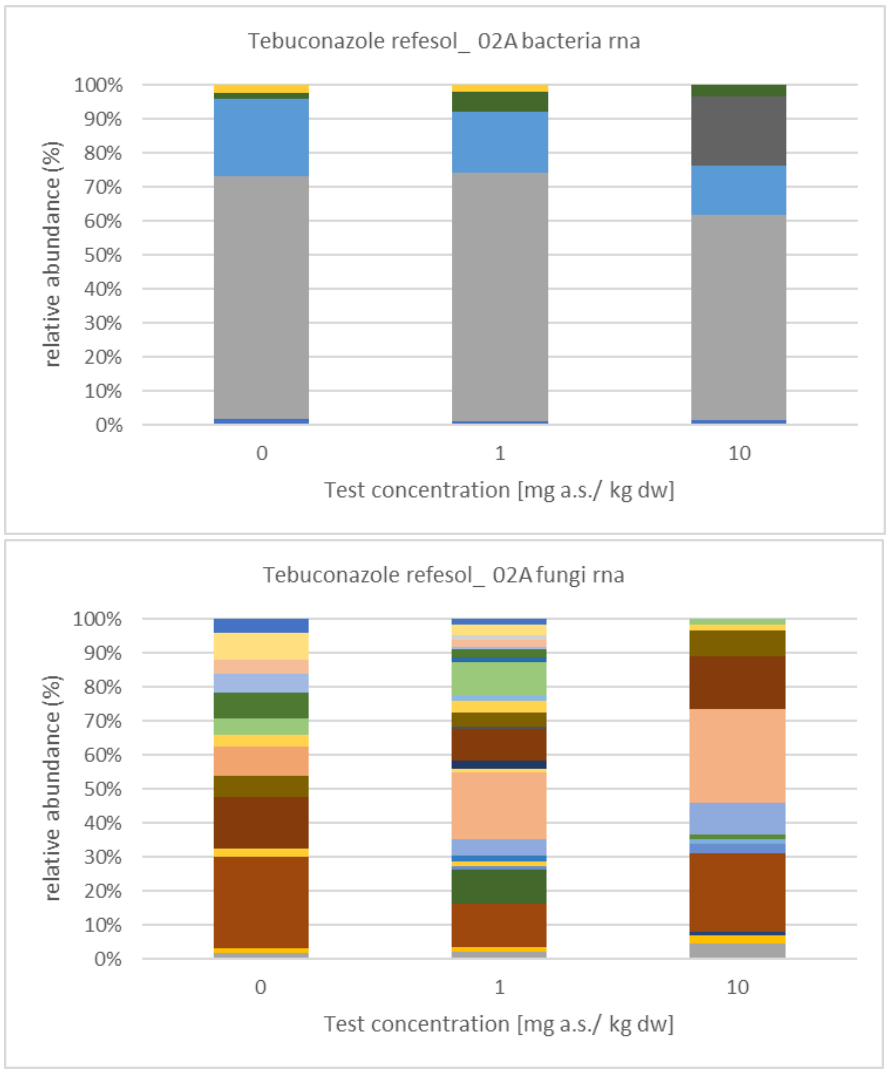
2.4.2.4.3 Soil microbial community structure (ARISA)

The evaluation of ARISA data showed changes in the bacterial and fungal community composition which can be observed in the relative abundances plot (Figure 33) and CA analysis (Figure 34). The changes were observed at DNA and RNA level, in particular at a concentration of 10 mg tebuconazole/kg dw soil. Despite the dispersion observed in the sample replicates (Figure 34), it can be observed that the changes in the community composition are associated to a change in the number OTUs and its composition, being reflected in the Shannon index. The decrease in the Shannon diversity index for the fungal community at RNA level appears to be related to a reduction in OTUs richness (or the number of observed taxa) and evenness indicating some variation in taxa abundance caused by the tebuconazole treatment (Table 38). No changes in this index were observed for the bacteria at both DNA and RNA levels.

In the relative abundance plot (Figure 33), the shifts in microbial composition are visualized as changes in the proportion of specific OTUs, reflecting altered taxa prevalence under the influence of tebuconazole, being in particular evident at a concentration of 10 mg a.s./kg dw soil, at RNA level. Similarly, the CA analysis (Figure 34) highlights these compositional shifts by clustering samples according to their community structure, with noticeable separation of treated samples from the controls. Despite dispersion within replicates, the overall trend shows that the treated samples exhibit distinct microbial profiles compared to the control.

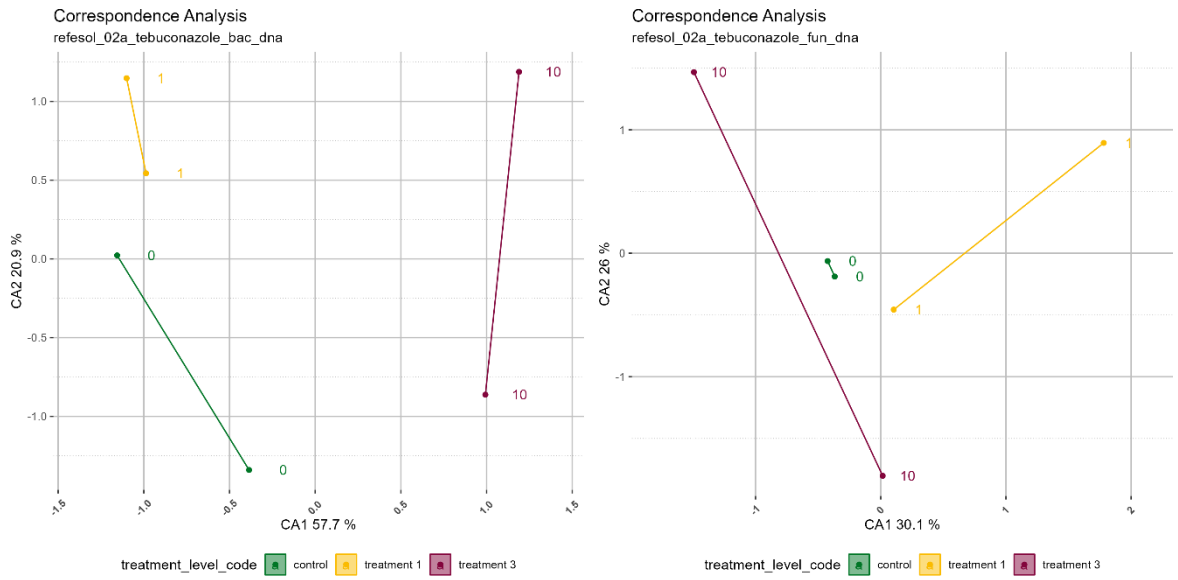
Figure 33: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 1 and 10 mg tebuconazole/kg soil dw in RefeSol 02A after 28 days.

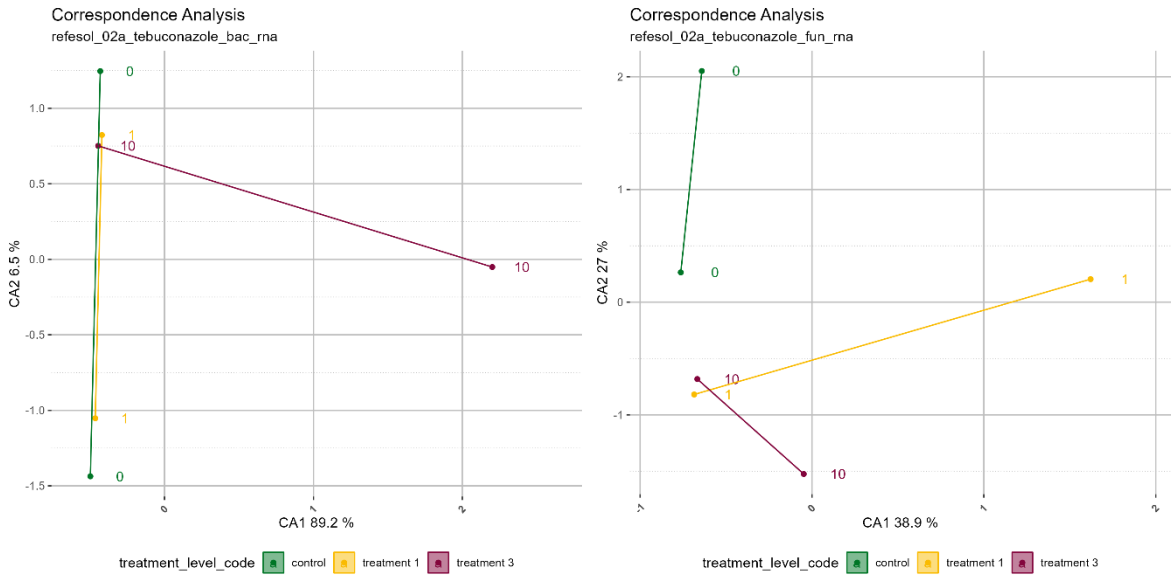




Source: Own illustration, Fraunhofer IME

Figure 34: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to tebuconazole if 1 and 10 mg/kg dw soil in RefeSol 02A.





Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), bacteria RNA (bottom left), and fungi RNA (bottom right).

Table 38: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 1 mg/kg and 10 mg/kg tebuconazole in Refesol 02A at day 28. Values represent the average of 2 replicates

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	15.0	0.8	2.3
	1 mg/kg	16.0	0.8	2.4
	10 mg/kg	19.5	0.9	2.5
DNA fungi	Control	29.5	0.9	2.9
	1 mg/kg	22.0	0.9	2.6
	10 mg/kg	21.0	0.9	2.6
RNA bacteria	Control	8.0	0.7	1.5
	1 mg/kg	8.0	0.7	1.5
	10 mg/kg	9.0	0.7	1.6
RNA fungi	Control	12.0	0.9	2.2
	1 mg/kg	17.0	0.9	2.5
	10 mg/kg	9.5	0.8	1.9

2.4.2.4.4 Integrated evaluation

Summary

Based on the results with tebuconazole in RefeSol 02A after approx. 100 days of exposure, the exoenzymatic activity of the ß-glucosidase (ISO 20130) was still inhibited by more than 25% (30% stimulation at 5 mg a.s./kg dw soil), indicating a chronic effect on the soil enzyme. The two other test methods (MicroResp™, ISO 15685) appeared less sensitive than the ISO 20130.

The spore germination test with *F. mosseae* showed no statistically significant effect at the two chosen test concentrations (PEC, 10x PEC).

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to tebuconazole exposure. The results indicate changes on the structural bacterial and fungal diversity in particular at 10 mg a.s./kg dw soil. Fungal diversity showed changes already at 1 mg/kg.

Based on the results, Table 39 summarizes the main results per test system.

Table 39: LOECs for effecs of tebuconazole in RefeSol 02A soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ISO 10832	ARISA CA	ARISA Alpha diversisy
D28-SI	>10	>10	1	1	>10	10	1
D28-I	>10	>10	>10	5	>10	>10	10
ETO-SI	>10	>10	1	1	>10	10	1
ETO-I	>10	>10	>10	1	>10	10	10
ERO-SI	-	>10	>10	>10	>10	-	-
ERO-I	-	>10	>10	>10	>10	-	-

[a]: Please note that the test duration for the spore germination test with *F. mosseae* (ISO 10832) was 14 days, and therefore, the values presented are the D14-I and D14-SI values.

2.4.3 Pyraclostrobin

2.4.3.1 Standard test results

In loamy sand, pyraclostrobin inhibited nitrification (OECD 216) by 48 and 69% after 14 days of exposure and by 20 to 36% after 28 days at test concentrations of 2.4 and 24 mg product/kg dw soil. The exposure period was increased to 56 days, as described in the OECD 216, and no inhibition could be observed after the prolongation. No inhibition above 10% was found

throughout the test in loamy silt. In the OECD 216 limit test, no effects above 25% (stimulation or inhibition) were found after 14 and 28 days of exposure in the three chosen soils (Table 40).

2.4.3.2 Lufa 2.1.

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity due to pyraclostrobin are presented in Table 40.

2.4.3.2.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

The AOB (ISO 15685) appeared to be not affected by pyraclostrobin at the chosen test concentrations of 3 to 15 mg a.s./kg dw soil. At test initiation, the activity of the AOB was inhibited by 16 % at the highest test concentration, which is still below the threshold value of 25% as stated in the OECD 216 and the observed effects on the nitrification as described previously. After 14 days, an effect of 39% (stimulation) occurred at 15 mg a.s./kg dw soil. However, after 28 days of exposure, no difference of more than 25% compared to the control was determined with the ISO 15685.

Throughout the exposure period of 28 days (including measurements at test initiation and day 14), no effects above 25% on the SIR (MicroResp™) were determined with any of the used eight substrates.

Regarding the enzyme activity (ISO 20130), an effect of more than 25% compared to the control occurred only on the activity of the arylsulfatase at 30 mg a.s./L (-31%), while after 14 days, only the ß-glucosidase was inhibited by more than 25% at a concentration of 15 mg a.s./kg dw soil (28%). After 28 days of exposure, the arylamidase was inhibited by 16 to 21% and the urease by 13 to 15% without a concentration dependency. Independent of the low effects (<25%), the test was prolonged to investigate if, after long-term incubation, effects might occur. Here, after 56 days, only the urease activity was inhibited by 29% to 39% at 3 to 30 mg a.s./kg dw soil, respectively. After 84 days, no effect above 25% on any enzyme activity was observed.

Table 40: Effects of pyraclostrobin with application rates of 3, 15 and 30 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Lufa 2.1. as test soil.

Test day	Test system	Test details	Inhibition [%]		
			3 mg/kg	15 mg/kg	30 mg/kg
Test initiation D0	ISO 15685	-	9	-3	16
	MicroResp™	Deionized water	0	8	-3
		D-(+)-Glucose	-2	3	-1
		L-Cysteine hydrochloride	2	6	4
		L-Malic acid	0	1	5
		y-Amino butyric acid	4	12	15
		N-Acetyl glucosamine	0	1	4
		Citric acid	-10	-4	-1
		L-Alanine	-1	5	0

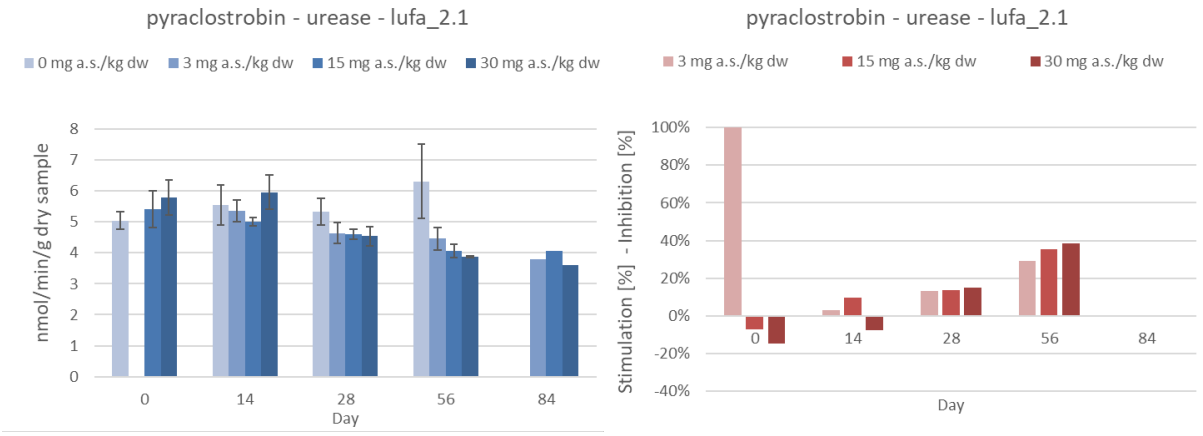
Test day	Test system	Test details	Inhibition [%]		
			3 mg/kg	15 mg/kg	30 mg/kg
	ISO 20130	Phosphatase	10	22	18
		ß-Glucosidase	-2	14	11
		Arylamidase	4	6	-1
		Urease	20	-7	-15
		Arylsulfatase	-12	-22	-31
D14	ISO 15685	-	1	-39	-20
	MicroResp™	Deionized water	5	-2	-9
		D-(+)-Glucose	7	5	2
		L-Cysteine hydrochloride	4	2	-5
		L-Malic acid	6	12	1
		y-Amino butyric acid	10	19	13
		N-Acetyl glucosamine	6	6	0
		Citric acid	1	0	-4
		L-Alanine	10	13	9
	ISO 20130	Phosphatase	13	8	5
		ß-Glucosidase	17	28	1
		Arylamidase	-1	14	-8
		Urease	3	10	-8
		Arylsulfatase	17	17	6
D28	ISO 15685	-	1	-20	-12
	MicroResp™	Deionized water	-2	0	-8
		D-(+)-Glucose	-4	-4	-5
		L-Cysteine hydrochloride	-3	0	-4
		L-Malic acid	8	-9	-4
		y-Amino butyric acid	0	13	10
		N-Acetyl glucosamine	-1	1	-2
		Citric acid	-15	-11	-10

Test day	Test system	Test details	Inhibition [%]		
			3 mg/kg	15 mg/kg	30 mg/kg
		L-Alanine	-4	0	-3
	ISO 20130	Phosphatase	1	6	2
		ß-Glucosidase	-4	1	4
		Arylamidase	19	16	21
		Urease	13	14	15
		Arylsulfatase	19	9	0
D56	ISO 20130	Phosphatase	6	10	15
		ß-Glucosidase	-1	6	-8
		Arylamidase	4	3	8
		Urease	29	36	39
		Arylsulfatase	11	21	19
D84	ISO 20130	Phosphatase	-3	0	6
		ß-Glucosidase	-4	15	**8**
		Arylamidase	13	3	20
		Urease	19	13	23
		Arylsulfatase	5	-7	-7
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	*12*
D28			-	-	*6*

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

Results of the ISO 20130 regarding the effect of pyraclostrobin on the exoenzymatic activity were chosen exemplarily to demonstrate certain effects on the soil microorganisms and their function. On day 28, no effect on the urease activity was observed; however, after 56 days, a LOEC of 3 mg a.s./kg dw soil was determined. However, there was a comparable effect between 29% and 39% at the three test concentrations (Figure 35). Nevertheless, calculating an EC_{10} and EC_{50} would be possible after 56 days. After 84 days, the effect on the urease activity was below 25% again.

Figure 35: Exoenzyme activity (left) and corresponding inhibition (right) due to pyraclostrobin for urease (ISO 20130) at application rates of 3, 15 and 30 mg/kg dw soil in the test with Lufa 2.1.



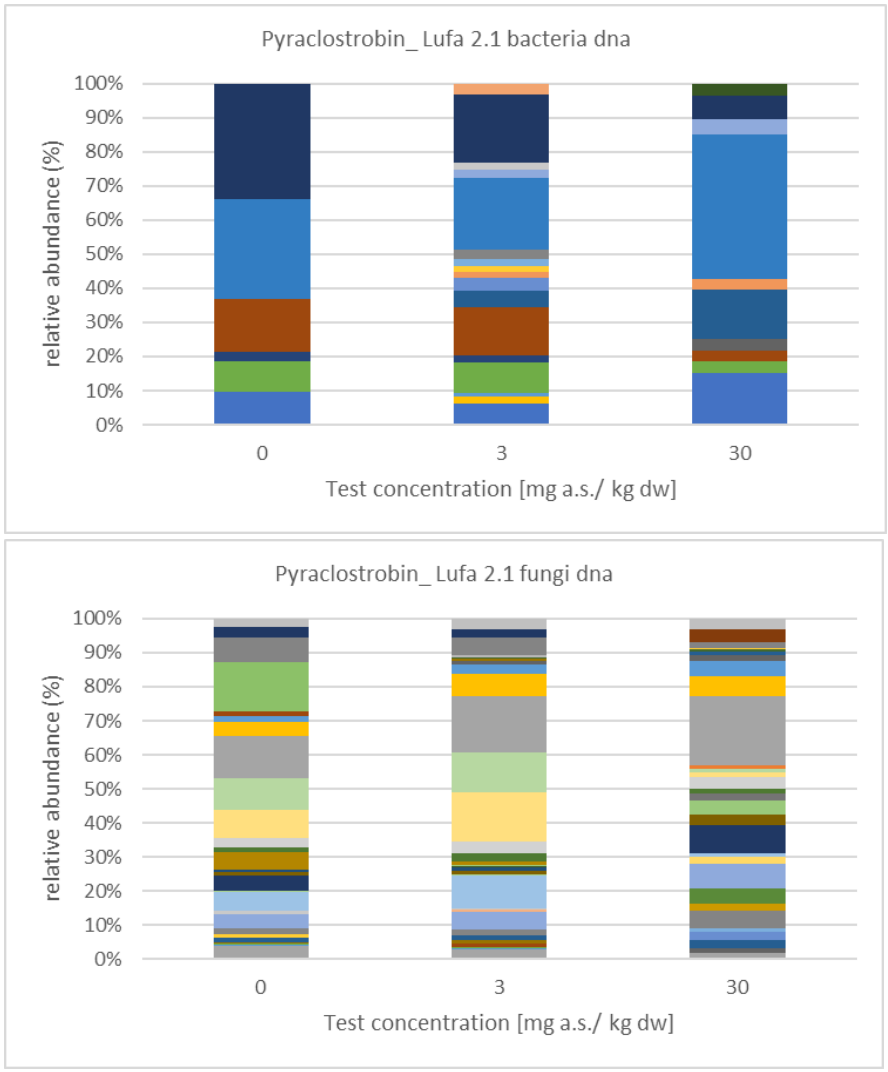
Source: Own illustration, Fraunhofer IME

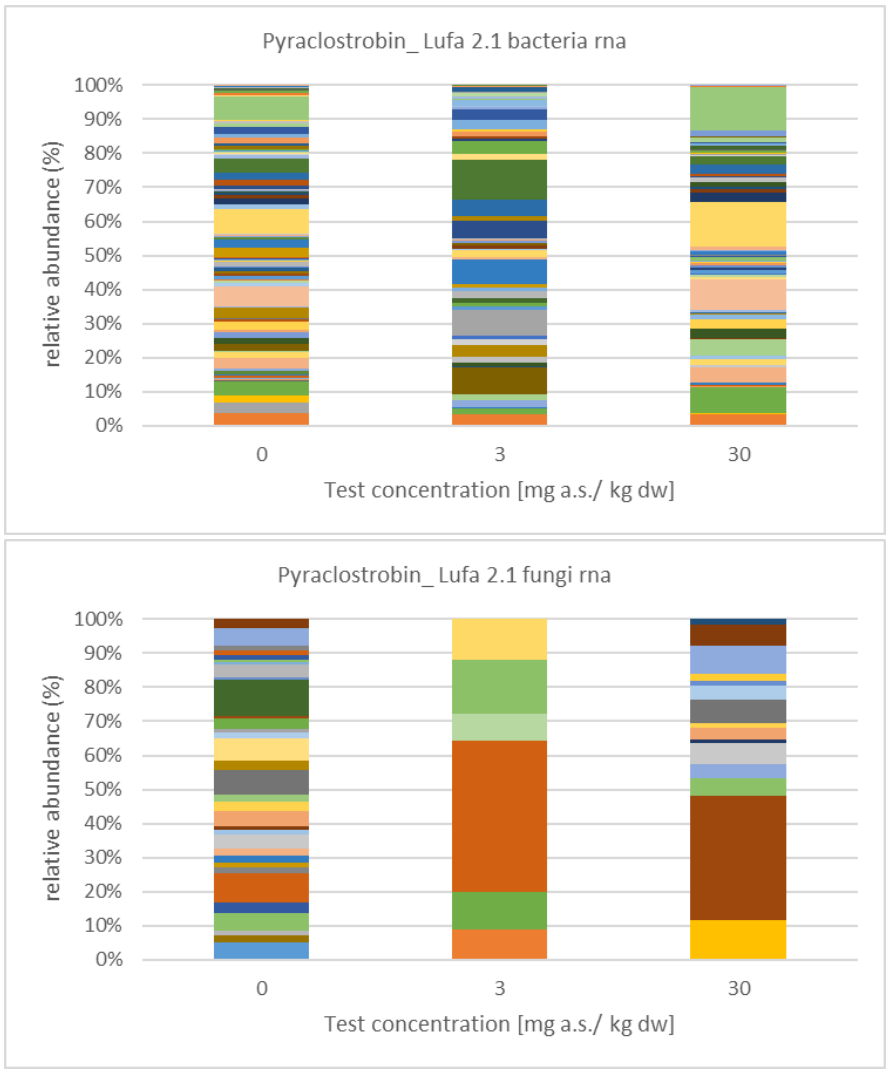
2.4.3.2.2 Soil microbial community structure (ARISA)

The evaluation of ARISA data showed changes in the bacterial and fungal community composition which can be observed in the relative abundances plot (Figure 36) and the CA analysis (Figure 37). The changes were observed at DNA and RNA level, in particular at a concentration of 30 mg pyraclostrobin/kg dw soil. It can be observed that the changes in the community composition are associated to a decrease in the number of OTUs observed and some variation in taxa abundance caused by the pyraclostrobin treatment, being reflected in the Shannon index (Table 41), in particular by a reduction of the index (not concentration-dependent) observed at 3 mg a.s./kg dw soil and at 30 mg a.s./kg dw soil.

In the relative abundance plot (Figure 36), the shifts in microbial composition are visualized as changes in the proportion of specific OTUs, reflecting altered taxa prevalence under the influence of pyraclostrobin. Similarly, the CA analysis (Figure 37) highlights these compositional shifts by clustering samples according to their community structure, with noticeable separation of treated samples from the controls, in particular at the concentration of 30 mg a.s./kg dw soil. Despite dispersion within replicates, the overall trend shows that the treated samples exhibit distinct microbial profiles compared to the control.

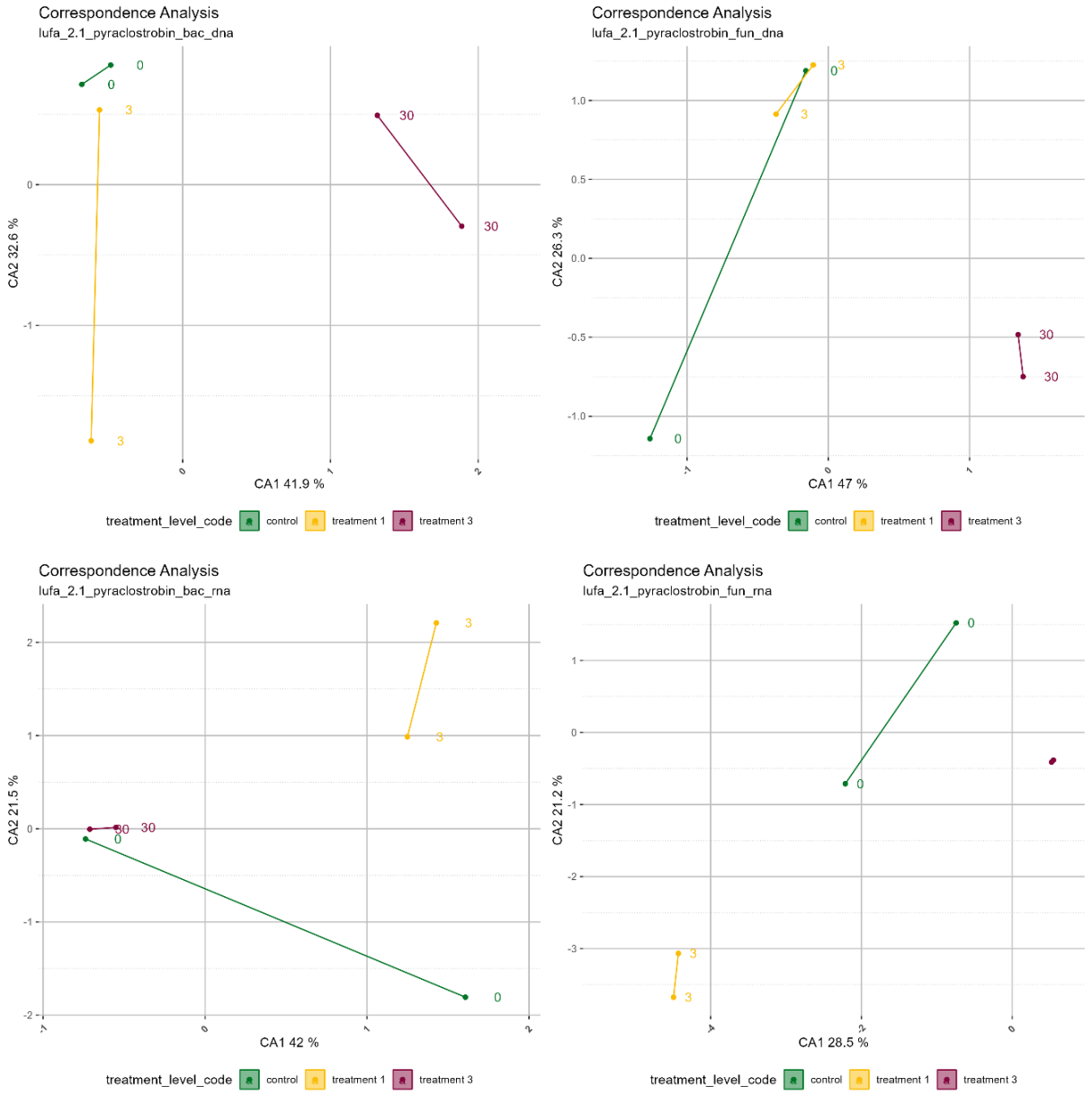
Figure 36: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 3 and 30 mg pyraclostrobin/kg soil dw in Lufa 2.1. after 28 days.





Source: Own illustration, Fraunhofer IME

Figure 37: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to pyraclostrobin concentrations of 3 and 30 mg/kg dw soil in Lufa 2.1.



Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), bacteria RNA (bottom left), and fungi RNA (bottom right).

Table 41: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 3 mg/kg and 30 mg/kg pyraclostrobin in Lufa 2.1 at day 28. Values represent the average of 2 replicates

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	5.5	0.9	1.5
	3 mg/kg	12.5	0.9	2.3
	30 mg/kg	7.5	0.8	1.7

DNA fungi	Control	22.5	0.9	2.7
	3 mg/kg	23.5	0.9	2.7
	30 mg/kg	26.0	0.9	2.9
RNA bacteria	Control	49.5	0.9	3.4
	3 mg/kg	33.5	0.9	3.2
	30 mg/kg	46.0	0.8	3.2
RNA fungi	Control	18.5	0.9	2.6
	3 mg/kg	3.5	0.9	1.2
	30 mg/kg	11.0	0.8	2.0

2.4.3.2.3 Integrated evaluation

Summary

Based on the results, after approximately 100 days of exposure at the three chosen test concentrations, none of the used test methods (MicroResp™, ISO 15685, ISO 20130) and the thereby observed endpoints were inhibited by more than 25%. Again, the results indicate that, even if no effects of more than 25% are observed after 28 days, this does not mean that the test substance might not influence the soil microorganisms after a longer incubation period, as seen for the urease activity after 56 days.

The effect of pyraclostrobin on the spore germination of *F. mosseae* in accordance to ISO 10832 could not be determined due to the poor spore germination of the AMF in LUFA 2.1.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to pyraclostrobin exposure. The results indicate changes on the structural bacterial and fungal diversity at both concentrations tested, more pronounced at 3 mg a.s./kg dw soil.

Based on the results, Table 42 summarizes the main results per test system.

Table 42: LOECs for effects of pyraclostrobin in Lufa 2.1 soil

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	>30	>30	>30	>30	3	3
D28-I	>30	>30	>30	>30	3	3
ETO-SI	>30	15	>30	3	3	3

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
ETO-I	>30	>30	>30	3	3	3
ERO-SI	-	>30	>30	>30	-	-
ERO-I	-	>30	>30	>30	-	-

2.4.3.3 Refesol 04A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to pyraclostrobin are presented in Table 43.

2.4.3.3.1 Soil microbial function (ISO 15685, MicroRespTM, ISO 20130)

The activity of the AOB (ISO 15685) was not affected by pyraclostrobin by more than 25% after 14 and 28 days. The ISO 15685 was not performed during the prolonged exposure period.

In the tests with RefeSol 04A, the MicroResp™ system showed an effect of more than 25% neither at day 14 nor day 28 on basal respiration and most of the substrates used for the SIR at the chosen test concentrations. However, after 28 days of exposure, 51% and 29% stimulation occurred at the two lower test concentrations of 3 mg a.s./kg dw soil and 15 mg a.s./kg dw soil, respectively, for the substrate citric acid. Therefore, after 56 days of exposure within the MicroResp™ system, only citric acid was used as a substrate, and an effect of 26% was determined at 3 mg a.s./kg dw. Therefore, the test was prolonged for a further 28 days, and after 84 days of exposure, no effect above 25% could be detected at any of the observed concentrations.

The enzyme activity determined with the ISO 20130 appeared more sensitive than the other two test systems. After 14 days of exposure, pyraclostrobin increased the arylamidase activity by 31% at 75 mg a.s./kg dw soil. The phosphatase activity was inhibited by 29% at the lowest test concentration of 3 mg a.s./kg dw soil, while the urease was inhibited by 28% to 45% at the three lowest test concentrations. After 28 days of exposure, the urease was most affected, with a concentration-dependent inhibition between 38 and 68%, while the arylsulfatase was stimulated by 59% at the lowest test concentration and 25% at the highest test concentration. Therefore, the effect on the two enzymes was determined after 56 days of exposure. Arylsulfatase and urease were still affected, while phosphatase was not. Therefore, the test was further prolonged, and after a total exposure duration of 84 days, the arylsulfatase activity was still affected by more than 25%. While at the lowest concentration of 3 mg a.s./kg dw soil, an inhibition of 44% was found, at higher concentrations of 30 and 75 mg a.s./kg dw soil, the inhibition was at 28% for each concentration. The results indicate a long-term effect at 3 mg a.s./kg dw soil concentration.

Problems occurred during the measurement of the urease activity that could not be solved within the given time. Therefore, the data presented in Table 43 is not reliable. Nevertheless, the determined effects after 56 days also indicate a long-term effect of pyraclostrobin on the exoenzymatic activity in the soil.

Table 43: Effects of pyraclostrobin with application rates of 3, 15, 30 and 75 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Refesol 04A as test soil.

Test day	Test system	Test details	Inhibition [%]			
			3 mg/kg	15 mg/kg	30 mg/kg	75 mg/kg
D14	ISO 15685	-	8	16	17	14
	MicroResp™	Deionized water	0	-4	-2	0
		D-(+)-Glucose	5	2	2	1
		L-Cysteine hydrochloride	8	7	5	4
		L-Malic acid	8	8	6	4
		y-Amino butyric acid	4	5	2	4
		N-Acetyl glucosamine	4	5	4	3
		Citric acid	-2	2	2	-2
		L-Alanine	1	1	5	2
	ISO 20130	Phosphatase	29	15	-1	-1
		ß-Glucosidase	12	13	13	-1
		Arylsulfatase	-11	1	1	-23
		Arylamidase	7	-5	18	-31
		Urease	28	45	30	-24
D28	ISO 15685	-	7	9	20	6
	MicroResp™	Deionized water	-9	0	0	1
		D-(+)-Glucose	-10	-1	2	-4
		L-Cysteine hydrochloride	-10	-6	-7	8
		L-Malic acid	-1	-2	-1	-5
		y-Amino butyric acid	-4	6	-3	-5
		N-Acetyl glucosamine	-2	-16	-2	5
		Citric acid	-51	-29	1	-1
		L-Alanine	-18	-10	3	5
	ISO 20130	Phosphatase	4	9	13	2
		ß-Glucosidase	11	21	13	19

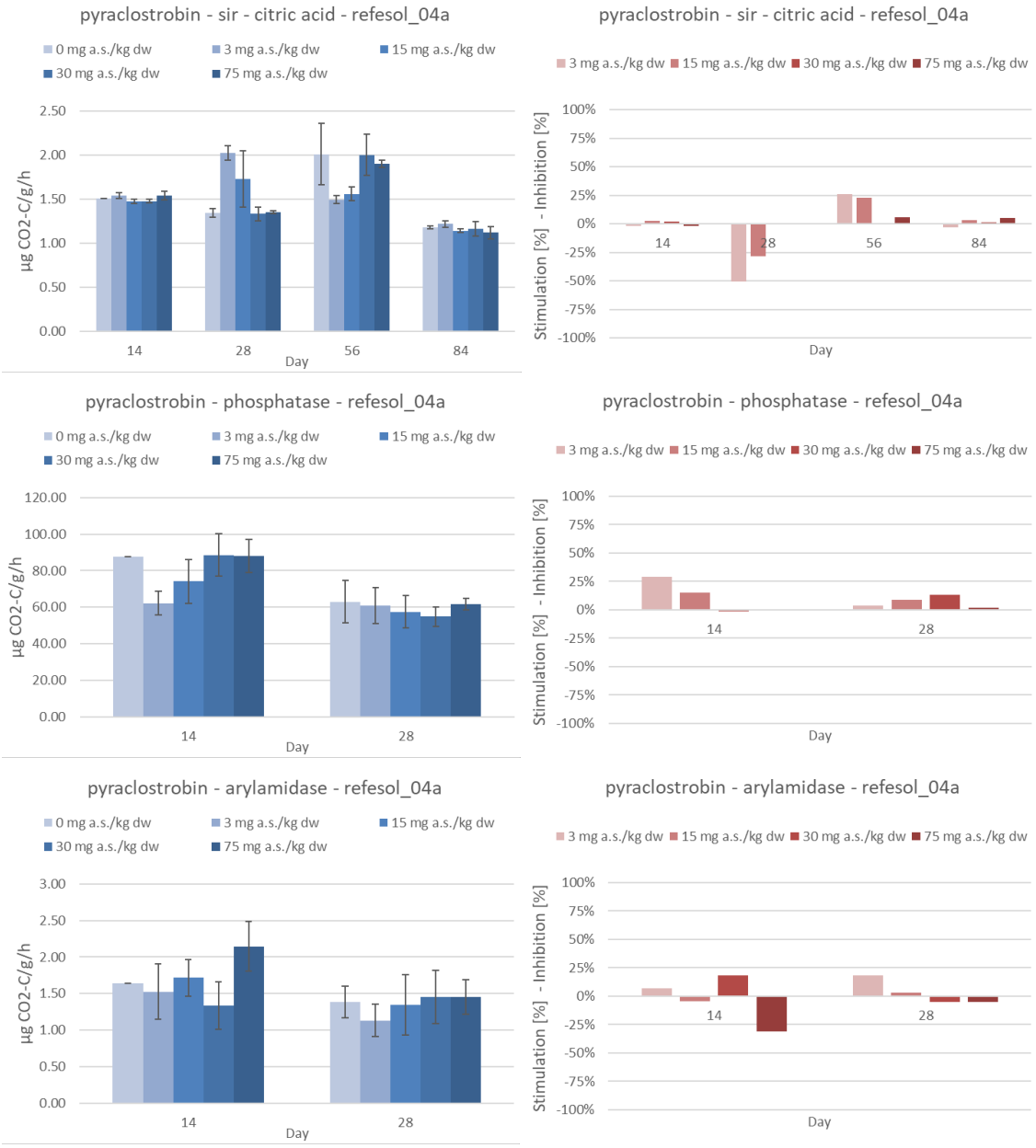
Test day	Test system	Test details	Inhibition [%]			
			3 mg/kg	15 mg/kg	30 mg/kg	75 mg/kg
		Arylsulfatase	-59	10	7	-3
		Arylamidase	18	3	-5	-5
		Urease	38	53	58	68
D56	MicroResp™	Citric acid	26	23	0	6
	ISO 20130	Urease	24	-36	1	-15
		Arylsulfatase	34	3	22	25
D84	MicroResp™	Citric acid	-3	3	1	5
	ISO 20130	Urease	-456*	-342*	-209*	-112*
		Arylsulfatase	44	10	28	28
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	14	-
D28			-	-	30	-

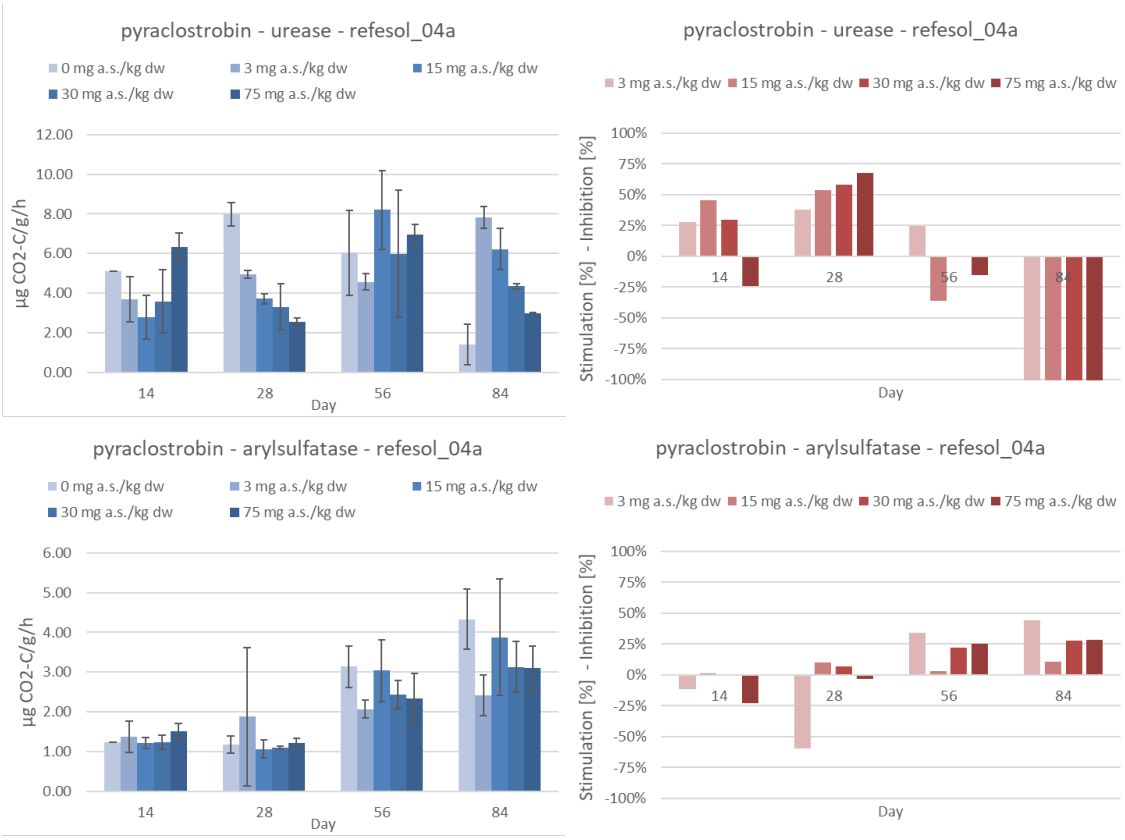
Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

*Note: When determining the urease activity, problems occurred with the colour change in the samples during the test procedure. In general, determination of urease activity was more difficult to perform with RefeSol 04A than with Lufa 2.1. In the present experiment, no valid measurement could be carried out on day 84. The results should, therefore, be viewed with caution.

For example, the representative results of the test methods with the observed effects are shown in Figure 40, which provides a detailed picture of the development of the observed effects. For example, using MicroResp™ with the substrate citric acid on day 28 kind of an adverse stimulation (hormesis) was found, which switched to the strongest inhibition at the lowest test concentration and a decrease of the inhibition with increasing concentrations at day 56, while there was no effect observed after 84 days.

Figure 38: Example results for effects of pyraclostrobin on microbial function at application rates of 3, 15, 30 and 75 mg/kg dw soil. Substrate-induced respiration (MicroResp™; upper figure) and exoenzymatic activity (ISO 20130; mid and lowest figure).



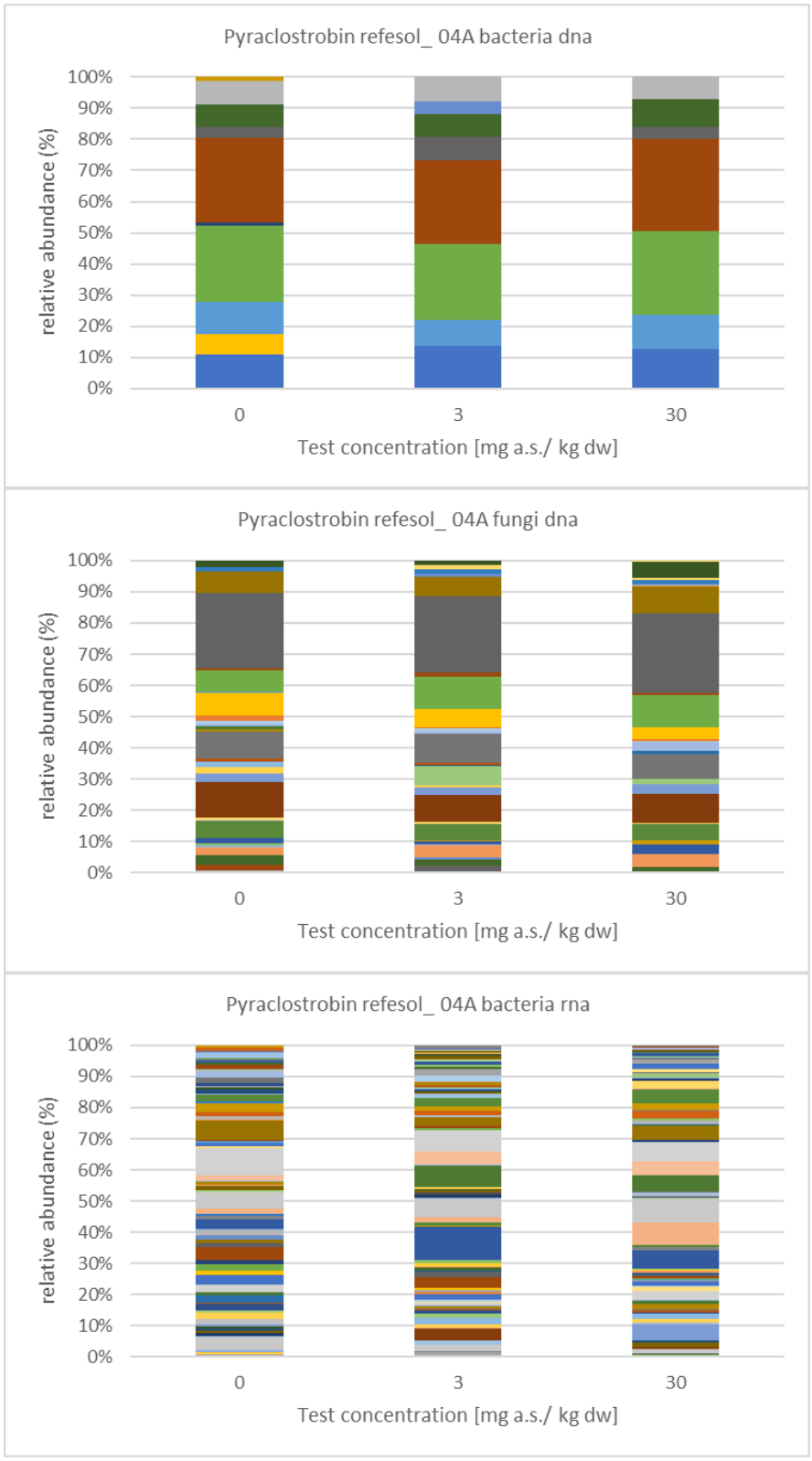


Source: Own illustration, Fraunhofer IME

2.4.3.3.2 Soil microbial community structure (ARISA)

The evaluation of ARISA data showed discrete changes in the bacterial and fungal community composition which can be observed mostly in the CA analysis (Figure 40). The changes were observed at DNA and RNA level, but more noticeable at bacterial RNA level at a concentration of 20 mg epyraclostrobin/kg dw soil. The Shannon index (Table 44) indicates minor or no changes based on the data for bacterial and fungal DNA and bacterial RNA at both concentrations tested (3 mg a.s./kg dw soil and 30 mg a.s./kg dw soil). Data for fungal RNA could not be determined due to unreliable results and the low concentration achieved during extractions.

Figure 39: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 3 and 30 mg pyraclostrobin/kg soil dw in RefeSol 04A after 28 days.



Source: Own illustration, Fraunhofer IME

Figure 40: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to pyraclostrobin concentrations of 3 and 30 mg/kg dw soil in RefeSol 04A.

Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), and bacteria RNA (bottom left).

Table 44: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 3 mg/kg and 30 mg/kg pyraclostrobin in Refesol 04A at day 28. Values represent the average of 2 replicates

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	7.5	0.9	1.8
	3 mg/kg	7.0	0.9	1.8
	30 mg/kg	6.0	0.9	1.6

DNA fungi	Control	24.0	0.8	2.7
	3 mg/kg	24.0	0.8	2.6
	30 mg/kg	21.5	0.8	2.6
RNA bacteria	Control	45.5	0.9	3.5
	3 mg/kg	45.0	0.9	3.5
	30 mg/kg	49.5	0.9	3.5

2.4.3.3.3 Integrated evaluation

Summary

Based on the results with pyraclostrobin in RefeSol 04A, after approx. 100 days of exposure, the exoenzymatic activity of the arylsulfatase (ISO 20130) was still inhibited by more than 25%, while the determined urease activity also indicated an inhibition at the chosen test concentration. The two other test methods (MicroResp™, ISO 15685) appeared less sensitive than the ISO 20130 for the fungicide pyraclostrobin in RefeSol 04A.

The effect of pyraclostrobin on the spore germination of *F. mosseae* in accordance with ISO 10832 could not be determined due to the poor spore germination of the AMF in RefeSol 04A.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to pyraclostrobin exposure. The results indicate changes on the structural bacterial and fungal diversity at both concentrations tested.

Based on the results, Table 45 summarizes the main results per test system.

Table 45: LOECs for effects of pyraclostrobin in RefeSol 04A soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	30	>75	3	3	3	>30
D28-I	30	>75	>75	3	3	>30
ETO-SI	30	>75	3	3	3	>30
ETO-I	30	>75	3	3	3	>30
ERO-SI	-	>75	>75	3	-	-
ERO-I	-	>75	>75	3	-	-

2.4.3.4 Refesol 02A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to ethofumesate are presented in Table 46.

2.4.3.4.1 Soil microbial function (ISO 15685, MicroRespTM, ISO 20130)

After 28 days of incubation, compared to the control, the AOB (ISO 15685) activity was not affected by more than 25% at the three chosen test concentrations. Therefore, the ISO 15685 was not performed on days 56 and 84.

In the tests with RefeSol 02A, a stimulation of more than 25% SIR compared to the control was not found using the seven different substrates for the SIR and deionized water for basal respiration after 28 days of exposure. Therefore, MicroResp™ was not performed on days 56 and 84.

The enzyme activity of all the observed enzymes determined within the ISO 20130 was affected by more than 25% after 28 days of exposure. While ß-glucosidase and arylsulfatase were stimulated, phosphatase, arylamidase and urease activity were inhibited. There was no concentration-dependent effect except for phosphatase. Therefore, the ISO 20130 was also performed after 56 days of exposure. Here, phosphatase activity was still inhibited by more than 25% at the two highest test concentrations, while the arylamidase was inhibited by 58% at 15 mg a.s./kg dw soil. The arylsulfatase was stimulated by more than 25% at all test concentrations but without a concentration-response relationship. No effect was observed on the activity of the ß-glucosidase and urease activity. After 84 days of exposure, the exoenzyme activity of all tested enzymes, except the urease activity, was inhibited by more than 25%. No concentration-response relationship was observed, but the results indicate a long-term effect of pyraclostrobin on the exoenzymatic activities

Table 46: Effects of pyraclostrobin with application rates of 3, 15 and 30 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using RefeSol 02A as test soil.

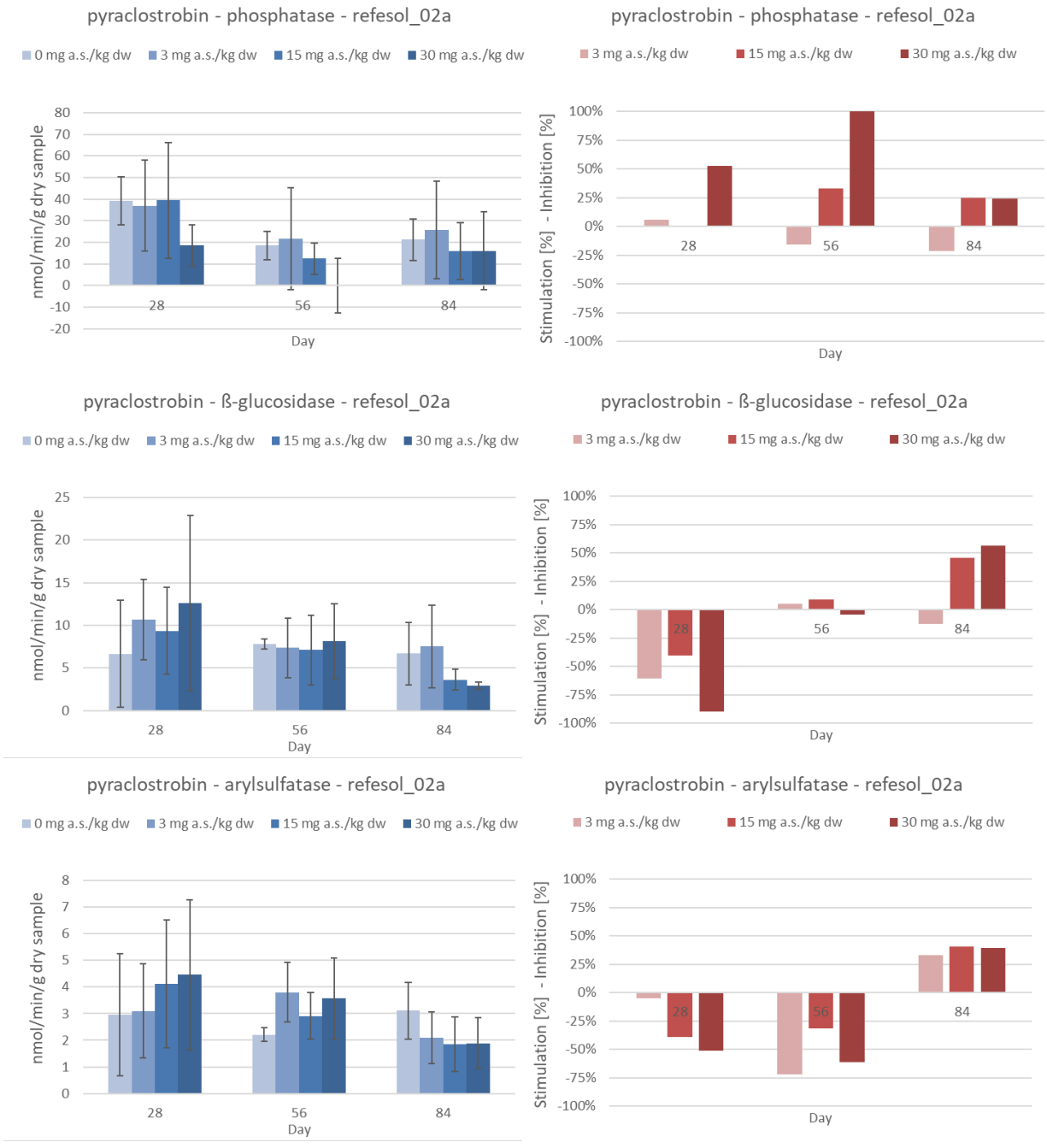
Test date	Test system	Test details	Inhibition [%]		
			3 mg/kg	15 mg/kg	30 mg/kg
D28	ISO 15685	-	1	8	21
	MicroResp™	Deionized water	15	3	15
		D-(+)-Glucose	12	0	14
		L-Cysteine hydrochloride	8	-2	15
		L-Malic acid	5	-5	16
		y-Amino butyric acid	0	-12	20
		N-Acetyl glucosamine	-6	-18	19
		Citric acid	-8	-23	19
		L-Alanine	-7	-24	22
	ISO 20130	Phosphatase	6	-1	53

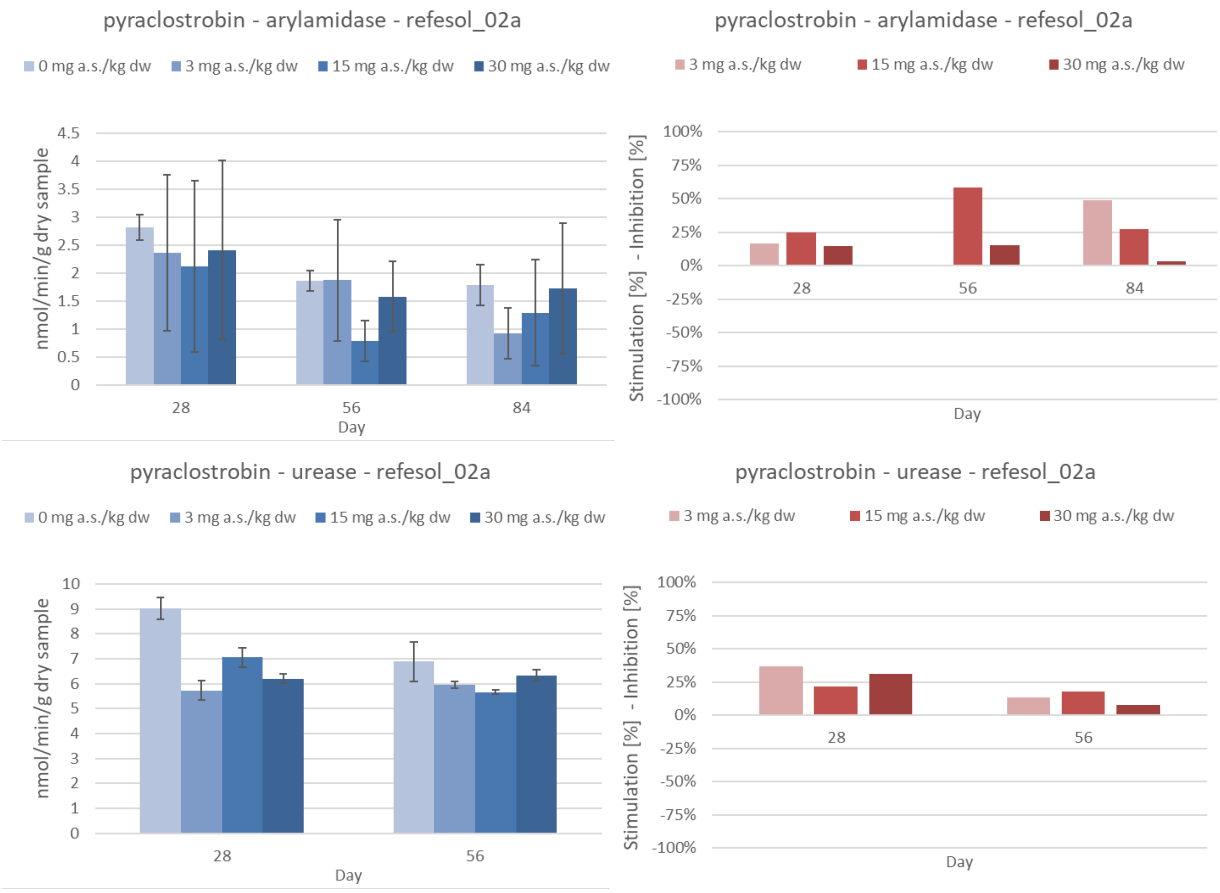
Test date	Test system	Test details	Inhibition [%]		
			3 mg/kg	15 mg/kg	30 mg/kg
		ß-Glucosidase	-61	-41	-89
		Arylsulfatase	-5	-39	-51
		Arylamidase	16	25	14
		Urease	37	22	31
D56	ISO 20130	Phosphatase	-16	33	100
		ß-Glucosidase	5	9	-5
		Arylsulfatase	-72	-32	-61
		Arylamidase	0	58	16
		Urease	14	18	8
D84	ISO 20130	Phosphatase	-21	25	24
		ß-Glucosidase	-13	46	57
		Arylsulfatase	33	41	39
		Arylamidase	49	28	3
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	*-16*
D28			-	-	*-2*

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

Results of the ISO 20130 regarding the effect of pyraclostrobin on the exoenzymatic activity were chosen exemplarily to demonstrate certain effects on the soil microorganisms and their function (Figure 41).

Figure 41: **Exemplary results for effects of pyraclostrobin on the microbial function in RefeSol 02A at application rates of 3, 15 and 30 mg/kg dw soil. Exoenzymatic activity (ISO 20130) of phosphatase (upper figure), ß-glucosidase (2nd figure), arylsulfatase (3rd figure), arylamidase (4th figure) and urease (5th figure) and the corresponding effects.**



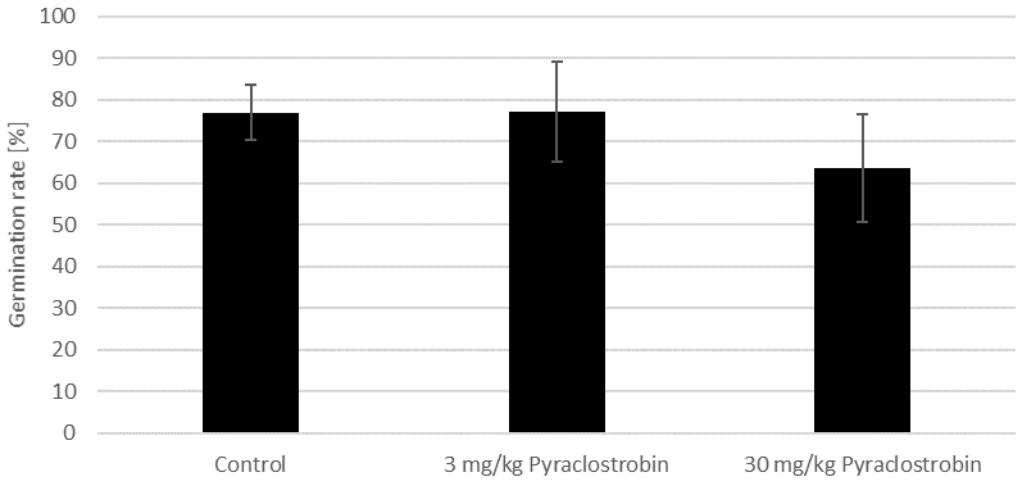


Source: Own illustration, Fraunhofer IME

2.4.3.4.2 Arbuscular mycorrhizal fungi (ISO 10832)

For the test with the fungicide pyraclostrobin, besides the control, two concentrations of 3 mg a.s./kg dw soil (PEC) and 30 mg a.s./kg dw soil (10x PEC) were tested. The test fulfilled the validity criteria. The lower test concentration of 3 mg a.s./kg dw soil did not affect the spore germination after 14 days of exposure. However, there was a statistically significant difference to the control at the highest test concentration of 30 mg a.s./kg dw soil with an inhibition of 13.6% (Figure 42). The NOEC appeared to be at a test concentration of 3 mg a.s./kg dw soil.

Figure 42: Results of the spore germination test with pyraclostrobin concentrations of 3 and 30 mg/kg dw soil in RefeSol 02A after 14 days of incubation.



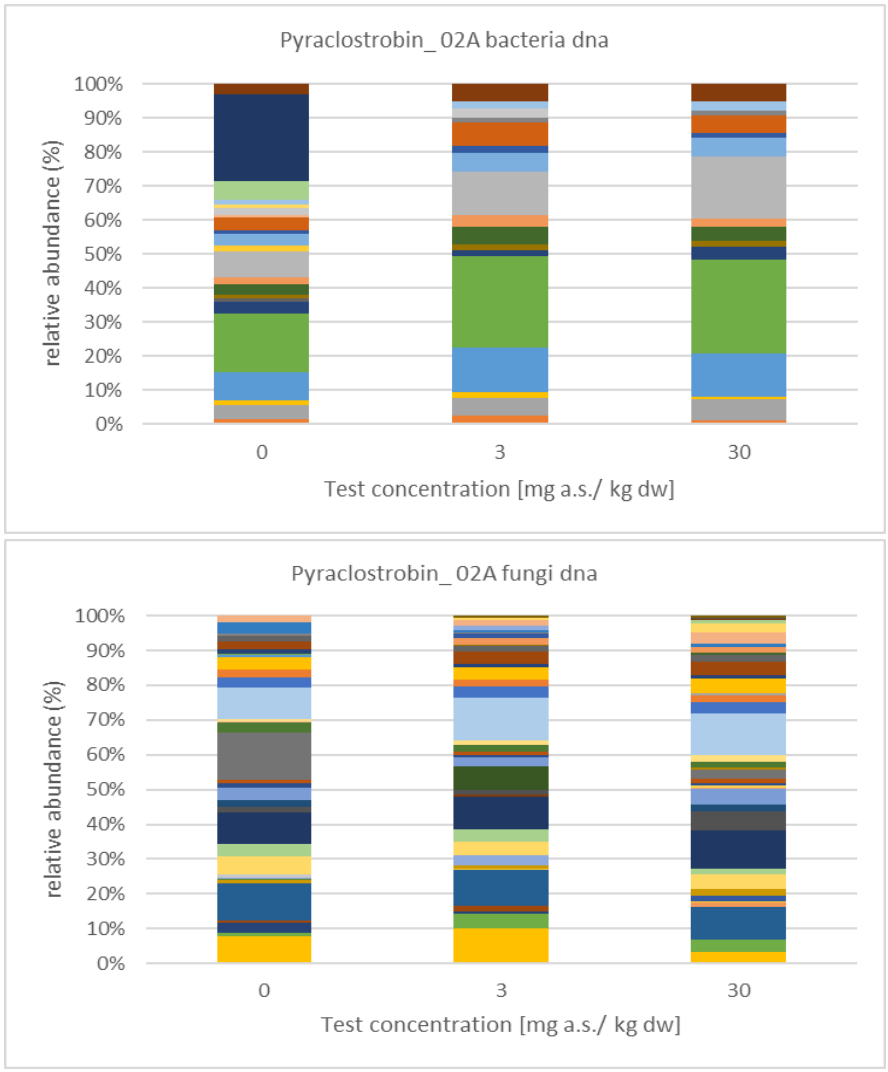
Source: Own illustration, Fraunhofer IME

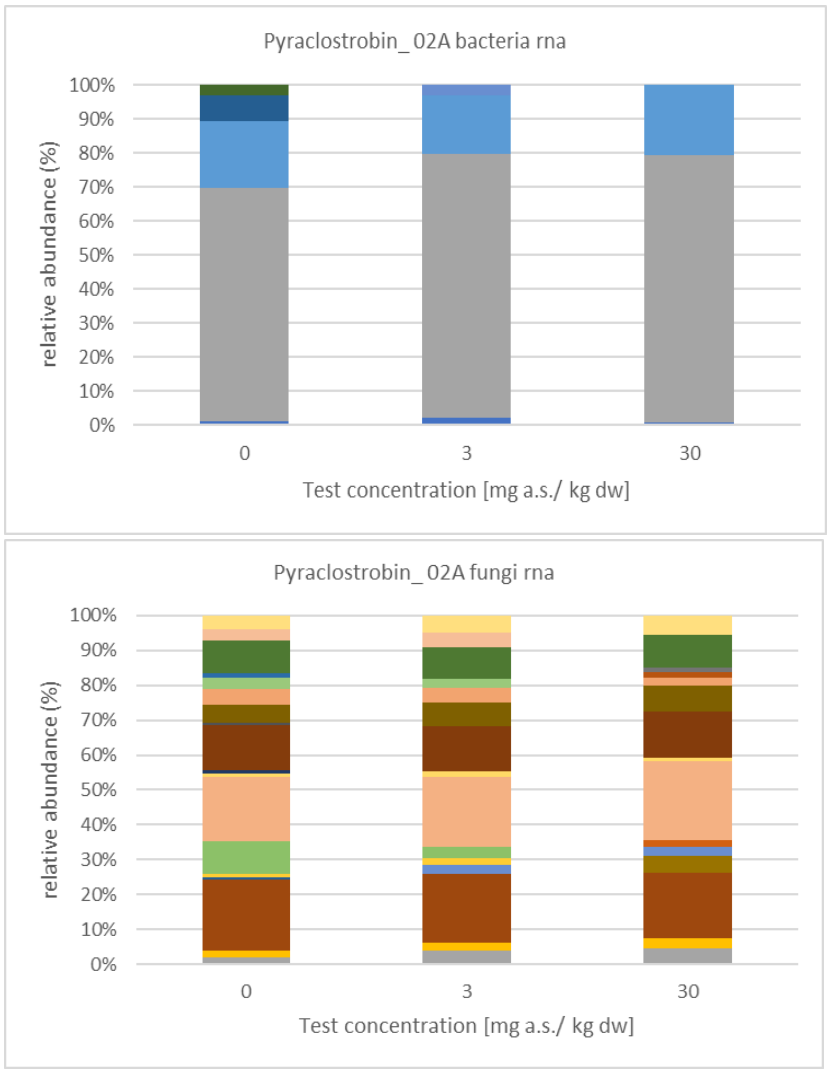
2.4.3.4.3 Soil microbial community structure (ARISA)

The evaluation of ARISA data showed changes in the bacterial and fungal community composition which can be observed and the CA analysis (Figure 44). The changes were observed at DNA and RNA level, in particular at a concentration of 3 and 30 mg pyraclostrobine/kg dw soil. Despite the dispersion observed in the sample replicates clear clusters are observed.

The Shannon index decrease at a concentration of 3 and 30 mg a.s./kg dw soil of pyraclostrobin (Table 47), suggesting a reduction in microbial biodiversity and a shift towards dominance by certain taxa following the application of pyraclostrobin at the specified concentrations, which can also be observed in the relative abundance plots (Figure 43). Effects are more pronounced for bacterial and fungal RNA data than DNA data.

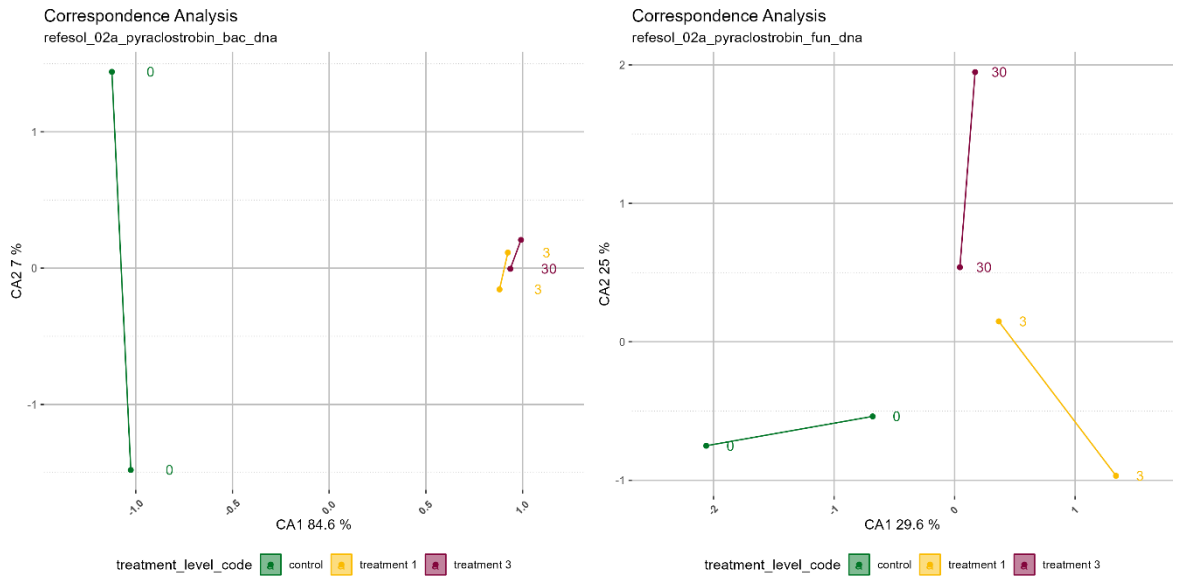
Figure 43: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 3 and 30 mg pyraclostrobin/kg soil dw in RefeSol 02A after 28 days.

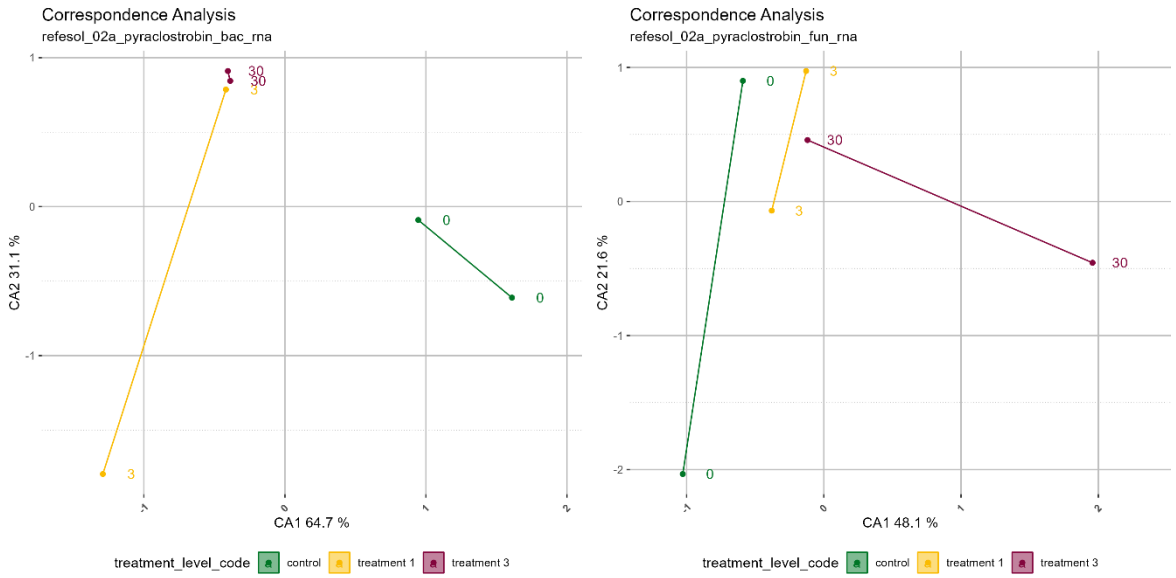




Source: Own illustration, Fraunhofer IME

Figure 44: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to pyraclostrobin concentrations of 3 and 30 mg/kg dw soil in RefeSol 02A.





Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), bacteria RNA (bottom left), and fungi RNA (bottom right).

Table 47: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 3 mg/kg and 30 mg/kg pyraclostrobin in Refesol 02A at day 28. Values represent the average of 2 replicates

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	21.0	0.8	2.5
	3 mg/kg	16.5	0.9	2.4
	30 mg/kg	15.0	0.8	2.3
DNA fungi	Control	27.0	0.9	2.9
	3 mg/kg	26.5	0.9	2.9
	30 mg/kg	32.0	0.9	3.1
RNA bacteria	Control	9.0	0.7	1.6
	3 mg/kg	7.0	0.7	1.4
	30 mg/kg	5.0	0.8	1.2
RNA fungi	Control	14.5	0.9	2.4
	3 mg/kg	12.5	0.9	2.3
	30 mg/kg	11.5	0.9	2.2

2.4.3.4.4 Integrated evaluation

Summary

Based on the results with the fungicide pyraclostrobin in RefeSol 02A, after approximately 100 days of exposure, the exoenzymatic activities (ISO 20130) were still inhibited by more than 25%, indicating a chronic effect on the soil enzymes. The two other test methods (MicroResp™, ISO 15685) appeared to be less sensitive compared to the ISO 20130 for the fungicide.

The spore germination test (ISO 10832) with *F. mosseae* showed a statistically significant effect (13.6%) at the highest test concentration of 30 mg a.s./kg dw soil after 14 days of exposure. The observed effect was lower than the effects observed on the exoenzymes. However, the results highlight the relevance of testing fungi next to soil microorganisms in more complex soils, as RefeSol 02A.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to pyraclostrobin exposure. The results indicate changes on the structural bacterial and fungal diversity at both concentrations tested.

Based on the results, Table 48 summarizes the main results per test system.

Table 48: LOECs for effects of pyraclostrobin in RefeSol 02A soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ISO 10832	ARISA CA	ARISA Alpha diversity
D28-SI	>30	>30	>30	3	30	3	3
D28-I	>30	>30	>30	3	30	3	3
ETO-SI	>30	>30	>30	3	30	3	3
ETO-I	>30	>30	>30	3	30	3	3
ERO-SI	-	>30	>30	3	-	-	-
ERO-I	-	>30	>30	3	-	-	-

[a]: Please note that the test duration for the spore germination test with F. mosseae (ISO 10832) was 14 days, and therefore, the values presented are the D14-I and D14-SI values.

2.4.4 Propamocarb hydrochloride

2.4.4.1 Standard test results

The UBA data contained for propamocarb hydrochloride results of a study in accordance with the OECD 216 with one test concentration (288.8 mg a.s./kg dw soil). Two test soils (loamy to clayey sand, sandy loam) were used, and measurements of total N were performed after 14, 28,

56 and 90 days. In the loamy to clayey sand, an effect of 36% occurred after 28 days and remained stable until day 90. In the loamy sand, after 28 days, the effects were in a comparable range of 33%, but they decreased afterwards and were below 25% after 90 days. In the OECD 216 limit test, the fungicide propamocarb hydrochloride led to an inhibition of the nitrogen transformation of 50% at 30 mg a.s./kg dw soil after 28 days of exposure in Lufa 2.1 (Table 49, Table 50). No effect was observed at the same concentration in the two other test soils (Table 53, Table 56).

2.4.4.2 Lufa 2.1.

Due to an error in calculating appropriate test concentrations based on the application rate, the test concentrations of 0.003, 0.015 and 0.03 mg a.s./kg dw were factor 1000 lower than required. The test was repeated with higher test concentrations of 3, 15, 30 and 75 mg a.s./kg dw soil. Both test results will be presented in the following chapter. However, the routines for creating the data bases was restricted to a unique combination of soil and test item per method. Thus, only the 2nd test was included in the database.

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity due to propamocarb hydrochloride are presented in Table 49.

2.4.4.2.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

In the 1st test, even using low test concentrations of 0.003 to 0.03 mg a.s./kg dw soil, throughout the whole exposure period of 84 days, the AOB was affected by more than 25%. There was no clear dose-response relationship, and the effect varied between the three test concentrations at every measurement point. However, after 84 days of exposure still, an effect of 11% (inhibition), -25% (stimulation) and -37% (stimulation) was found at the three test concentrations, indicating a potential chronic effect over time.

Throughout the exposure period of 28 days, mainly effects below 25% on the SIR (MicroResp™) were observed. At test initiation, the activity of citric acid was stimulated by 27% at 0.03 mg a.s./kg dw soil, while after 14 days, L-Malic acid and citric acid were stimulated by more than 25% both at the two lower test concentrations of 0.003 and 0.015 mg a.s./kg dw soi. After 28 days of exposure, only the SIR at the substrates L-Malic acid and citric acid were affected by more than 25%. However, a strong inhibition, as observed in the nitrogen transformation (see above), was not observed after 28 days. The test was not prolonged for a further 28 to 56 days.

The five chosen enzymes' exoenzymatic activity (ISO 20130) was not affected by more than 25% at either measurement day (day 0, 14, 28, 56 and 84).

Table 49: 1st test - Effect of propamocarb hydrochloride with application rates of 0.003, 0.015 and 0.03 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Lufa 2.1. as test soil.

Test day	Test system	Test details	Inhibition [%]		
			0.003 mg/kg	0.015 mg/kg	0.03 mg/kg
Test initiation D0	ISO 15685	-	11	-8	3
	MicroResp™	Deionized water	-3	0	-6
		D-(+)-Glucose	-3	-2	-7
		L-Cysteine hydrochloride	-6	-3	-10*

Test day	Test system	Test details	Inhibition [%]		
			0.003 mg/kg	0.015 mg/kg	0.03 mg/kg
		L-Malic acid	-8	-5	-11
		y-Amino butyric acid	1	17	11
		N-Acetyl glucosamine	-7	1	-8
		Citric acid	-13	-13	-27
		L-Alanine	-6	0	-9
	ISO 20130	Phosphatase	2	10	2
		ß-Glucosidase	7	13	23
		Arylamidase	15	6	11
		Urease	0	2	7
		Arylsulfatase	11	15	4
D14	ISO 15685	-	-2	10*	1
	MicroResp™	Deionized water	-9	-7	0
		D-(+)-Glucose	-6	-1	1
		L-Cysteine hydrochloride	-46	-38	3
		L-Malic acid	-8	11	16
		y-Amino butyric acid	-17	-9	-1
		N-Acetyl glucosamine	-32	-34	-10
		Citric acid	-8	-1	1
		L-Alanine	-2	10*	1
	ISO 20130	Phosphatase	-1	-6	12
		ß-Glucosidase	10	-1	4
		Arylamidase	3	3	1
		Urease	11	21	2
		Arylsulfatase	22	-9	21
D28	ISO 15685	-	34	-18	9
	MicroResp™	Deionized water	-10	-5	-7
		D-(+)-Glucose	-8	-7	-6

Test day	Test system	Test details	Inhibition [%]		
			0.003 mg/kg	0.015 mg/kg	0.03 mg/kg
		L-Cysteine hydrochloride	-4	-2	-6
		L-Malic acid	-10	-59	-7
		y-Amino butyric acid	2	15	11
		N-Acetyl glucosamine	-4	-4	-9
		Citric acid	-25	-18	-12
		L-Alanine	-7	-1	-5
	ISO 20130	Phosphatase	5	10	12
		ß-Glucosidase	-3	0	-11
		Arylamidase	6	-7	-12
		Urease	4	3	-10
		Arylsulfatase	1	-1	-5
D56	ISO 15685	-	14	-35	-6
	ISO 20130	Phosphatase	-9	7	4
		ß-Glucosidase	-7	11	18
		Arylamidase	-23	-22	-24
		Urease	5	8	3
		Arylsulfatase	-11	3	-3
D84	ISO 15685	-	11	-25	-37
	ISO 20130	Phosphatase	-3	0	1
		ß-Glucosidase	-10	-7	4
		Arylamidase	12	-8	-1
		Urease	11	6	17
		Arylsulfatase	0	0	-6
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	*11*
D28			-	-	*-4*

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

In the 2nd test an additional 4th test concentration was chosen to observe whether the determination of an EC_x based on three or more test concentrations would also be possible concerning an endpoint calculation. Even using four test concentrations ranging from 3 to 75 mg a.s./kg dw soil, no clear dose-response relationship could be established for any of the three test methods.

The AOB (ISO 15685) was strongly affected by inhibition of up to 94% (day 28, 30 mg a.s./kg dw soil). The threshold of 25% was exceeded at 15 to 75 mg a.s./kg dw soil at day 14 and at the two highest test concentrations of 30 and 75 mg a.s./kg dw soil after 28 days of exposure. Therefore, the test duration was extended to 56 days of exposure. On day 56, a general decrease of the previously observed inhibitions was observed. Only at 30 mg a.s./kg dw soil was an effect above 25% observed. However, after a further 28 days of exposure, at day 84 again at the three highest test concentrations (15 – 75 mg a.s./kg dw soil), the activity of the AOB was inhibited by 40 to 52% without dose-response.

Throughout the exposure period of 28 days, mainly effects below 25% on the SIR (MicroResp™) were observed. However, after 28 days of exposure, the activity of citric acid was inhibited by 31% to 39% at concentrations ranging from 3 to 75 mg a.s./kg dw soil. Therefore, the test was prolonged, and after 56 days of exposure at 3 and 15 mg a.s./kg dw, soil stimulations above 25% were observed for citric acid. After 84 days, no effect above 25% was observed for the various substrates.

After 14 and 28 days of exposure to propamocarb hydrochloride, the exoenzymatic activity (ISO 20130) of the enzymes phosphatase, ß-glucosidase and arylamidase was affected by more than 25% but without dose-response relationship. Therefore, the exposure was prolonged, and after 56 days of exposure, neither of the three exoenzymatic activities (ISO 20130) of the enzymes phosphatase, ß-glucosidase and arylamidase were affected by more than 25%.

Table 50: 2nd test - Effect of propamocarb hydrochloride with application rates of 3, 15, 30 and 75 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Lufa 2.1. as test soil.

Test day	Test system	Test details	Inhibition [%]			
			3 mg/kg	15 mg/kg	30 mg/kg	75 mg/kg
D14	ISO 15685	-	-7	48	93	64
	MicroResp™	Deionized water	-2	-5	5	0
		D-(+)-Glucose	-3	-4	1	2
		L-Cysteine hydrochloride	-6	-5	3	2
		L-Malic acid	0	3	5	0
		y-Amino butyric acid	0	2	5	1
		N-Acetyl glucosamine	2	2	2	2
		Citric acid	4	2	0	1
		L-Alanine	3	2	3	3
	ISO 20130	Phosphatase	0	5	30	14
		ß-Glucosidase	-23	25	51	-8
		Arylamidase	28	33	2	15
		Urease	-13	7	13	11

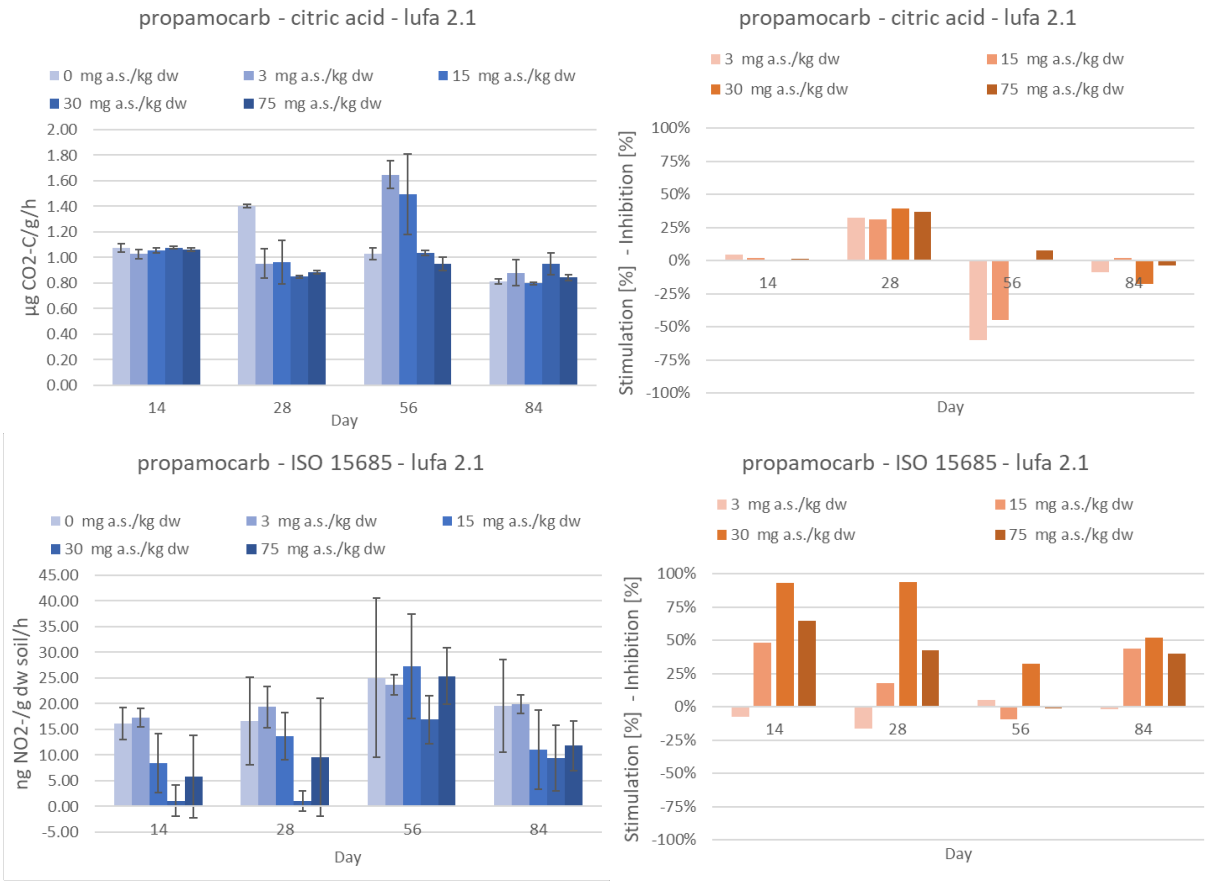
Test day	Test system	Test details	Inhibition [%]			
			3 mg/kg	15 mg/kg	30 mg/kg	75 mg/kg
		Arylsulfatase	-6	10	5	-6
D28	ISO 15685	-	-16	18	94	43
	MicroResp™	Deionized water	7	8	-8	-12
		D-(+)-Glucose	23	27	20	18
		L-Cysteine hydrochloride	11	14	5	2
		L-Malic acid	17	21	17	15
		y-Amino butyric acid	13	14	8	5
		N-Acetyl glucosamine	11	13	8	5
		Citric acid	32	31	39	37
		L-Alanine	13	16	14	12
	ISO 20130	Phosphatase	26	14	5	6
		ß-Glucosidase	25	17	-8	15
		Arylamidase	-5	-56	-76	-61
		Urease	-3	24	18	-5
		Arylsulfatase	22	11	-16	8
D56	ISO 15685	-	5	-9	33	-1
	MicroResp™	Deionized water	1	9	8	14
		D-(+)-Glucose	-20	-13	8	13
		L-Cysteine hydrochloride	-16	-3	6	11
		L-Malic acid	-24	-8	3	3
		y-Amino butyric acid	-15	-1	3	9
		N-Acetyl glucosamine	-20	-7	1	11
		Citric acid	-60	-45	0	8
		L-Alanine	-31	-15	-3	7
	ISO 20130	Phosphatase	3	7	4	-2
		ß-Glucosidase	-17	10	-5	-12
		Arylamidase	-2	5	5	-15

Test day	Test system	Test details	Inhibition [%]			
			3 mg/kg	15 mg/kg	30 mg/kg	75 mg/kg
		Urease	-	-	-	-
		Arylsulfatase	-	-	-	-
D84	ISO 15685	-	-2	43	52	40
	MicroResp™	Deionized water	-8	-1	-21	-4
		D-(+)-Glucose	-8	-1	-21	-4
		L-Cysteine hydrochloride	-4	2	-20	-6
		L-Malic acid	-4	3	-19	-3
		y-Amino butyric acid	-6	4	-24	-5
		N-Acetyl glucosamine	4	7	-13	-2
		Citric acid	-1	3	-18	-6
		L-Alanine	-9	2	-17	-4
D14	OECD 216 (reference)	N-Transformation	-	-	0	-
D28			-	-	50	-

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

Results of MicroResp™ and the ISO 15685 regarding the effect of propamocarb hydrochloride on the substrate-induced respiration using citric acid and on the ammonium oxidizing bacteria were chosen exemplarily to demonstrate certain effects on the soil microorganisms and their function (Figure 45).

Figure 45: SIR with citric acid (upper left) and potential nitrification (lower left) and corresponding inhibition (SIR: upper right; Potential nitrification: lower right) due to propamocarb hydrochloride at application rates of 3, 15, 30 and 75 mg/kg dw soil at day 28 in the 2nd test with Lufa 2.1.



Source: Own illustration, Fraunhofer IME

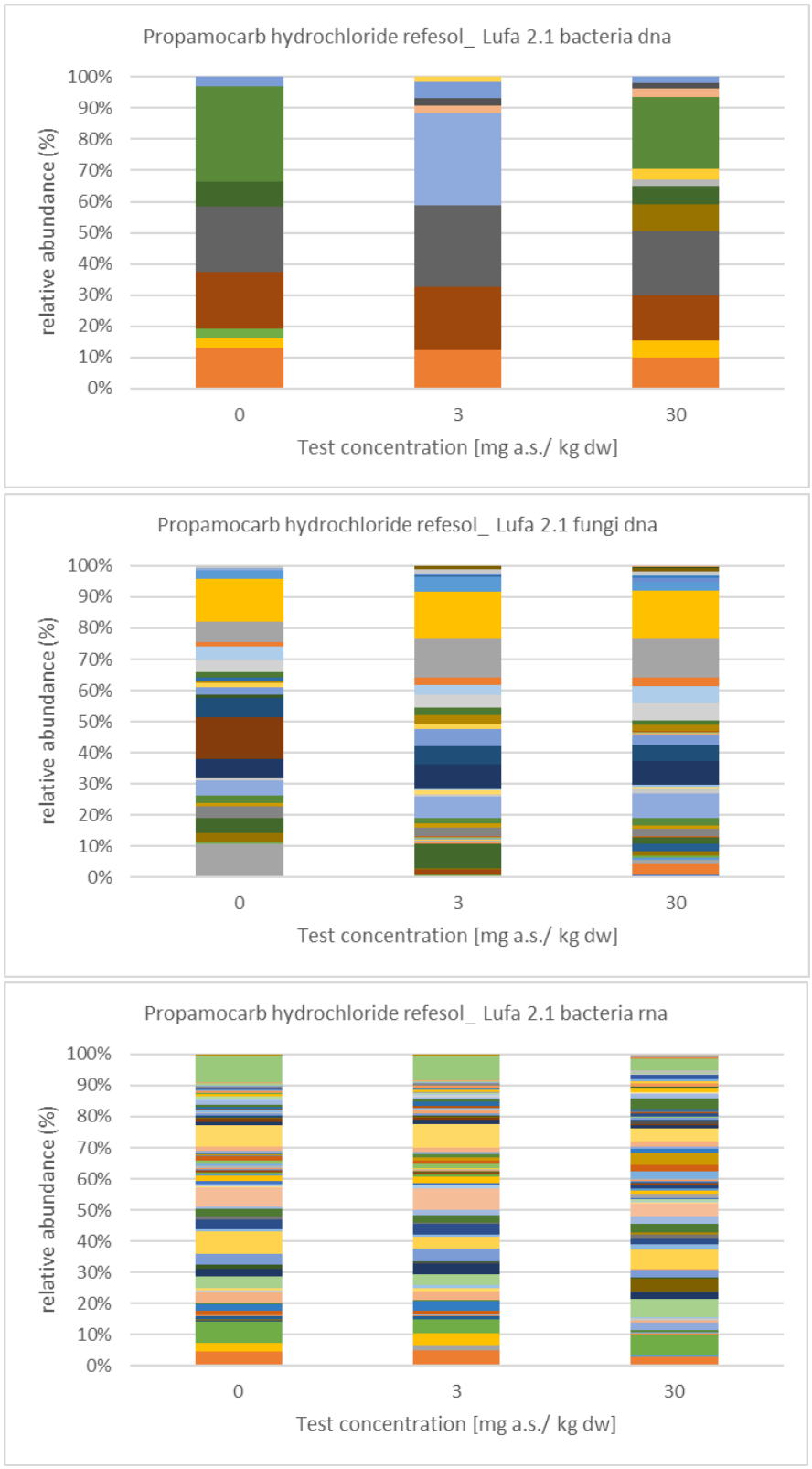
2.4.4.2.2 Soil microbial community structure (ARISA)

The evaluation of ARISA data showed changes in the bacterial and fungal community composition which can be observed in the relative abundances plot (Figure 46) and the CA analysis (Figure 47). The changes were observed at DNA and RNA level, at 3 and 30 mg propamocarb hydrochloride /kg dw soil. Despite dispersion within replicates, the overall trend shows that the treated samples exhibit distinct microbial profiles compared to the control.

In the relative abundance plot (Figure 46), the shifts in microbial composition are visualized as changes in the proportion of specific OTUs, reflecting altered taxa prevalence under the influence of ethofumesate. Similarly, the CA analysis (Figure 47) highlights these compositional shifts by clustering samples according to their community structure, with noticeable separation of treated samples from the controls. Despite dispersion within replicates, the overall trend shows that the treated samples exhibit distinct microbial profiles compared to the control.

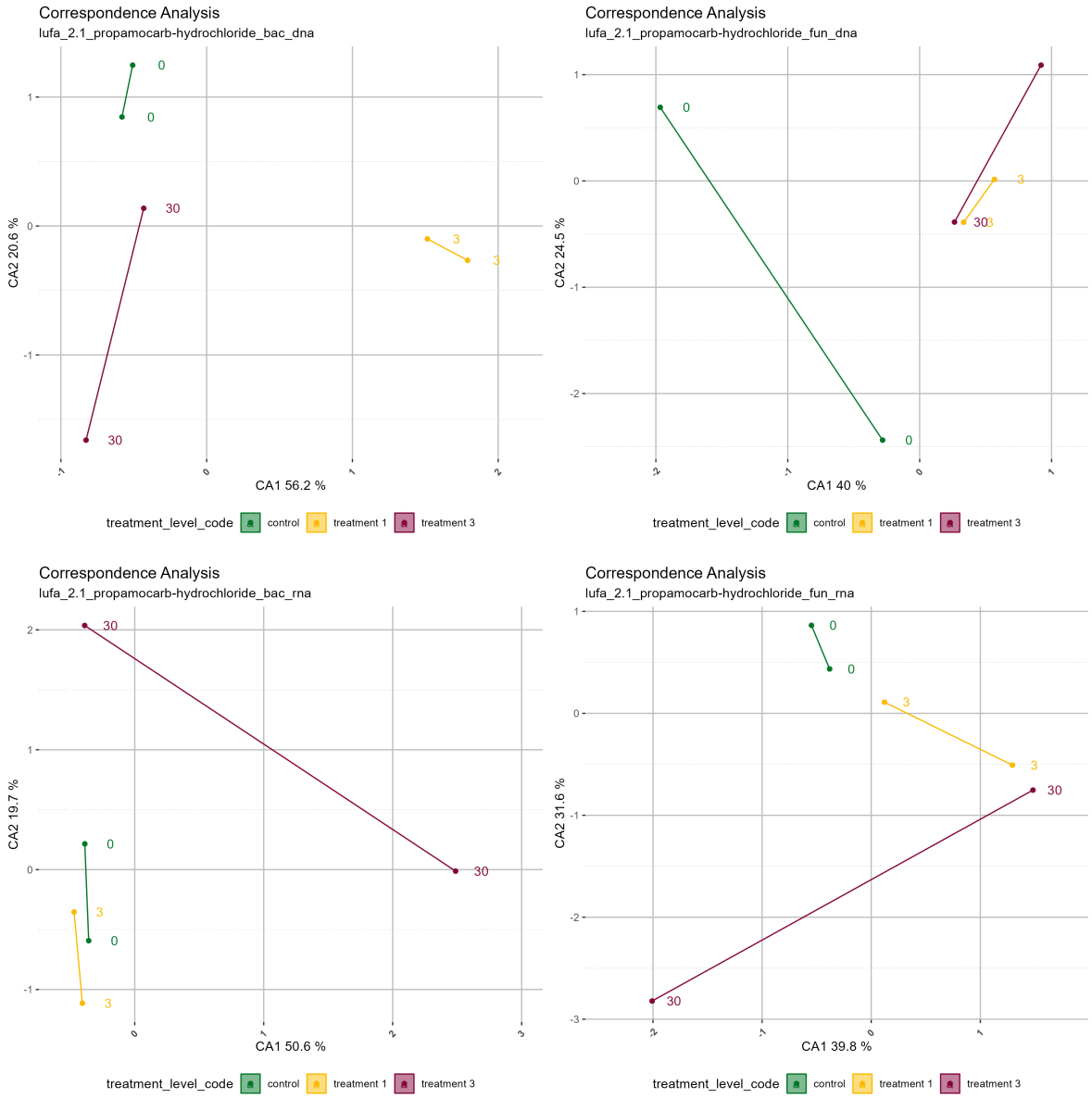
The Shannon index shows a slight increase in bacterial and fungal diversity at the DNA level at a concentration of 30 mg a.s./kg dw soil of propamocarb hydrochloride (Table 51). The observed increases in the Shannon index suggest an increased microbial diversity following the application of propamocarb at the specified concentration.

Figure 46: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 3 and 30 mg propamocarb hydrochloride/kg soil dw in Lufa 2.1. after 28 days.



Source: Own illustration, Fraunhofer IME

Figure 47: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to propamocarb hydrochloride concentrations of 3 and 30 mg/kg dw soil in Lufa 2.1.



Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), bacteria RNA (bottom left) and fungi RNA (bottom right).

Table 51: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 3 mg/kg and 30 mg/kg propamocarb hydrochloride in Lufa 2.1 at day 28. Values represent the average of 2 replicates.

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	6.5	0.9	1.7
	3 mg/kg	6.5	0.9	1.6
	30 mg/kg	9.5	0.9	2.1
DNA fungi	Control	19.0	0.9	2.6

	3 mg/kg	27.0	0.9	2.9
	30 mg/kg	29.5	0.9	2.9
RNA bacteria	Control	54.0	0.9	3.5
	3 mg/kg	55.5	0.9	3.6
	30 mg/kg	49.5	0.9	3.5
RNA fungi	Control	14.0	0.8	2.1
	3 mg/kg	13.5	0.8	2.0
	30 mg/kg	13.5	0.8	2.2

2.4.4.2.3 Integrated evaluation

Summary

The results of the 1st test (concentration range: 0.003 – 0.03 mg a.s./kg dw soil) and the 2nd test (concentration range: 3 – 75 mg a.s./kg dw soil) are in good agreement. In the 1st test, with lower test concentrations, effects were mainly below 25%. Suppose effects above 25% occurred in the test with AOB (ISO 15685) as stimulation of the activity. The two other test systems appeared to be mainly unaffected. In the 2nd test with higher test concentrations, the AOB appeared to be the most sensitive group, and strong effects occurred in soil with 15 to 75 mg a.s./kg dw. However, the SIR was also affected by more than 25% stimulation at different test concentrations depending on the exposure duration. After 84 days of exposure, still, at 30 mg a.s./kg dw soil, various substrates used for SIR measurements indicated a chronic long-term effect on the soil microorganisms and the associated nutrient cycles.

The effect of propamocarb hydrochloride on the spore germination of *F. mosseae* in accordance with ISO 10832 could not be determined due to the poor spore germination of the AMF in LUFA 2.1.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to propamocarb hydrochloride exposure. The results indicate changes on the structural bacterial and fungal diversity at 3 and 30 mg a.s./kg dw soil.

Based on the results, Table 52 summarizes the main results per test system.

Table 52: LOECs for effects of propamocarb hydrochloride in Lufa 2.1 soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	30	30	3	3	3	3
D28-I	30	>75	3	3	3	3

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
ETO-SI	30	15	3	3	3	3
ETO-I	30	15	3	3	3	3
ERO-SI	-	15	>75	>75	-	-
ERO-I	-	15	>75	>75	-	-

2.4.4.3 Refesol 04A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to propamocarb hydrochloride are presented in Table 53.

2.4.4.3.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

The AOB have been affected by more than 25% on days 14, 28 and 56. There was no clear dose-response relationship. However, after 14 and 28 days of exposure, a comparable inhibition of the AOB activity was observed at the two highest test concentrations. Therefore, the test was prolonged to 56 days. Here, the AOB activity was inhibited by 56% at 30 mg a.s./kg dw soil, indicating a further prolongation of the test. Unfortunately, due to an error in the evaluation sheet, the inhibition was not recognised in time, and after 84 days of exposure, the ISO 15685 was not carried out. Nevertheless, the results indicate a high sensitivity of the nitrifying bacteria to the fungicide propamocarb hydrochloride.

Throughout the exposure period of 28 days, no effects above 25% on the SIR (MicroResp™) were observed at any of the used substrates. Therefore, the test was not performed beyond 28 days of exposure.

The exoenzymatic activity (ISO 20130) of ß-glucosidase, arylsulfatase and urease were affected by more than 25% after 28 days of exposure. This was stimulation in almost all cases, and the lowest concentration of 3 mg a.s./kg dw soil inhibited the urease activity of 100%. The measurement of urease activity was performed in parallel to the day 84 measurements of the experiment with pyraclostrobin. The problems described above also occurred in the urease measurement presented here. The results are, therefore, unreliable; the measurement of urease activity after 56 days showed no effect of more than 25% at the selected test concentrations. The effect on ß-glucosidase and arylsulphatase activity was below 25% after 56 days, so the test was terminated.

Table 53: Effects of propamocarb hydrochloride with application rates of 3, 15, 30 and 75 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Refesol 04A as test soil.

Test day	Test system	Test details	Inhibition [%]			
			3 mg/kg	15 mg/kg	30 mg/kg	75 mg/kg
D14	ISO 15685	-	12	5	54	41

Test day	Test system	Test details	Inhibition [%]			
			3 mg/kg	15 mg/kg	30 mg/kg	75 mg/kg
	MicroResp™	Deionized water	4	-1	-3	3
		D-(+)-Glucose	8	3	-2	-5
		L-Cysteine hydrochloride	7	2	-6	-8
		L-Malic acid	8	1	-1	0
		y-Amino butyric acid	6	-1	-2	2
		N-Acetyl glucosamine	8	2	1	4
		Citric acid	9	1	1	4
		L-Alanine	7	-1	-2	2
	ISO 20130	Phosphatase	17	-9	-7	5
		ß-Glucosidase	-36	-39	-66	1
		Arylsulfatase	-4	8	3	-14
		Arylamidase	-38	-48	-7	-6
		Urease	54	92	93	8
D28	ISO 15685	-	-22	3	36	36
	MicroResp™	Deionized water	2	10	2	2
		D-(+)-Glucose	5	11	0	0
		L-Cysteine hydrochloride	4	8	-1	0
		L-Malic acid	0	1	-10	-1
		y-Amino butyric acid	5	8	0	1
		N-Acetyl glucosamine	8	12	1	3
		Citric acid	12	17	8	11
		L-Alanine	4	11	2	5
	ISO 20130	Phosphatase	-12	-18	-17	-21
		ß-Glucosidase	-43	-44	-39	-28
		Arylsulfatase	-23	-33	-37	-23
		Arylamidase	-15	7	10	7
		Urease	106*	-242*	-100*	-209*

Test day	Test system	Test details	Inhibition [%]			
			3 mg/kg	15 mg/kg	30 mg/kg	75 mg/kg
D56	ISO 15685	-	-27**	-25**	56**	-2
	ISO 20130	ß-Glucosidase	8	0	-2	-2
		Arylsulfatase	-3	8	1	5
		Urease	4	19	14	-21
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	19	-
D28			-	-	1	-

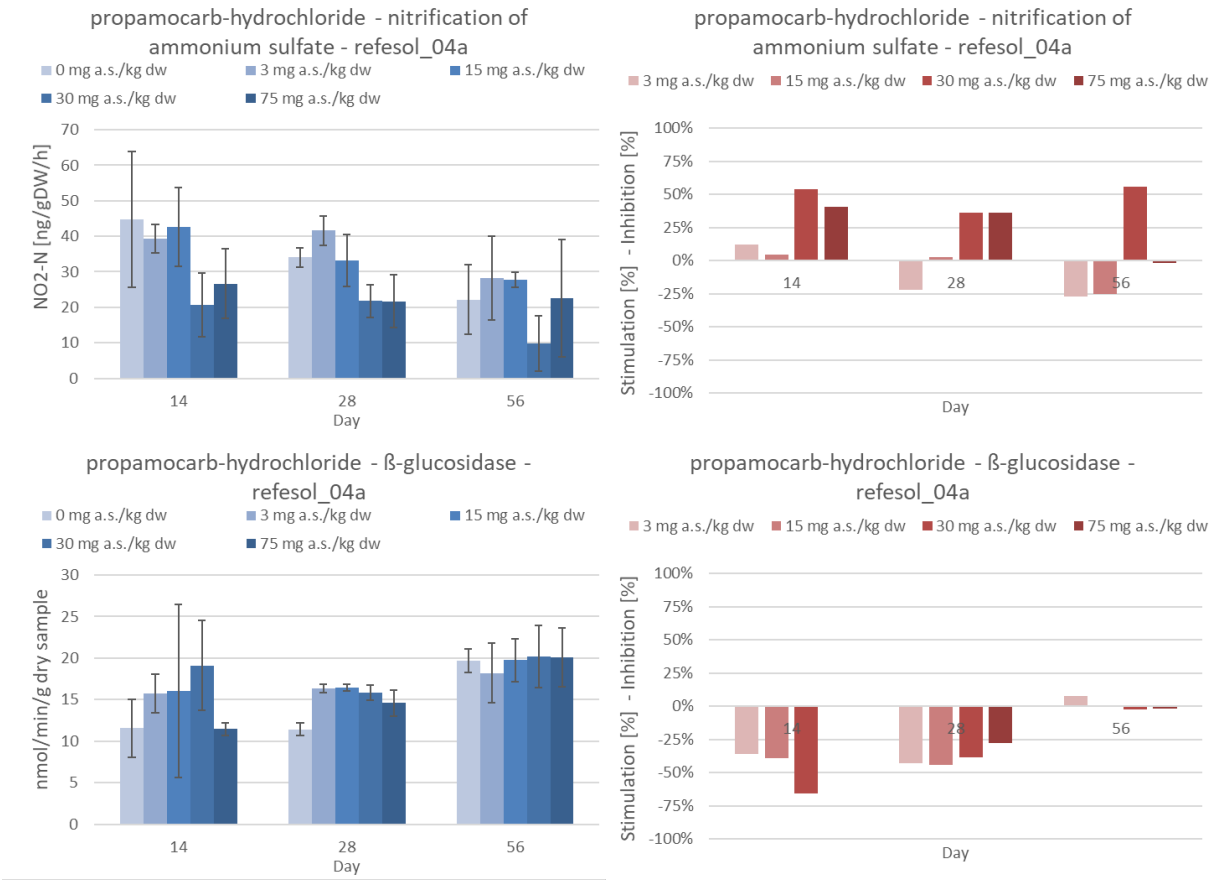
Red colour: Deviations compared to the control were either 25% (increase) or -25% (decrease).

*Note: When determining the urease activity, problems occurred with the colour change in the samples during the test procedure. In general, determination of urease activity was more difficult to perform with RefeSol 04A than with Lufa 2.1. In the present experiment, no valid measurement could be carried out on day 84.

**Note: Due to an error in the Excel spreadsheet, the results first indicated no effect above 25% on the activity of the AOB. However, the error was found and corrected while re-evaluating the data, but the test had already been discarded.

As an example, representative results of test methods with observed effects are shown in Figure 48 to obtain a detailed picture of the development of the observed effects.

Figure 48: Example results for effects of propamocarb hydrochloride on the microbial function at application rates of 3, 15, 30 and 75 mg/kg dw soil. Potential nitrification (ISO 15685; upper figure) and exoenzymatic activity (ISO 20130; lower figure).



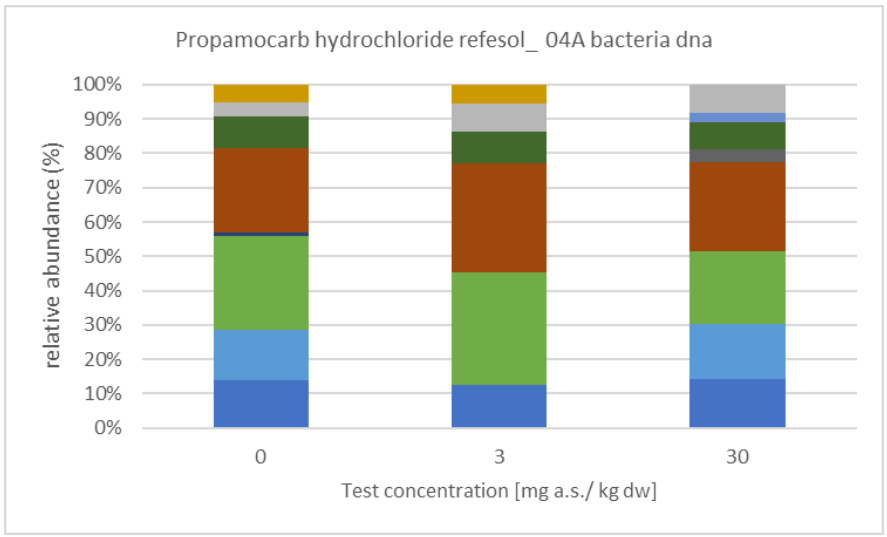
Source: Own illustration, Fraunhofer IME

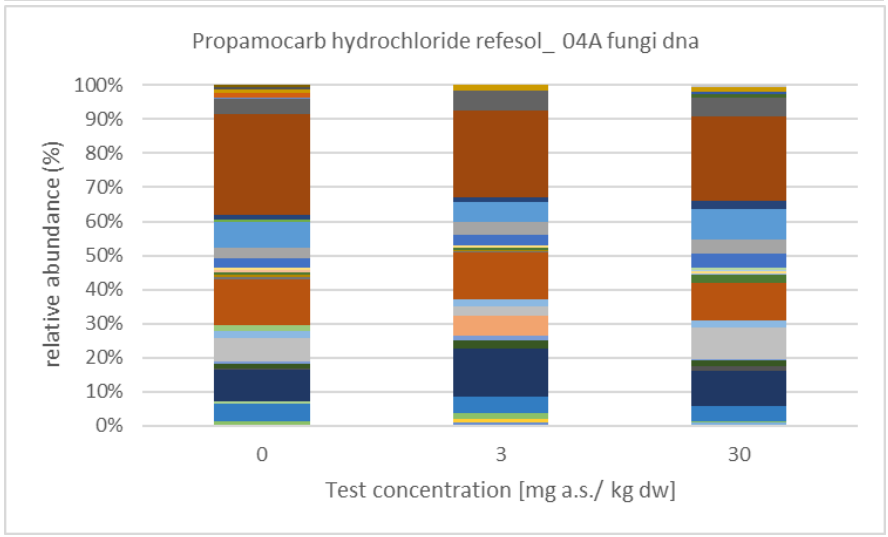
2.4.4.3.2 Soil microbial community structure (ARISA)

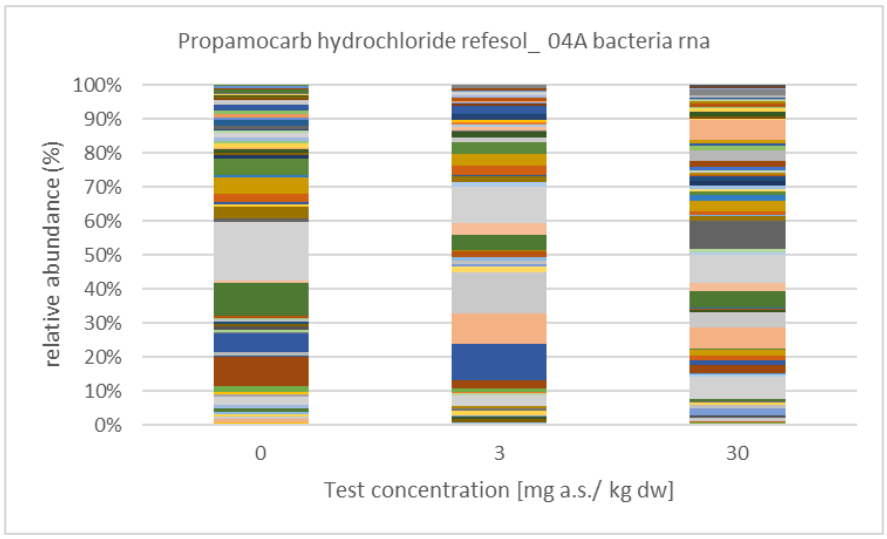
The evaluation of ARISA data revealed only minor changes in both bacterial and fungal community composition, as illustrated in the relative abundance plot (Figure 49) and the CA plot (Figure 50) at a concentration of 3 and 30 mg propamocarb hydrochloride/kg dry weight soil. The dispersion within replicates, make difficult to assess trends observed. Information regarding diversity and community composition based on RNA data could not be retrieved due to data inconsistency due to the low RNA concentration obtained for the 04A soil samples.

The Shannon index (Table 54) indicates no changes in bacterial and fungal diversity at DNA and RNA levels (only for bacteria) at both concentrations tested (3 mg a.s./kg dw soil and 30 mg a.s./kg dw soil). This information suggests that propamocarb hydrochloride do not have a big impact on the microbial communities.

Figure 49: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 3 and 30 mg propamocarb hydrochloride/kg soil dw in RefeSol 04A after 28 days.

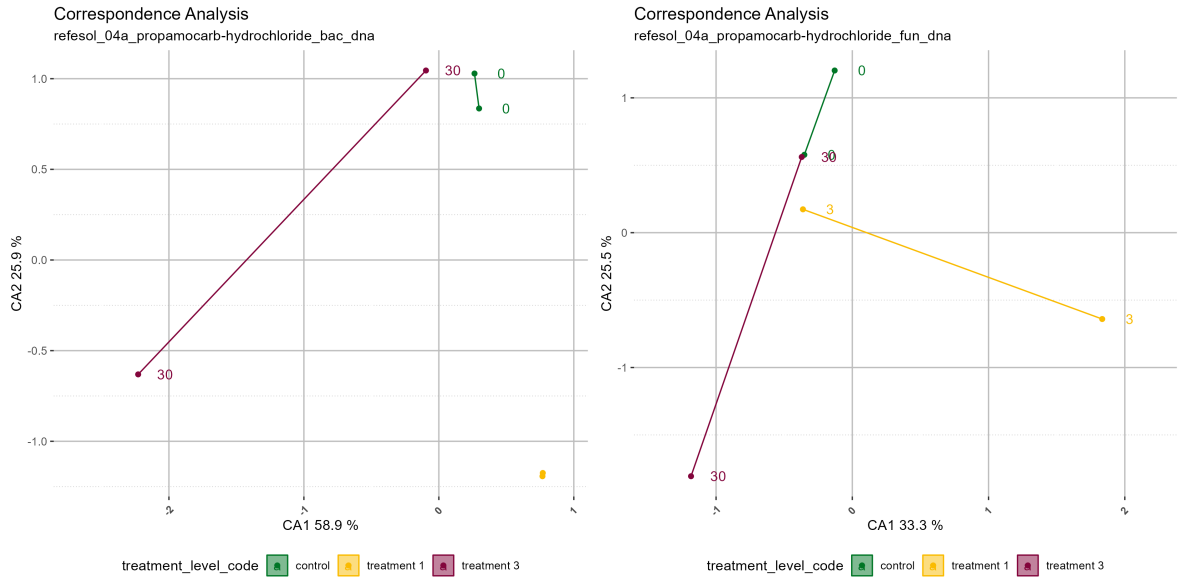


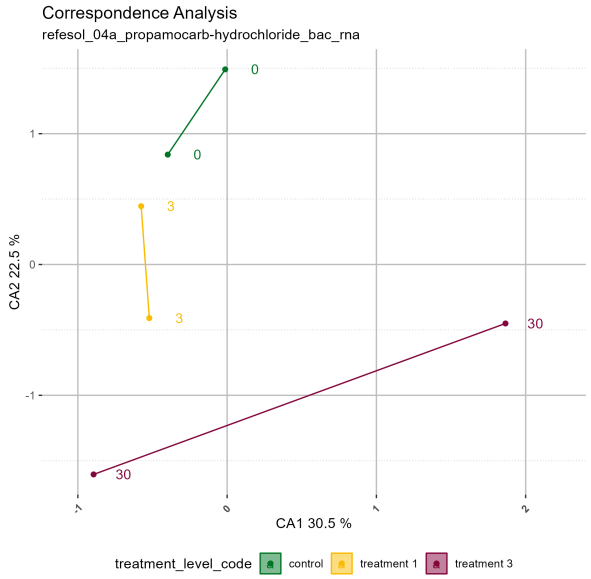




Source: Own illustration, Fraunhofer IME

Figure 50: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to propamocarb hydrochloride concentrations of 3 and 30 mg/kg dw soil in RefeSol 04A.





Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), and bacteria RNA (bottom left).

Table 54: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 3 mg/kg and 30 mg/kg propamocarb hydrochloride in Refesol 04A at day 28. Values represent the average of 2 replicates.

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	7.0	0.9	1.8
	3 mg/kg	6.0	0.9	1.6
	30 mg/kg	7.0	0.9	1.8
DNA fungi	Control	22.0	0.8	2.4
	3 mg/kg	20.5	0.8	2.5
	30 mg/kg	20.5	0.8	2.5
RNA bacteria	Control	38.0	0.9	3.2
	3 mg/kg	30.5	0.9	3.1
	30 mg/kg	40.0	0.9	3.3

2.4.4.3.3 Integrated evaluation

Summary

After 56 days of exposure to propamocarb hydrochloride at the four chosen test concentrations, only the AOB activity was still inhibited by more than 25%, indicating that the ISO 15685 represents the most sensitive method within the three used test methods (MicroResp™, ISO 15685, ISO 20130) in RefeSol 04A.

The effect of propamocarb hydrochloride on the spore germination of *F. mosseae* in accordance with ISO 10832 could not be determined due to the poor spore germination of the AMF in RefeSol 04A.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to propamocarb hydrochloride exposure. The results suggest that propamocarb hydrochloride do not have a big impact on the microbial communities.

Based on the results, Table 55 summarizes the main results per test system.

Table 55: LOECs for effects of propamocarb hydrochloride in RefeSol 04A.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	>30	30	>75	3	>30	>30
D28-I	>30	30	>75	>75	>30	>30
ETO-SI	>30	3	>75	3	>30	>30
ETO-I	>30	30	>75	>75	>30	>30
ERO-SI	-	3	>75	>75	-	-
ERO-I	-	30	>75	>75	-	-

2.4.4.4 Refesol 02A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to ethofumesate are presented in Table 56.

2.4.4.4.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

After 28 days of incubation, compared to the control, the AOB (ISO 15685) activity was not affected by more than 25% at the three chosen test concentrations. Therefore, the ISO 15685 was not performed on days 56 and 84.

In the tests with RefeSol 02A, stimulation of the SIR of more than 25% compared to the control was found at the highest test concentration of 30 mg a.s./kg dw soil for L-malic acid and citric acid after 28 days of exposure. Therefore, the test was prolonged to 56 days. Here, while the SIR with the previously mentioned substrates was still stimulated, the other substrates used, except D-(+)-glucose, were stimulated by more than 25% compared to the control. Only basal respiration was not affected by more than 25%. After 84 days of exposure, the observed stimulation of more than 25% compared to the control remained only for the substrate citric acid at the highest test concentration, indicating a long-term effect.

The enzyme activity of the phosphatase and urease was not affected by more than 25% after 28 days. However, the arylsulfatase and ß-glucosidase activity were stimulated by more than 25%, while the activity of the arylamidase was inhibited without a concentration-response relationship. After 56 days of exposure, the enzymatic activities of the ß-glucosidase, the arylsulfatase and the arylamidase were inhibited by more than 25%, but again, no concentration-response could be observed. The ISO 20130 was prolonged to day 84, and still inhibitory effects on the three exoenzymes could be determined with up to 51% inhibition of the arylamidase activity at the lowest test concentration of 3 mg a.s./kg dw soil.

Table 56: Effects of propamocarb hydrochloride with application rates of 3, 15 and 30 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using RefeSol 02A as test soil.

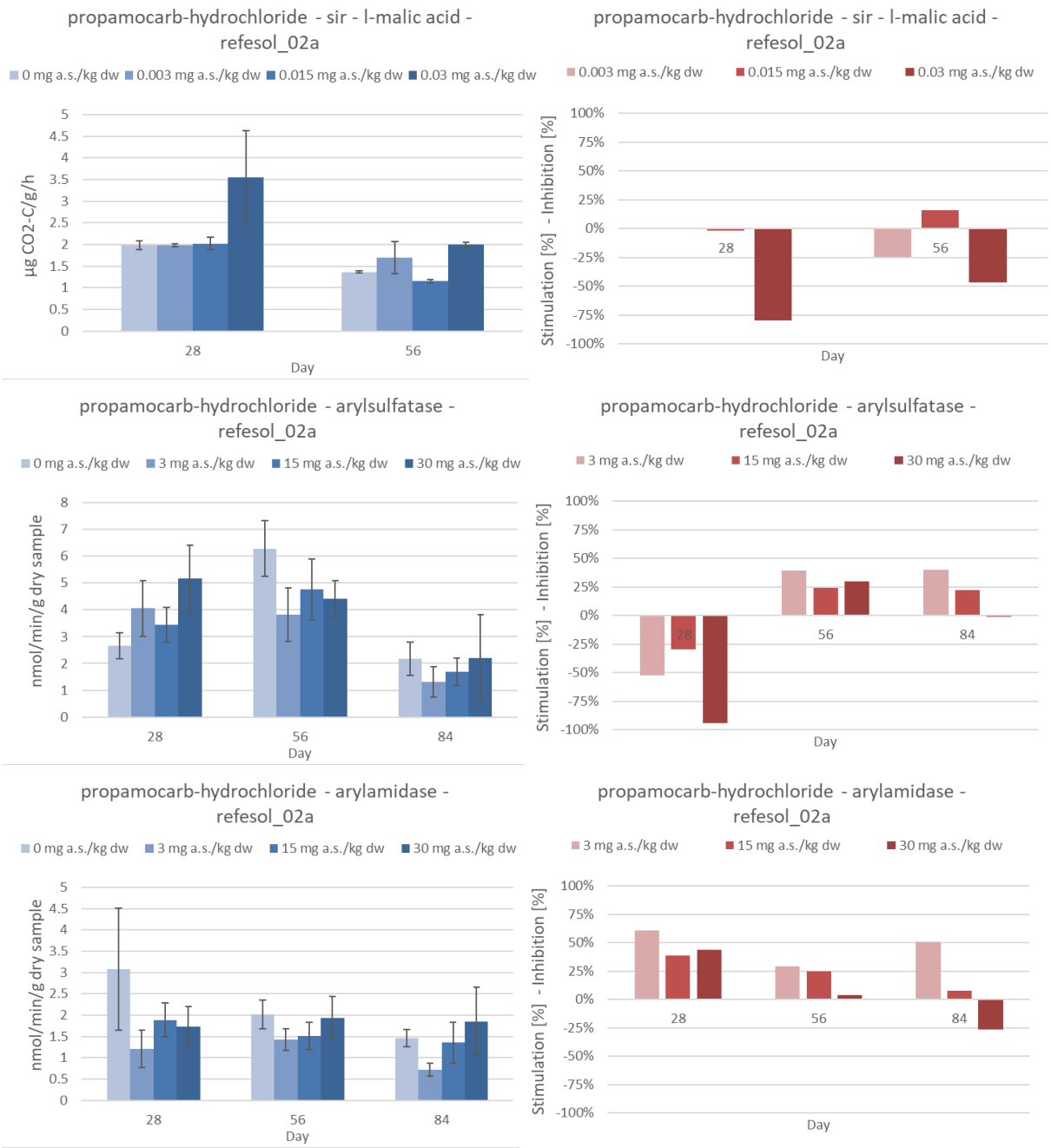
Test date	Test system	Test details	Inhibition [%]		
			3 mg/kg	15 mg/kg	30 mg/kg
D28	ISO 15685	-	0	5	12
	MicroResp™	Deionized water	4	-1	22
		D-(+)-Glucose	-6	0	-22
		L-Cysteine hydrochloride	-4	-1	-6
		L-Malic acid	0	-2	-79
		y-Amino butyric acid	1	-2	21
		N-Acetyl glucosamine	-1	-3	7
		Citric acid	-5	-4	-153
		L-Alanine	-4	-2	-15
	ISO 20130	Phosphatase	-12	7	1
		ß-Glucosidase	-33	-15	-34
		Arylsulfatase	-52	-29	-94
		Arylamidase	61	39	44
		Urease	-2	3	-8
D56	MicroResp™	Deionized water	6	20	-6
		D-(+)-Glucose	-24	20	-21
		L-Cysteine hydrochloride	-23	16	-36
		L-Malic acid	-25	16	-47
		y-Amino butyric acid	-5	13	-47

Test date	Test system	Test details	Inhibition [%]		
			3 mg/kg	15 mg/kg	30 mg/kg
		N-Acetyl glucosamine	-7	15	-48
		Citric acid	-63	18	-164
		L-Alanine	-19	14	-44
	ISO 20130	ß-Glucosidase	31	35	30
		Arylsulfatase	39	24	30
		Arylamidase	30	25	4
D84	MicroResp™	Deionized water	7	6	8
		D-(+)-Glucose	9	5	8
		L-Cysteine hydrochloride	10	6	5
		L-Malic acid	10	7	5
		y-Amino butyric acid	9	8	7
		N-Acetyl glucosamine	10	9	4
		Citric acid	10	10	-50
		L-Alanine	8	10	-18
	ISO 20130	ß-Glucosidase	31	-14	-24
		Arylsulfatase	40	22	-1
		Arylamidase	51	8	-27
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	*-14*
D28			-	-	*-4*

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

Results of the substrate-induced respiration (MicroResp™, L-malic acid) and the enzymatic activity (ISO 20130, arylsulfatase and arylamidase) are presented exemplarily to demonstrate certain effects on the soil microorganisms and their function from the exposure to propamocarb hydrochloride (Figure 51).

Figure 51: **Exemplary results for effects of propamocarb hydrochloride on the microbial function at application rates of 3, 15 and 30 mg/kg dw soil in RefeSol 02A. Substrate-induced respiration (MicroResp™; activity upper left figure, effect upper right figure) and exoenzymatic activity (ISO 20130; activity mid and lower left figures, effect mid and lower right figure).**

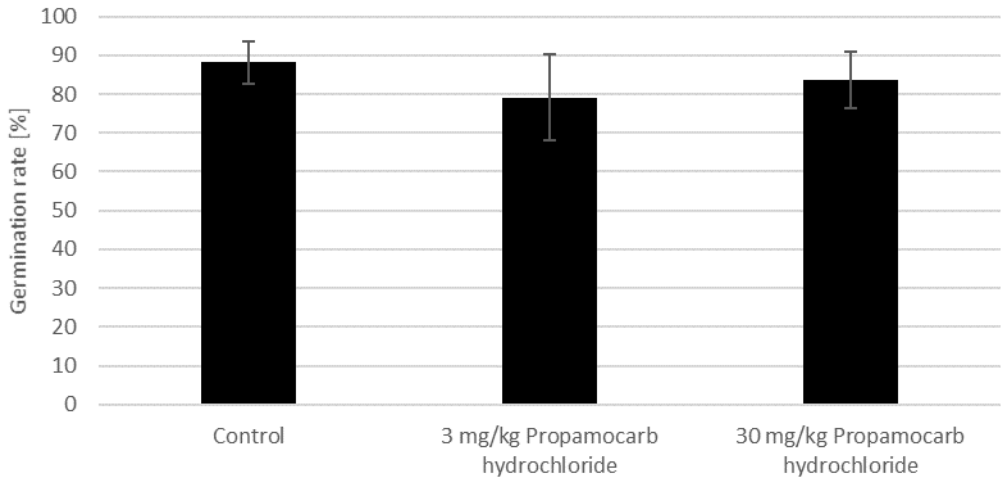


Source: Own illustration, Fraunhofer IME

2.4.4.4.2 Arbuscular mycorrhizal fungi (ISO 10832)

For the fungicide propamocarb hydrochloride test, besides the control, two concentrations of 3 mg a.s./kg dw soil (PEC) and 30 mg a.s./kg dw soil (10x PEC) were tested. The test fulfilled the validity criteria. The chosen test concentrations did not affect the spore germination after 14 days of exposure. Therefore, the NOEC appeared to equal or above the highest test concentration of 30 mg a.s./kg dw soil.

Figure 52: Results of the spore germination test with propamocarb hydrochloride concentrations of 3 and 30 mg/kg dw soil in RefeSol 02A after 14 days of incubation.



Source: Own illustration, Fraunhofer IME

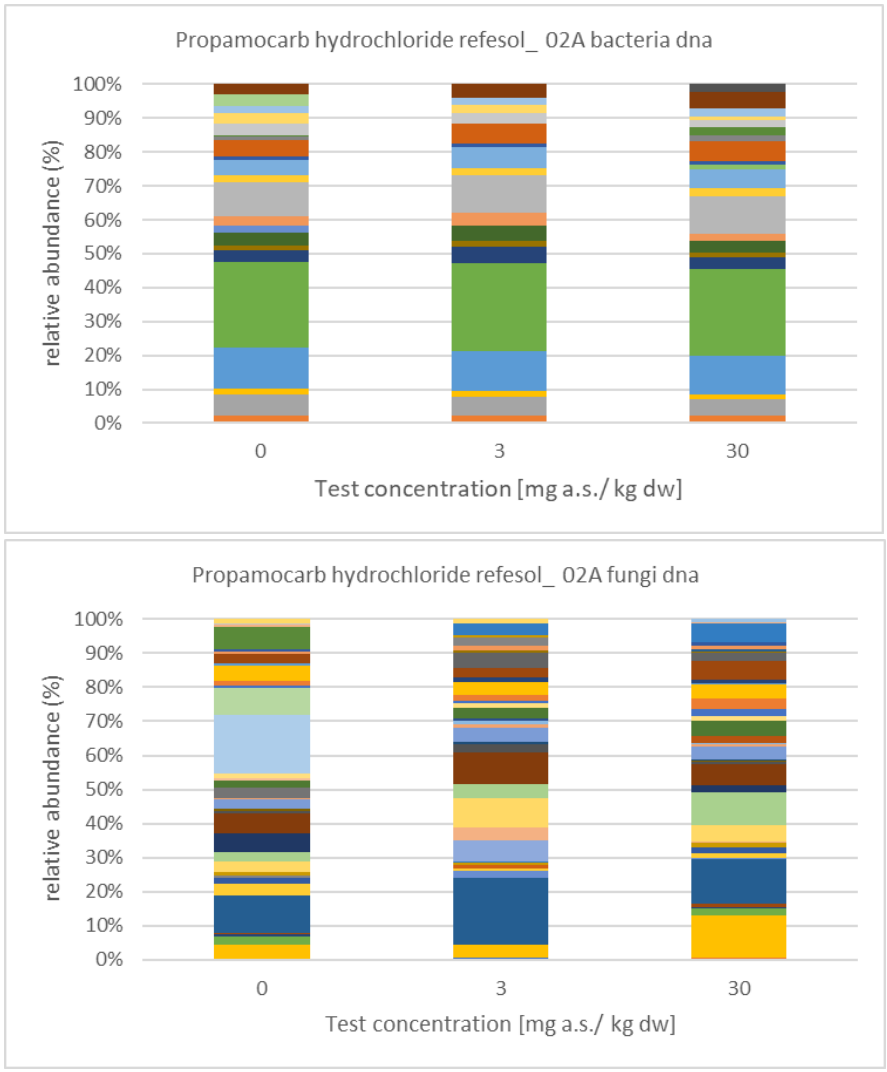
2.4.4.4.3 Soil microbial community structure (ARISA)

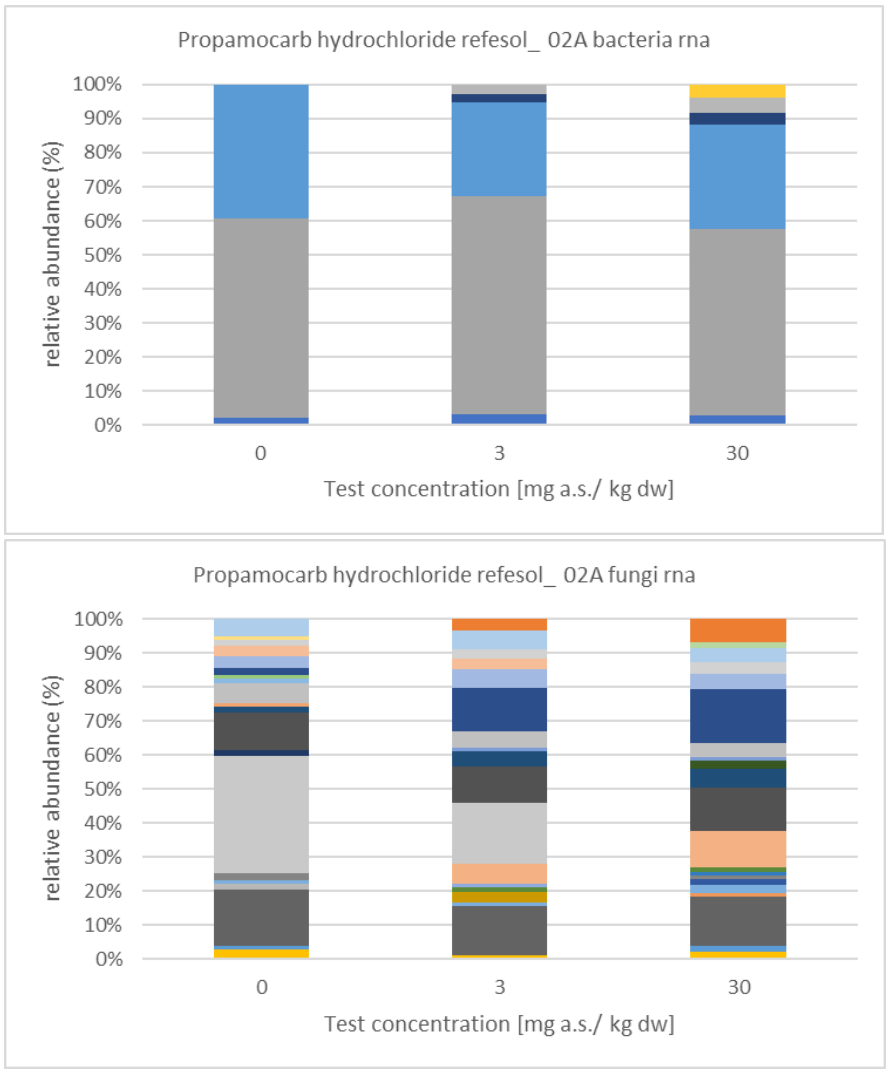
The evaluation of ARISA data revealed shifts in both bacterial and fungal community composition, as illustrated in the relative abundance plot (Figure 53) and the CA plot (Figure 54). These changes were evident at both the DNA and RNA levels, at 3 and 30 mg propamocarb hydrochloride/kg dry weight soil, indicating a clear response of the microbial communities to this treatment.

In the relative abundance plot (Figure 53), the shifts in microbial composition are visualized as changes in the proportion of specific OTUs, reflecting an increase in the number of OTUs prevalence under the influence of propamocarb hydrochloride. Similarly, the CA analysis (Figure 54) highlights these compositional shifts by clustering samples according to their community structure, with noticeable separation of treated samples from the controls. Despite dispersion within replicates, the overall trend shows that the treated samples exhibit distinct microbial profiles compared to the control.

The increase in the Shannon diversity index appears to be more closely related to an increase in taxa richness caused by the propamocarb hydrochloride treatment, rather than change in the even distribution of OTUs (Table 57). This increase suggests an increase of biodiversity within the communities, which is further supported by the clustering pattern in the CA plot.

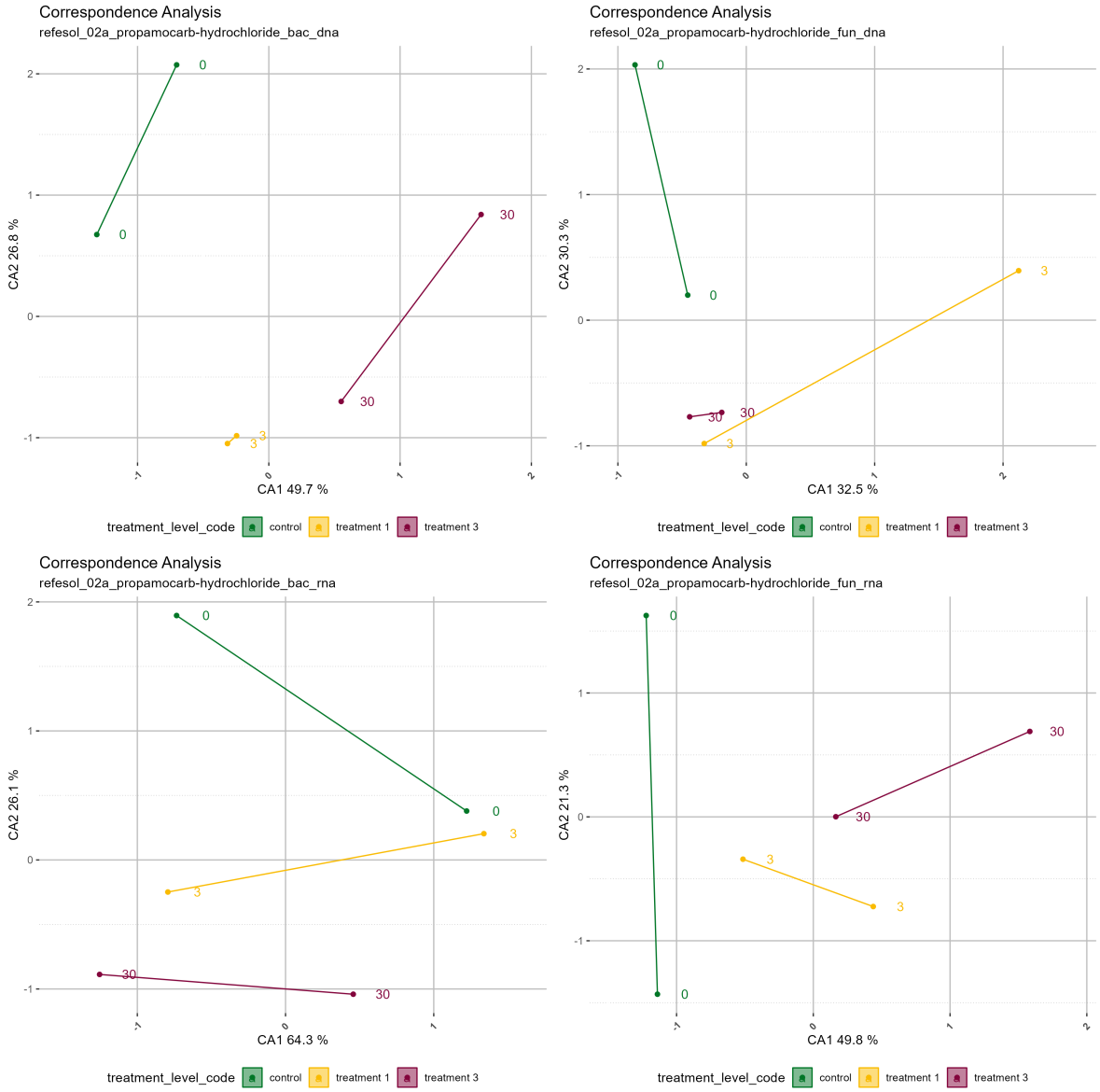
Figure 53: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 3 and 30 mg propamocarb hydrochloride/kg soil dw in RefeSol 02A after 28 days.





Source: Own illustration, Fraunhofer IME

Figure 54: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to propamocarb hydrochloride concentrations of 3 and 30 mg/kg dw soil in RefeSol 02A.



Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), bacteria RNA (bottom left), and fungi RNA (bottom right).

Table 57: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 3 mg/kg and 30 mg/kg propamocarb hydrochloride in Refesol 02A at day 28. Values represent the average of 2 replicates

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	20.5	0.9	2.6
	3 mg/kg	18.0	0.9	2.5
	30 mg/kg	21.0	0.9	2.6

DNA fungi	Control	25.0	0.9	2.7
	3 mg/kg	23.0	0.9	2.7
	30 mg/kg	28.5	0.9	2.9
RNA bacteria	Control	5.0	0.9	1.4
	3 mg/kg	8.0	0.8	1.6
	30 mg/kg	12.0	0.7	1.8
RNA fungi	Control	13.5	0.8	2.1
	3 mg/kg	14.0	0.9	2.4
	30 mg/kg	18.0	0.9	2.6

2.4.4.4.4 Integrated evaluation

Summary

Based on the results with the fungicide propamocarb hydrochloride in RefeSol 02A after approximately 100 days of exposure, the exoenzymatic activities (ISO 20130) were still inhibited by more than 25%, indicating a chronic effect on the soil enzymes. The two other test methods (MicroResp™, ISO 15685) appeared to be less sensitive compared to the ISO 20130 for the fungicide. However, the SIR with citric acid was also affected by more than 25% after 84 days of exposure.

The spore germination test (ISO 10832) with *F. mosseae* showed no statistically significant effect at the chosen test concentrations of 3 and 30 mg a.s./kg dw soil after 14 days of exposure and, therefore, was also less sensitive than, e.g. the ISO 20130.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to propamocarb hydrochloride exposure. The results suggest that propamocarb hydrochloride have an impact on the fungal and bacterial communities in a concentration-dependent manner by increasing the number of taxa observed (OTUs).

Based on the results, Table 58 summarizes the main results per test system.

Table 58: LOECs for effects of propamocarb hydrochloride in RefeSol 02A soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ISO 10832	ARISA CA	ARISA Alpha diversity
D28-SI	>30	>30	>30	3	>30	3	30
D28-I	>30	>30	30	3	>30	3	30
ETO-SI	>30	>30	3	3	>30	3	30

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ISO 10832	ARISA CA	ARISA Alpha diversity
ETO-I	>30	>30	>30	3	>30	3	30
ERO-SI	-	>30	30	3	-	-	-
ERO-I	-	>30	>30	3	-	-	-

[a]: Please note that the test duration for the spore germination test with *F. mosseae* (ISO 10832) was 14 days, and therefore, the values presented are the D14-I and D14-SI values.

2.4.5 Tiamulin hydrogen fumarate

2.4.5.1 Standard test results

The UBA data contained for tiamulin hydrogen fumarate results of a study in accordance with the OECD 216 with two test concentrations (0.36 and 3.6 mg a.s./kg dw soil) over 90 days. The test soil was a sandy loam. Effects were based on nitrate production, and measurements were performed after 28 and 90 days. After 28 days at 3.6 mg a.s./kg dw soil, a difference from the control of 102.45% was found, while there was no difference at the lower concentration of 0.36 mg a.s./kg dw soil. After the test was prolonged to 90 days, the effect decreased at the higher test concentration, and no significant difference from the control was found. At the lower concentration, the stated effects deviate. Here, a difference from the control of 14.97% is stated, which would not be relevant considering a threshold of 25%. In the OECD 216 limit test, the veterinary pharmaceutical tiamulin hydrogen did not affect the nitrogen transformation after 14 and 28 days of exposure in the three test soils at the chosen test concentrations of 3.6 mg a.s./kg dw soil.

2.4.5.2 Lufa 2.1.

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity due to tiamulin hydrogen fumarate are presented in Table 59.

2.4.5.2.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

At test initiation, no effect on the AOB (ISO 15685) was observed at the chosen test concentrations of 0.36 to 7.2 mg a.s./kg dw soil. From day 14 until day 84, a steady concentration-dependent inhibitory effect was observed with inhibition of 15%, 17% and 19% at 3.6 mg a.s./kg dw soil and 46%, 41% and 47% at 7.2 mg a.s./kg dw soil, respectively.

At test initiation, after 14 and 28 days for the SIR (MicroResp™), no difference from the control of more than 25% was found with any of the used substrates. Therefore, the test system was not used during the test prolongation to day 56 or 84.

An inhibitory effect on the activity of ß-glucosidase (32%) and arylsulfatase (26%) was found at test initiation at the lowest test concentration of 0.36 mg a.s./kg dw soil, while there was no effect on the other tested enzymes. Until day 14, the observed effects decreased, and no effect was above 25% on the tested enzymes. The strongest effect on the enzymatic activity was found after 28 days. Here, at 3.6 mg a.s./kg dw soil, the activity of ß-glucosidase (32%) and urease (32%) was affected, but no effect was determined at the other test concentrations. For the

arylsulfatase activity at the two lower test concentrations of 0.36 and 3.6 mg a.s./kg dw soil, 26% and 58% were inhibited, respectively. No effect occurred at the highest test concentration of 7.2 mg a.s./kg dw soil. Therefore, the test was also performed after 56 days of exposure, but no more than 25% effect on the activity of the observed enzymes was found.

Table 59: Effect of tiamulin hydrogen fumarate with application rates of 0.36, 3.6 and 7.2 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Lufa 2.1. as test soil.

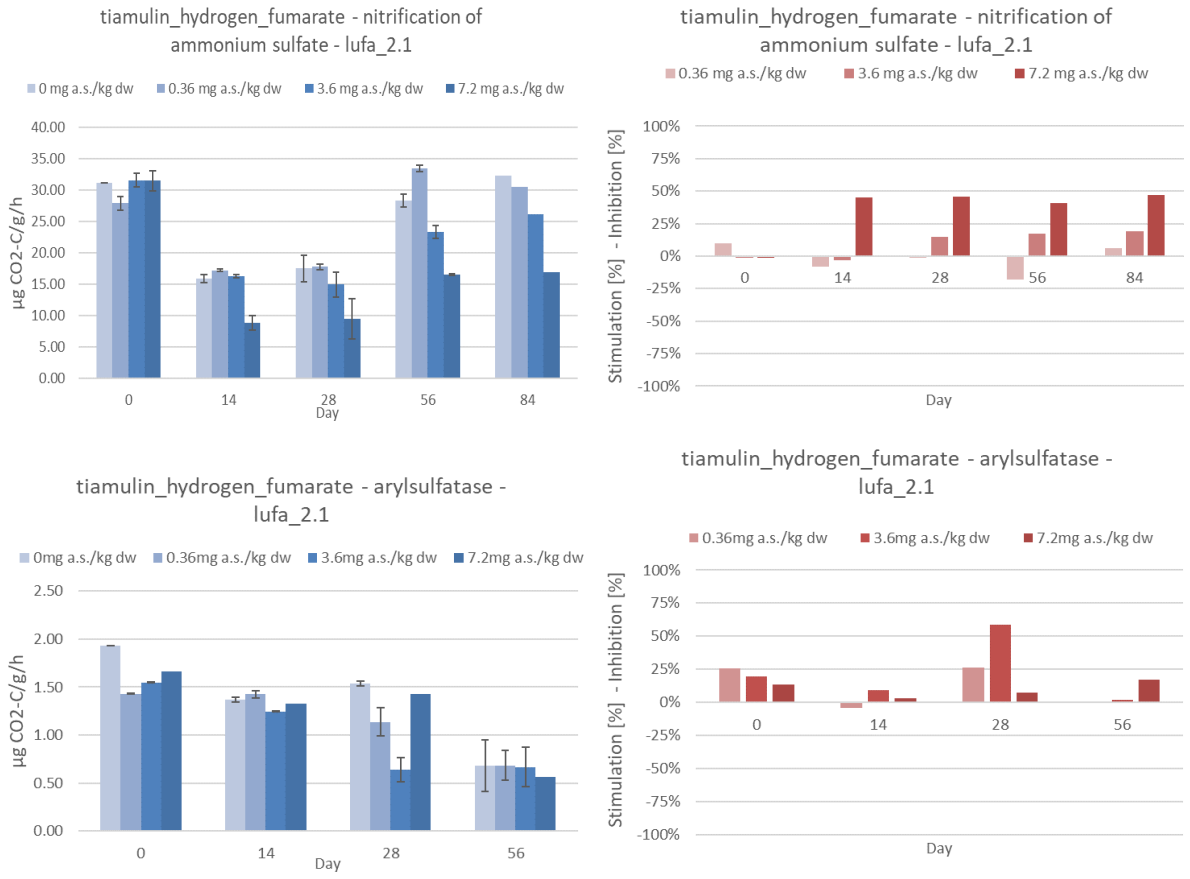
Test day	Test system	Test details	Inhibition [%]		
			0.36 mg/kg	3.6 mg/kg	7.2 mg/kg
Test initiation D0	ISO 15685	-	10	-1	-1
	MicroResp™	Deionized water	-1	-7	-6
		D-(+)-Glucose	-7	-10	-11
		L-Cysteine hydrochloride	-7	-10	-12
		L-Malic acid	-12	-11	-15
		y-Amino butyric acid	-10	-12	-14
		N-Acetyl glucosamine	-10	-12	-13
		Citric acid	-10	-14	-13
		L-Alanine	-12	-7	-9
	ISO 20130	Phosphatase	8	11	9
		ß-Glucosidase	33	16	8
		Arylsulfatase	26	20	14
		Arylamidase	4	-2	10
		Urease	23	20	8
D14	ISO 15685	-	-8	-3	45
	MicroResp™	Deionized water	6	9	11
		D-(+)-Glucose	6	7	8
		L-Cysteine hydrochloride	6	6	7
		L-Malic acid	4	7	7
		y-Amino butyric acid	4	5	8
		N-Acetyl glucosamine	3	4	5
		Citric acid	3	1	2

Test day	Test system	Test details	Inhibition [%]		
			0.36 mg/kg	3.6 mg/kg	7.2 mg/kg
		L-Alanine	5	8	8
	ISO 20130	Phosphatase	4	17	20
		ß-Glucosidase	-2	15	10
		Arylsulfatase	-4	9	3
		Arylamidase	-21	3	1
		Urease	-9	9	10
D28	ISO 15685	-	-1	15	46
	MicroResp™	Deionized water	-4	-6	-5
		D-(+)-Glucose	-4	-6	-2
		L-Cysteine hydrochloride	-3	-5	-3
		L-Malic acid	-6	-5	-4
		y-Amino butyric acid	-7	-6	-3
		N-Acetyl glucosamine	-6	-5	-4
		Citric acid	-12	-7	-7
		L-Alanine	-8	-6	-5
	ISO 20130	Phosphatase	8	0	-7
		ß-Glucosidase	19	32	20
		Arylsulfatase	26	58	7
		Arylamidase	-3	6	14
		Urease	-8	32	21
D56	ISO 15685	-	-18	17	41
	ISO 20130	Phosphatase	3	13	22
		ß-Glucosidase	2	8	21
		Arylsulfatase	0	2	17
		Arylamidase	-6	3	6
		Urease	6	3	15
D84	ISO 15685	-	6	19	47
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	*18*
D28			-	-	*1*

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

ISO 15685 and ISO 20130 results regarding the effect of tiamulin hydrogen fumarate on the ammonium oxidizing bacteria and the exoenzymatic activity were chosen exemplarily to demonstrate certain effects on the soil microorganisms and their function (Figure 55).

Figure 55: Exemplary results of the potential nitrification (ISO 15685; upper figure) and the exoenzymatic activity (ISO 20130; lower figure) for tiamulin hydrogen fumarate at application rates of 0.36, 3.6 and 7.2 mg/kg dw soil in the test with Lufa 2.1.



Source: Own illustration, Fraunhofer IME

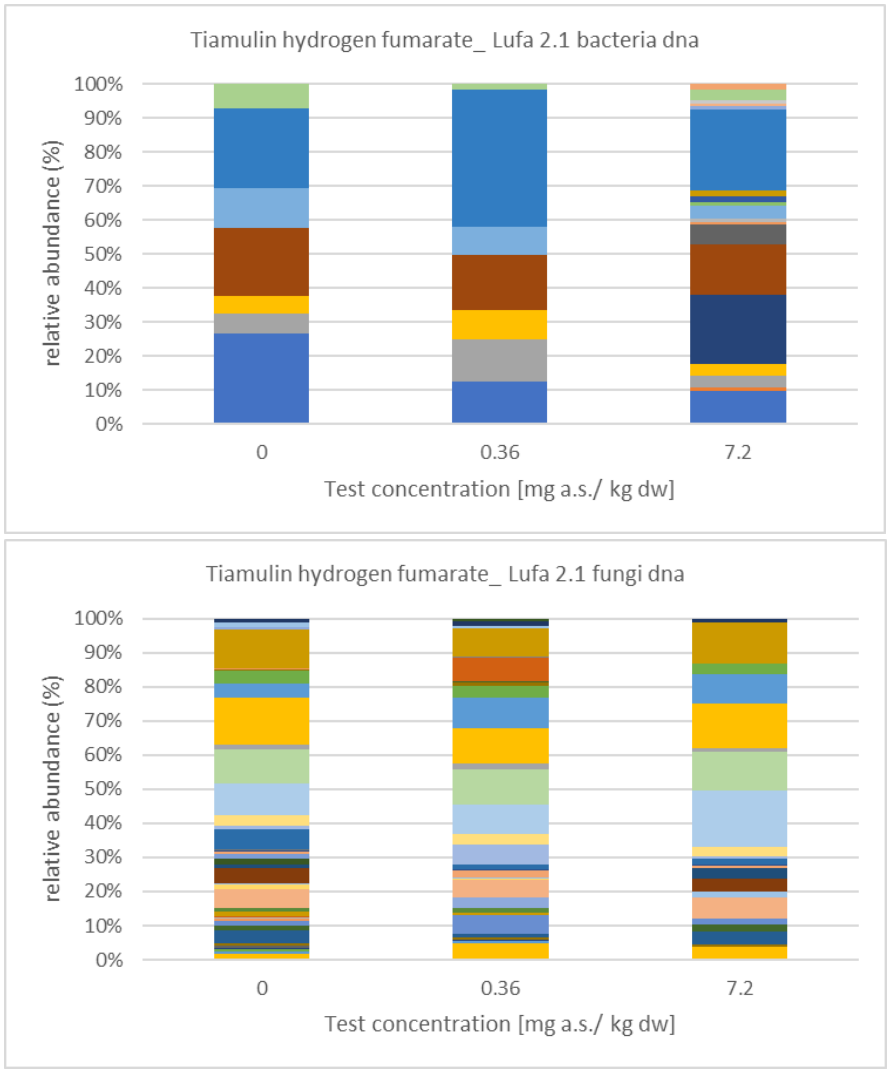
2.4.5.2.2 Soil microbial community structure (ARISA)

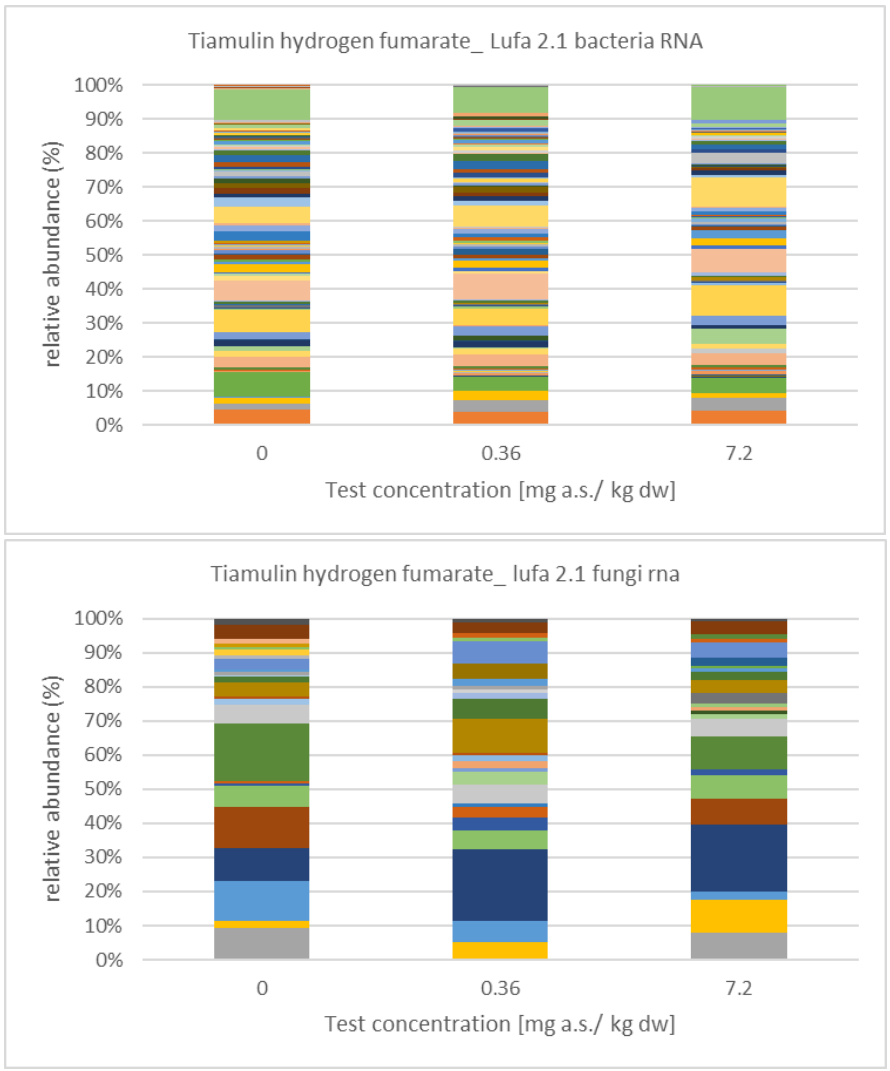
The evaluation of ARISA data revealed shifts in both bacterial and fungal community composition, as illustrated in the relative abundance plot (Figure 56) and the CA plot (Figure 57). The data did not show a reproducible pattern between DNA and RNA levels. No concentration-response for fungal and bacterial DNA was found. A tendency for a concentration-response (ranking along the x-axis) was observable for bacterial DNA but not fungal DNA (Figure 57).

In the relative abundance plot (Figure 56), the shifts in microbial composition could not be clearly interpreted due to the high number of OTUs in the samples. However, CA analysis (Figure 57) highlights these compositional shifts by clustering samples according to their community structure, with noticeable separation of treated samples from the controls.

The Shannon diversity index showed an increase which is related to an increase in the bacterial richness at DNA level at a concentration of Tiamulin hydrogen fumarate of 7.2 mg a.s./kg dw soil. The fungal index slightly decreased at the same concentration, for both DNA and RNA (Table 60).

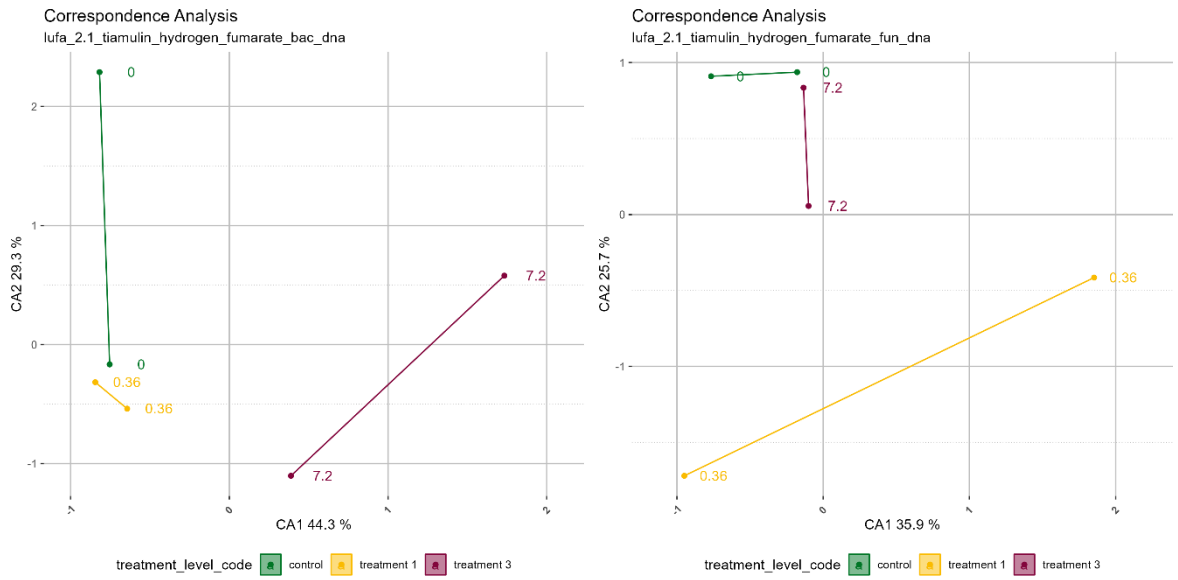
Figure 56: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 0.36 and 7.2 mg tiamulin hydrogen fumarate/kg soil dw in Lufa 2.1. after 28 days.

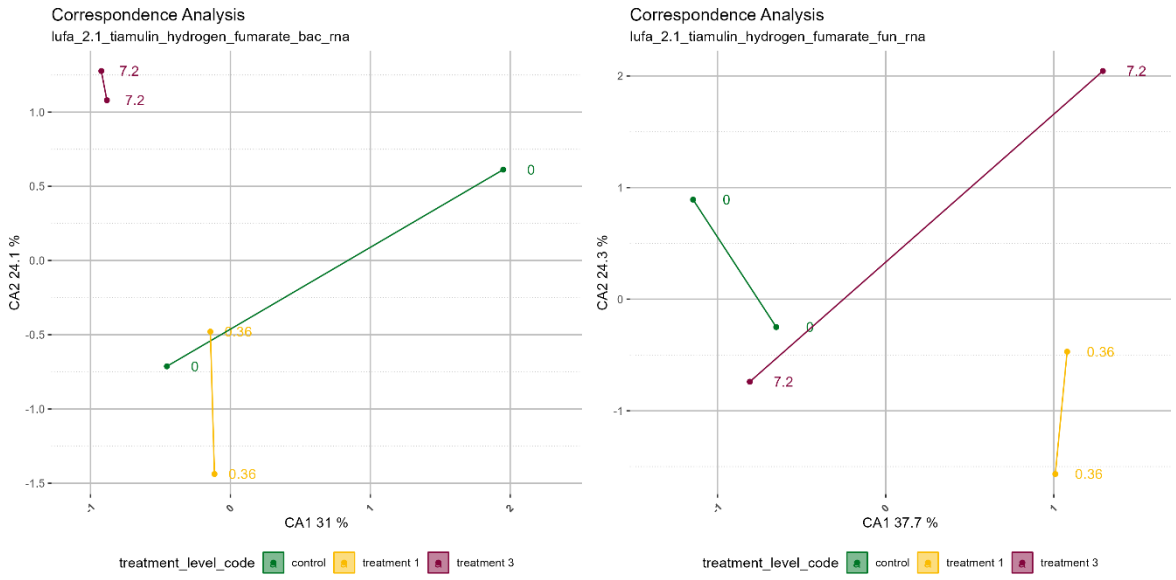




Source: Own illustration, Fraunhofer IME

Figure 57: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to tiamulin hydrogen fumarate concentrations of 0.36 and 7.2 mg/kg dw soil in Lufa 2.1.





Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), bacteria RNA (bottom left) and fungi RNA (bottom right).

Table 60: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 0.36 and 7.2 mg/kg tiamulin hydrogen fumarate in Lufa 2.1 at day 28. Values represent the average of 2 replicates.

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	5.5	0.9	1.5
	0.36 mg/kg	6.0	0.9	1.6
	7.2 mg/kg	12.5	0.9	2.1
DNA fungi	Control	27.5	0.9	2.9
	0.36 mg/kg	26.5	0.9	2.9
	7.2 mg/kg	19.0	0.9	2.6
RNA nacteria	Control	60.0	0.9	3.6
	0.36 mg/kg	69.0	0.9	3.8
	7.2 mg/kg	60.5	0.9	3.5
RNA fungi	Control	18.0	0.9	2.5
	0.36 mg/kg	18.0	0.9	2.5
	7.2 mg/kg	15.5	0.9	2.4

2.4.5.2.3 Integrated evaluation

Summary

For the veterinary pharmaceutical tiamulin hydrogen fumarate, the ammonium oxidizing bacteria appeared to be the most sensitive group of soil microorganisms considering the three applied test methods (ISO 15685, ISO 20130 and MicroResp™). Here, even the establishment of a dose-response relationship was possible. While the SIR with eight different substrates was not affected at all within 28 days of exposure, the activity of the exoenzymes ß-glucosidase, arylsulfidase and urease were affected by more than 25% but without dose-response relationship until day 28, but no effect above 25% was found at day 56.

The effect of tiamulin hydrogen fumarate on the spore germination of *F. mosseae* in accordance with ISO 10832 could not be determined due to the poor spore germination of the AMF in LUFA 2.1.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to tiamulin hydrogen fumarate exposure. The results suggest that tiamulin hydrogen fumara have an impact on the fungal and bacterial communities at a concentration of 7.2 mg a.s./kg dw soil.

Based on the results, Table 61 summarizes the main results per test system.

Table 61: LOECs for effects of tiamulin hydrogen fumarate in Lufa 2.1 soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	>7.2	7.2	>7.2	0.36	7.2	7.2
D28-I	>7.2	7.2	>7.2	0.36	7.2	7.2
ETO-SI	>7.2	7.2	>7.2	0.36	7.2	7.2
ETO-I	>7.2	7.2	>7.2	0.36	7.2	7.2
ERO-SI	-	7.2	>7.2	>7.2	-	-
ERO-I	-	7.2	>7.2	>7.2	-	-

2.4.5.3 Refesol 04A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to tiamulin are presented in Table 62.

2.4.5.3.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

The activity of the AOB (ISO 15685) was stimulated in a concentration-dependent manner from 38% at the lowest test concentration of 0.36 mg a.s./kg dw soil to 104% at 14.4 mg a.s./kg dw soil after 14 days of exposure. After 28 days of exposure, the stimulation decreased significantly in all treatments. At the three lower test concentrations, an inhibition between 8 and 38% was found, while at the highest test concentration, the stimulation decreased to 11%. Therefore, the ISO 15685 was performed at day 56, but no further effect on the AOB activity above 25% was determined at test concentrations ranging from 0.36 to 14.4 mg a.s./kg dw soil.

The MicroResp™ system showed no effects above 25% on basal respiration and the SIR at the chosen test concentrations after 14 days of exposure. However, after 28 and 56 days of exposure, the SIR (all substrates) was stimulated above 25%, with a clear tendency to have stronger effects at lower test concentrations. The basal respiration was not affected by more than 25% at any of the chosen test concentrations. After 84 days of exposure, the effects could not be determined for the various substrates at the four test concentrations.

The enzyme activity (ISO 20130) appeared to be the most sensitive method compared to the other two test systems. After 14 days of exposure, tiamulin hydrogen fumarate inhibited the urease activity by 65% to 100%. However, the strongest inhibition was found at the lowest test concentration, while the lowest was found at the highest. A similar pattern was observed in terms of stimulation for arylsulfatase (20% to 70%). The ß-glucosidase activity was stimulated between 46% and 28% without any concentration dependency. Neither phosphatase nor arylamidase activity differed by more than 25% from the control activity. After 28 days of exposure, the urease activity was still inhibited between -6% and 71% in a concentration-dependent manner. The results of the ß-glucosidase differed significantly. While there was an inhibition of 52% at the lowest test concentration of 0.36 mg a.s./kg dw soil, a stimulation of 32% was found at a concentration of 3.6 mg a.s./kg dw soil and no differences from the control of more than 25% at the other test concentrations. Phosphatase and arylamidase activity was not affected. Due to the effects at day 28, the test was also performed after 56 days for the three affected enzymes. No effect on the arylamidase activity was found, but the ß-glucosidase and urease activity were still affected by more than 25%, but without a specific dose-response relationship. After 84 days of exposure, only at the urease activity, an effect of more than 25% was measured with stimulations of 99% and 86% compared to the control treatment at 7.2 and 14.4 mg a.s./kg dw soil.

Table 62: Effects of tiamulin hydrogen fumarate with application rates of 0.36, 3.6, 7.2 and 14.4 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Refesol 04A as test soil.

Test day	Test system	Test details	Inhibition [%]			
			0.36 mg/kg	3.6 mg/kg	7.2 mg/kg	14.4 mg/kg
D14	ISO 15685	-	-38	-81	-89	-104
	MicroResp™	Deionized water	-9	-11	-7	-8
		D-(+)-Glucose	-11	-11	4	0
		L-Cysteine hydrochloride	-5	-8	5	-1
		L-Malic acid	-6	-9	5	-1

Test day	Test system	Test details	Inhibition [%]			
			0.36 mg/kg	3.6 mg/kg	7.2 mg/kg	14.4 mg/kg
		y-Amino butyric acid	-1	-4	7	1
		N-Acetyl glucosamine	3	0	9	2
		Citric acid	2	-1	15	8
		L-Alanine	0	-1	16	8
	ISO 20130	Phosphatase	-15	-19	-23	-22
		ß-Glucosidase	-33	-28	1	-46
		Arylsulfatase	-70	-53	-48	-20
		Arylamidase	-21	-7	-18	8
		Urease	100	94	91	65
D28	ISO 15685	-	8	21	38	-11
	MicroResp™	Deionized water	-14	-11	-16	-11
		D-(+)-Glucose	-30	-21	-20	-13
		L-Cysteine hydrochloride	-19	-27	-18	-12
		L-Malic acid	-21	-25	-21	-14
		y-Amino butyric acid	-25	-20	-19	-18
		N-Acetyl glucosamine	-21	-16	-19	-13
		Citric acid	-31	-20	-25	-17
		L-Alanine	-21	-16	-26	-15
	ISO 20130	Phosphatase	-2	-10	14	17
		ß-Glucosidase	52	-32	9	16
		Arylsulfatase	14	13	22	-1
		Arylamidase	-17	-14	5	-46
		Urease	-6	34	71	69
D56	ISO 15685	-	-11	-13	21	7
	MicroResp™	Deionized water	-11	-16	-23	-16
		D-(+)-Glucose	-68	-70	-30	-26
		L-Cysteine hydrochloride	-56	-64	-32	-24

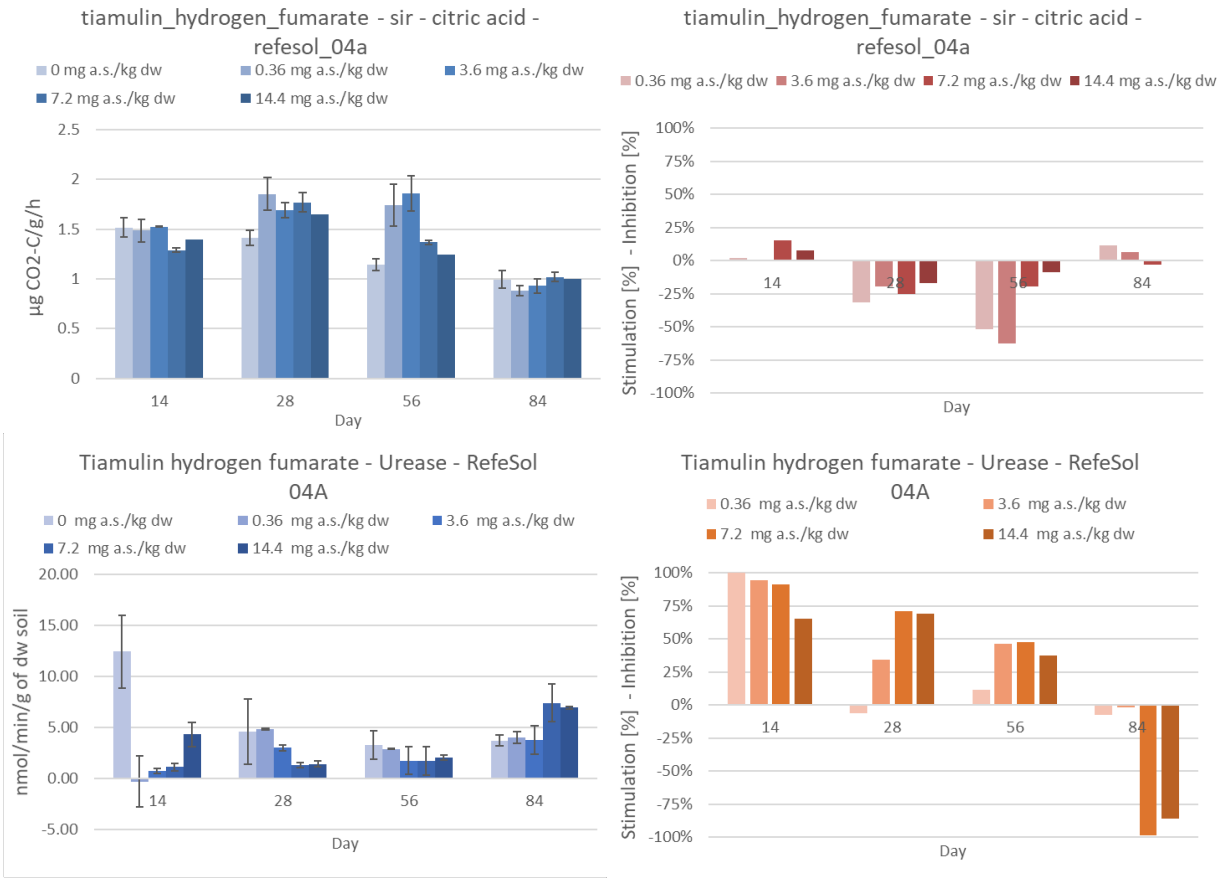
Test day	Test system	Test details	Inhibition [%]			
			0.36 mg/kg	3.6 mg/kg	7.2 mg/kg	14.4 mg/kg
		L-Malic acid	-57	-64	-37	-18
		y-Amino butyric acid	-43	-52	-28	-15
		N-Acetyl glucosamine	-44	-55	-24	-12
		Citric acid	-52	-63	-20	-9
		L-Alanine	-35	-43	-11	-3
	ISO 20130	ß-Glucosidase	-12	-43	-12	-44
		Arylamidase	21	5	9	15
		Urease	12	47	47	37
D84	MicroResp™	Deionized water	3	-1	-12	-11
		D-(+)-Glucose	3	-2	-8	-9
		L-Cysteine hydrochloride	4	-2	-8	-8
		L-Malic acid	20	11	8	4
		y-Amino butyric acid	9	4	-6	-8
		N-Acetyl glucosamine	9	7	-2	-2
		Citric acid	11	6	-3	-1
		L-Alanine	6	1	-7	-5
	ISO 20130	ß-Glucosidase	-8	-19	-3	-2
		Urease	-7	-2	-99	-86
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	-15	-
D28			-	-	7	-

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

For example, some effects are shown in Figure 60 to obtain a better picture of certain effects. The concentration-response of ß-glucosidase at day 28 is difficult to explain because the largest inhibition was found at 2 mg a.s./kg dw soil, decreasing with increasing exposure. Thus, the LOEC für inhibition is estimated to be > 100 mg/kg. The lowest estimated LOEC is at 2 mg a.s./kg dw soil for stimulation on day 14. On day 84, stimulation at 100 mg a.s./kg dw soil was above 25%. Significance was not tested, but the LOEC was estimated to be 100 mg a.s./kg dw soil.

Urease was inhibited sometimes above 25 %, but this was never related to a plausible concentration-response relation. Thus, no clear effects were found.

Figure 58: Exemplary results for effects of tiamulin hydrogen fumarate at application rates of 0.36, 3.6, 7.2 and 14.4 mg/kg dw soil on microbial function. MicroResp™ – SIR with the substrate citric acid (upper figure) and exoenzymatic activity of urease (ISO 20130, lower figure) and the corresponding effects.

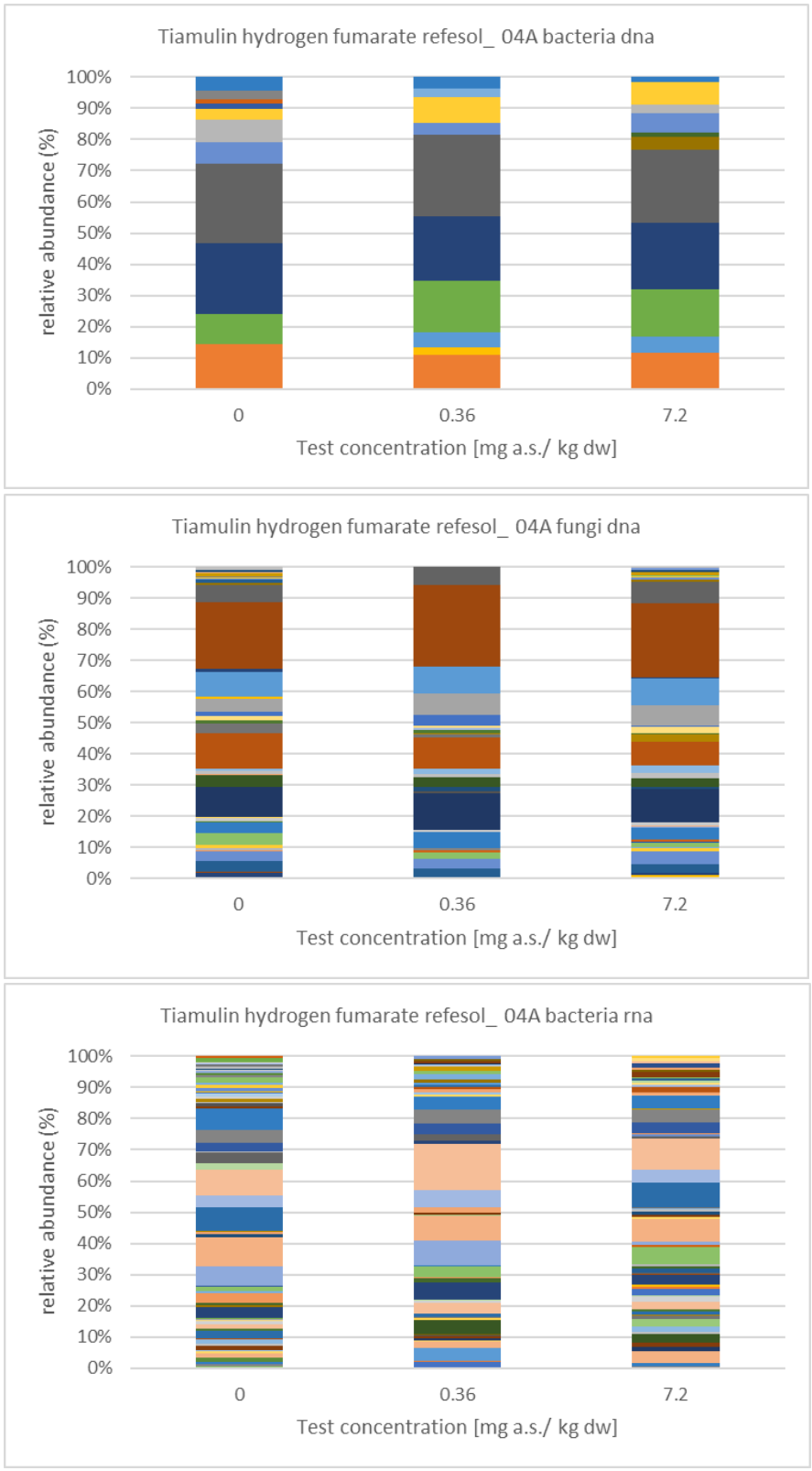


Source: Own illustration, Fraunhofer IME

2.4.5.3.2 Soil microbial community structure (ARISA)

The analysis of ARISA data did not show evident changes in bacterial or fungal profiles. The relative abundance plots (Figure 73) are complex to interpret due to the high number of OTUs, and while apparent changes are observed in the CA plots, these are not very meaningful due to the large dispersion in the data (Figure 74). Additionally, the Shannon index indicated no changes in bacterial or fungal diversity at the tested concentrations of 0.36 and 7.2 mg a.s./kg dw soil of tiamulin hydrogen fumerate (Table 63).

Figure 59: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 0.36 and 7.2 mg tiamulin hydrogen fumarate/kg soil dw in RefeSol 04A after 28 days.



Source: Own illustration, Fraunhofer IME

Figure 60: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to tiamulin hydrogen fumarate concentrations of 0.36 and 7.2 mg/kg dw soil in RefeSol 04A.

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), and bacteria RNA (bottom left).

Table 63: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 0.36 and 7.2 mg/kg tiamulin hydrogen fumarate in Refesol 04A at day 28. Values represent the average of 2 replicates.

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	9.0	0.9	1.9
	0.36 mg/kg	8.0	0.9	1.9
	7.2 mg/kg	8.5	0.9	2.0
DNA fungi	Control	31.0	0.8	2.8

	0.36 mg/kg	21.0	0.8	2.5
	7.2 mg/kg	29.5	0.8	2.8
RNA bacteria	Control	38.0	0.9	3.3
	0.36 mg/kg	32.0	0.9	3.1
	7.2 mg/kg	38.0	0.9	3.4

2.4.5.3.3 Integrated evaluation

Summary

For the veterinary pharmaceutical tiamulin hydrogen fumarate in RefeSol 04A, the exoenzymatic activity of urease appeared to be the most sensitive parameter considering the three applied test methods (ISO 15685, ISO 20130 and MicroResp™). However, tiamulin hydrogen fumarate also had a strong effect on the SIR with most of the chosen substrates until 56 days of exposure, but no effect was determined after 84 days.

The effect of tiamulin hydrogen fumarate on the spore germination of *F. mosseae* in accordance with ISO 10832 could not be determined due to the poor spore germination of the AMF in RefeSol 04A.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to tiamulin hydrogen fumarate exposure. The results suggest that tiamulin hydrogen fumarate has an impact on the fungal and bacterial communities at concentrations of 0.36 and 7.2 mg a.s./kg dw soil.

Based on the results, Table 64 summarizes the main results per test system.

Table 64: LOECs for effects of tiamulin hydrogen fumarate in RefeSol 04A.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	>7.2	7.2	0.36	0.36	0.36	>7.2
D28-I	>7.2	7.2	>14.4	0.36	0.36	>7.2
ETO-SI	>7.2	7.2	0.36	0.36	0.36	>7.2
ETO-I	>7.2	7.2	>14.4	0.36	0.36	>7.2
ERO-SI	-	>14.4	>14.4	7.2	-	-
ERO-I	-	>14.4	>14.4	>14.4	-	-

2.4.5.4 Refesol 02A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to ethofumesate are presented in Table 65.

2.4.5.4.1 Soil microbial function (ISO 15685, MicroRespTM, ISO 20130)

After 28 days of incubation, the activity of the AOB (ISO 15685) and the SIR (MicroRespTM) were not affected by more than 25% at the three chosen test concentrations compared to the control. Therefore, neither test system was performed on days 56 and 84.

The enzyme activity (ISO 20130) of the arylsulfatase, ß-glucosidase and urease were not affected by more than 25% after 28 days. The phosphatase activity was strongly increased compared to the control, showing stimulations of up to 388% at 3.6 mg a.s./kg dw soil. The arylamidase activity was stimulated by 32% at the highest test concentration of 7.2 mg a.s./kg dw soil. Therefore, the effect of tiamulin hydrogen fumarate on the enzymatic activity of phosphatase and arylamidase was also determined after 56 days of exposure. Here, only the phosphatase was still affected by more than 25% with a stimulation of 26% at 3.6 mg a.s./kg dw soil. After 84 days of exposure, even this enzymatic activity was no longer affected due to the veterinary pharmaceutical.

Table 65: Effects of tiamulin hydrogen fumarate with application rates of 0.36, 3.6 and 7.2 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using RefeSol 02A as test soil.

Test date	Test system	Test details	Inhibition [%]		
			0.36 mg/kg	3.6 mg/kg	7.2 mg/kg
D28	ISO 15685	-	-2	-8	1
	MicroResp™	Deionized water	-6	-21	-2
		D-(+)-Glucose	-6	-21	3
		L-Cysteine hydrochloride	-8	-23	5
		L-Malic acid	-7	-22	6
		y-Amino butyric acid	-6	-21	5
		N-Acetyl glucosamine	-4	-20	1
		Citric acid	-3	-17	-11
		L-Alanine	-5	-20	-20
	ISO 20130	Phosphatase	-220*	-388*	-226*
		ß-Glucosidase	1	-6	-20
		Arylsulfatase	8	-18	-8
		Arylamidase	-9	3	-32
		Urease	16	18	4
D56	ISO 20130	Phosphatase	-24	-26	-17

Test date	Test system	Test details	Inhibition [%]		
			0.36 mg/kg	3.6 mg/kg	7.2 mg/kg
		ß-Glucosidase	-10	-20	2
		Arylamidase	8	20	18
D84	ISO 20130	Phosphatase	-11	-13	-22
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	*-17*
D28			-	-	*-8*

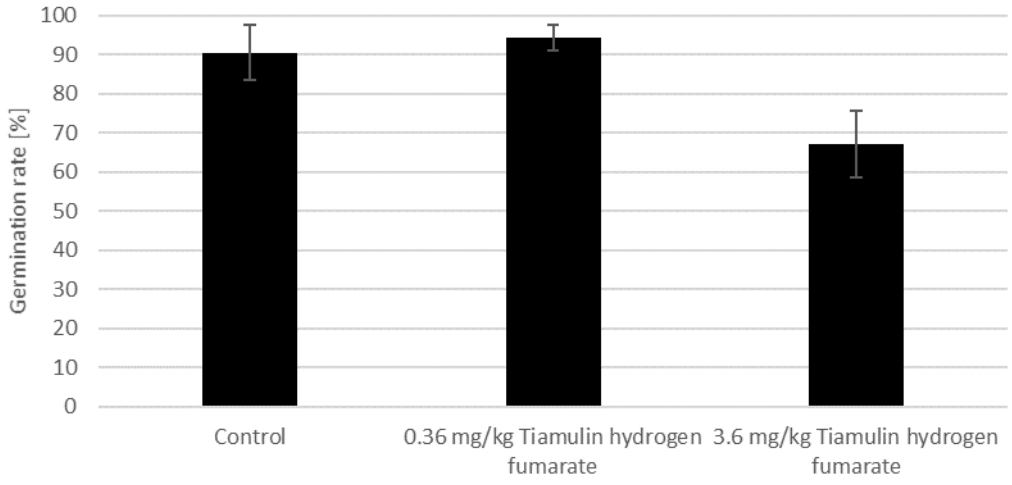
Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

*There has been only a low activity for the control treatments in 3 of 4 replicates. Therefore, the results are unreliable and will not be coloured in red.

2.4.5.4.2 Arbuscular mycorrhizal fungi (ISO 10832)

For the test with the veterinary pharmaceutical tiamulin hydrogen fumarate, two concentrations of 0.36 and 3.6 mg a.s./kg dw soil were tested against a control. The test fulfilled the validity criteria. The spore germination was statistically significantly affected by the highest test concentration of 3.6 mg a.s./kg dw soil with inhibition of 29.9% after 14 days of exposure. The NOEC appeared at the lower test concentration of 0.36 mg a.s./kg dw soil.

Figure 61: Results of the spore germination test with tiamulin hydrogen fumarate at 0.36 and 3.6 mg/kg dw soil in RefeSol 02A after 14 days of incubation.



Source: Own illustration, Fraunhofer IME

2.4.5.4.3 Soil microbial community structure (ARISA)

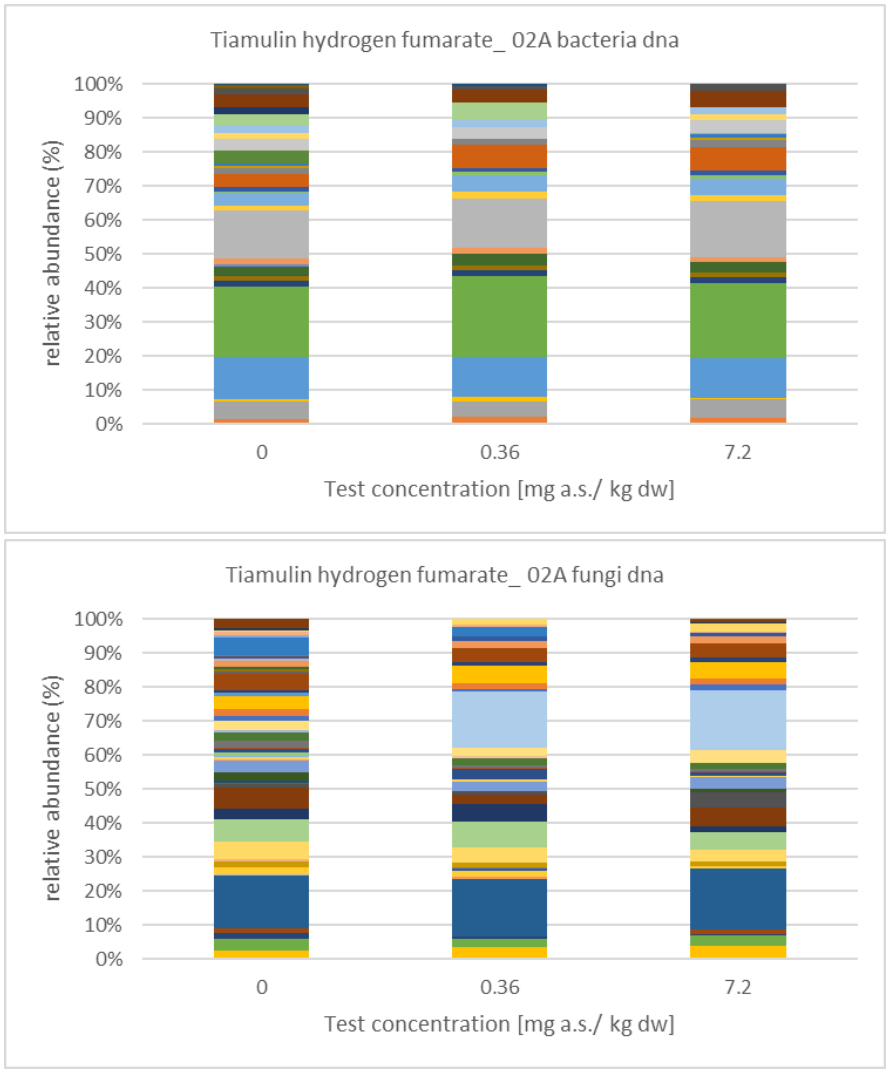
The evaluation of ARISA data showed changes in the bacterial and fungal community composition which can be observed in the relative abundances plot (Figure 77) and the CA analysis (Figure 78). The changes were observed at DNA and RNA level, in particular at 0.36 and 7.2 mg tiamulin hydrogen fumerate/kg dw soil, indicating a clear response of the microbial communities to this treatment.

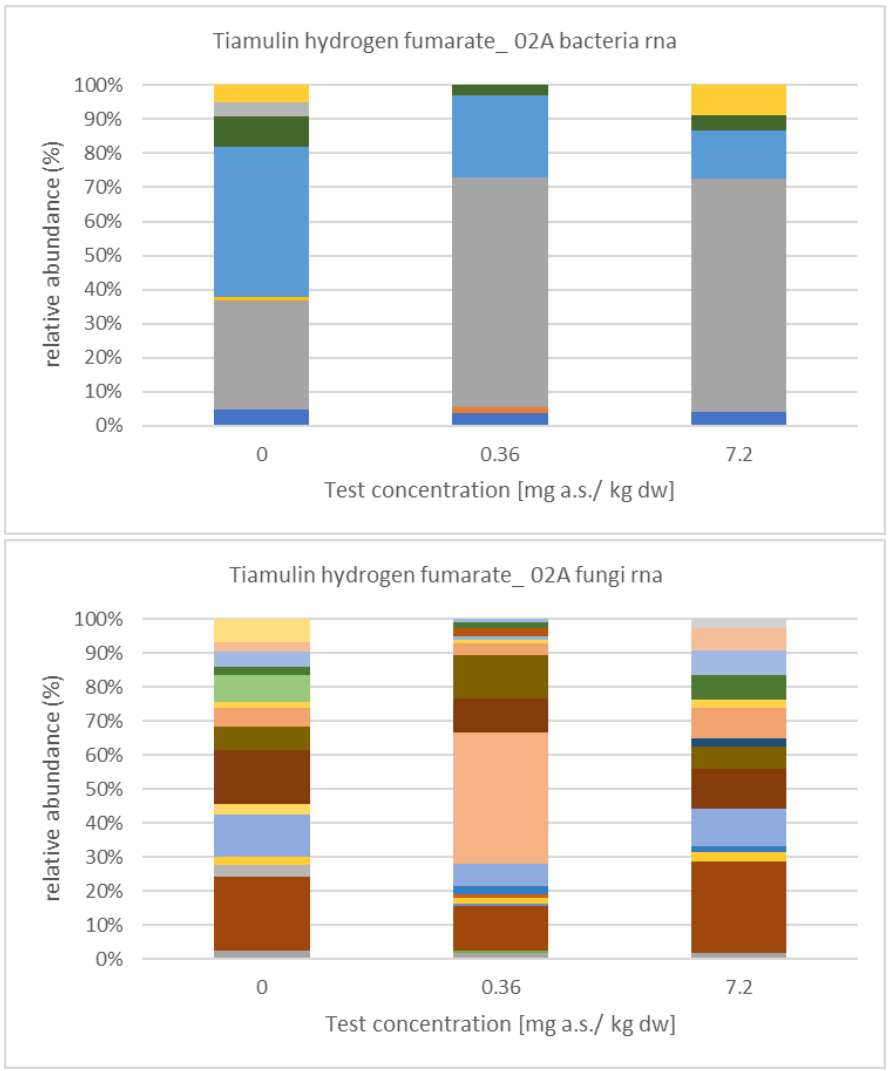
In the relative abundance plot (Figure 77), the shifts in microbial composition are visualized as changes in the proportion of specific OTUs, reflecting altered taxa prevalence under the influence of tiamulin hydrogen fumerate. Similarly, the CA analysis (Figure 78) highlights these compositional shifts by clustering samples according to their community structure, with

noticeable separation of treated samples from the controls. Despite dispersion within replicates, the overall trend shows that the treated samples exhibit distinct microbial profiles compared to the control.

The decrease in the Shannon diversity index appears to be related to a reduction in the number of OTUs observed caused by the tiamulin hydrogen fumerate treatment, added to small changes in the even distribution of OTUs (Table 66).

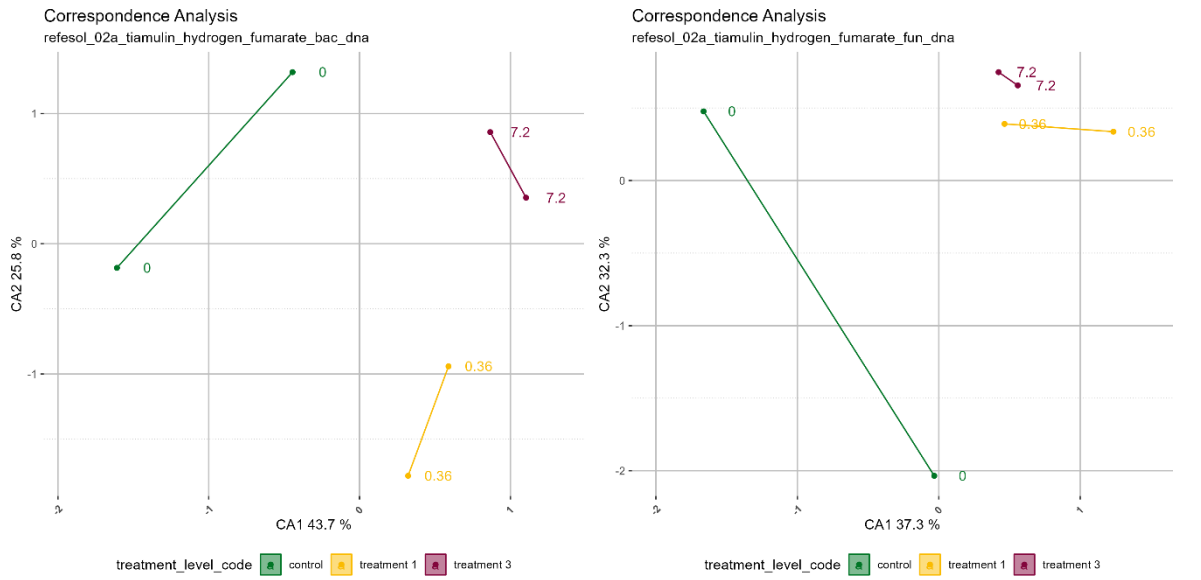
Figure 62: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 0.36 and 7.2 mg tiamulin hydrogen fumarate/kg soil dw in RefeSol 02A after 28 days.

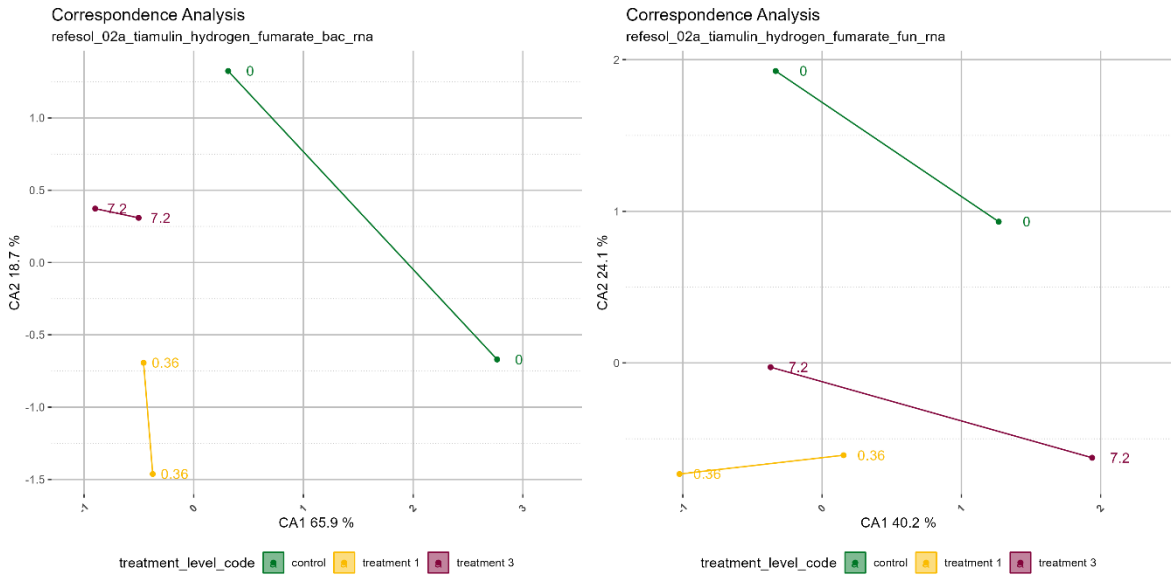




Source: Own illustration, Fraunhofer IME

Figure 63: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to tiamulin hydrogen fumarate concentrations of 0.36 and 7.2 mg/kg dw soil in RefeSol 02A.





Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), bacteria RNA (bottom left), and fungi RNA (bottom right).

Table 66: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 0.36 and 7.2 mg/kg tiamulin hydrogen fumarate in Refesol 02A at day 28. Values represent the average of 2 replicates.

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	26.5	0.8	2.8
	0.36 mg/kg	20.5	0.9	2.6
	7.2 mg/kg	22.0	0.8	2.6
DNA fungi	Control	37.5	0.9	3.2
	0.36 mg/kg	29.5	0.8	2.9
	7.2 mg/kg	26.0	0.9	2.8
RNA bacteria	Control	10.0	0.8	1.8
	0.36 mg/kg	8.0	0.7	1.6
	7.2 mg/kg	10.0	0.7	1.7
RNA fungi	Control	12.0	0.9	2.2
	0.36 mg/kg	13.0	0.8	2.0
	7.2 mg/kg	11.0	0.9	2.0

2.4.5.4.4 Integrated evaluation

Summary

Based on the results of the veterinary pharmaceutical tiamulin hydrogen fumarate in RefeSol 02A after approximately 100 days of exposure, none of the three chosen test methods (ISO 15685, MicroResp™, ISO 20130) to observe the effect on soil microorganisms indicated an effect by more than 25% on the respective group of microorganisms (nitrifiers, enzymes) or function (SIR).

The spore germination test (ISO 10832) with *F. mosseae* showed a statistically significant effect (29.9%) at a test concentration of 3.6 mg a.s./kg dw soil after 14 days of exposure. Therefore, for the veterinary pharmaceutical, the spore germination test with the AMF appears to be the most sensitive organism, and the results highlight the relevance of testing fungi next to soil microorganisms in more complex soils, as RefeSol 02A, at least for veterinary pharmaceuticals.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to tiamulin hydrogen fumarate exposure. The results suggest that tiamulin hydrogen fumarate has an impact on the fungal and bacterial communities at a concentration of 0.36 and 7.2 mg a.s./kg dw soil.

Based on the results, Table 67 summarizes the main results per test system.

Table 67: LOECs for effects of tiamulin hydrogen fumarate in RefeSol 02A soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ISO 10832	ARISA CA	ARISA Alpha diversity
D28-SI	>7.2	>7.2	>7.2	7.2	3.6	0.36	0.36
D28-I	>7.2	>7.2	>7.2	>7.2	3.6	0.36	0.36
ETO-SI	>7.2	>7.2	>7.2	0.36	3.6	0.36	0.36
ETO-I	>7.2	>7.2	>7.2	>7.2	3.6	0.36	0.36
ERO-SI	-	>7.2	>7.2	>7.2	-	-	-
ERO-I	-	>7.2	>7.2	>7.2	-	-	-

[a]: Please note that the test duration for the spore germination test with *F. mosseae* (ISO 10832) was 14 days, and therefore, the values presented are the D14-I and D14-SI values.

2.4.6 Didecyldimethylammonium chloride (DDAC)

2.4.6.1 Standard test results

The UBA data contained for didecyldimethylammonium chloride (DDAC) results of two studies following the OECD 216. In the first study, three test concentrations of 10, 100 and 1000 mg

a.s./kg dw soil) were tested in two test soils (sandy loam, low humic content sand). Nitrate, nitrite, ammonium formation rates and CO_2 formation rates were measured. The highest inhibition was found after five days of exposure in the sandy loam with 24.8% at 1000 mg a.s./kg dw soil. The 2nd study tested the effect of eight concentrations between 50 and 6400 mg a.s./kg dw soil on the N-transformation (OECD 216) in sandy loam. Here, an EC_{50} value of 135.6 mg a.s./kg dw soil was derived after 28 days of exposure. In Lufa 2.1 at 300 mg a.s./kg dw soil, an inhibition of 51% and 61% after 14 and 28 days of exposure was in line with the previously described results. In RefeSol 04A, only after 14 days of exposure was inhibition (67%) observed, while after 28 days of exposure, no effect above 25% was determined. No effect on the nitrogen transformation was found in RefeSol 02A.

2.4.6.2 Lufa 2.1.

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity due to propamocarb hydrochloride are presented in Table 68.

2.4.6.2.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

At test initiation and the following determination points (days 14, 28, 56), a concentration-dependent effect on the activity of the AOB (ISO 15685) was observed at the chosen test concentrations. At 30 mg a.s./kg dw soil, the inhibition ranged from 14% to 33%, with the highest inhibition at day 28, while at the highest test concentration of 300 mg a.s./kg dw soil at test initiation, the inhibition was at 83% and increased to 100% at day 14, 28 and 56. After 84 days of exposure, no AOB inhibition occurred at any of the test concentrations. In addition, no effect was observed at the lowest test concentration of 3 mg a.s./kg dw soil. The results agree with the UBA data results indicating an EC_{50} of 136 mg a.s./kg dw soil after 28 days.

The MicroResp™ system showed no effects above 25% on basal respiration and the SIR at the chosen test concentrations after 14 and 28 days of exposure. Therefore, the MicroRespTM system was not performed on day 56 and day 84.

The enzyme activity of ß-glucosidase, phosphatase and urease was not inhibited at test initiation, while at 30 mg a.s./kg dw soil, the arylsulfatase was inhibited by 52%. The arylamidase was inhibited by 26% at 300 mg a.s./kg dw soil. After 14 days of incubation, only the ß-glucosidase was inhibited in a concentration-dependent manner from 20% to 35%. The other enzyme activities were comparable to the control treatment. On day 28, only the arylsulfatase was affected. A stimulation of 39% compared to the control was found. Due to this, only the effect of DDAC on the activity of this specific enzyme was observed after 56 days, and no effect was found. Due to this, after 84 days, the ISO 20130 was no longer performed.

Table 68: Effect of DDAC with application rates of 3, 30 and 300 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Lufa 2.1. as test soil.

Test day	Test system	Test details	Inhibition [%]		
			3.0 mg/kg	30 mg/kg	300 mg/kg
Test initiation D0	ISO 15685	-	0	18	83
	MicroResp™	Deionized water	1	6	3
		D-(+)-Glucose	-6	-4	2
		L-Cysteine hydrochloride	-12	2	2

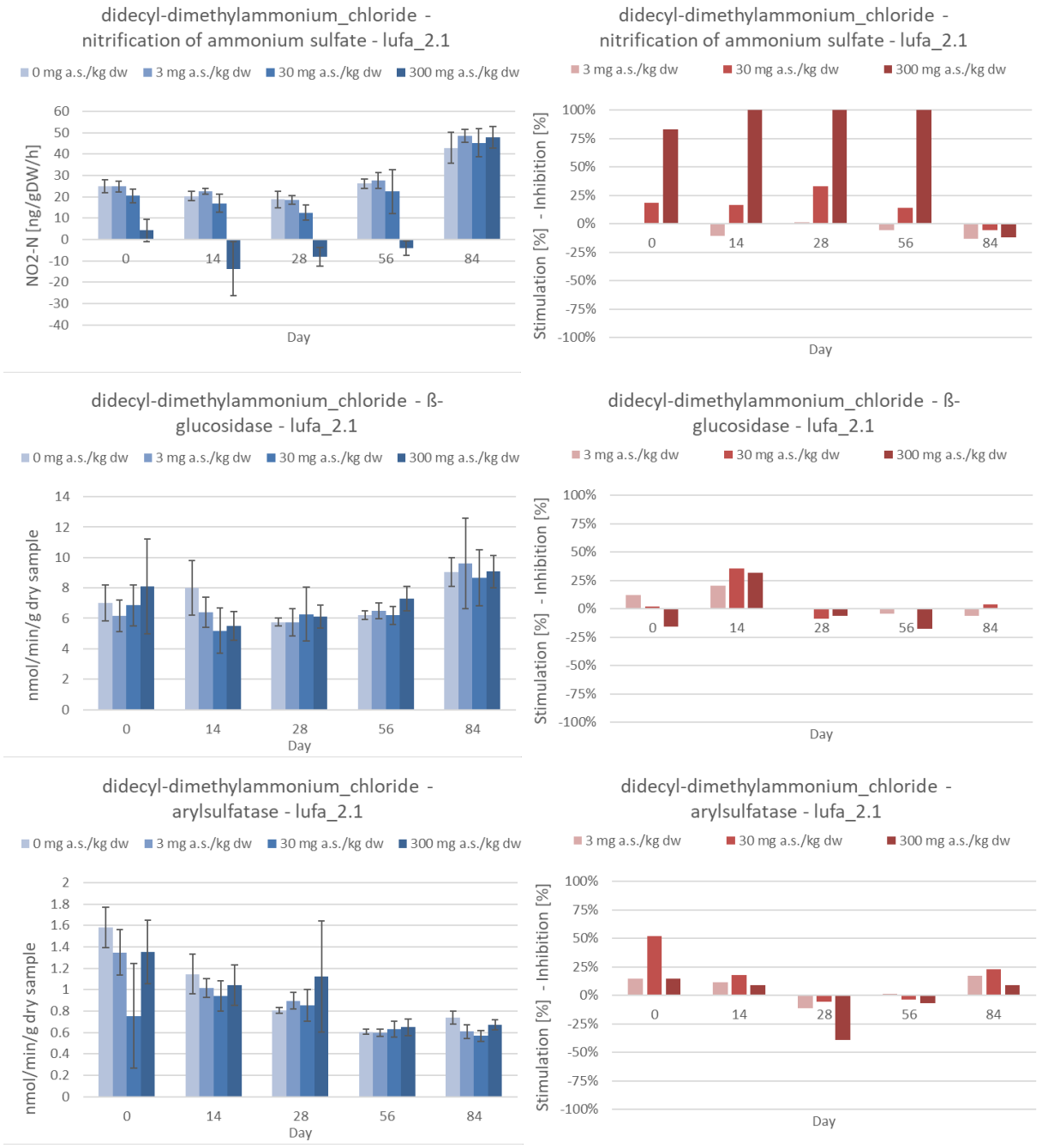
Test day	Test system	Test details	Inhibition [%]		
			3.0 mg/kg	30 mg/kg	300 mg/kg
		L-Malic acid	-14	0	0
		y-Amino butyric acid	-7	1	0
		N-Acetyl glucosamine	-7	0	2
		Citric acid	-7	-4	0
		L-Alanine	-4	4	5
	ISO 20130	Phosphatase	-6	-4	-10
		ß-Glucosidase	12	2	-16
		Arylsulfatase	15	52	15
		Arylamidase	8	17	26
		Urease	10	20	16
D14	ISO 15685	-	-11	16	100
	MicroResp™	Deionized water	-7	-1	-2
		D-(+)-Glucose	-5	-6	-5
		L-Cysteine hydrochloride	-4	-1	-1
		L-Malic acid	-4	-2	-3
		y-Amino butyric acid	-3	0	-2
		N-Acetyl glucosamine	-2	0	-1
		Citric acid	-2	-5	-7
		L-Alanine	-3	0	-5
	ISO 20130	Phosphatase	2	13	2
		ß-Glucosidase	20	35	31
		Arylsulfatase	11	18	9
		Arylamidase	-6	5	-13
		Urease	-3	5	-14
D28	ISO 15685	-	1	33	100
	MicroResp™	Deionized water	5	1	5
		D-(+)-Glucose	2	3	6

Test day	Test system	Test details	Inhibition [%]		
			3.0 mg/kg	30 mg/kg	300 mg/kg
		L-Cysteine hydrochloride	6	6	7
		L-Malic acid	5	6	6
		y-Amino butyric acid	7	6	5
		N-Acetyl glucosamine	7	7	6
		Citric acid	7	4	2
		L-Alanine	6	7	2
	ISO 20130	Phosphatase	7	5	14
		ß-Glucosidase	0	-9	-6
		Arylsulfatase	-11	-6	-39
		Arylamidase	3	9	-7
		Urease	-2	1	-1
D56	ISO 15685	-	-5	14	100
	ISO 20130	Phosphatase	-10	-13	-7
		ß-Glucosidase	-4	1	-17
		Arylsulfatase	2	-4	-7
		Arylamidase	2	2	9
		Urease	n.d.	n.d.	n.d.
D84	ISO 15685	-	-13	-5	-12
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	*51*
D28			-	-	*61*

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

ISO 15685 and ISO 20130 results regarding the effect of DDAC on the ammonium oxidizing bacteria and the exoenzymatic activity were chosen exemplarily to demonstrate certain effects on the soil microorganisms and their function (Figure 64).

Figure 64: Exemplary results of the potential nitrification (ISO 15685; upper figure) and the exoenzymatic activity (ISO 20130; mid and lower figure) for DDACat application rates of 3, 30 and 300 mg/kg dw soil in the test with Lufa 2.1.



Source: Own illustration, Fraunhofer IME

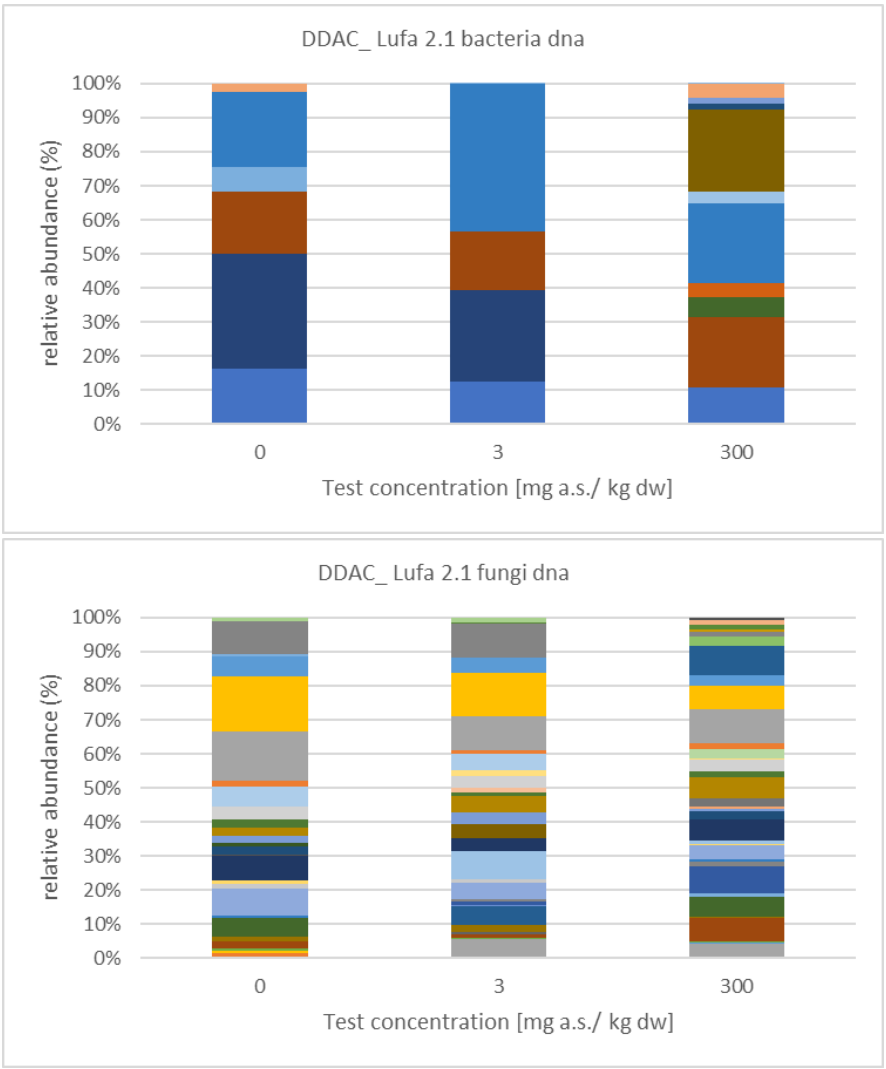
2.4.6.2.2 Soil microbial community structure (ARISA)

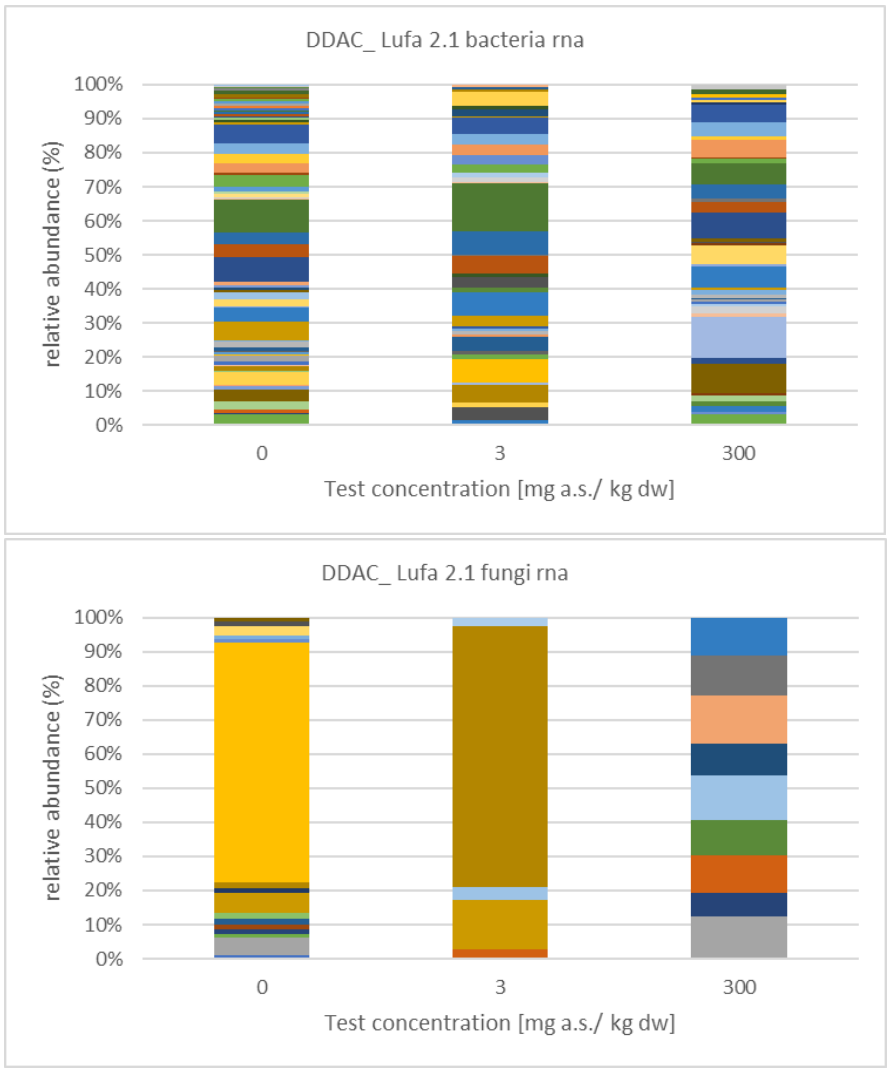
The evaluation of ARISA data revealed changes in the bacterial and fungal community composition, as demonstrated in the relative abundance plots (Figure 65) and CA analysis (Figure 66). These changes were detected at DNA level at concentrations of 3 and 300 mg didecyldimethylammonium chloride /kg dw soil, with the most pronounced effects observed at 300 mg/kg dw soil, indicating a strong microbial community response to this treatment. However, interpreting the changes in the CA plots at RNA level is complex due to high data dispersion.

In the relative abundance plots (Figure 65), shifts in microbial composition are evident as changes in the composition and proportion of specific OTUs, reflecting altered taxa prevalence under the influence of didecyldimethylammonium chloride. Similarly, the CA analysis at DNA level (Figure 66) highlights these compositional shifts, clustering samples based on community structure and showing a clear separation between treated samples and controls.

The Shannon index increases at the DNA level and decreases at the RNA level for both bacteria and fungi, suggesting inconsistent changes in diversity (Table 69). Nonetheless, it is evident that the application of didecyldimethylammonium chloride at both concentrations impacted the community composition.

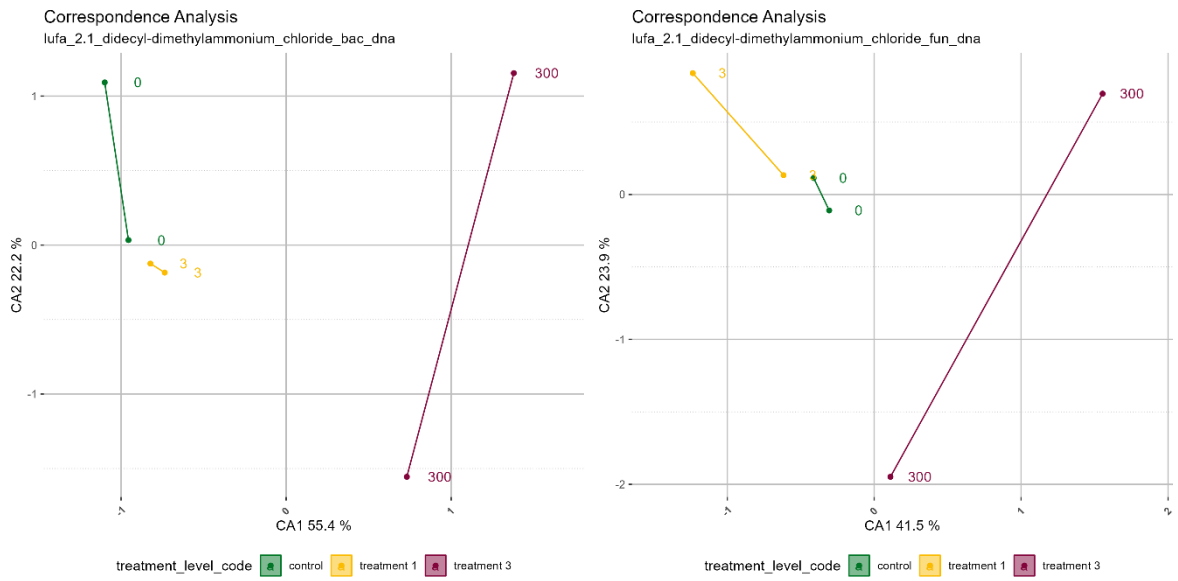
Figure 65: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 3.0 and 300 mg DDAC/kg soil dw in Lufa 2.1. after 28 days.

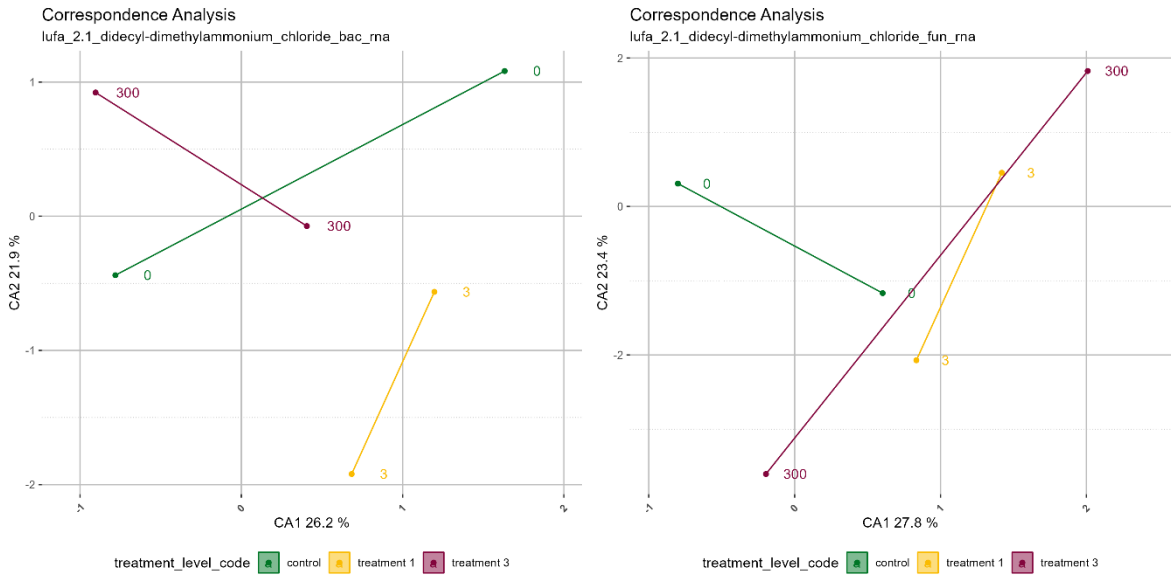




Source: Own illustration, Fraunhofer IME

Figure 66: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to DDAC at application rates of 3 and 300 mg/kg dw soil in Lufa 2.1.





Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), bacteria RNA (bottom left) and fungi RNA (bottom right).

Table 69: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 3 and 300 mg/kg didecyldimethylammonium chloride in Lufa 2.1 at day 28. Values represent the average of 2 replicates.

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	5.0	1.0	1.5
	3 mg/kg	4.0	0.9	1.3
	300 mg/kg	7.5	0.9	1.8
DNA fungi	Control	21.0	0.9	2.7
	3 mg/kg	21.5	0.9	2.8
	300 mg/kg	25.5	0.9	2.9
RNA bacteria	Control	37.5	0.9	3.2
	3 mg/kg	24.0	0.9	2.9
	300 mg/kg	25.0	0.9	2.9
RNA fungi	Control	9.0	0.6	1.2
	3 mg/kg	3.0	0.7	0.7
	300 mg/kg	4.5	1.0	1.3

2.4.6.2.3 Integrated evaluation

Summary

For the biocide DDAC, the ammonium oxidizing bacteria appeared to be the most sensitive group of soil microorganisms considering the three applied test methods (ISO 15685, ISO 20130 and MicroResp™). Here, even the establishment of a dose-response relationship was possible. While the SIR with eight different substrates was not affected at all within 28 days of exposure, the activity of the exoenzymes ß-glucosidase, arylsulfidase and arylamidase were affected by more than 25% but without a dose-response relationship, until day 14 to 28, but no effect above 25% was found at day 56.

The effect of DDAC on the spore germination of *F. mosseae* in accordance with ISO 10832 could not be determined due to the poor spore germination of the AMF in LUFA 2.1.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to didecyldimethylammonium chloride. The results suggest that didecyldimethylammonium chloride has an impact on the fungal and bacterial communities at a concentration of 3 and 300 mg a.s./kg dw soil.

Based on the results, Table 70 summarizes the main results per test system.

Table 70: LOECs for effects of DDAC in Lufa 2.1 soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	300	30	>300	>300	3	3
D28-I	300	30	>300	300	3	3
ETO-SI	300	30	>300	30	3	3
ETO-I	300	30	>300	30	3	3
ERO-SI	-	>300	>300	>300	-	-
ERO-I	-	>300	>300	>300	-	-

2.4.6.3 Refesol 04A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to DDAC are presented in Table 71.

2.4.6.3.1 Soil microbial function (ISO 15685, MicroRespTM, ISO 20130)

After 28 days of exposure, a concentration-dependent effect on the activity of the AOB (ISO 15685) was observed at the chosen test concentrations ranging from 3% to 17% and 84%

inhibition at 3, 30 and 300 mg a.s./kg dw soil. Therefore, the test was prolonged and even after 56- and 84-days exposure at the highest test concentration of 300 mg a.s./kg dw soil, the AOB activity was inhibited by 51% and 68%, respectively. Again, the results are in good agreement with the results of the UBA data, indicating an EC_{50} of 135.6 mg a.s./kg dw soil after 28 days, as already found using Lufa 2.1 soil.

The MicroResp™ system showed at the chosen test concentrations no effects above 25% on the basal respiration and the SIR with seven different substrates after 28- and 56-days exposure. Due to an error in the evaluation sheet, the effects were stronger at first, and the test was prolonged for 84 days. Here, even if after 28 and 56 days of exposure, no effect above 25% occurred, effects on the SIR with glucose, L-cysteine hydrochloride at 30 mg a.s./kg dw soil and citric acid at 3 mg a.s./kg dw soil were observed.

After 28 days of exposure, the enzyme activity (ISO 20130) of phosphatase and urease was not affected by more than 25%. However, stimulation of the enzyme activity at the highest test concentration (ß-glucosidase, arylsulfatase and arylamidase) and the lowest test concentration (arylamidase) was determined. However, after 56 days of exposure, none of the five enzymes was inhibited or stimulated by more than 25% compared to the control. Due to this, after 84 days, the ISO 20130 was no longer performed.

Table 71: Effects of DDAC with application rates of 3, 30 and 300 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using Refesol 04A as test soil.

Test day	Test system	Test details	Inhibition [%]		
			3.0 mg/kg	30 mg/kg	300 mg/kg
D28	ISO 15685	-	3	17	84
	MicroResp™	Deionized water	-7	-1	-8
		D-(+)-Glucose	-2	-3	8
		L-Cysteine hydrochloride	0	-5	7
		L-Malic acid	-1	-4	5
		y-Amino butyric acid	-6	-5	0
		N-Acetyl glucosamine	-3	-4	2
		Citric acid	-1	-6	6
		L-Alanine	-4	-6	0
	ISO 20130	Phosphatase	-2	5	-11
		ß-Glucosidase	-2	-3	-29
		Arylsulfatase	-5	0	-26
		Arylamidase	-25	7	-32
		Urease	-21	-2	2
D56	ISO 15685	-	3	13	51

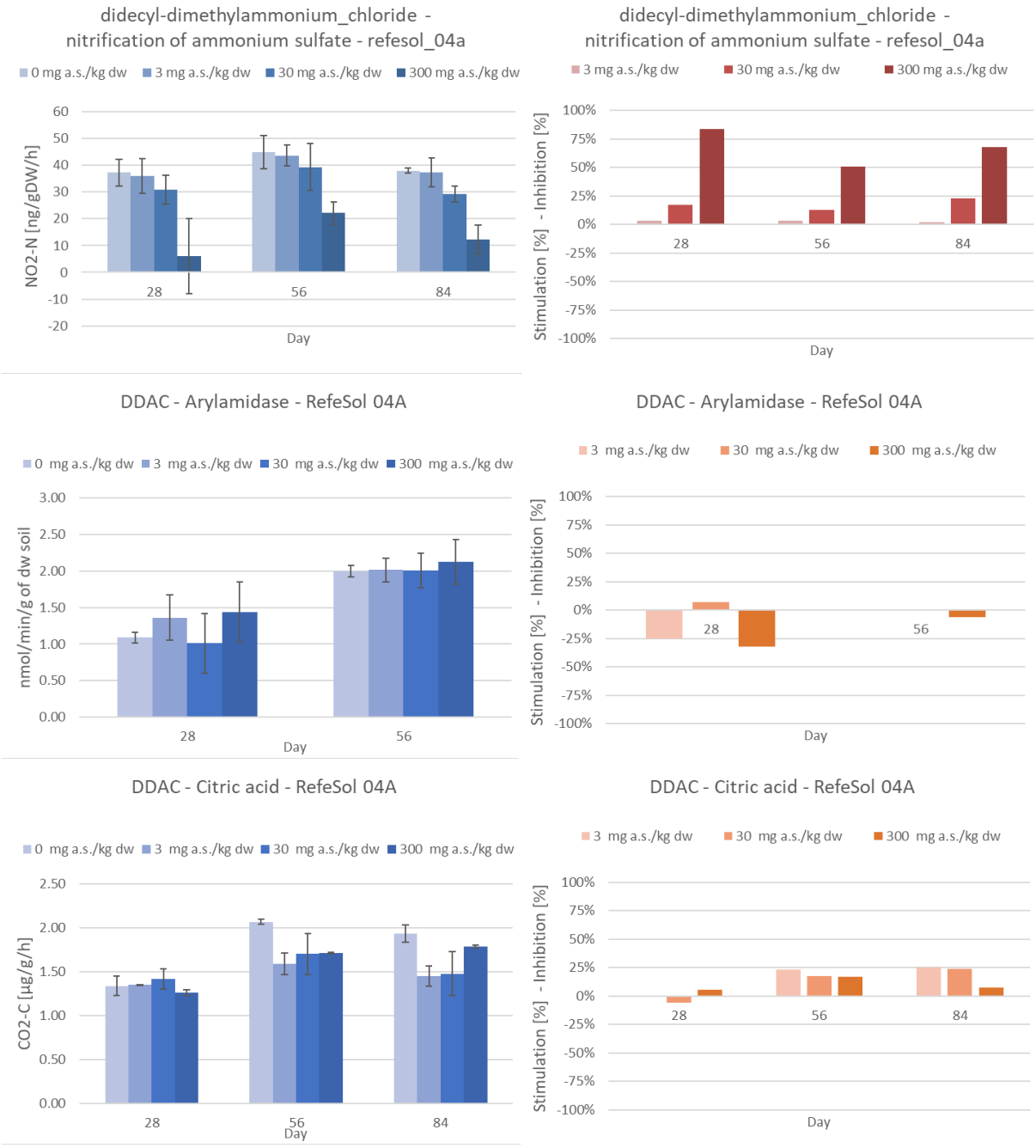
Test day	Test system	Test details	Inhibition [%]		
			3.0 mg/kg	30 mg/kg	300 mg/kg
	MicroResp™*	Deionized water	-11	-11	1
		D-(+)-Glucose	22	21	16
		L-Cysteine hydrochloride	21	21	9
		L-Malic acid	20	18	11
		y-Amino butyric acid	1	1	6
		N-Acetyl glucosamine	10	10	10
		Citric acid	23	18	17
		L-Alanine	8	8	13
	ISO 20130	ß-Glucosidase	-6	21	10
		Arylsulfatase	11	1	-13
		Arylamidase	-1	-1	-6
D84	ISO 15685	-	2	23	68
	MicroResp™*	Deionized water	-9	-3	-10
		D-(+)-Glucose	24	27	6
		L-Cysteine hydrochloride	23	27	-2
		L-Malic acid	22	24	1
		y-Amino butyric acid	3	9	-5
		N-Acetyl glucosamine	12	17	0
		Citric acid	25	24	8
		L-Alanine	10	15	2
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	*67*
D28			-	-	*16*

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

*Note: On day 28, there was a wrong entry into the evaluation sheet for MicroResp™, and due to this, the effects appeared to be much stronger and the test was also carried out on days 56 and 84, even if the effects after re-evaluation were found to be below 25%.

Results of the ISO 15685 and ISO 20130 regarding the effect of DDAC on the ammonium oxidizing bacteria and the exoenzymatic activity were chosen exemplarily to demonstrate certain effects on the soil microorganisms and their function (Figure 64).

Figure 67: Exemplary results of the potential nitrification (ISO 15685; upper figure), the exoenzymatic activity (ISO 20130; mid figure) and the SIR with citric acid (MicroResp™) for DDAC at application rates of 3, 30 and 300 mg/kg dw soil in the test with RefeSol 04A.



Source: Own illustration, Fraunhofer IME

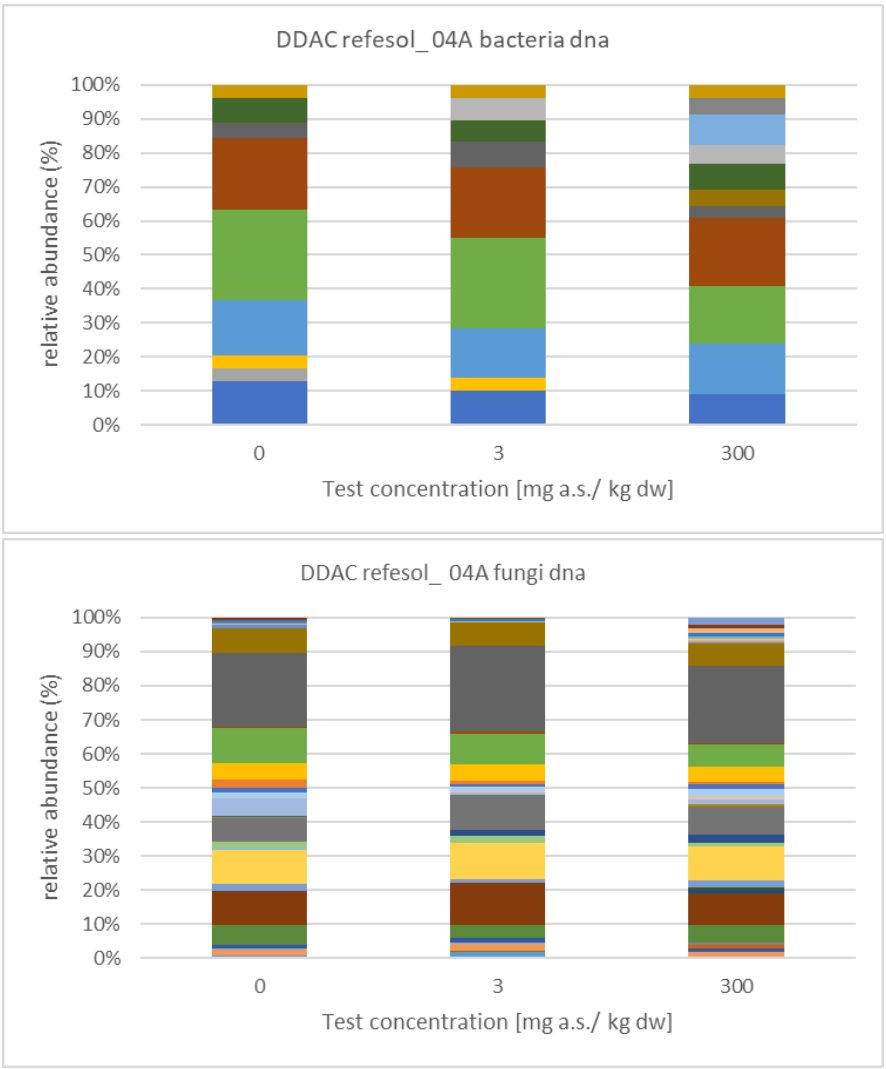
2.4.6.3.2 Soil microbial community structure (ARISA)

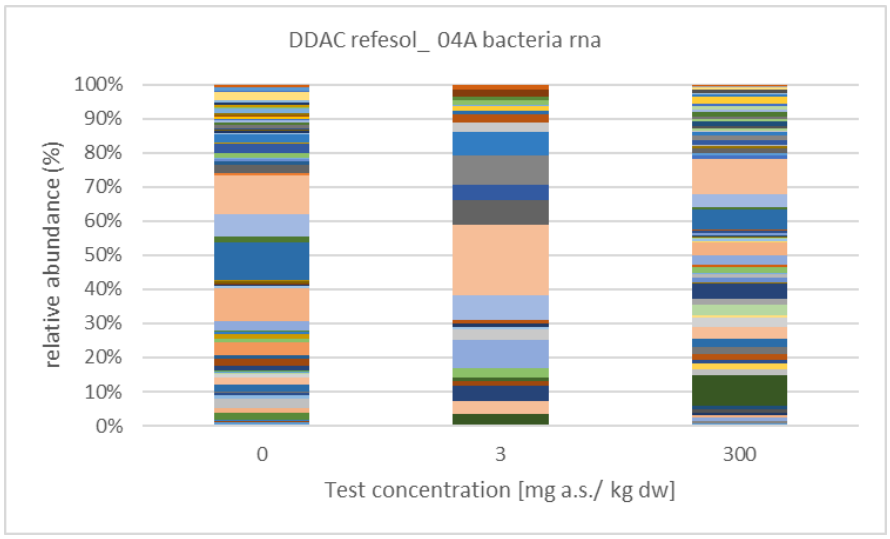
The evaluation of ARISA data revealed changes in the bacterial and fungal community composition, as demonstrated in the relative abundance plots (Figure 68) and CA analysis (Figure 69). These changes were detected at DNA and RNA level (bacteria) at concentrations of 3 and 300 mg didecyldimethylammonium chloride /kg dw soil, with the most pronounced effects observed at 300 mg/kg dw soil, indicating a strong microbial community response to this treatment. Data for fungal RNA, though existing, was not reliable as a consequence of the low concentration achieved during extractions.

In the relative abundance plots (Figure 68), shifts in microbial composition, though complex, are evident as changes in the composition and proportion of specific OTUs, reflecting altered taxa prevalence under the influence of didecyldimethylammonium chloride. Similarly, the CA analysis at DNA level (Figure 69) and RNA level (bacteria) highlights these compositional shifts, clustering samples based on community structure and showing a clear separation between treated samples and controls, which can be visualized despite big data dispersion.

The Shannon index increases at the DNA level for both bacteria and fungi, suggesting an increase in diversity at a concentration of 300 mg a.s./kg dw soil, while the bacterial diversity at the RNA level remains unchanged (Table 72).

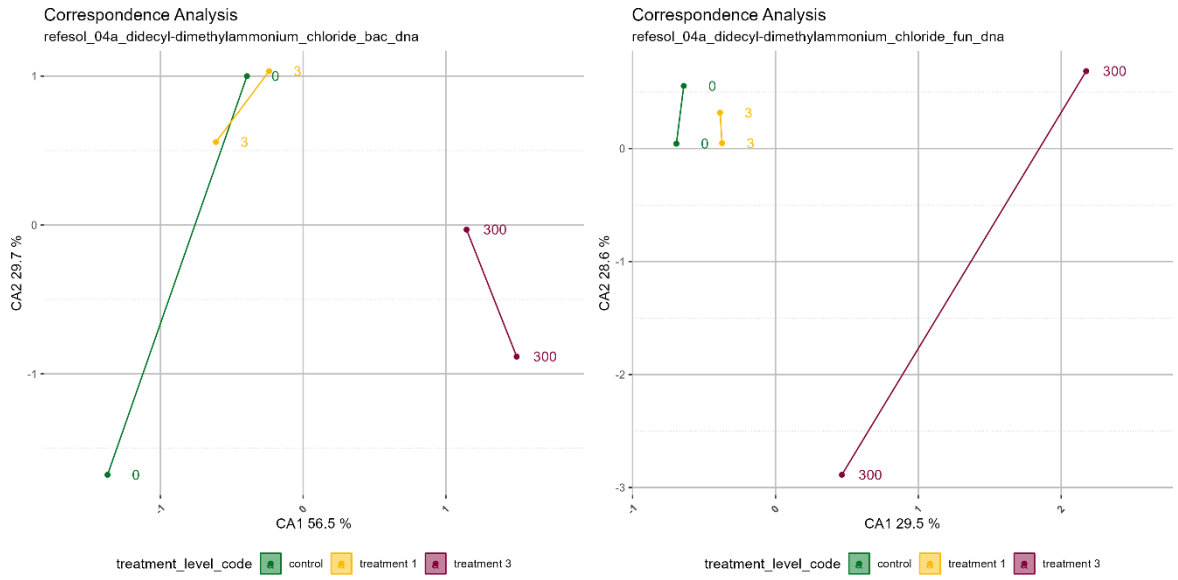
Figure 68: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 3 and 300 mg DDAC/kg soil dw in RefeSol 04A after 28 days.

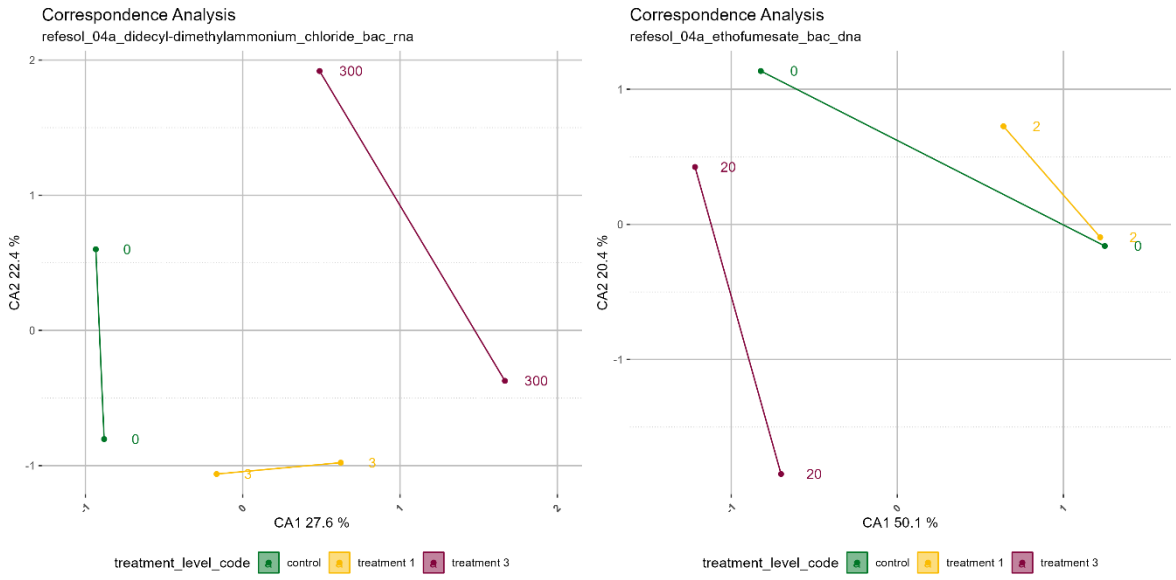




Source: Own illustration, Fraunhofer IME

Figure 69: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to DDAC concentrations of 3 and 300 mg/kg dw soil in RefeSol 04A.





Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), and bacteria RNA (bottom left).

Table 72: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 3 and 300 mg/kg didecyldimethylammonium chloride in Refesol 04A at day 28. Values represent the average of 2 replicates.

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	7.5	0.9	1.9
	3 mg/kg	8.5	0.9	2.0
	300 mg/kg	10.5	0.9	2.2
DNA fungi	Control	23.5	0.8	2.6
	3 mg/kg	21.5	0.8	2.5
	300 mg/kg	26.0	0.8	2.7
RNA bacteria	Control	41.5	0.9	3.3
	3 mg/kg	18.5	0.9	2.6
	300 mg/kg	41.5	0.9	3.4

2.4.6.3.3 Integrated evaluation

Summary

For the biocide DDAC in RefeSol 04A, the ammonium oxidizing bacteria (ISO 15684) appeared to be the most sensitive group of soil microorganisms considering the three applied test methods (ISO 15685, ISO 20130 and MicroResp™). Here, even the establishment of a dose-response relationship was possible. The SIR with eight different substrates was only slightly affected after 84 days of exposure, while no effects on the SIR had been determined after 28 and 56 days. The enzymatic activity (ISO 20130) was the least sensitive test system.

The effect of DDAC on the spore germination of *F. mosseae* in accordance with ISO 10832 could not be determined due to the poor spore germination of the AMF in RefeSol 04A. T

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to didecyldimethylammonium chloride. The results suggest that didecyldimethylammonium chloride has an impact on the fungal and bacterial communities at a concentration of 3 and 300 mg a.s./kg dw soil.

Based on the results Table 73 summarizes the main results per test system.

Table 73: LOECs for effects of DDAC in RefeSol 04A.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ARISA CA	ARISA Alpha diversity
D28-SI	>300	300	>300	3	3	3
D28-I	>300	300	>300	>300	3	3
ETO-SI	300	300	3	3	3	3
ETO-I	300	300	3	3	3	3
ERO-SI	-	300	3	>300	-	-
ERO-I	-	300	3	>300	-	-

2.4.6.4 Refesol 02A

The observed inhibitions of the AOB (ISO 15685), the SIR (MicroResp™) and the enzymatic activity (ISO 20130) due to ethofumesate are presented in Table 74.

2.4.6.4.1 Soil microbial function (ISO 15685, MicroResp™, ISO 20130)

After 28 days of incubation, compared to the control, the AOB (ISO 15685) activity was not affected by more than 25% at the three chosen test concentrations. Therefore, the ISO 15685 was not performed on days 56 and 84.

In the tests with RefeSol 02A, stimulation of the SIR of more than 25% compared to the control was found for all of the used substrates but not for the basal respiration after 28 days of exposure. Effects were observed at the lowest test concentration of 3 mg a.s./kg dw soil (except D-(+)-glucose and L-cysteine hydrochloride) and at 30 mg a.s./kg dw soil but not at the highest test concentration of 300 mg a.s./kg dw soil. After that, the test was prolonged to 56 days. No further effect above 25% could be observed, and the test was terminated.

The enzyme activity determined with the ISO 20130 was affected by more than 25% (stimulation and/or inhibition) for all observed enzymes after 28 days of exposure. The strongest effect after 28 days was observed for the phosphatase activity with an inhibition

ranging from 34% to 63% compared to the control, while for ß-glucosidase and arylsulfatase at the highest test concentration, strong stimulations of 134% and 82%, respectively, were found. Therefore, the ISO 20130 was also performed after 56 days of exposure. Again, except for urease, all enzymatic activities were affected by more than 25% compared to the control, but no concentration-effect relationship could be observed. The strongest inhibition (up to 100% at 30 mg a.s./kg dw soil) still was observed on the phosphatase activity, while the ß-glucosidase and arylsulfatase activity was still stimulated at the highest test concentration (64% and 46%, respectively). After 84 days of exposure, there was still more than 25% effect on all enzymatic activities. The phosphatase activity was inhibited by 53% at the highest test concentration, indicating a slight concentration-response. A comparable result was observed for ß-glucosidase and arylsulfatase with inhibitions at the highest test concentration of 32% and 27%, respectively. The highest inhibition was found at the lowest test concentration of 3 mg a.s./kg dw soil with 52% for the arylamidase.

Table 74: Effects of DDAC with application rates of 3, 30 and 300 mg/kg dw soil in four test systems (ISO 15685, MicroResp™, ISO 20130 and OECD 216) using RefeSol 02A as test soil.

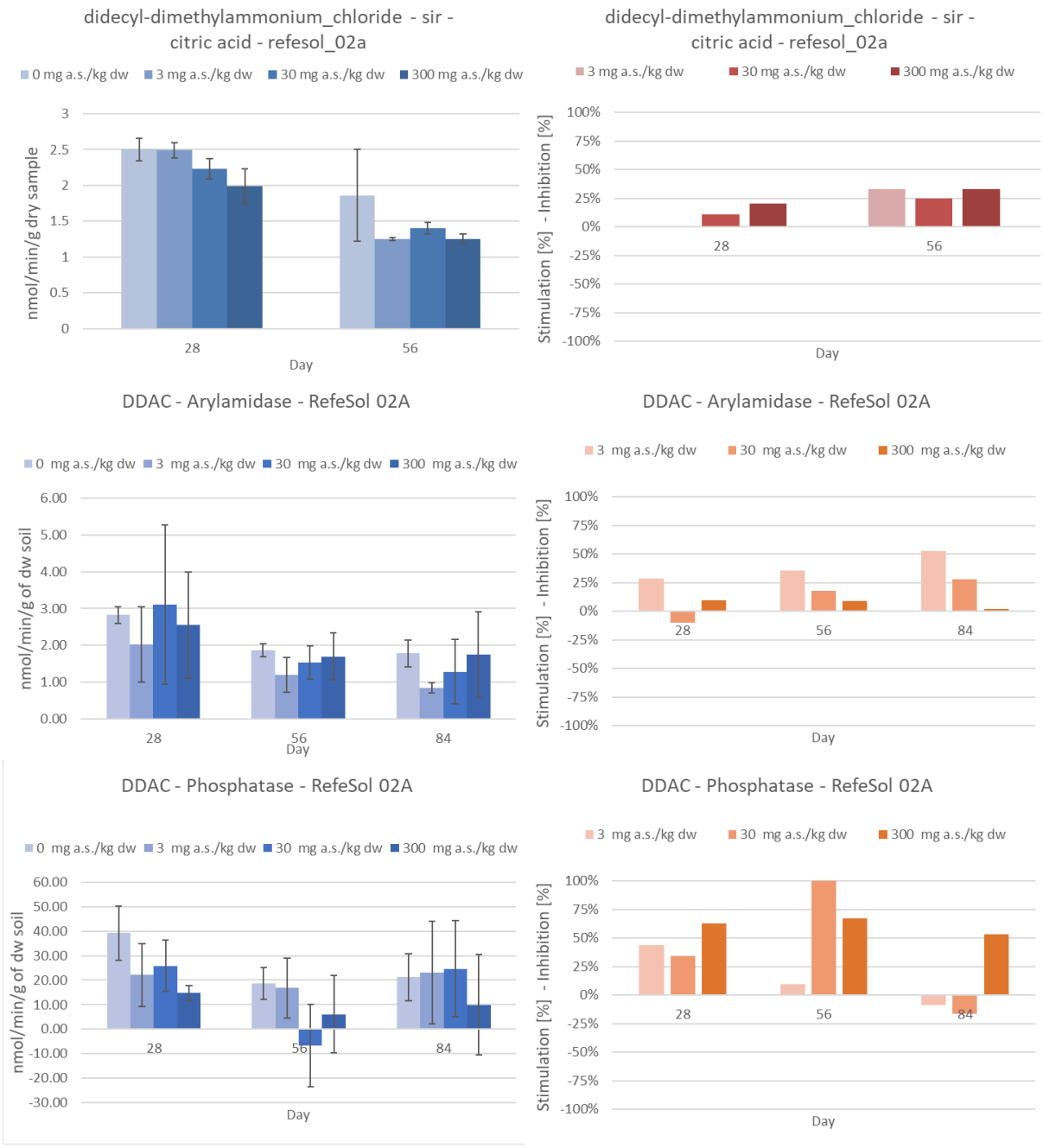
Test date	Test system	Test details	Inhibition [%]		
			3 mg/kg	30 mg/kg	300 mg/kg
D28	ISO 15685	-	-1	1	17
	MicroResp™	Deionized water	0	-11	-4
		D-(+)-Glucose	-11	-31	-17
		L-Cysteine hydrochloride	-17	-32	-18
		L-Malic acid	-26	-37	-21
		y-Amino butyric acid	-28	-32	-19
		N-Acetyl glucosamine	-35	-34	-19
		Citric acid	-49	-34	-19
		L-Alanine	-41	-30	-14
	ISO 20130	Phosphatase	43	34	63
		ß-Glucosidase	-23	-23	-134
		Arylsulfatase	-11	-58	-82
		Arylamidase	29	-10	10
		Urease	18	25	9
D56	MicroResp™	Deionized water	10	4	17
		D-(+)-Glucose	9	0	10
		L-Cysteine hydrochloride	13	3	15

Test date	Test system	Test details	Inhibition [%]		
			3 mg/kg	30 mg/kg	300 mg/kg
		L-Malic acid	10	2	14
		y-Amino butyric acid	16	4	18
		N-Acetyl glucosamine	15	5	17
		Citric acid	11	1	11
		L-Alanine	17	5	19
	ISO 20130	Phosphatase	10	100	67
		ß-Glucosidase	16	-22	-64
		Arylsulfatase	-45	-13	-46
		Arylamidase	36	18	9
		Urease	1	4	-9
D84	ISO 20130	Phosphatase	-9	-16	53
		ß-Glucosidase	-8	-21	32
		Arylsulfatase	-20	-6	27
		Arylamidase	52	28	2
D14	*OECD 216 (reference)*	*N-Transformation*	-	-	*-17*
D28			-	-	*4*

Red colour: Inhibition> 25% or < - 25% (indicating a stimulation).

Results of the substrate-induced respiration (MicroResp™, citric acid) and the enzymatic activity (ISO 20130, phosphatase and arylamidase) are presented exemplarily to demonstrate certain effects on the soil microorganisms and their function from the exposure to DDAC (Figure 70).

Figure 70: **Exemplary results for effects of tiamulin hydrogen fumarate on the microbial function at application rates of 3, 30 and 300 mg/kg dw soil in RefeSol 02A. Substrate-induced respiration (MicroResp™; activity upper left figure, effect upper right figure) and exoenzymatic activity (ISO 20130; activity mid and lower left figures, effect mid and lower right figure).**



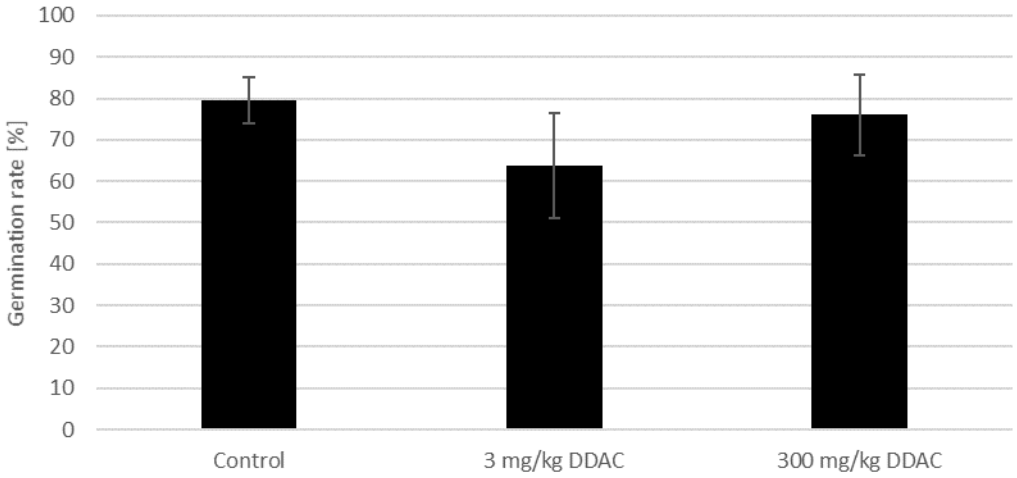
Source: Own illustration, Fraunhofer IME

2.4.6.4.2 Arbuscular mycorrhizal fungi (ISO 10832)

For the spore germination test (ISO 10832) with the biocide DDAC, two concentrations of 3 and 300 mg a.s./kg dw soil were tested against a control. The test fulfilled the validity criteria. The spore germination was not statistically significantly affected by the two test concentrations. At the lower test concentration of 3 mg a.s./kg dw soil, an inhibition of 16.1% was observed after 14 days of exposure (Figure 71). Due to the quite high inhibition at 3 mg a.s./kg dw soil but the lack of statistical significance and a concentration-response relationship, the test was repeated.

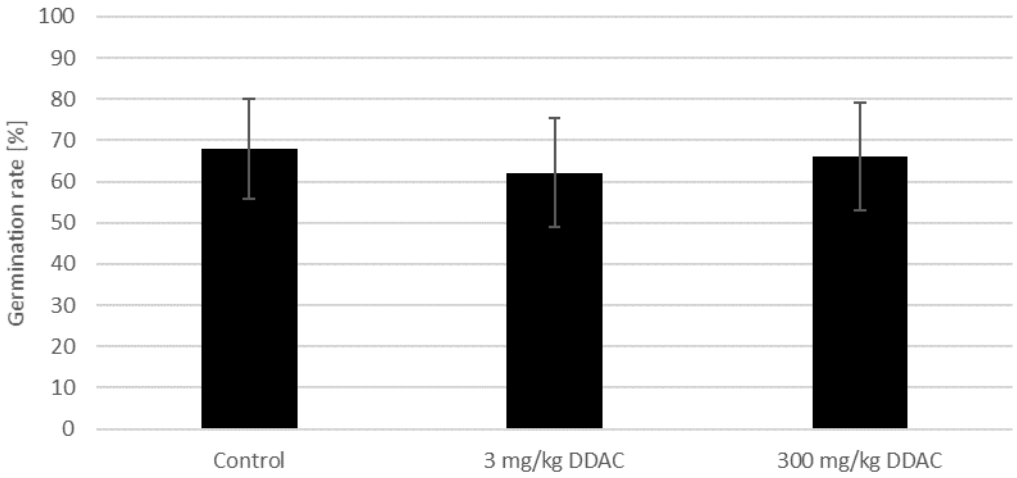
The 2nd test with DDAC did not fulfil the validity criteria since the spore germination was 68%, slightly below the 75% stated as the validity criterion in ISO 10832. Since the value of 75% was established for artificial soil, the still high spore germination of 68% in the natural soil RefeSol 02A was considered the results of the 2nd test valid. The spore germination was not statistically significantly affected by the two test concentrations. At the lower test concentration of 3 mg a.s./kg dw soil, an inhibition of 6.3% was observed after 14 days of exposure (Figure 72), which was lower than in the 1st test. The result at the highest test concentration was comparable in both tests. Therefore, the results show that DDAC did not significantly impact the spore germination of *F. mosseae* after 14 days of exposure.

Figure 71: Results of the 1st spore germination test with DDAC concentrations of 3 and 300 mg/kg dw soil in RefeSol 02A after 14 days of incubation.



Source: Own illustration, Fraunhofer IME

Figure 72: Results of the 2nd spore germination test with DDAC concentrations of 3 and 300 mg/kg dw soil in RefeSol 02A after 14 days of incubation.



Source: Own illustration, Fraunhofer IME

2.4.6.4.3 Soil microbial community structure (ARISA)

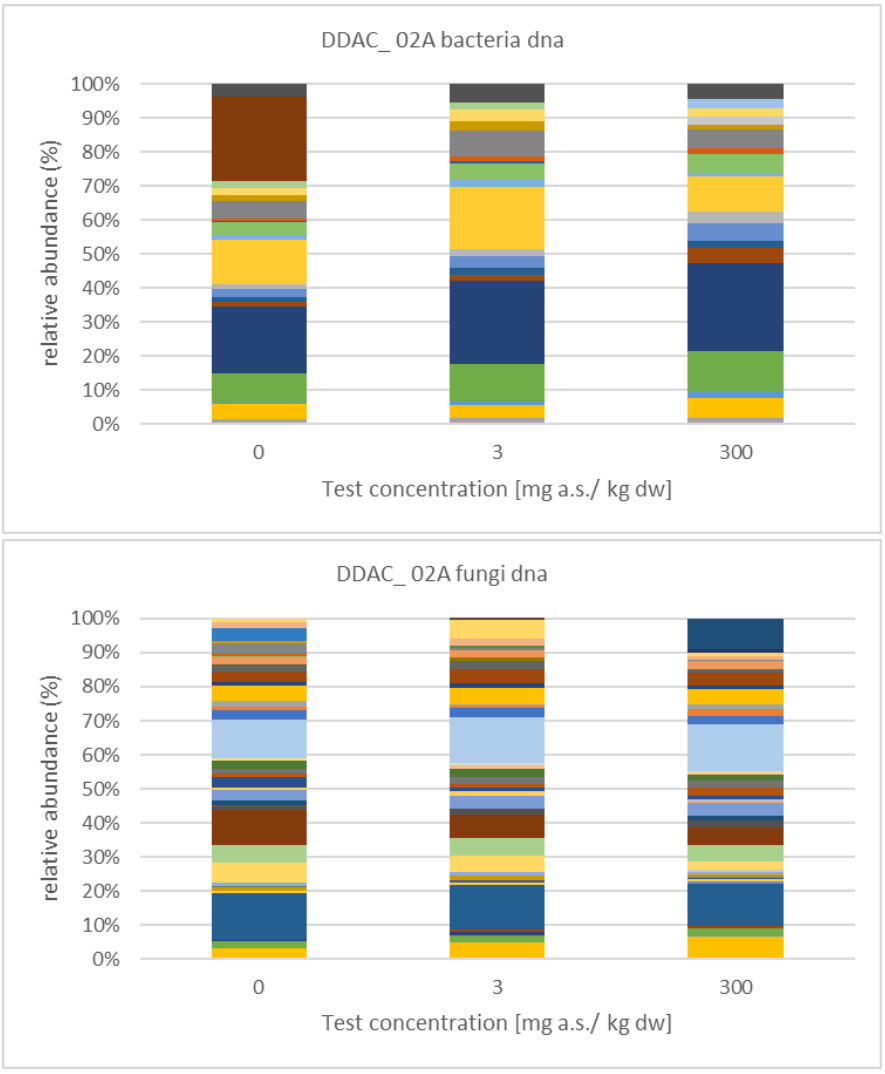
The evaluation of ARISA data revealed changes in the bacterial and fungal community composition, as demonstrated in the relative abundance plots (Figure 73) and CA analysis (Figure 74). These changes were observed at concentrations of 3 and 300 mg

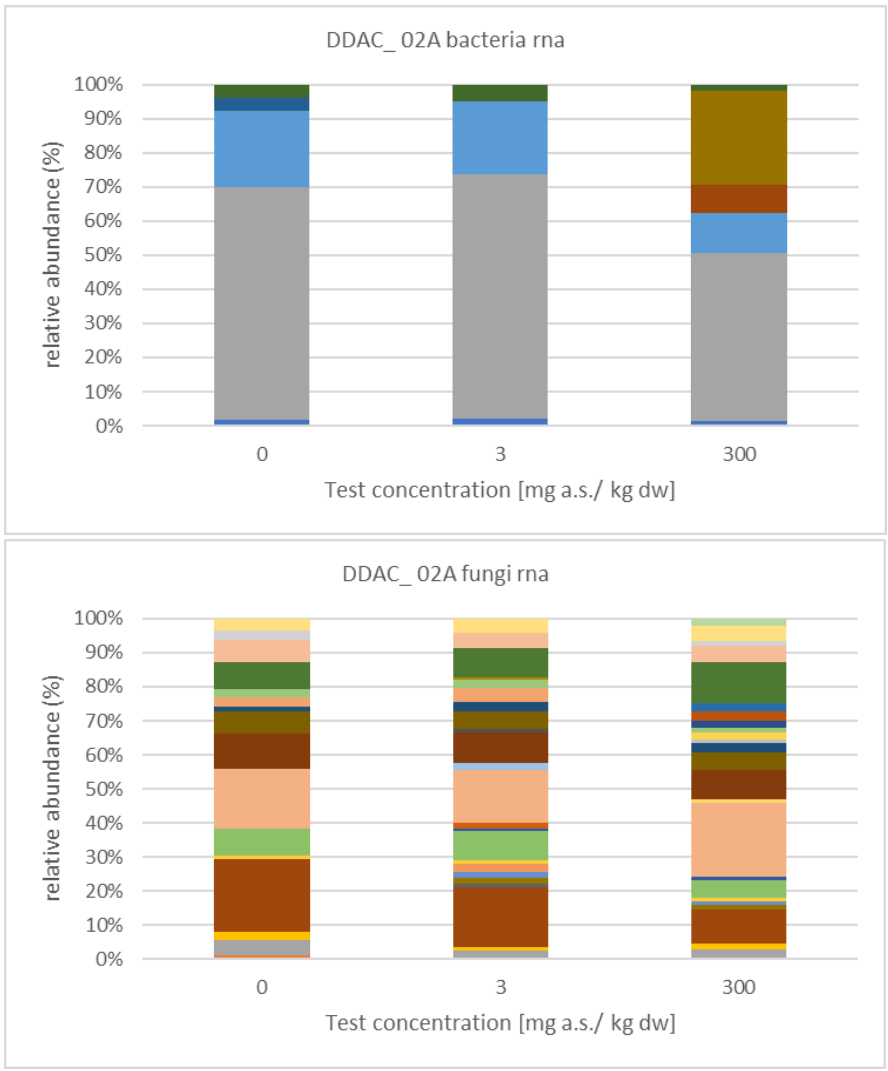
didecyldimethylammonium chloride/kg dw soil, but without a reproducible pattern between DNA and RNA levels

In the relative abundance plots (Figure 73), shifts in microbial composition, though complex to visualize, evidence some changes in the composition and proportion of specific OTUs, reflecting altered taxa prevalence under the influence of didecyldimethylammonium chloride. Similarly, the CA analysis at DNA and RNA level highlights these compositional shifts, clustering samples based on community structure and showing a clear separation between treated samples and controls, which can be visualized despite big data dispersion (Figure 74).

The Shannon indices indicate no change in bacterial and fungal diversity at the DNA level at both test concentrations of 3 and 300 mg a.s./kg dw soil. A slight increase of the Shannon index at 300 mg a.s./kg dw soil was determined for bacterial RNA, which is related to an increase in the OTUs richness (Table 75).

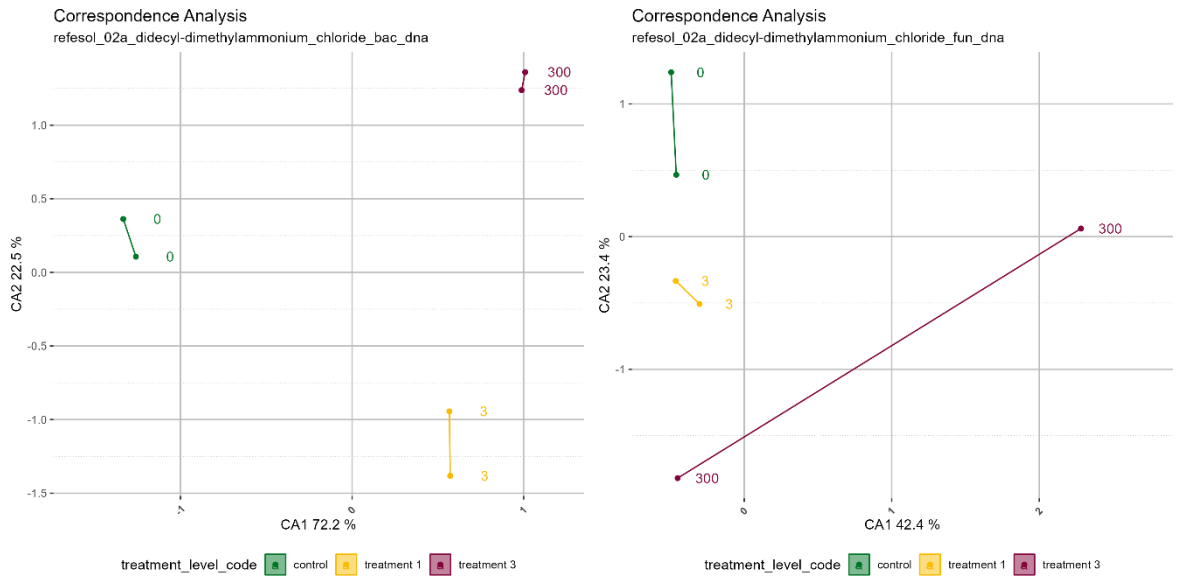
Figure 73: Alpha – diversity: Relative abundance of bacterial and fungal ARISA-fragments. Data correspond to exposure to 3.0 and 300 mg DDAC/kg soil dw in RefeSol 02A after 28 days.

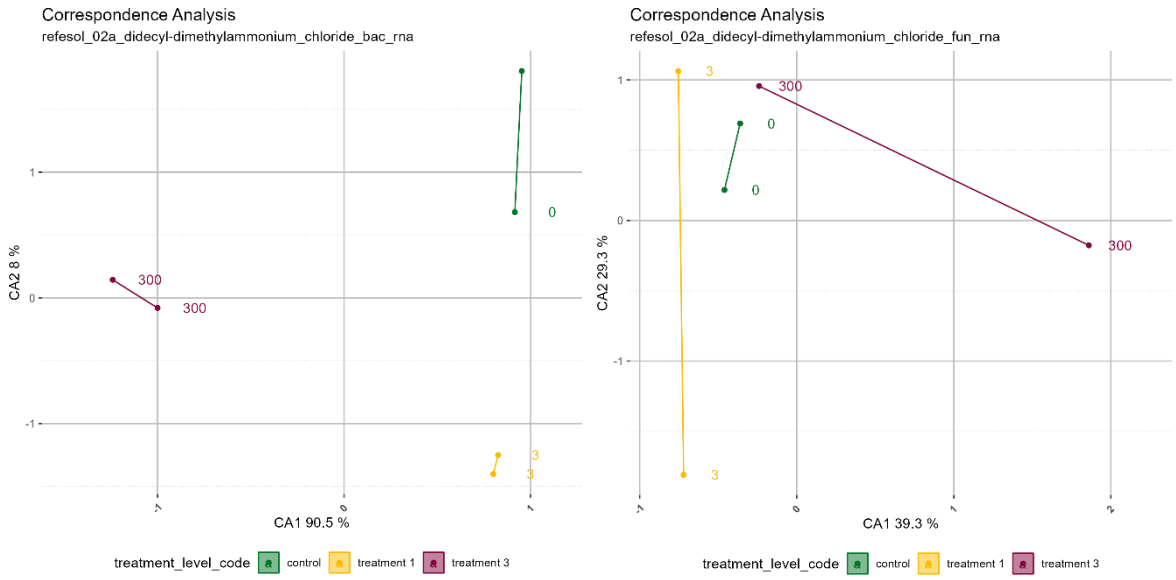




Source: Own illustration, Fraunhofer IME

Figure 74: Sample scores of correspondence analysis of ARISA data for 28 days of exposure to DDAC concentrations of 3 and 300 mg/kg dw soil in RefeSol 02A.





Source: Own illustration, Fraunhofer IME

Explained variance per multivariate dimensions given at the axes. Presentation of results for bacteria DNA (top left), fungi DNA (top right), bacteria RNA (bottom left), and fungi RNA (bottom right).

Table 75: Alpha diversity indices based on the DNA and RNA of the test's bacterial and fungal ARISA profiles with 3 and 300 mg/kg didecyldimethylammonium chloride in Refesol 02A at day 28. Values represent the average of 2 replicates.

Sample	Treatment	OTUs observed	Eveness	Shannon diversity
DNA bacteria	Control	18.5	0.8	2.4
	3 mg/kg	19.0	0.8	2.5
	300 mg/kg	18.5	0.9	2.5
DNA fungi	Control	34.0	0.9	3.1
	3 mg/kg	33.0	0.9	3.1
	300 mg/kg	30.5	0.9	3.0
RNA bacteria	Control	10.0	0.7	1.6
	3 mg/kg	8.0	0.7	1.5
	300 mg/kg	12.0	0.8	2.0
RNA fungi	Control	13.5	0.9	2.3
	3 mg/kg	17.0	0.9	2.6
	300 mg/kg	17.5	0.9	2.5

2.4.6.4.4 Integrated evaluation

Summary

Based on the results with DDAC in RefeSol 02A, after approximately 100 days of exposure, the exoenzymatic activities (ISO 20130; except urease) were still inhibited by more than 25%, indicating a chronic effect on the soil enzymes, at least at the highest test concentration. The two other test methods (MicroResp™, ISO 15685) appeared less sensitive than the ISO 20130.

The spore germination test with *F. mosseae* showed no statistically significant effect at the two chosen test concentrations.

The combination of analysis performed with the ARISA data provide a comprehensive view of how bacterial and fungal communities respond to didecyldimethylammonium chloride. The results suggest that didecyldimethylammonium chloride have an impact on the fungal and bacterial communities at a concentration of 3 and 300 mg a.s./kg dw soil.

Based on the results, Table 76 summarizes the main results per test system.

Table 76: LOECs for effects of tiamulin hydrogen fumarate in RefeSol 02A soil.

Option	OECD 216	ISO 15685	MicroResp™	ISO 20130	ISO 10832	ARISA CA	ARISA Alpha diversity
D28-SI	>300	>300	3	3	>300	3	3
D28-I	>300	>300	>300	3	>300	3	3
ETO-SI	>300	>300	3	3	>300	3	3
ETO-I	>300	>300	>300	3	>300	3	3
ERO-SI	-	>300	>300	3	-	-	-
ERO-I	-	>300	>300	3	-	-	-

[a]: Please note the test duration for the spore germination test with *F. mosseae* (ISO 10832) was 14 days; therefore, the values presented are the D14-I and D14-SI values.

2.5 Evaluation of the suitability of the different test methods for assessing effects on microbial function and structure

The test methods used in chapter 2 were compared with respect to the following criteria:

- Variability of controls in tests on bacterial function
- Minimum Detectable Differences in tests on bacterial function and spore germination
- Treatment related trends in ARISA
- Sensitivity of the tests, i.e. the chance to find an effect and the treatment level where effects were found
- Suitability different natural test soils for tests with pesticides and microorganisms and their influence on the test outcome in comparison to artificial soils

2.5.1 Variability of controls

Variability of controls is an often used as validity criterion for ecotoxicological tests. In OECD TG 216 it is required that 'the variation between replicate control samples should be less than ± 15%'. It is not clear which measure of variation is requested. Here we used the coefficient of variation (standard deviation divided by the mean) and calculated its means over all test items and soils for the tests on microbial function on each sampling date. ISO 15685 and ISO 20130 did often not fulfil the 15 % criterion while the mean coefficients of the MicroResp method and OECD 216 were all below 15%.

Table 77: Mean coefficients of variation [%] for the different methods to measure effects on function of the soil community.

Method / endpoint	Day 0	Day 14	Day 28	Day 56	Day 84	Mean
OECD216		**14.4**	**4.3**			**9.4**
ISO15685	**26.3**	**17.1**	**15.2**	**25.8**	**18.1**	**18.7**
ISO20130	**11.6**	**17.5**	**25.1**	**15.1**	**17.2**	**18.8**
arylamidase	9.0	16.4	15.6	11.5	10.7	13.3
arylsulfatase	13.6	20.7	21.7	13.2	18.4	18.1
phosphatase	9.1	11.4	41.6	17.3	16.0	23.2
ß-glucosidase	18.6	21.0	27.2	12.2	19.7	20.1
urease	6.3	18.5	19.0	25.8	21.6	19.5
MicroResp	**5.2**	**6.8**	**5.6**	**4.8**	**4.1**	**5.5**
basal respiration	3.1	5.7	5.0	3.4	3.6	4.4
sir - citric acid	12.9	7.9	6.6	9.0	3.4	7.5
sir - l-alanine	4.7	9.1	5.2	5.4	4.1	5.9
sir - l-cysteine hydrochloride	2.9	5.1	5.1	3.9	3.3	4.4

Method / endpoint	Day 0	Day 14	Day 28	Day 56	Day 84	Mean
sir - l-malic acid	3.8	6.5	5.9	5.1	7.3	5.9
sir - n-acetyl glucosamine	2.7	5.2	4.7	3.6	3.9	4.3
sir - y-amino butyric acid	9.1	8.2	7.2	3.4	3.2	6.3
sir -d-(+)-glucose	2.8	7.1	5.0	4.5	3.9	5.0

Note that the number of available data per method and data varies. Values in red are larger than 15%.

In the ISO 10832 no validity criterion for the variability of the controls is given but it is required that the germination rate in the control should be at least 75%. The CV of the control germination rate in the six tests varied between 6 and 11 % with a mean of 8 %.

No validity criteria exist for testing effects on microbial structure using ARISA and coefficients of variation of e.g. the Shannon index were not calculated since only two replicates were tested.

2.5.2 Minimum Detectable Differences

As a statistical measure and indicator value of the power of a test system to detect differences between controls and treatments, the minimum detectable difference (MDD, converted to per cent of the control treatment) between the controls and all single treatment levels of all experimental transformation and inhibition studies was calculated. The two-sided Dunnett test was conducted for all data sets. For the intended comparison the different experimental approaches here, the data were not transformed and no pre-tests on normality and variance homogeneity were conducted.

For all test methods with sufficient data and replication (OECD 216, MicroResp™, ISO 20130, ISO 15685) at hand and their different measurement endpoints, the MDD were computed using data on the arithmetic means and standard deviations while knowing the number of replicates (usually four). For the purpose of an overall methodological comparison of statistical sensitivities between experimental approaches and measurement endpoints, it was considered unnecessary to systematically test for formal prerequisites of applicability of statistical approaches for hypothesis testing using distributions of the t-family, such as Shapiro–Wilks test or variance homogeneity (e.g. Welch test). This approach was chosen for two reasons: It was assumed that the statistical power of the aforementioned methods to detect deviations from the Null-hypothesis (e.g. normality given) would be low given the low number of replicates (similarly true for the MDD-computation, admittedly); in particular when alpha-inflation of the repeated testing would be considered violations of the base hypothesis would be hard to detect. Further, there was no reason to assume that violations of the prerequisites would be more frequent in one method compared to the other and thus a systematic bias between the methods had to be considered.

The analysis of the MDD was based on the approach of Brock et al. (2015). However, the equation used was adjusted somewhat: Brock et al. set the statistical test power (beta-error) to 50 % by default for the testing procedure. However, since this does not correspond to the general conventions of a desired test power of ecotoxicological testing (Duquesne et al. 2020), the power was fixed at 80 % (type-II error of 20 %).

Equation 1: Equation for calculating minimum detectable differences between controls and treatment groups.

$$MDD = (\bar{x}_0 - \bar{x}_1) = (t_{1-\alpha,df,k} + z_{1-\beta,df}) \sqrt{\frac{s_0^2}{n_0} + \frac{s_1^2}{n_1}}$$

Legend of the equation above: $\bar{x}_0$: arithmetic mean of the control group; $\bar{x}_1$: arithmetic mean of the treatment group; $t_{1-\alpha,df,k}$: critical t-value from Dunnett´s table work with given significance threshold α, degrees of freedom df, overall number of comparisons/treatment groups; $t_{1-\beta}$: t-value from the standard normal distribution for a given beta error; s_0^2: variance of the control group; s_1^2: variance of the treatment group; n_0: number of replicates of the control group; n_1: number of replicates of the treatment group.

The means of control and treatment groups and their standard deviations were taken from the project-related database. The control mean was used to convert the absolute value of MDD into a percentage for ease of interpretation. The significance threshold was set to α = 5%, the degrees of freedom were calculated using the information on control and treatment group replication (usually n = 4), and the number of comparisons was taken from the number of treatment groups exclusive to the control group and the corresponding critical t-values for a two-sided Dunnett test were extracted from the original publication (Dunnett 1955).

The consideration of beta error was implemented by calculating the t-value of a standard normal distribution at 20 % type-II error probability.

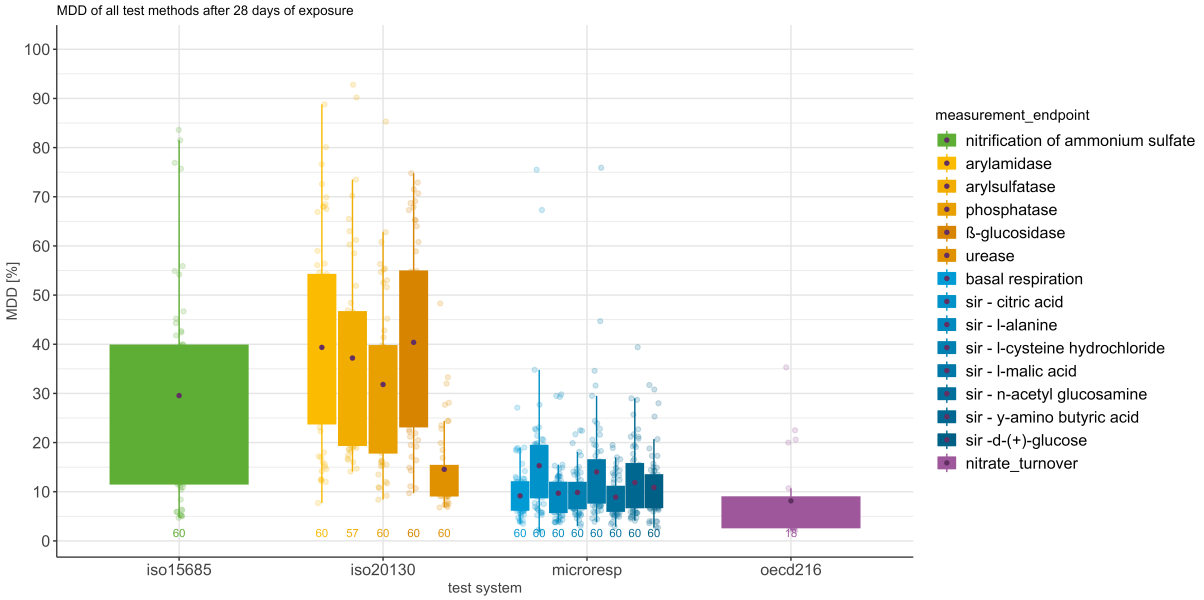
The variances of controls and treatment groups were considered separately in the equation above and computed from the sample standard deviation by squaring.

The MDD computation was implemented in R-scripts and looped over four experimental approaches (methods), the studies (combination of soil x test item in a given approach), the sampling day (including day zero), the measurement endpoints (if more than one as for MicroResp™ and ISO 20130) and the treatment levels tested in a study. In total, 2500 MDDs could be computed, which was the base for drawing the boxplots showing the distribution of MDDs in Figure 75.

The MDD indicated the power of the test systems as a proxy for ranking two or more test systems that could fit the same level in a tiered approach. However, it should be considered that the degrees of freedom (depending on the number of replicates and test concentrations) were not equal for all data sets and were often lower than in a test conducted in the context of the risk assessment for a specific substance. Since the aim was to rank the test methods based on their power, it was considered acceptable.

The four methods on bacterial function for which the MDD derivation was feasible show relative MDD values (from each available treatment compared to the control) between 10 and 50 %. The ISO 15685 and ISO 20130 delivered the highest MDD values, meaning that small effects cannot be detected and statistically detectable effects will likely be higher than for the other two methods, MicroResp™ and OECD 216. Between measured endpoints of ISO 20130, urease showed, on average, the lowest MDDs.

Figure 75: Overview of distribution of minimum detectable differences of four experimental methods, separated by different endpoints measured after 28 days of exposure.



Source: Own illustration, Darwin statistics

Overall, median MDD values for the transformation and inhibition data were below 35 % (Table 78) for all measurement endpoints and, compared to higher-tier methods in aquatic and soil ERA (mesocosms, field tests, terrestrial model ecosystems), relatively low.

Table 78: MDD values [%] of tests on microbial function. Dunnett, two-sided, Power 0.8, alpha = 0.05, all dates, substances and test soils tested.

method	measurement_endpoint	median_mdd	max_mdd	min_mdd
iso15685	nitrification of ammonium sulfate	31.3	124.4	4.7
iso20130	arylamidase	31.9	219.0	9.0
iso20130	arylsulfatase	23.5	199.6	6.1
iso20130	phosphatase	21.3	572.4	5.3
iso20130	urease	23.7	156.9	6.9
iso20130	ß-glucosidase	34.4	253.6	7.8
microresp	basal respiration	8.0	27.1	1.8
microresp	sir - citric acid	14.6	189.2	2.0
microresp	sir - l-alanine	9.6	35.6	2.2
microresp	sir - l-cysteine hydrochloride	8.8	36.1	1.7
microresp	sir - l-malic acid	11.2	78.4	2.7
microresp	sir - n-acetyl glucosamine	8.4	21.2	2.3
microresp	sir - y-amino butyric acid	9.3	46.0	1.9
microresp	sir -d-(+)-glucose	9.6	39.3	2.1
oecd216	nitrate_turnover	13.2	79.4	2.1

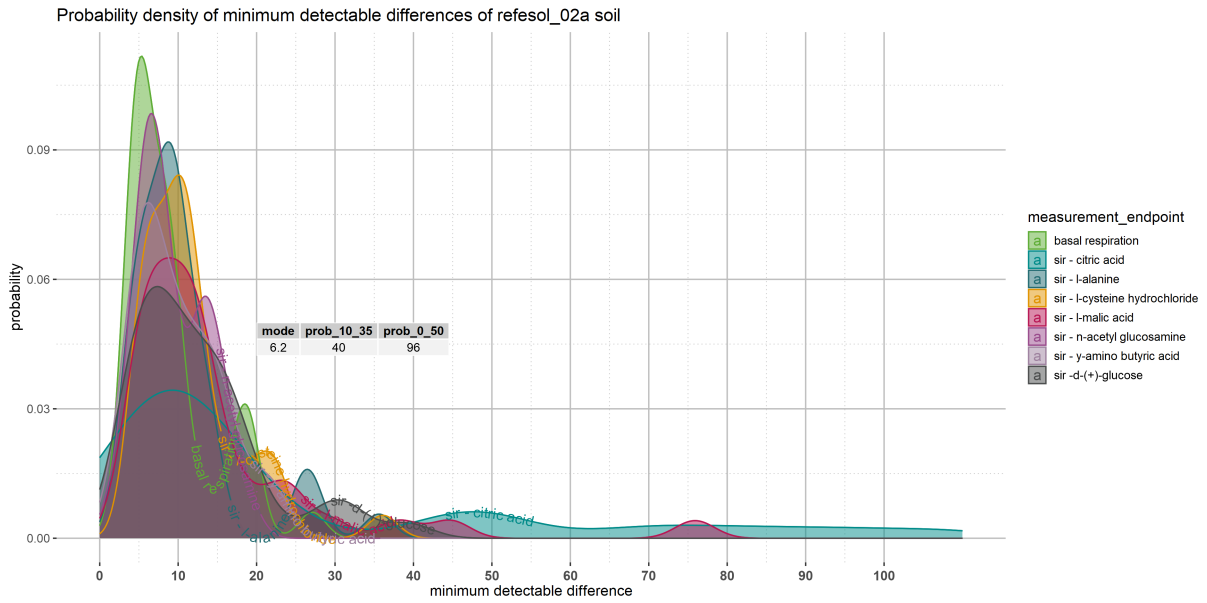
The results of MDD computation for the MicroResp™ method show high probabilities of obtaining MDDs between 10 and 35 %, classified as 'small effect' according to EFSA PPR panel (2017) in relation to higher tier tests with in-soil organisms. However, for laboratory tests with

soil microoroganisms, the relevant deviation from treatment to control is 25 %. MDDs lower than 25 % were observed for the majority of measurement endpoints exept ISO 15685 (nitrification) and ISO 20130 (arylamidase).

MDDs of the MicroResp™ method were almost always below 50 %. The most likely value (*mode*) was 6.2 % over all measurement endpoints for the MicroResp™ method. The measurement endpoint with the lowest mean MDD was the transformation of the substrate *n-acetyl glucosamine* with a median MDD of 8.4 %.

The median MDDs for effects on enzymatic activity (ISO 20130) or nitrification (ISO 15685) were below 35%.

Figure 76: Probability density plot for comparing eight measurement endpoints showing the sensitivity of MicroResp™ studies of overall MDD results in RefeSol 02A soil.



Source: Own illustration, Darwin Statistics

The six mycorrhiza fungi germination tests (the 2. DDAC test with control germination < 75% was not considered here) conducted in RefeSol 02A were evaluated using the ToxRat software, which allowed to conduct also the pre-tests on outliers (Dixon-Hardley or Hampel outlier test depending of normal distribution), normal distribution (Shapiro-Wilks test), variance homogeneity (Levene's test) and trend. Prior to the evaluation the data was arc-sin transformed based on the recommendations of Green et al. (Green et al. 2018). In all cases, the hypotheses of normal distribution and variance homogeneity were not rejected for the arc-sin transformed germination rates. The one-sided Williams tests was conducted because the aim was to assess inhibition in treatments compared to the control. MDDs varied between 8 and 13 % and thus, allowed to detect negligible to small effects according to the classification in EFSA PPR panel (2017). However, this relates to the transformed data only.

Table 79: MDDs [%] obtained in the ISO 10832 (AMF) tests using 6 replicates for the control and the two concentrations tested.

Test item	Concentration 1	Concentration 2
Ethofumesate	10.6	11.2
Tebuconazole	8.5	8.9

Test item	Concentration 1	Concentration 2
Pyraclostrobin	12.4	13.0
Propamocarb	9.1	9.5
Tiamulin	7.8	7.8
DDAC	9.8	10.3

Note that the MDDs are related to the arcsin transformed germination rates.

2.5.3 Sensitivity of the tests

As outlined in section 2.3.6, different options for the derivation of endpoints from toxicity tests with microorgansism are possible, depending on the direction of the deviation, the relevant effect size and time frame or the possible inclusion of a recovery option. For the comparison of the sensitivity of the different tests described in chapter 2 mainly the data for day 28 was used, because for this day, a complete data set was available. However, note that the duration of the spore germination with *F. mosseae* (ISO 10832) test is 14 days but the test was also considered here in the comparison.

We compared the LOECs estimated in section 2.4 with two options, considering according to OECD 216 any deviation from the control (reduction and stimulation), and considering only inhibition within the treatment compared to the control. Note that for the spore germination test (ISO 10832) only inhibition is observed and that in the correspondence analysis of the ARISA data the direction of effects cannot be determined asOTUs can react differently.

Considering reduction and/or stimulation of microbial functions of equal relevance, ISO 20130 (enzyme activity) and ARISA correspondence analysis (CA) provided in 14 of the 18 cases the lowest LOECs.

If only inhibition is considered as an adverse effect, the pattern regarding the sensitivity of the different methods is similar. The enzyme activity test (ISO 20130) provided the lowest LOEC in 11 cases, ARISA CA in 12 cases (Table 81). In three cases, the enzyme activity alone was the most sensitive method providing the lowest LOEC value, while the ARISA CA method was the most sensitive method once within the 13 cases.

Finally, the various methods were considered which were prolonged up to day 84 since they showed inhibitions at the previous measurement dates were compared for the option that a recovery of an inhibition and/or stimulation. The so called ERO-SI option clearly outlined the ISO 20130 on the enzymatic activity as the most sensitive of the three test methods on microbial function (ISO 15685, ISO 20130 and MicroResp™).

These results have to be considered with care because not all tests were conducted with the same number of test concentrations and only a limited set of substances has been tested. OECD 216 was conducted as limit test and measurements were performed only at day 14 and 28, since the test was performed just to verify the data provided by the UBA. The results of the OECD 216 limit tests indicate that the chosen test substances do not show the expected high effects to soil microorganisms. This might be explained by differences in the test substances itself since formulations including co-formulants like e.g. safeners have been used in some of the regulatory studies dataset, provided by UBA. In the studies of this project, the active substances were used. It was anticipated that the testing of the pure active substance would be a worst-case test and effects should be even higher than effects of a formulation, including bystanders. Another explanation might be differences in the physico-chemical properties of the used test soil, which are described more in detail in chapter 2.5.4

ISO 10832 and ARISA were conducted with only two concentrations, while the other tests used at least three or four test concentrations. Thus, LOECs could have changed, if additional concentrations would have been tested. It should also be noted that the LOECs were estimated (see section 2.4) due to the large number of tests and the low number of replicates (ARISA) and/or test concentrations per test. Especially the LOECs estimated for the ARISA CA are uncertain because only the ordination of two replicates in controls and the two chosen test concentrations could be visually compared.

However, the data do allow the detection of a trend (see Table 80 - Table 82), showing that enzymatic activity test (ISO 20130) is more sensitive than the other microbial function tests and less affected by the type of the test soil. However, the results on the effect of the test substances on the activity of various enzymes in the soil (ISO 20130) is difficult to assess. Effects ≥25% were observed regularly, but responses were not always comparable through substances and soils and there was a lack of concentration-response relationships. Furthermore, the observed effects changed during the course of the experiment after 14, 28, 56- or 84-days exposure, e.g. between a stimulation at one point and an inhibition at the next measurement point. For some of the substances after 56 days no effect ≥25% was observed. However, if the test was extended to 84 days, effects ≥25% were observed. Therefore, an interpretation of the results appears to be difficult and does not seem to follow the common interpretation of ecotoxicological tests with higher organisms.

From the theory of functional redundancy, structure can be expected to be more sensitive than function. To assess the sensitivity of ARISA, experiments with more replicates, more test concentrations and in the best case also more sampling dates would be needed to make a final decision on the reliability of the results and the applicability of the method in an environmental risk assessment.

The ARISA fingerprinting method identified shifts in the microbial community, indicating changes even in cases where other functional tests as the ISO 15685, ISO 20130 and/or MicroResp™ showed no discernible response. These highlights both the soil's resilience and the microorganisms' capacity to adapt and compensate affected functions. Nonetheless, while functions remained unaffected, observable changes in community patterns persisted. It is important to consider that biodiversity in a soil is important for its stability and ability to better respond to environmental stress, so if changes in the soil community reduce diversity, the soil ecosystem may become more vulnerable to disturbances in a long-term (Meena et al. 2020, de Faccio Carvalho et al. 2024).

It is important to consider the complexity and limitations of the technique before to be considered as a lower-tier technique for risk assessment. One major challenge with ARISA, and in general with any molecular technique is the DNA/RNA extraction method employed. Different methods, such as bead-beating or column-based extraction, vary in their efficiency to lyse cells from different taxa, leading to biases in the community composition detected. For example, bead-beating may more effectively break down through cell walls of certain bacteria, while column-based methods may favor the extraction of DNA from other microbial groups. These biases can significantly impact diversity indices, as they may either over- or underestimate the presence of specific taxa, leading to potential misinterpretation of community structure and diversity. Therefore, careful selection and standardization of DNA extraction and PCR-based methods are critical for ensuring their accuracy and reliability as tools for risk assessment, as variations in protocols can significantly influence the detection of microbial diversity and potentially affecting ecological interpretations.

One major challenge with ARISA is the interpretation of diversity indices—while it can provide measures like the Shannon or Simpson indices, where similar values for these indices can be

generated by very different communities, masking important shifts in community structure. This can lead to false conclusions, where changes in the ecosystem might go unnoticed due to the inability of these indices to capture the full picture.

In contrast to the limitations of diversity indices, multivariate analysis (such as beta diversity) provides a more comprehensive approach for assessing changes in microbial community structure, capturing differences in community composition between samples, allowing for a detailed analysis of shifts across the entire community, rather than relying on aggregate indices. This is particularly useful in cases where diversity indices (e.g., Shannon) may fail to detect subtle but important changes in less abundant species.

Table 80: Estimated LOECs [mg a.s./kg dw] for day 28 considering both, inhibition and stimulation for all tested methods, substances and soils

Test item	Soil	OECD 216	ISO 15685	Micro-Resp™	ISO 20130	ISO 10832	ARISA CA	ARISA Alpha diver-sity	Ratio OECD LOEC to $LOEC_{min}$
DDAC	Lufa 2.1	300	30	>300	300	-	3	>300	100
Ethofumesate	Lufa 2.1	>10	>20	>20	10	-	20	20	>1
Propamocarb	Lufa 2.1	30	30	3	3	-	3	3	10
Pyraclostrobin	Lufa 2.1	>30	>30	>30	>30	-	3	3	>10
Tebuconazole	Lufa 2.1	>10	5	5	>10	-	1	1	>10
Tiamulin	Lufa 2.1	>7.2	7.2	>7.2	0.36	-	7.2	7.2	>20
DDAC	Refe-Sol 02A	>300	>300	3	3	>300	3	3	>100
Ethofumesate	Refe-Sol 02A	>10	>20	2	2	20	2	2	>5
Propamocarb	Refe-Sol 02A	>30	>30	>30	3	>30	3	30	>10
Pyraclostrobin	Refe-Sol 02A	>30	>30	>30	3	30	3	3	>10
Tebuconazole	Refe-Sol 02A	>10	>10	1	1	10	10	1	>10
Tiamulin	Refe-Sol 02A	>7.2	>7.2	>7.2	7.2	3.6	0.36	0.36	>20
DDAC	Refe-Sol 04A	>300	300	>300	3	-	3	3	>100
Ethofumesate	Refe-Sol 04A	>10	>100	>100	2	-	2	>20	>5
Propamocarb	Refe-Sol 04A	>30	30	>75	3	-	>30	>30	>10
Pyraclostrobin	Refe-Sol 04A	30	>75	3	3	-	3	>30	10

Test item	Soil	OECD 216	ISO 15685	Micro-Resp™	ISO 20130	ISO 10832	ARISA CA	ARISA Alpha diver-sity	Ratio OECD LOEC to $LOEC_{min}$
Tebuconazole	Refe-Sol 04A	>50	>50	10	1	-	1	1	>50
Tiamulin	Refe-Sol 04A	>7.2	7.2	0.36	0.36	-	0.36	>7.2	>20
Total cases with lowest LOEC		0	0	6	14	0	14	10	

Table 81: Estimated LOECs [mg a.s./kg dw] for day 28 considering only inhibition

Test item	Soil	OECD 216	ISO 15685	Micro-Resp™	ISO 20130	ISO 10832	ARISA CA	ARISA Alpha diversity	OECD-LOEC / $LOEC_{min}$
DDAC	Lufa 2.1	≤300	30	>300	300		3	3	100
Ethofumesate	Lufa 2.1	>10	>20	>20	10	-	20	20	>1
Propamocarb	Lufa 2.1	30	>75	3	3		3	3	10
Pyraclostrobin	Lufa 2.1	>30	>30	>30	>30		3	3	>10
Tebuconazole	Lufa 2.1	>10	>10	>10	>10		1	1	>10
Tiamulin	Lufa 2.1	>7.2	7.2	>7.2	0.36		7.2	7.2	>20
DDAC	RefeSol 02A	>300	>300	>300	3	>300	3	3	>100
Ethofumesate	RefeSol 02A	>10	>20	>20	>20	20	2	2	>5
Propamocarb	RefeSol 02A	>30	>30	30	3	>30	3	30	>10
Pyraclostrobin	RefeSol 02A	>30	>30	>30	3	30	3	3	>10
Tebuconazole	RefeSol 02A	>10	>10	>10	5	>10	>10	>10	10
Tiamulin	RefeSol 02A	>7.2	>7.2	>7.2	>7.2	3.6	0.36	0.36	>20
DDAC	RefeSol 04A	>300	300	>300	>300	-	3	3	>100
Ethofumesate	RefeSol 04A	>10	>100	>100	2	-	2	>20	>5
Propamocarb	RefeSol 04A	>30	30	>75	>75	-	>30	>30	>10
Pyraclostrobin	RefeSol 04A	30	>75	>75	3	-	3	>30	10
Tebuconazole	RefeSol 04A	>50	>50	>50	1	-	1	1	>50
Tiamulin	RefeSol 04A	>7.2	7.2	7.2	0.36	-	0.36	>7.2	>20
Total cases with lowest LOEC		0	1	1	11	0	12	9	

Table 82: Estimated LOECs [mg a.s./kg dw] considering recovery of stimulation or inhibition for the recovery option (ERO-SI) for the three functional tests ISO 15685, Micro-Resp and ISO 20130

Test item	Soil	ISO 15685	MicroResp™	ISO 20130
DDAC	Lufa 2.1	>300	>300	>300
Ethofumesate	Lufa 2.1	>20	>10	20
Propamocarb	Lufa 2.1	15	>75	>75
Pyraclostrobin	Lufa 2.1	>30	>30	>30
Tebuconazole	Lufa 2.1	>10	>10	1
Tiamulin	Lufa 2.1	7.2	>7.2	>7.2
DDAC	RefeSol 02A	>300	>300	3
Ethofumesate	RefeSol 02A	>20	2	10
Propamocarb	RefeSol 02A	>30	30	3
Pyraclostrobin	RefeSol 02A	>30	>30	3
Tebuconazole	RefeSol 02A	>10	>10	>10
Tiamulin	RefeSol 02A	>7.2	>7.2	>7.2
DDAC	RefeSol 04A	300	3	>300
Ethofumesate	RefeSol 04A	>100	>100	20
Propamocarb	RefeSol 04A	3	>75	>75
Pyraclostrobin	RefeSol 04A	>75	>75	3
Tebuconazole	RefeSol 04A	>50	>50	>50
Tiamulin	RefeSol 04A	>14.4	>14.4	7.2
Total cases with lowest LOEC		3	2	7

Note that for OECD 216, only data for a single date (day 28 or day 14) are available. Thus, this test was not included here.

2.5.4 Effects of soil type

To evaluate the effects on soil type on the sensitivity of the different test methods, it was counted how often a LOEC could be estimated (i.e. an effect was found) (Table 83). This was done for day 28 for both protection options (considering only inhibition or both, stimulation or inhibition as adverse). The spore germination test with AMF (ISO 10832) was not considered here because it was only conducted in Refesol 02A.

For OECD 216 and ISO 15685 the RefeSol 02A was not suitable because no effects were detected – in contrast to the tests in other soils. The MicroResp™ method detected only rarely an inhibition of respiration on day 28 but more often a stimulation. For enzymatic activity (ISO

20130) and ARISA the soil type did not indicate major differences between the effects observed with the six test substances.

The results of the OECD 216 limit tests indicate that the chosen test substances do not show the expected high effects to soil microorganisms. This might be explained by differences in the physico-chemical properties of the used test soil (e.g. sand content, pH, C_{org}) as the references provided by the UBA used different soils with varying properties compared to the conducted studies within this project. There are various publications indicating that soil properties as texture, pH and organic carbon will have an impact on the ecotoxicity of the test substance used for organic and inorganic substances (Amorim et al. 2005, Criel et al. 2008, Schlich and Hund-Rinke 2015, Aderjan et al. 2023). Even the project results show that there can be significant differences using the same test methods and test concentrations but different soils. For example, the ISO 15685 appears to be a sensitive test method using the worst-case soil Lufa 2.1 (low pH, high sand content), but is not sensitive anymore if a more complex soil as RefeSol 02A (higher pH, high silt content) is used.

Table 83: Cases where on day 28 an inhibition (d28-I) or a stimulation or an inhibition (d28-SI) was found. For each method, the soil with the most effect detection is set in bold.

Test system	d28-I Lufa 2.1	d28-I RefeSol 02A	d28-I RefeSol 04A	d28-SI Lufa 2.1	d28-SI RefeSol 02A	d28-SI RefeSol 04A
OECD 216	**2**	0	1	**2**	0	1
ISO 15685	2	0	**3**	**4**	0	3
MicroResp™	**1**	**1**	**1**	2	**3**	**3**
ISO 20130	4	**5**	4	4	**6**	**6**
ARISA CA	**6**	5	5	**6**	**6**	5
ARISA Alpha diversity	**6**	5	2	**5**	**5**	2
Total cases	21	16	16	18	20	20

2.5.5 Statistical considerations

2.5.5.1 Univariate data sets (microbial function, mycorrhiza germination)

For the risk assessment of plant protection products, the OECD 216 test is conducted in a hypothesis test design with two concentrations recommended in relation to the application rate. For the derivation of the regulatory endpoint, a difference from treatment to the control of 25 % is used as threshold, independently from the presence of statistically significant effects. Testing the application rate and up to the 10fold application rate instead of a series of test concentrations is a pragmatic approach under the assumption that the response is monotonous. For non-agricultural substances a regression approach with at least five test concentrations is requested as well as the calculation of EC_{10}, EC_{25} and EC_{50} values for the description of the inhibition of N-transformation. In the tests on microbial function conducted within this project, the concentration response was often not monotonous but the tests were also not designed for a regression approach since no test was conducted with at least five test concentrations. Nevertheless, for a direct effect, the monotonous concentration response is the most plausible response. Stimulation at low concentrations and increasing inhibition at higher concentrations (hormesis) was sometimes observed within this project, in the tests described above. Reasons

for a hormesis can be explained for example by reduced density-dependence (lower intra-species competition) resulting in better conditions of the less sensitive groups. Cases with stronger inhibition at lower concentrations than at higher concentrations are difficult to explain and cannot be analysed by the usual concentration response functions.

A disadvantage of a limit test is the missing information on the threshold concentration, e.g. the EC_{25} if the PEC_{max} results in effects above 25%. In such cases, no information on the effect of other test concentrations are available. In addition, a limit test is driven be the effect observed for a single test concentration (or dose) and the uncertainty due to variability and error. In contrast to this, a regression approach calculates the effect concentration for a specific effect magnitude based on larger data set.

A second assumption of the current approach is that up to 25 % difference between treatment and control relate to acceptable effects on microoroganisms in the field. No explanation for the selection of the 25 % is available but in principle, the definition of an effect magnitude considered of ecological relevance seems to be more practicable compared to focussing on statistical significance, only. The calculation of the MDDs (section 2.7.2) showed a strong variation of the statistical power. In the OECD 216 tests conducted, the MDD varied between 2 to 79 % and therefore also the possibility of significance detection (Table 78). Thus, conclusions on acceptability of effects based on statistical significance would not be comparable between different tests conducted with the same method (here: OECD 216), because NOECs in different tests can be related to different effect magnitudes. A recent example for setting an acceptable effect magnitude is given in the refined bee guidance document (Adriaanse et al. 2023). The new approach in this guidance is to use the concentration response functions calculated for the laboratory tests to calculate the expected effect at a given environmental concentration and to compare this to a level of concern, in this case 10 % deviation between treatment and control.

In general, regression approaches are recommended due to their larger flexibility regarding critical effect magnitudes. Moreover, test concentrations for regression approaches cover a range of application rates. Effect concentrations allow a higher comparability between test outcomes compared to NOEC or LOECs derived by hypothesis tests. Limit tests might be sufficient, if information on effects for lower exposure levels is not needed.

2.5.5.2 Multivariate data sets (community structure, ARISA)

ARISA provides a method to switch from functional endpoints to structural endpoints of microbial communities. The technique could be applied to samples taken in a test following OECD 216/217 design but also in Terrestrial Model Ecosystems (TMEs, indoor or outdoor), and field studies. The method provides similar data sets as aquatic mesocosms and field tests with soil invertebrates. I.e., the quantitative measures of operational taxonomic units (OTU) observed with ARISA are considered equivalent to the abundance of taxa observed in aquatic mesocosms or field tests with soil invertebrates. It is presupposed that peaks not only represent presence-absence data on OTUs but that the peak area can be integrated as a proxy of abundance at various levels of exposure over time. Thus, the data per OTU can be evaluated in an univariate way, i.e., similar to evaluating significant differences in taxa abundance between treatment groups and controls.

When analyzing ARISA data, several statistical considerations are crucial to ensure accurate interpretation of microbial diversity and community structure:

- Data preprocessing and normalization:

ARISA generates peaks representing different OTUs (Operational Taxonomic Units). To account for technical variability in peak size due to factors like computer interpolation, dye migration

discrepancies, and run-to-run variations, peaks within a size range are grouped (binned) into window frames that match the imprecision of OTU size calling (de Faria et al. 2021). In this project, an imprecision of ± 1bp was used based on the publication of Ramette (2009). Additonally and given that different samples might yield varying total peak intensities, data normalization (e.g., relative abundance or rarefaction) is necessary to ensure comparability between samples.

- Univariate analysis (alpha diversity):

Diversity can be described by different indices (alpha diversity), e.g. number of taxa (or here, OTUs), evenness (which focussed on the distribution of abundances) or a combination of both. The Shannon index considers both, number of taxa and the distribution of abundance over these taxa. The Shannon index is calculated for each sample, independently from all other samples. Thus, these indices can be analysed by univariate analysis such as hypothesis testing and regression analysis. However, the interpretation of EC_x values for evenness or Shannon index is difficult compared to EC_x values for functional endpoints, life history traits, abundance, biomass, or population growth rates. In addition, it must be kept in mind that the same or similar indices can be given by very different communities and thus, shifts in community structure can be hidden. Finally, direct effects on abundant taxa can increase evenness and thus also the Shannon index. Therefore, effects of treatments might be masked. Changes of diversity indices indicate effects on the community structure but the absence of changes of diversity indicies might not be a 'real' absence of effects on the community structure. Concluding, the analysis of diversity indices is not as powerful approach for the assessment of effects on community structure.

- Multivariate analysis (beta diversity)

Multivariate approaches, e.g. ordination analysis might be more suitable for the effect detection on community structure. Since in this project, only two concentrations (plus control) with two replicates each were analysed by ARISA, no statistical testing was possible and only the similarity between the six samples are shown in ordination plots. The original reference publication of the ARISA method (Ranjard et al. 2001) mentions the Principal Component Analysis (PCA) as a possible method to compare the structural similarity between treated and untreated communities. The PCA forms the basis for the Principal Response Curves (PRC) method that is the currently recommended multivariate approach to the evaluation of community-level studies like aquatic mesocosm studies EFSA PPR panel (2013), oligochaetes (e.g., enchytraeids (Moser et al. 2007)) or soil microarthropod semi-field tests (Scholz-Starke et al. 2013). PRC are an ordination technique developed originally to evaluate community data sets of mesocosm studies and display the response of the community standardised to the control over time (van den Brink and Ter Braak 1997). In addition, species weights are calculated, which rank the taxa (respectively species or OTUs) based on the correspondence of their response to the principal (community) response. The method also provides the variance proportions explained by the treatment (test item), time, and variability between replicates (residual variance) and the significance of the part of the variance displayed by the PRCs. Post-hoc significance tests can assess the effects on the different sampling dates and calculate NOEC values for the community level. However, the number of test concentrations and / or replicates must be sufficient to allow a permutation test.

- Replication and statistical power:

Variability in ARISA data within replicates can arise from several technical and biological factors, and it is a recognized issue in studies when using PCR-based techniques. Key sources of variability include differences in DNA extraction efficiency, PCR amplification biases, and (capillary)electrophoresis conditions (differences between run to run). For example, PCR

stochasticity can cause variations in the amplification of different taxa, leading to differences in peak profiles between replicates, even when analyzing the same sample (Danovaro et al., 2006, Slabbert et al., 2010). Additionally, DNA extraction methods can introduce variability by favoring some taxa over others, affecting the OTU representation across replicates (Díaz et al. 2020). These phenomena were observed in this project, where different extraction methods were applied based on the suitability of the soils. Consequently, direct comparisons between the different soils used is not appropriate. However, it's important to note that ARISA was used in this project to screen its potential as a technique, which led to an experimental design that prioritized a broader range of soils and substances rather than emphasizing in replication. For regulatory applications, a more rigorous design with enhanced quality control and replication would be mandatory to ensure reliability and standardization.

2.5.6 Summary of the evaluation

The most promising insight from the data analysis of the three functional tests on microbial respiration (MicroResp™), enzym activity (ISO 20130) and nitrification (ISO 15685) results were that adding one more treatment level, as done for the RefeSol 04A soil, could enhance the probability that a proper dose-response curve could be modelled. It is highly recommended that at least four treatment levels should be studied regulary in addition to the control.

N-Transformation (OECD 216) and nitrification (ISO 15685) both address the use of nitrogen sources by the microbial community. No major differences in the performance of both tests were found within this project. The test on nitrification according to ISO 15685 test seems to be slightly more sensitive but this could have been caused by the fact that the test on N-Transformation according to OECD 216 was conducted only as limit test. Thus, there is no strong reason to replace or amend the test current set for micoroorgansims, which is N-Transformation (OECD 216) by the performed test on nitrification (ISO 15685).

However, the example tests suggest that N-transformation is often not a sensitive endpoint. Other tests, especially the test on enzyme activity (ISO 20130), were up to a factor of > 100 more sensitive compared to the test on N-transformation (OECD 216). To ensure a sufficient protection of micorroganisms exposed to pesticides, at least one more test on microbial function must be added to the first tier of the risk assessment as well as the standard test battery. For consistent evaluation of pesticides and actives, the test on N-Transformation (OECD 216) could be kept, additionally.

The test on microbial respiration (MicroResp™) showed lower variability of controls and also smaller MDDs than the test on enzymatic activity (ISO 20130) but the latter was clearly more sensitive with usually lower estimated LOECs in the 18 tests conducted here. Thus, we recommend to test enzyme activity (ISO 20130) supplementary to N-transformation (OECD 216).

Fungi and especially mycorrhiza play an important role for soil fertility, but are not covered in tests on soil microbial function. With ISO 10832, a standardized test guideline is available to test effects on germination of AMF. For the six test analysed items including fungizides, AMF-test was less sensitive in comparison to the tests on bacterial function. In addition, as described in chapter 2.3.5.4.4.6, the experiments conducted within this project outlined that the guideline ISO 10832 needs some revision, if tests with natural soil are tested. Nevertheless, it is recommended as it is the only available test covering effects on the development of arbuscular mycorrhizal fungi. At the moment, within the project ERAMYC (FKZ: 3720 64 4070) a test system with the aim to observe effects of test substances on the symbiotic phase of AMF is currently developed; a ring-test for the validation of the method between a set of laboratories is ongoing. The test

method which will be established might be a suitable alternative, covering more relevant endpoints for AMFs.

Tests on microbial community structure using ARISA are not recommended yet as standard tests before more information on variability and reproducibility are available.

Supplementing N-Transformation (OECD 216) by the enzyme activity test (ISO 20130) and germination of AMF (ISO 10832) would ensure more protectivity in regulatory decisions but also require additional effort in the standard risk assessment. However, at least enzyme activity (ISO 20130) would not mean a full additional test, since preparation of soil samples of the experimental setup and application of test items can be shared with the conduction of the currently used test on N-Transformation (OECD 216). AMF are not yet considered in the environmental risk assessment of plant protection products (EFSA PPR panel, 2017), biocides or veterinary pharmaceuticals, whereby EFSA PPR panel (2017) raises a knowledge gap regarding the effect of AMF towards plant protection products. Accordingly, further research is needed aiming to observe toxicity of actives towards AMF – also on the symbiontic phase. The results obtained within the project show that tested active substances showed effects on AMF by using the spore germination test following ISO 10832, even if other methods used in the project showed a greater sensitivity. However, this does not mean that this is always the case for all tested substances and AMF - species. Toxicity of actives towards AMF might also be triggered by physical and chemical properties of actives, tested species, used host plants and soil substrates. Accordingly, the recommendation is to increase the knowledge of actives toxicity towards AMF – also on the symbiontic phase.

2.6 Proposal of procedures complementing the current EU plant protection product risk assessment practice for soil microorganisms

2.6.1 Current state

Currently, the European plant protection product risk assessment for soil microorganisms is defined in the Uniform principles, EU 546/2011 of 10 June 2011 as follows:

"2.5.2.6. *Where there is a possibility of non-target soil micro-organisms being exposed, no authorisation shall be granted if the nitrogen or carbon mineralisation processes in laboratory studies are affected by more than 25 % after 100 days, unless it is clearly established through an appropriate risk assessment that under field conditions there is no unacceptable impact on microbial activity after use of the plant protection product in accordance with the proposed conditions of use, taking account of the ability of micro-organisms to multiply.*"

The valid guidance document for the risk assessment for microorganisms exposed to plant protection products is still the outdated SANCO Terrestrial Guidance Document (SANCO 2002). It refers to a 'litter bag test under field conditions' in case effects on soil microorganisms are larger than 25 % after 100 d in an OECD 216 or 217 test. According to the SANCO guidance, the litter bag test as higher tier option can also be triggered by laboratory tests with earthworms, mites or springtails. However, due to less suitability of using a functional test (the litter bag test) for the relieve of effects on structural endpoints (in-soil invertebrates), the scientific community as well as EFSA decided to not accept the litter bag test as higher tier option for in-soil invertebrates anymore. If there is an indicated risk at the first step of the risk assessment for in-soil invertebrates, terrestrial model ecosystems field tests are used to refine the risk assessment.

According to OECD 216 with regards to pesticides, two concentrations (application rate + up to the 10fold application rate) should be tested on in-soil microorganisms. The assessment targets the application rate as well as the in-crop situation and accepts temporary effects on microorganism if their durations is restricted to less than 100 days. If effects above 25 % in the

lower treatment level (i.e. the maximum predicted concentration) are measured at day 28, the OECD 216 can be prolonged up to 100 days. The rationale for the selection of 25 % as trigger or the extension of the test up to 100 days is not explained in the guidance document (SANCO 2002) nor in the uniform principles. The current approach assumes a 1:1 relationship (and thus, low uncertainty) for extrapolation from the laboratory test to the field situation because an effect up to 25 % over less than 100 day in the laboratory tests with the maximum PEC is considered to be safe without the application of an 'assessment' or 'safety' factor.

The evaluations within this project showed that test outcomes from tests on microorganisms were up to factor 100 more sensitive compared to the test outcomes from the currently used test on N-transformation (OECD 216).

2.6.2 Exisiting proposals addressing the state of the science on risk assessment of chemicals for in-soil organisms

As a background document for preparing a new guidance document for the risk assessment of soil organisms, the EFSA PPR panel published 2017 a Scientific Opinion (SO) on the state of the science which covers also soil microorganisms (EFSA, 2017).

As a basic step for proposing risk assessment schemes, the SO suggests specific protection goals for the different group of soil organisms. Specific Protection Goals (SPG)s are proposed for two groups of microorganisms, Mycorrhiza and other Fungi and Protozoa (EFSA, 2017, Table 16) and Bacteria and Archaea (EFSA, 2017, Table 17). The SO differentiates between the in-field where temporary effects are accepted and the off-field situation with only negligible effects considered acceptable. The main difference between the SPGs for eukaryotes and prokaryotes is that for the latter also large effects over days are considered acceptable in-field. Within the SO, effect magnitudes are scaled as follows:

- "Large effects: pronounced reduction, corresponding to effects above 65%;
- Medium effects: reduction comparable to median effect size (i.e. corresponding to median effect class of 50%; effects between 35% and 65%);
- Small effects: reduction above No Effect Level and below medium effects (above 10% and below 35%);
- Negligible effects: reduction up to No Effect Level (comparable to 10%)".

This scaling was done independent from the taxonomic group but it is clarified in the SO that is related to the assessment endpoints ("namely which magnitude of effect might be tolerable for in-soil organisms as drivers of ecosystem services in order to still meet the proposed SPG options"). For the measurement endpoints of specific tests (e.g. inhibition of germination) which have to be related to the assessment endpoints thresholds can be different.

Table 84: Specific Protection Goal (SPG) options for microorganisms (mycorrhiza, other fungi, protozoa, soil, bacteria, archaea) as proposed by the EFSA PPR panel (2017). Adapted and simplified from EFSA PPR panel (2017).

Area	Ecosystem Service	Ecological Entity	Attribute	Magnitude and temporal scale
In-field	Biodiversity, genetic resources, cultural services	Community	Structure / diversity	Small effects up to months Medium effects up to weeks For bacteria and archaea only: Large effects up to days

Area	Ecosystem Service	Ecological Entity	Attribute	Magnitude and temporal scale
In-field	Nutrient cycling, pest control, natural attenuation, soil structure, food web support	Functional group	Abundance / biomass / activity	Small effects up to months Medium effects up to weeks For bacteria and archaea only: Large effects up to days
Off-field	Biodiversity and all ecosystem services	Community	Structure / diversity	Negligible effects/temporal not relevant

This SPGs are just suggestions since the setting of SPGs is a task to the risk management (i.e. the European Commission (2011)) but has not been done yet for soil organisms. Nevertheless, they can be used as a starting point for further developments of the risk assessment for soil organisms.

The EFSA PPR panel proposed a tiered approach for the risk assessment of in-soil organisms (EFSA PPR panel 2017). As outlined in the SO, the basic principles of a tiered approach are that all tiers address the same protections goals and that lower tiers are more conservative but less realistic than higher tiers. In order to assess whether the different tiers provide the desired level of protectiveness, a reference tier is needed. While the reference tier would be the real situation in the agricultural landscape, the more practical surrogate reference tier for in-soil organisms are field or terrestrial model ecosystem or (mesocosm) studies, which could be supplemented by population models, once they are developend and validated (see section 7.2 in the SO).

The part of the tiered risk assessment scheme dealing with microorganisms as proposed by the EFSA PPR panel (2017) is shown here in Figure 77:

To supplement the current tier 1 (lower tier) assessment for microorganisms, the panel recommended to include also a test with mycorrhiza. Note that in the scheme, the mycorrhiza fungi are not covered under microorganisms but in the right part of the scheme together with soil fauna.

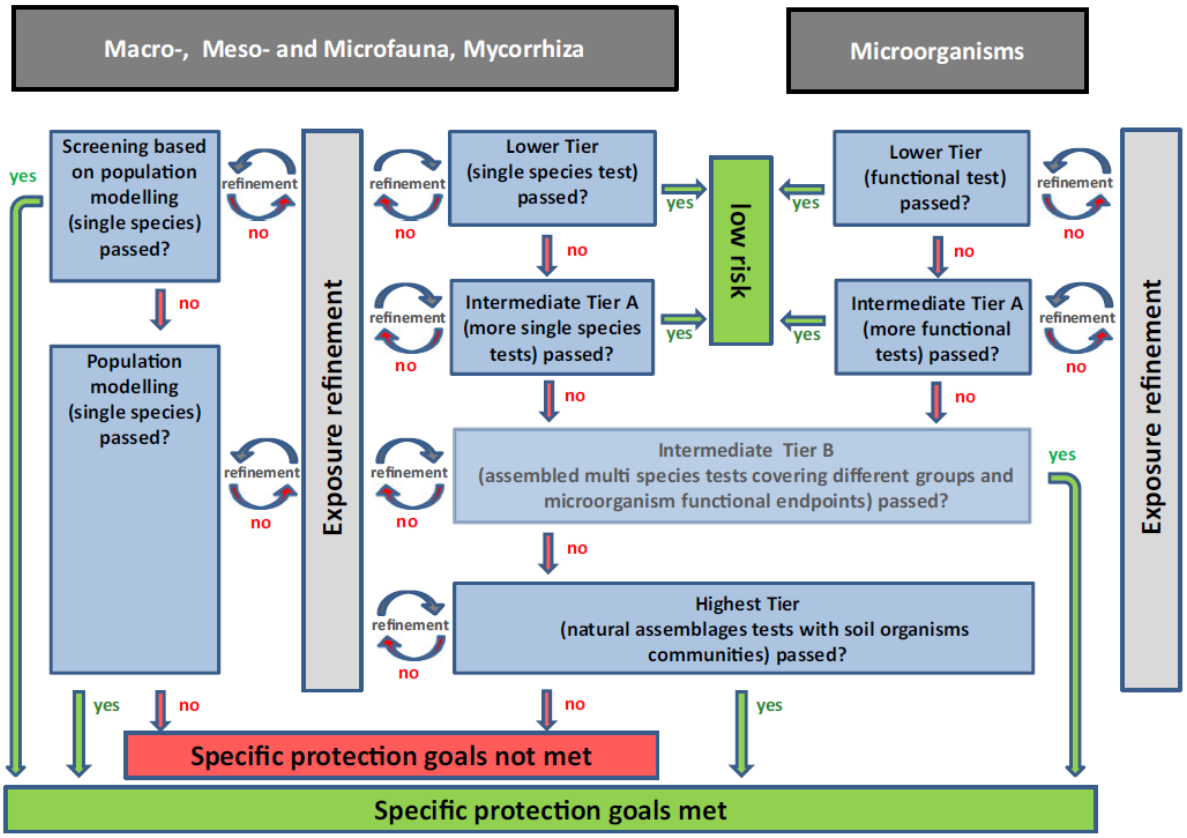
A classical intermediate tier (e.g. tier 2 in aquatic risk assessment) is to test additional species and, if a sufficient number of species is tested, to calculate Species Sensitivity Distributions. The panel considers this also as an option or the soil fauna. However, for mycorrhiza fungi and other microorganisms this is not practical, since the number of mycorrhiza species which can be tested is limited yet and other microorganisms are not tested on taxon level. Instead, the panel suggests to conduct additional "f*unctional tests (BIOLOG®, MicroResp®, see Section 9.3.2), [which] give more detailed information on the capacity of microorganisms to degrade different types of compounds, and the effect of pollutants on degradation*".

For the next tier (intermediate B in Figure 77) "*interactions between species and groups of in-soil organisms and the structural and functional responses of endpoints for microorganisms' communities may be jointly investigated*". The term "*assembled multi-species tests covering different groups and microorganism functional endpoints*" suggests that this could be laboratory microcosms including a (simplified) soil food web with some introduced faunal taxa and a microbial community. Note that the focus of such tests is still on the function of the microbial community. However, the intermediate Tier B is shown in grey in the figure indicating that such testst with assembled multi-species systems are not really recommended by the panel. '*Since experience with this type of assembled terrestrial communities is so far almost completely lacking – and therefore the proper calibration of the outcome of these experiments with the reference tier couldbe very difficult – this step of the proposed risk assessment scheme is considered to be*

conceptually relevant but problematic in the implementation until further experience is available' (EFSA PPR panel 2017).

The highest tier (and thus the surrogate reference tier) proposed are tests with *"natural assemblages tests with soil organism communities*". These corresponds to field tests as refinement option for an identified lower tier risk on earthworms, springtails or mites. While the current practise is to focus only on the groups which triggered the field test (e.g. earthworms), the panel recommended "*to assess jointly populations of macro-, meso- and microfauna and structural/functional responses of microbial communities, in order to be able to detect indirect effects of intended uses of PPPs on in-soil organisms, i.e. disruption of food webs or significant shifts in niche occupancy*".

Figure 77: Flow chart of a risk assessment scheme for in-soil organisms as proposed by EFSA PPR panel (2017).



Source: EFSA PPR panel (2017), Figure 13

Additionally, to EFSA PPR panel (2017), EFSA published on concept paper (EFSA 2021) for the application of OMICs and Bioinformatics approaches for the next generation risk assessment as well as one roadmap for the integration of environmental microbiomes in the risk assessment (Debode et al. 2024). Both publications highlight the necessity to include effects on the community structure of in-soil microoganis through the application of chemicals in the risk assessment.

2.6.3 Proposal of an ERA scheme for microorganisms

We agree with EFSA (EFSA PPR panel 2017, EFSA 2021, Debode et al. 2024) that the current scheme, based only on the OECD 216 test on N-transformation, should be supplemented by additional functional and structural methods.

The proposed specific protection goals (EFSA PPR panel (2017)) address also the diversity of the microbial community (Table 84) and not just the function. Moreover, all tiers of the risk assessment scheme should address the protection goals. Therefore, we suggest to consider in addition to microbial function also the structure of the microbial community already in the standard tier. This is indicated by two boxes for the standard and the intermediate tier in the proposed scheme (Figure 78.)

For the standard tier, the evaluations of this project show, that the currently used test on N-Transformation (OECD 216) is not protective in several cases in comparison to other tests on microbial function. Therefore, the standard test set should be supplemented by an additional functional test in tier 1, as already suggested by EFSA PPR panel (2017). Based on the results of this project, a test on enzymatic activity (ISO 20130) should be included in the standard test set for microorganisms. Effects detected on microbial respiration with the test system MicroResp™ did not show a higher sensitivity compared to OECD 216 and is not suggested for the inclusion in the standard test battery.

The current trigger defined in the Uniform Principles (EU No 546/2011) for acceptable effects in the first (or standard) tier is a deviation in the treatment compared to the control for N- or C-transformation below 25% after 100 days. This decision criterion may also be applied to the ISO 20130, if the current practice should be followed and only the maximum PEC should be tested. Alternatively, the tests could also be evaluated using the specific protection goals captured in EFSA PPR panel (2017), i.e. considering magnitude and duration of effects. If in future, also the risk for off-field areas should be assessed, it would be useful to replace the current limit approach to a concentration response design as recommended already in the OECD TG 216 for non-agricultural chemicals. The derivation of a NOEC or EC_x would allow the assessment of different exposure scenarios instead of only the worst case in-field scenario.

The inclusion of tests with mycorrhiza fungi in the lower tier is suggested to address effects on microbial structure, even if the results of this project did not indicate a higher sensitivity of germination rate of AMF, compared to the conducted tests on bacterial function. However, advanced tests on AMF (including e.g.; the symbiotic phase) or tests on other AMF -species might show different sensitivity of AMF than bacterial function tested within this project, if exposed to chemicals. For single species tests with other in-soil organisms, (i.e. earthworm, springtails, mites), the decision criterion on acceptability of effects is based on a toxicity exposure ratio (TER). If the TER = NOEC / PEC_{soil} is less than 5, no acceptable risk is indicated. However, this acceptablitity criterion needs to be calibrated and validated. More experience with Mycorrhiza tests is needed to define a decision criterion for this group. In the best case, the lower tier assessment would be calibrated using data from the reference tier, i.e. field tests, but in contrast to soil fauna, such data are not available for Mycorrhiza and other microorganisms yet.

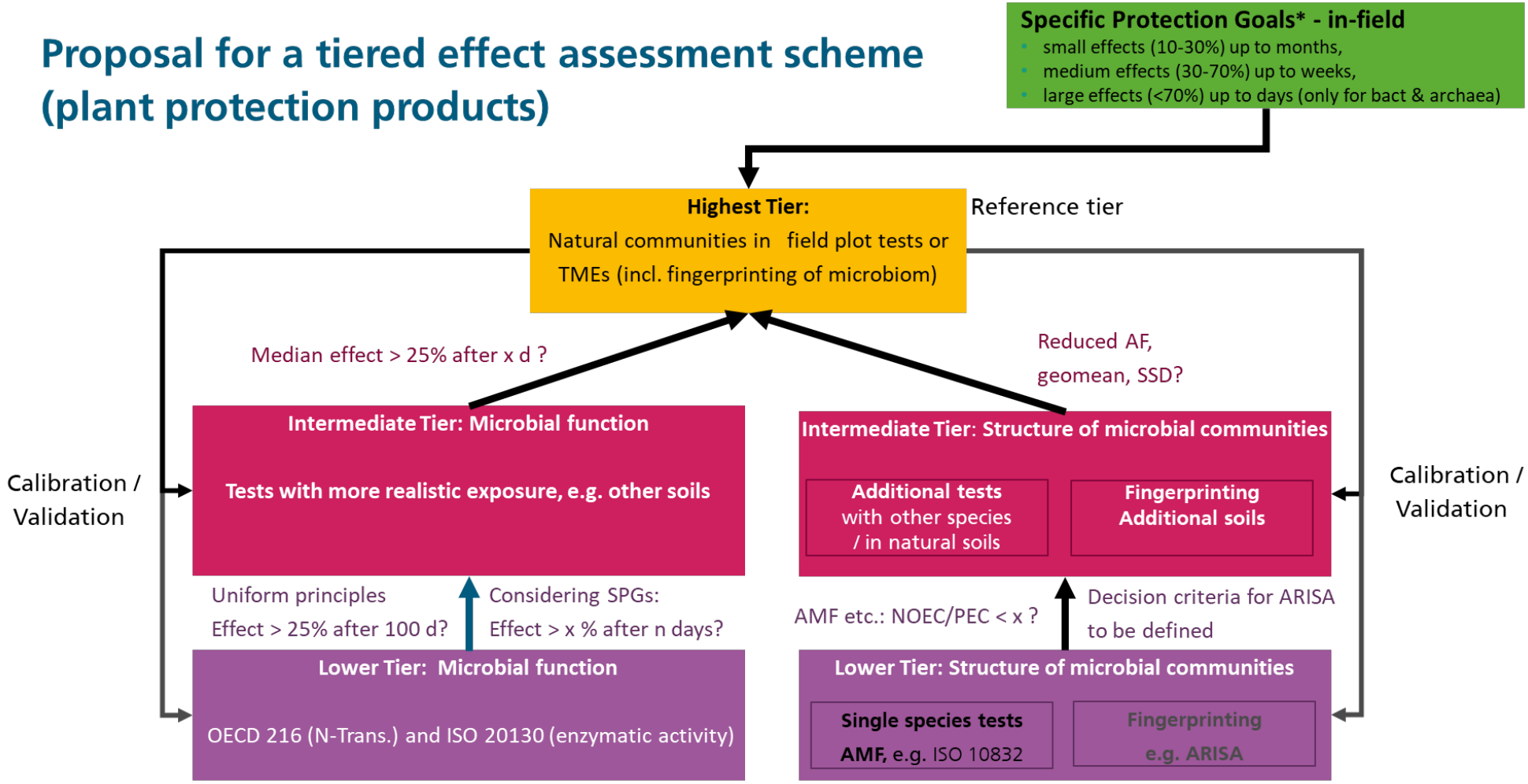
ARISA or more sophisticated fingerprinting methods could provide information on the effects of test items on the structure of the microbial community. In the project, ARISA was used mainly for financial reasons. In future, other methods could provide also taxonomic information instead of only the relative abundance of OTUs. However, for the use of fingerprinting methods standardisation, defined assessment endpoints and decision criteria are needed which requires a better understanding e.g. of the variability between replicates, the variability over time and the most relevant assessment endpoints.

In the intermediate tier, we suggest to test the same microbial functions as in the first tier but with more soils to reduce the uncertainty on the representativeness of the test soil (and the associated community) for the situation in the field. If the decision criterion is still 25 % effect at the end of the test for the PEC, the mean or median effect observed in the different soils could be used as refinement option.

For refinement of risks indicated by AMF tests, additional AMF species or additional soils could be tested in analogy to testing other organism groups. As done for other groups, e.g. aquatic organisms, the standard assessment factor could be lowered. Risk for the community structure indicated by fingerprinting methods could be refined by testing additional soils.

Finally, full fauna field tests, including at least earthworms, mesofauna and microorganisms, conducted under realistic, i.e. outdoor environmental conditions, represent the highest and thus, the reference tier in the EFSA Scientific Opinion (EFSA PPR panel, 2017). To our knowledge, in the past such tests on sinlge organism groups, like e.g. earthworms, were only conducted to refine risk indicated by lower tier data for earthworms, springtails or mites, but not for microorganisms. However, soil samples can be taken during a field test (using field plots or terrestrial model ecosystems) and can then be analysed on function and structure of the microbial community with the same methods as used in lower tiers. Within this project, the ARISA method was used to give insight into effects on the community structure through pesticides exposure by comparing the alpha-diversity as well as the correspondence analysis. The combination of both measures enabled the observation of effects, raised by chemicals exposure. Using ARISA or further developed fingerprinting methods identifying effects of pesticides on the community structure is also in line with EFSA`s vision for the next generation risk assessment (EFSA, 2021). One future key task for the integration of community responses from microorganism is the standardisation of methods as well as the identification of the natural ‘baseline’ (EFSA, 2024) and clear definition of the ‘No observed effect level’.

Figure 78: Proposed tiered approach of a risk assessment scheme for effects of plant protection products on in-soil microorganisms



Blue criterion: current practice. Red criteria: to be defined, ideally by calibration to the reference tier. Source: Own illustration, Fraunhofer IME

3 Antibiotic resistance

3.1 Introduction

“Single-species toxicity tests with microorganisms play an important role during the ERA of antibiotics“ (Brandt et al. 2015). The advantage of these methods is that they are standardized, well defined, rapid and robust and results from different laboratories are thus more comparable than for community tests. An important consideration for all single-species tests is that even single species of bacteria may contain both antibiotic-resistant and sensitive strains and the choice of test strain is thus critical (Dias et al. 2015). We therefore propose that proper strain selection should be included in standard guidelines for single-species toxicity testing with bacteria to be used for ERA. Hence, thorough genomic and phenotypic characterization of a broad range of standard test strains is needed in order to select sensitive strains for ecotoxicity testing.

3.2 Material and methods

3.2.1 Selection of bacterial strains

3.2.1.1 Strains available at IME and sequencing results

In order to make a proper strain selection for WP3, the availability of genomic information of the strains available at IME was revised. From the 22 strains (19 species) revised, 16S rRNA (partial) sequence information and whole genome information is available for ten and nine species, respectively (see Table 85). Additionally, five strains (*Janibacter terrae* DSM 13953; *Arthrobacter* sp. DSM 917; *Lactobacillus plantarum* DSM 20205; *Pseudomonas fluorescens* ATCC 17571; *Pseudomonas putida* DSM 291) have been sent for whole genome sequencing with the aim to determine their suitability for antibiotic resistance testing. These strains were selected for whole genome sequencing as they are relevant soil bacteria.

Table 85: Availability of genomic information of the soil-relevant species at Fraunhofer IME.

Species name	Code	Cultivation conditions	16S rRNA gene (partial)	Complete genome available
Acidovorax facilis	DSM 550	Medium 1, 30°C		X
Actinomadura spadix	DSM 43459	Medium 65, 28°C	X	
Arthrobacter sp.	NRLL B 03652	Medium 92, 30°C	X	
Asticcacaulis excentricus	ATCC 15261	Medium 595, 30°C	X	
Bacillus amyloliquefaciens	DSM 7	Medium 1, 30°C	X	X
Bacillus amyloliquefaciens	DSM 1061	Medium 1, 30°C	X	X
Bacillus amyloliquefaciens	DSM 1067	Medium 1, 30°C		
Bacillus circulans	DMS 11	Medium 1, 30°C		
Bacillus pumilus	ATCC-6631	Medium 1, 26°C	X	X
Bacillus subtilis	DSM 1090	Medium 1, 30°C		
Janibacter terrae	DSM 13953	Medium 92, 28°C		

Species name	Code	Cultivation conditions	16S rRNA gene (partial)	Complete genome available
Lactobacillus plantarum	DSM 20205	Medium 11, 30°C		X
Methylobacterium extorquens	DSM 1338	Medium 1 + 1% MeOH, 26°C		
Nitrosomonas europaea	DSM 28437	Medium 1583, 28°C		
Pseudomonas fluorescens	ATCC 17571		X	X
Pseudomonas putida	NRRL B 01245	Medium 1, 26°C		
Pseudomonas putida	DSM 50026	Medium 1, 28°C	X	X
Rhodococcus ruber	DSM 43338	M. 65 od. 53, 28°C		
Saccharopolyspora erythraea	ATCC-11635	Medium 65, 28°C		X
Spingobium xenophagum	DSM 11094	Medium 220, 28°C		
Streptomyces lydicus	NRLL-2433	M.65, 84, 252, 28°C	X	X
Streptomyces rimosus	NRRL 2234	Medium 65, 28°C	X	

The results of the whole genome sequencing are summarized in Table 86.

Table 86: Results of the bacterial phylogenomic study.

Name of the material sent for sequencing	DSMZ identification after sequencing*	Gram	Comment
Janibacter terrae DSM 13953	*Pseudomonas citronellolis*	-	Strain DSM 13953 (ID 21-405) might represent a new species within the genus *Pseudomonas*
Arthrobacter sp. DSM 917			Mixed culture, sequencing results unspecific
Lactobacillus plantarum DSM 20205	*Lactiplantibacillus plantarum*	+	Strain DSM 20205 might represent a strain of *Lactobacillus* species but might as well represent a new species within the genus *Lactiplantibacillus*
Pseudomonas flourescens ATCC-17571	*Pseudomonas gessardii*	-	Strain ATCC-17571 might represent a strain of the species *Pseudomonas gessardii*
Pseudomonas putida DSM 291	*Pseudomonas putida*	-	Identity confirmed

* Results based on Type Strain Genome Server (TYGS) analysis and checked by via the 16S rRNA gene sequence similarity.

3.2.1.2 Strain selection

Four strains, to be used in this study, have been selected in collaboration with UBA (personal communication with UBA, 28.10.2021). For the selection, the following criteria were considered:

- Soil relevant bacteria
- Maximal diversity among selected organism (gram+/-, different genera etc.)
- Availability of genomic information

Based on this the four strains selected for this WP are listed in Table 87.

Table 87: Strains selected for MIC determination.

Name	DSMZ No	Isolated from	Date of sampling	Gram stain
Arthrobacter sp.	917	Soil	before 08.02.1977	+
Lactiplantibacillus plantarum	Phylogenetic study/DSMZ report 405-21	No information	No information	+
Pseudomonas gessardii	Phylogenetic study/DSMZ report 405-21	No information	No information	-
Acidovorax facilis	550	Soil	before 10.04.1975	-

3.2.2 Selection of antimicrobial substances

The test-substance selection was done in agreement with UBA (personal communication, 12.10.2021), considering the sales data of antibiotics for 2019 (Wallmann et al. 2020). The list of the selected substances is shown in Table 88.

Table 88: List of test substances WP3.

Name	Molecular formula	Description	Antibiotic class
Colistin sulfate	$C_{45}H_{85}N_{13}O_{10} \cdot H_2SO_4$	Antibiotic	Polymyxin
Neomycin sulfate	$C_{23}H_{46}N_6O_{13} \cdot x\ H_2SO_4$	Antibiotic	Aminoglycosid
Chlortetracyclin hydrochloride	$C_{22}H_{24}Cl_2N_2O_8$	Antibiotic	Tetracyclin
Tiamulin hydrogen fumarate	$C_{32}H_{51}NO_8S$	Antibiotic	Pleuromutilin
Copper sulfate	$CuSO_4$	Heavy metal	na
TWEEN 20	$C_{58}H_{114}O_{26}$	Detergent	na

Na: not applicable

3.2.3 MIC determination

3.2.3.1 Preparation of bacterial suspensions for testing

The species *Lactiplantibacillus plantarum* (*L. plantarum*) strain DSM 20205 and *Pseudomonas gessardii* (*P. gessardii*) strain DSM 418 were recovered from glycerol stocks (preserved at -80°C) available at IME. *Acidovorax facilis* (*Ac. facilis*) strain DSMZ 550 and *Arthrobacter* sp. strain DSMZ 917 were ordered as lyophilized culture from DSMZ. Culture media and temperature used for reactivation of the cultures are described in Table 89.

Table 89: Conditions for reactivation of bacterial suspension for testing

	Temperatur [°C]	Culture media*
Acidovorax facilis	30°C	Media 1 (DSMZ)
Arthrobacter sp.	30°C	Trypticase Soy Yeast extract Medium
Lactiplantibacillus plantarum	30°C	MRS Medium

	Temperatur [°C]	Culture media*
Pseudomonas gessardii	26°C	Nutrient Broth

Once cells were reactivated they were transferred to Müller Hinton broth (MHB, Merck, Germany), according DIN EN ISO 20776-1, for maintenance until performance of the tests. Incubation of the cells was done in flask under agitation, at the respective suggested temperatures used during activation (see Table 89). Before the tests, cell suspensions were plated in Müller Hinton Agar (MHA, Merck, Germany) and incubated overnight under the described conditions.

3.2.3.2 Preparation of the inoculum for the test

The inoculation solution for the test was prepared by re-suspending individual colonies, selected from an 18-24 h agar plate, in fresh MHB media. The turbidity of the suspension, measured by optic density at 625 nm (OD625), was adjusted between 0.08 and 0.13 (equivalent to 0.5 McFarland turbidity standard) resulting in a suspension containing approximately 1-2 x 108 CFU/ml (for Escherichia coli ATCC® 25922). The adjusted inoculation solution was diluted 1:1000 to obtain a solution with approximately 1-2 x 106 CFU/ml.

3.2.3.3 Preparation of substances stock solutions

Stock solutions for each antibiotic were prepared and stored in the dark at 4°C until their use. The preparation of solutions for the pre-test and main test were prepared by doing serial dilutions from the stocks (see Table 90).

Table 90: Preparation of stock solutions.

Name	Potency	Active ingredient concentration	Weighing	Volumen H_2O	ρ (active substance) [mg/L]
Colistin sulfate	22986 IU/mg	1.12 mg/mg	9.434 mg	10 mL	1056.66
Chlortetracycline hydrochloride	931 IU/mg	0.93 mg/mg	17.906 mg	10 mL	1665.26
Copper Sulfate	249.69 g/mol	159.61 g/mol	0.3916 g	50 mL	5006.40
Neomycin trisulfate	---	0.695 mg/mg	21.595 mg	10 mL	1500.85
Tiamulin hydrogen fumarate	---	99.50%	26.778 mg	10 mL	2644.00
Name	**ϕ(Tween)**	**Molar Mass**			
TWEEN 20	100%	1227.72 g/mol	---		

3.2.4 Preliminary test

Before the performance of the main test, a range finder test using a broader concentration spectrum was conducted. The pre-test concentrations depended on the respective substance and tested organism (Table 91). Eleven dilution/concentrations were tested for each substance. The minimum inhibitory concentrations were determined and determined in triplicate during each of the pre-tests. The MIC values determined during the pre-test were used to define the concentration range of the main test (see Table 91).

Table 91: Concentration ranges of the substances used during pre-test and main test, and MIC values determined during the pre-test

		Pre-test		Main test
Substance name	**Strain**	**concentration range**	**MIC**	**concentration range**
Chlortetracylin hydrochloride*	*Ac. Facilis*	0.5-500	<0.5	0.031-4
	Arthrobacter sp.	0.5-500	8	0.5-64
	L. plantarum	0.5-500	4	0.5-64
	P. gessardii	0.5-500	1	0.125-16
Colistin sulfate*	*Ac. Facilis*	5x10-8 -500	50	2-256
	Arthrobacter sp.	5x10-8 -500	>500	4-512
	L. plantarum	5x10-8 -500	---	4-512
	P. gessardii	5x10-8 -500	0.5	0.031-4
Copper sulfate*	*Ac. Facilis*	5-2500	625	16-2048
	Arthrobacter sp.	5-2500	625	16-2048
	L. plantarum	4-2048	1024	16-2048
	P. gessardii	5-2500	1250	16-2048
Neomycin sulfate*	*Ac. Facilis*	5x10-8 -500	50	2-256
	Arthrobacter sp.	5x10-8 -500	>500	4-512
	L. plantarum	5x10-8 -500	5	0.125-16
	P. gessardii	5x10-8 -500	5 - 50	0.125-16
Tiamulin hydrogen fumarate*	*Ac. Facilis*	0.5-500	1	0.031-4
	Arthrobacter sp.	0.5-500	500	4-512
	L. plantarum	0.5-500	62.5	4-512
	P. gessardii	0.5-500	500	4-512
TWEEN 20**	*Ac. Facilis*	0.05-50	---	0.39-50
	Arthrobacter sp.	0.05-50	---	0.39-50
	L. plantarum	0.05-50	---	0.39-50
	P. gessardii	0.05-50	---	0.39-50

*concentration in mg/L, ** concentration in % w/v

3.2.5 Main test

In the main test, 96-well flat base polystyrene microtiter plates were used (Sarstedt 82.1581.001, Germany). For each substance, eight concentrations (A-H vertical wells) and ten replicates (1-10 horizontal wells) were tested. Dilutions of the substance were prepared from the stock solutions (information will be provided in the final report).

Three different controls were included in the plates (11-12 vertical wells):

Substance control: belongs to the substance, at each respective dilution/concentration without the inoculum (vertical well 11).

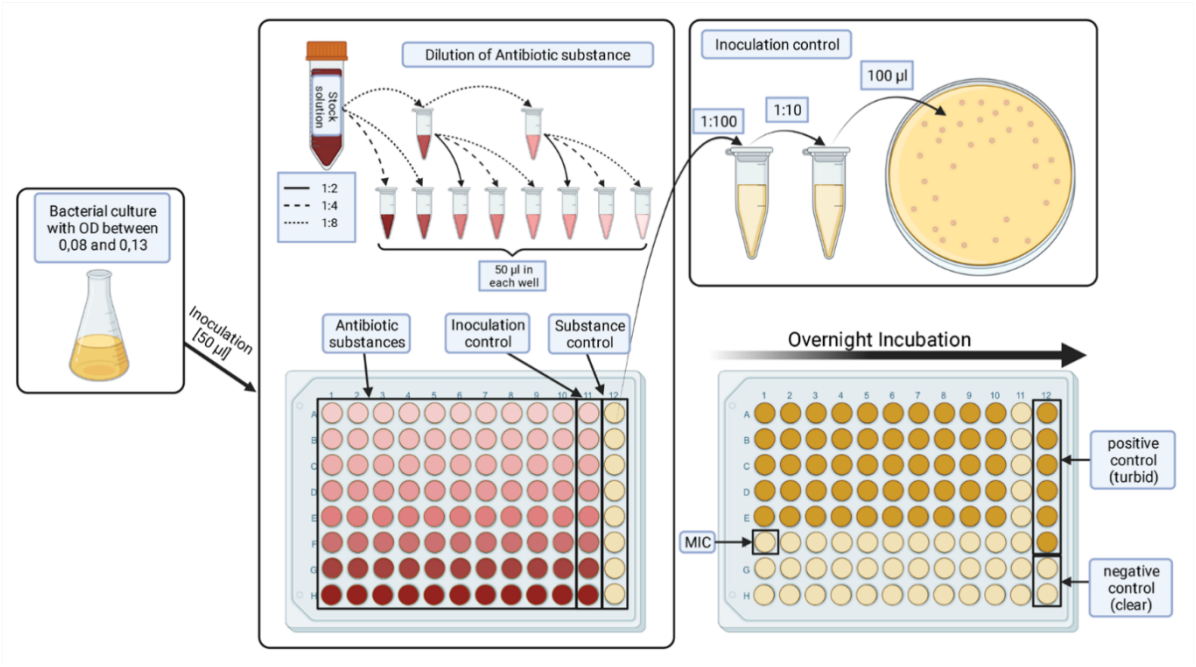
Inoculation control: belongs to the inoculation solution without the test substance (vertical well 12, raw 1-6). From this control, 10 µl were taken, diluted by a factor of 1000 in media and 100 µl plated onto MHA media, to check the number of CFU/ml (validity criteria). A correct inoculation size should result in a number of CFU's in between 20 and 80.

Media control: belongs to the culture media used (MHB) without substance or inoculation solution.

The test volume in each well was 100 µl consisting in: 50 µl inoculation solution (see 3.2.3.2) + 50 µl test substance at each respective concentration (column 1-10). Controls (column 11-12) were poured in a volume of 100 µl.

A summary of the test setup can be seen in Figure 79.

Figure 79: Schematic description of the minimum inhibitory concentration (MIC) using the microdilution method.



Source: Own illustration created with BioRender.com, Fraunhofer IME

3.2.6 Data evaluation

The EUCAST database[3] contains information about MIC distributions of microorganisms for different antibiotics. The data are representative of results obtained with MIC methods performed by or calibrated to reference broth microdilution according ISO-20776-2.

For evaluating if the MICs of the soil bacteria tested in this project are on average lower than the values reported for MICs from clinically relevance strains in the EUCAST data base, the weighted geometric mean of the reported MICs per species was calculated[4]. Since for all four antibiotics tested, the mean MIC of the soil bacteria was higher than the mean of the MICs of the clinical strains, no statistics was applied.Species Sensitivity Distributuons were plotted to visualize the location of the soil bacteria MICs in the total total MIC distribution by means of the ETX program (Van Vlaardingen et al. 2004), Version 2.3.1[5]. Since the objective was just the visualization of the distribution, Hazardous concentrations and goodness of fit are not reported.

Tables containing the information from EUCAST, plus the information of this project have been generated in Excel (see C Appendix 3).

3.3 Results

All tests were conducted successfully and have fulfilled the validity criteria:

- Inoculum control: between 0.08 and 0.13 at OD625 (equivalent to 0.5 McFarland turbidity standard) resulting in a suspension containing approximately 1- 2x10^8 CFU/ml
- No growth in the negative controls.
- No growth in the substance controls.

In Table 92, MIC results can be seen for each tested substance/strain. Values represent the findings of 10 replicates. Variability of MICs between the four bacterial strains tested spanned up to two orders of magnitude. No single strain was the most sensitive one but *Ac. facilis* provided the lowest MIC for two of the four antibiotics while *Arthrobacter sp.* was never the most sensitive strain. The minimum MICs of the four antibiotics tested were at least two orders of magnitude lower than for the two other substances ($CuSO_4$ and TWEEN 20) tested. The lowest MIC found was 0.06 mg/L for chlortetracylin hydrochloride.

Table 92: Minimum inhibitory concentration (MIC) of the tested substances and the tested bacteria strains.

Test substances	*Ac. facilis*	*Arthrobacter* sp.	*L. plantarum*	*P. gessardii*
Chlortetracylin hydrochloride *	0.06	8	4	1
Colistin sulfate*	64-128	no inh.	32	0.5-1
Neomycin sulfate*	32	no inh.	< 4	8
Tiamulin hydrogen fumarate *	1	512	64	512

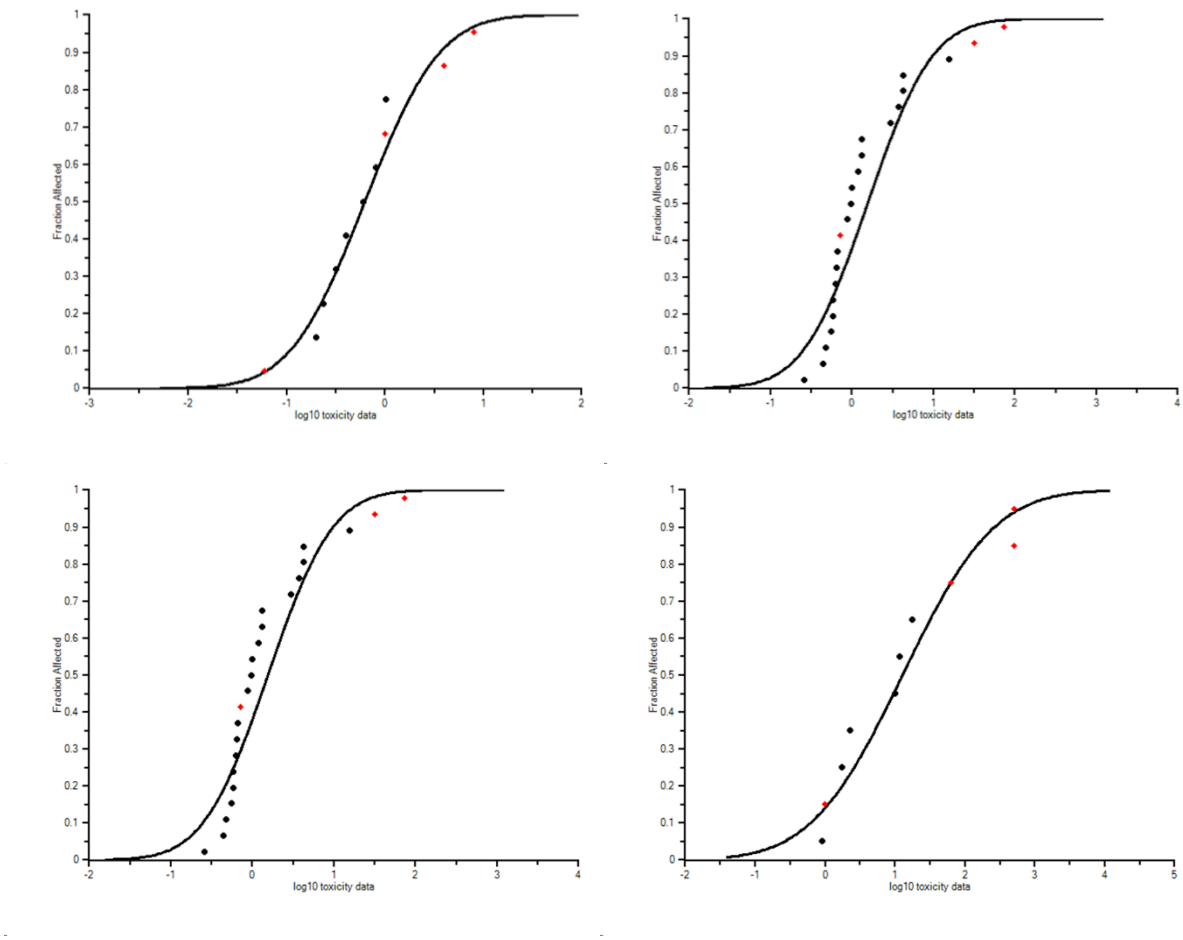
[3] https://mic.eucast.org/search/
[4] Actually, the weighted mean of the log-transformed MICs was back transformed to the concentration scale.
[5] Program download and manual available at: https://rvs.rivm.nl/onderwerpen/risicobeoordeling/modellen-voor-risicobeoordeling/ETX#:~:text=ETX%20is%20een%20computerprogramma%20waarmee%20op%20basis%20van

Test substances	*Ac. facilis*	*Arthrobacter* sp.	*L. plantarum*	*P. gessardii*
Copper sulfate*	512	1024	2048	1024
TWEEN 20**	no inh.	no inh.	no inh.	no inh.

*concentration in mg/L, ** concentration in % w/v, no inh. = not inhibition found, rRed numbers in bold: lowest MIC per test item

The SSD plots show that in general, the soil bacteria were not more sensitive than the clinical strains for the four antibiotics tested (Figure 80). The mean MIC of the soil bacteria was always higher than the one of the clinical strains (see Appendix 3). Only in one case, Chlortetracylin, a MIC from a soil bacterium was lower than all the MICs of the clinical strains. However, it should be noted that the plotted MICs of the clinical strains are weighted mean of many MIC values per species and that also for clinical strains such a low MIC was reported in several cases (see Appendix 3).

Figure 80: Species Sensitivity Distributions of Chlrotretracylin (top left), Colistin (top right), Neomycin (bottom left) and Tiamulin (bottom right) based on MIC values of listed in EUCAST (black dots) and the soil bacteria tested here.



Source: Own illustration, Fraunhofer IME

Note that *Arthrobacter* sp. showed not inhibition in the tests with Colistin and Neomycin (MIC > 512 mg/L and thus, was not included in the SSDs.

3.4 Risk characterization

The MICs summarized in Table 65 are given as concentrations in water and have to be converted to concentrations in soil to allow a comparison with PECsoil and to calculate risk quotients (RQ). The water concentrations can be transformed into predicted minimum inhibitory concentrations (pMICs) for soil using the equilibrium partitioning (EqP) method as used under the water framework directive (EC 2018) and REACH (ECHA 2008, ECHA 2017) and also applied for the calculation of PECs for sediment in phase II CVMP guideline (EMA 2016).

In this approach it is presumed that the interaction of the antimicrobial and the bacterium will take place in the pore water where also the AMR processes occur. The pMIC as determined for surface water also applies for pore water. From the pMIC for (pore)water and the K_{OC}, the associated concentration for soil can be calculated and used for the derivation of the pMICsoil. This is considered not to introduce an additional uncertainty as otherwise the similar (reverse) calculations would be applied for calculating an PECsurface water from the PECsoil.

Two additional substance specific data are needed to calculate risk quotients for soils, the organic carbon partitioning coefficient K_{OC} and the PECsoil. The European Public Assessment Reports (EPAR) on the EMA website[6] were searched for data of the four antibiotics. However, for none of the four substances a report was found. In addition, information was searched in the Publicly Available Assessment Reports (PuAR) included in the Product Information Database of the Veterinary Medicines Directorate of the UK[7]. However, the PuAR for products including chlortetracylin hydrochlorid[8], colistin sulfate[9] or neomycin sulfate[10] do not include data on physico-chemical properties of the active substances nor PEC values. Only for tiamulin fumarate the PuAR prepared by the BVL on the product TYAWALT 450 mg/g includes a K_{OC} and a PECSoil (BVL 2018) which allowed a risk assessment for soil.

The calculations of MICsoil is described in detail in Appendix E. For a worst-case assessment, the minimum MICwater and the lower of the two reported K_{OC} values of tiamulin fumarate were used. This resulted in an RQ of 116 which is about 30 times larger than the RQ of 3 reported in the PuAr based on the NOEC or 3600 µg/kg and an assessment factor of 10 for terrestrial plant growth. However, the RQ for MIC was calculated without the use of an assessment factor which would further increase the RQ. Thus, this case the antibiotic resistance testing indicates a higher risk than the standard assessment for soil organisms. Note that in this case the OECD 216 test as the only one addressing microorganisms indicated no significant effects of 27 000 µg/kg (BVL 2018).

Table 93: Risk characterization for minimum of the MIC values of the four antibiotics in Table 92.

Test item	min MIC_{Water} [mg/L]	min MIC_{Soil} [µg/kg]	PEC_{Soil} [µg/kg]	RQ_{MIC}	RQ_{Ecotox}
Chlortetracylin hydrochlorid	0.06	No data	No data	-	-
Colistin sulfate	0.5	No data	No data	-	-

[4] https://www.ema.europa.eu/en/medicines/what-we-publish-medicines-and-when/european-public-assessment-reports-background-and-context

[7] https://www.vmd.defra.gov.uk/ProductInformationDatabase

[8] https://www.vmd.defra.gov.uk/productinformationdatabase/files/UKPAR_Documents/UKPAR_1986814.PDF

[9] https://www.vmd.defra.gov.uk/productinformationdatabase/files/UKPAR_Documents/UKPAR_1987283.PDF

[10] https://www.vmd.defra.gov.uk/productinformationdatabase/files/UKPAR_Documents/UKPAR_1989870.PDF

Test item	min MIC_{Water} [mg/L]	min MIC_{Soil} [µg/kg]	PEC_{Soil} [µg/kg]	RQ_{MIC}	RQ_{Ecotox}
Neomycin sulfate	<4	No data	No data	-	-
Tiamulin fumarate	1	9.6	1109	116	3

RQ = PEC / MIC, RQsoil = risk quotients for soil in the PuAR. The RQ_{ecotox} is based on a NOEC of 3600 µg/kg for non-target plants as the lowest endpoint and an assessment factor of 10

For human pharmaceuticals, a default AF of 10 is applied to calculate PNECs, e.g., based on a NOEC from the activated sludge respiration inhibition test (OECD 209) to evaluate the anti-microbial effects of anti-microbial substances. For consistency, the same default AF could be applied to the MICs. However, the AF for MIC should consider the amount of strains tested. In the tests conducted here, the MICs per substance showed considerable variation between the four bacterial strains, and MICs varied over up to two orders of magnitude without a clear, most sensitive strain. Therefore, the minimum MIC per test item was used for the example risk assessment here.

4 Degradation performance of soil microorganisms

In work package 4, experiments were carried out to determine the degradation performance of microbial communities in agricultural soils after multiple exposure of chemicals. The effect of multiple applications of a chemical or the exposure to multiple chemicals on the microbial community were investigated. For this purpose, soil degradation tests were performed to determine degradation rates (DT50 values) for the respective chemicals under different exposure situations, e.g. as a single component or in presence of a further chemical.

4.1 Test strategy and test design

The tests were designed to investigate different situations of single and multiple chemical exposure at realistic concentrations and include the following steps:

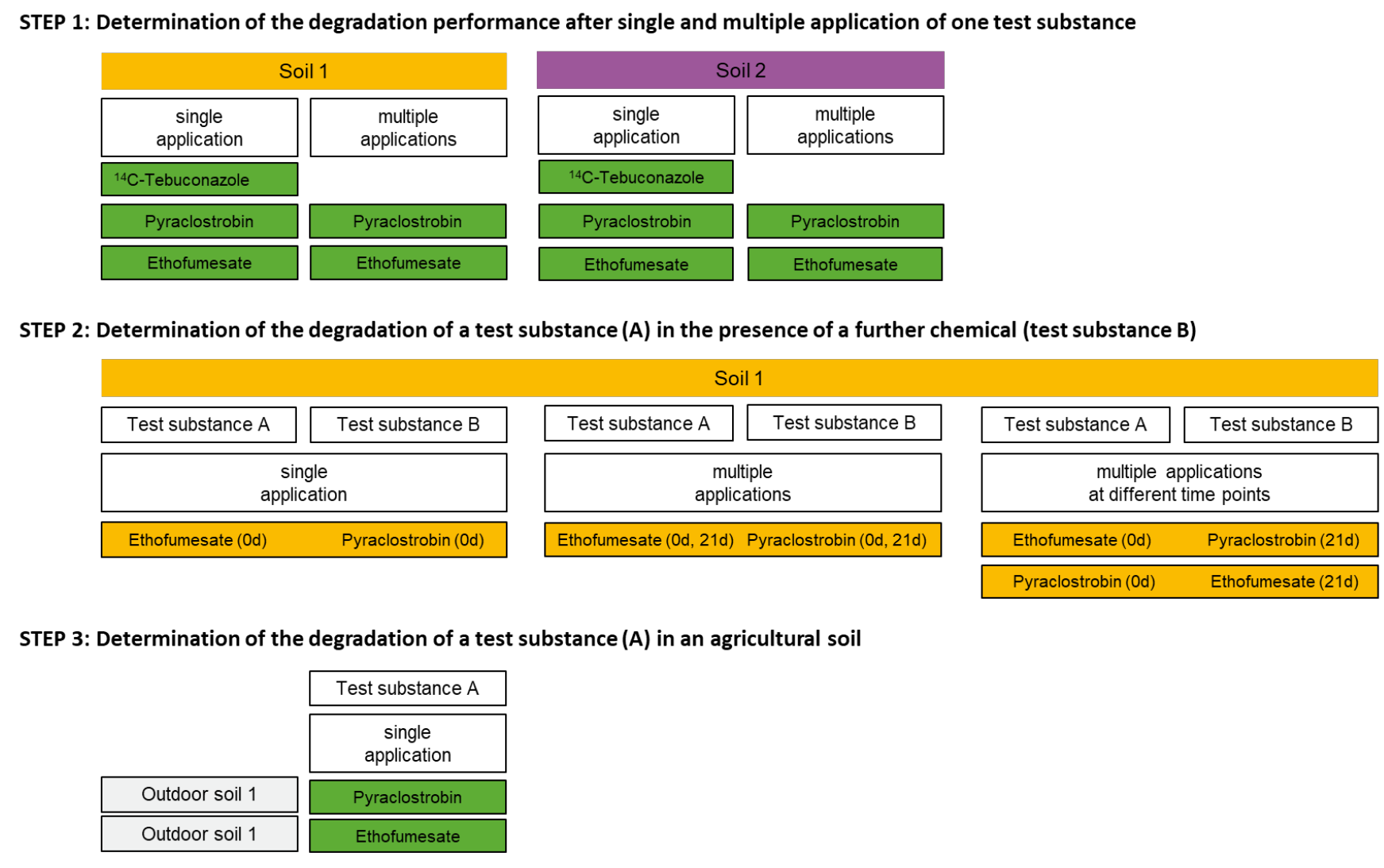
STEP 1: Determination of the degradation performance of soil microorganisms after a single application of a test substance compared to the repeated (multiple) application of the same test substance (simulating the repeated application of a pesticide with a designated interval).

STEP 2: Determination of the degradation performance of the soil microbial community of a test substance in the presence of one further chemical.

STEP 3: Determination of the degradation performance of soil microorganisms of a test substance in an outdoor agricultural soil already containing a realistic mixture of chemicals. The outdoor soil should have comparable soil properties to one of the soils used in step 1 or 2 to relate any degradation effect to the multiple chemicals additionally present in the soil.

The test strategy is summarised graphically in Figure 81.

Figure 81: Test design for determination of the degradation performance of soil microorganisms after multiple exposure to chemicals.

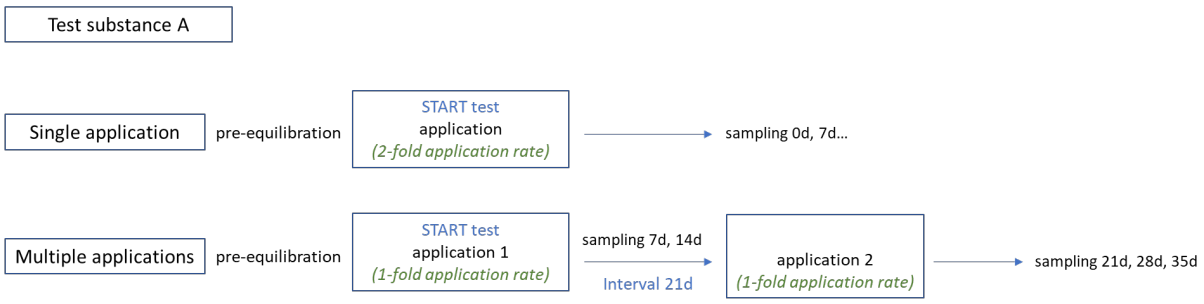


Source: Own illustration, Fraunhofer IME

As shown in Figure 81, two soils were used in STEP 1 experiments: RefeSol 02A ("soil 1") and LUFA 2.1 ("soil 2"). It was planned to investigate two test substances per soil during STEP 1. Each test substance should be applied once (single application) or two times (multiple applications). For the experiments including multiple applications, an interval of 21 days between the two application was scheduled. This time interval was chosen based on the most suggested good agricultural practice (GAP) for the test substance pyraclostrobin and tebuconazole.

The test design of STEP 1 experiments for each test substance and each soil is presented in Figure 82.

Figure 82: Test design for single and multiple applications of one test substance (STEP 1).



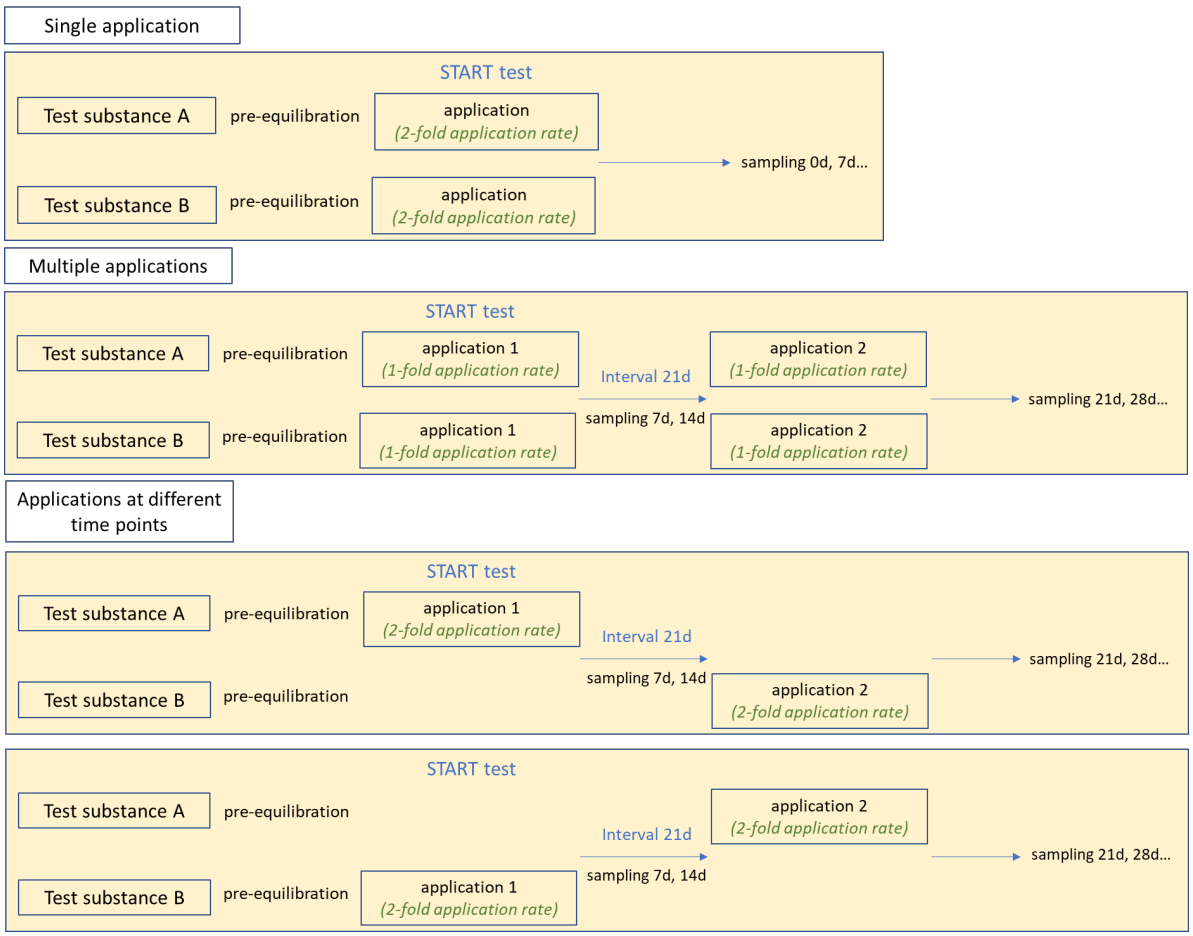
Source: Own illustration, Fraunhofer IME

Tebuconazole and pyraclostrobin were initially chosen as test substances for STEP 1 simulation testing. Pyraclostrobin was used as non-labelled test substance whereas tebuconazole was available as ^{14}C-labelled test substance to obtain additional information on mineralisation and non-extractable residues (NER). However, the experiments with single application of ^{14}C-labelled tebuconazole resulted in relatively long DT50 values in both soils which was considered as not appropriate for the aim of this project. After an additional 30 day-pretest with different application rates of tebuconazole which could not enhance the previously observed degradation rates, it was decided to use ethofumesate instead of tebuconazole as test substance within this project. Therefore, the experiments including single application were repeated with non-labelled ethofumesate and the degradation tests with multiple applications were directly carried out using non-labelled ethofumesate.

In STEP 2 exclusively one soil, the RefeSol 02A ("soil 1"), and the two test substances pyraclostrobin and ethofumesate were used for the simulation tests. The degradation performance of the soil microbial community from STEP 2 could be compared with STEP 1 test results on the basis of the DT50 values of the respective test substance. During STEP 2, the two chemicals were applied at the same time points (as a mixture) as a single or as multiple application and also separately at two different time points as single application. Thus, each test substance acted as a "stressor" on the degradation of the other test substance present in the soil sample. The application interval as well as the initial concentrations in the test sets were kept constant in STEP 1 and STEP 2 to establish comparable conditions throughout the project.

The test design of STEP 2 is shown in Figure 83.

Figure 83: Test design for single and multiple applications of a test substance in the presence of a further chemical (STEP 2)

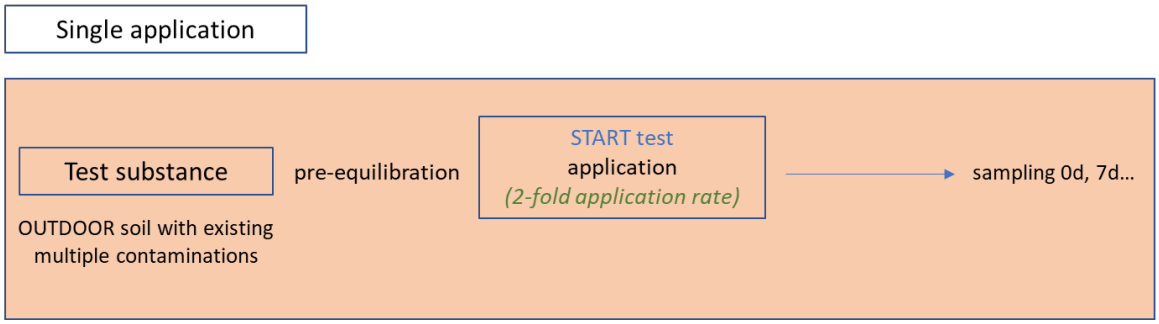


Source: Own illustration, Fraunhofer IME

In the last experimental STEP 3, the degradation of the test substances pyraclostrobin and ethofumesate by the soil microbial community was investigated in an outdoor soil containing a realistic mixture of chemicals. In order to maintain the soil characteristics as well as the soil microbial community as comparable as possible to soil RefeSol 02A used in STEP 1 and STEP 2, a soil under normal agricultural use was freshly sampled next to the original sampling site of RefeSol 02A used for STEP 1 and STEP 2 and was employed for STEP 3 tests. The test substances were applied separately as single application.

The test design of STEP 2 is shown in Figure 84.

Figure 84: Test design for single application of a test substance to an outdoor soil (STEP 3)



Source: Own illustration, Fraunhofer IME

4.2 Materials and methods

4.2.1 Soils

The simulation tests were carried out with two soils which were also used in work package 2. Standard soils of the RefeSol reference soils (RefeSol 02A and RefeSol 04-A) and from LUFA Speyer (Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, LUFA 2.1) were available for the experiments and were used for the method development. Generally, RefeSol soils and soils from LUFA Speyer are natural soils representative for commonly occuring soil types in Germany (i.e. Central Europe) which are long time available and therefore provide comparability of studies in the regulatory context. For soil degradation studies all soils can be sampled field fresh with active microflora on short-term. After discussion with UBA, it was decided to use RefeSol 02A and LUFA 2.1 for the soil degradation experiments. The physico-chemical properties of the two test soils are summarised in Table 13 in chapter 2.3.2.

Soil RefeSol 02A (and soil RefeSol 04-A) is standard agricultural soil available at the testing facility and was freshly sampled as required for the degradation test sets. Soil LUFA 2.1 was obtained from Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, Obere Langgasse 40, D-67346 Speyer (LUFA Speyer). A history of fertiliser and pesticide use for soils RefeSol 02A and LUFA 2.1 is shown in Table 167 and Table 168.

In addition, a soil with an existing multiple contamination reflecting soil cultivation was also employed. For this purpose, soil RefeSol 02A was freshly sampled on April 16, 2024 from a field which was in normal agricultural use including application of commercial plant protection products. A history of fertiliser and pesticide use is shown in Table 175.

4.2.2 Test substances

Test substances were chosen from the substances which were also used in work package 2 and described in chapter 2.3.1 (see Table 10).

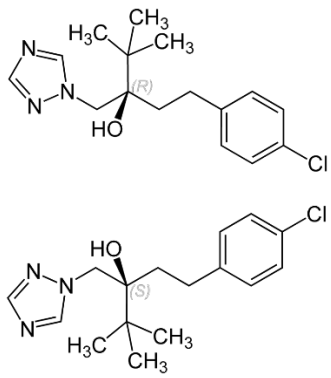
For simulation testing in work package 4, tebuconazole and pyraclostrobin were chosen at first as test substances for simulation testing. Pyraclostrobin was used as non-labelled test substance whereas tebuconazole was available as ^{14}C-labelled test substance to obtain additional information regarding mineralisation and non-extractable residues (NER) besides the DT50 values of the parent compound. However, first results of soil degradation tests with ^{14}C-labelled tebuconazole showed that tebuconazole was not appropriate for simulation testing in this project due to its high DT50 values. Therefore, it was decided to use non-labelled ethofumesate instead of tebuconazole as test substance for further testing. Ethofumsate was chosen on the

basis of literature data regarding degradation rate and possible contemporaneous use together with pyraclostrobin.

4.2.2.1 Tebuconazole

Information of the radiolabelled tebuconazole is summarised in Table 94.

Table 94: ^{14}C-labelled test substance tebuconazole.

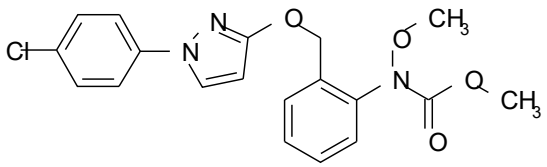
Name	[1,2,4-triazole-U-14C] tebuconazole
Chemical name	(RS)-1-tert-Butyl-1-(4-chlorphenethyl)-2-(1H-1,2,4-triazol-1-yl)ethanol
CAS number	107534-96-3
Molecular formula	C16H22ClN3O
Molecular weight	307.82 g/mol (non-labelled substance)
Structure	
Lot/Batch number	XXVII/48/A/1
Specific activity	5.768 MBq/mg
Radiochemical purity	99.90%
Chemical purity	98.98%
Supplier	Izotop, Institute of Isotopes Co. Ltd., Budapest, Hungary
Date of receipt	21 April 2022
Storage conditions	≤ - 18 °C in tightly closed containers

In addition to the radiolabelled tebuconazole, non-labelled tebuconazole and the known metabolite 1,2,4-triazole were purchased from Sigma-Aldrich.

4.2.2.2 Pyraclostrobin

The test substance pyraclostrobin was obtained as non-labelled test material from Sigma-Aldrich. Information on the test substance is presented in Table 95.

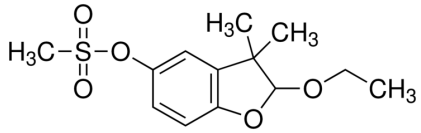
Table 95: Test substance pyraclostrobin.

Name	**Pyraclostrobin** **PESTANAL™, analytical standard**
Chemical name (IUPAC)	Methyl-*N*-(2-[1-(4-chlorphenyl)-1*H*-pyrazol-3-yl]oxymethylphenyl)-(*N*-methoxy)carbamate
CAS number	175013-18-0
Molecular formula	$C_{19}H_{18}ClN_3O_4$
Molecular weight	387.82
Structure	
Lot/Batch number	BCCG4302
Purity	99.9%
Expiry date	31 October 2026
Supplier	Supelco / Sigma-Aldrich
Date of receipt	25 May 2022
Storage condition	Store in cool place. Keep container tightly closed in a dry and well-ventilated place

4.2.2.3 Ethofumesate

The test substance ethofumesate was obtained as non-labelled test material from Sigma-Aldrich. Information on the test substance is presented in Table 96.

Table 96: Test substance ethofumesate.

Name	**Ethofumesate** **PESTANAL™, analytical standard**
Chemical name (IUPAC)	2-Ethoxy-3,3-dimethyl-2,3-dihydro-1-benzofuran-5-yl methanesulfonate
CAS number	26225-79-6
Molecular formula	$C_{13}H_{18}O_5S$
Molecular weight	286.34
Structure	
Lot/Batch number	BCCG3134
Purity	98.1%
Expiry date	31 July 2026

Name	Ethofumesate PESTANAL™, analytical standard
Supplier	Sigma-Aldrich
Date of receipt	14 December 2023
Storage condition	Tightly closed. Dry.

4.2.3 Liquid scintillation counting (LSC)

For the experiments with the radiolabelled test substance ^{14}C-tebuconazole, the radioactivity in liquid samples (e.g. application solutions, soil extracts, samples in Oxysolve after combustion) was determined by liquid scintillation counting (LSC). After mixing an aliquot of the solution of interest with an aliquot of a suitable liquid scintillation cocktail (e.g. Ultima Gold LLT for aqueous samples and Ultima Gold for organic samples), LSC measurements were performed using a Hidex liquid scintillation counter.

Each sample was measured for 5 minutes in duplicate in order to ensure reproducibility and the values reported are the mean values. Computer-constructed quench curves, derived from a commercially available series of sealed quench standards (from Packard), automatically convert cpm to dpm. The background radioactivity was determined by counting samples with no radioactivity within the same batch as the samples. The background was subtracted automatically from each sample after measurement.

4.2.4 Combustion analysis

During the degradation experiments using ^{14}C-tebuconazole, the radioactivity remaining in soil was determined by liquid scintillation counting (LSC) after combustion. For this purpose, the soil material remaining after extraction was air dried at room temperature. Five replicates of each sample were combusted using a Zinsser Oxidizer. The resulting $^{14}CO_2$ was trapped in Oxysolve C 400, and afterwards it was quantified by LSC. For this purpose, each vial (volume: 20 ml) was measured for 5 minutes by means of a Hidex or Packard Tri-Carb scintillation analyzer. The radioactivity of each sample was calculated using the mass data and the LSC data.

4.2.5 HPLC method

The analytical method, which was depeloped to analyse the test substances tebuconazole, its known metabolite 1,2,4-triazole and pyraclostrobin by HPLC including radiodection and UV-detection is summarised in Table 97.

Table 97: Radio-HPLC system and method for analysis of tebuconazole, 1,2,4-triazole and pyraclostrobin.

HPLC pump	LPG 3400 SD, Thermo Fisher Scientific
HPLC autosampler	WPS-3000 SL, Thermo Fisher Scientific
UV detector	Diode array Ultimate 3000, Thermo Fisher Scientific
Radiodetector	LB 514, Berthold
Chromatographic data system	Pyramid Valuestation with Chromeleon 7, Thermo Fisher Scientific

HPLC pump	LPG 3400 SD, Thermo Fisher Scientific		
Stationary phase	PerfectSil Target ODS-3 HD 5 µm 250 x 4.6 mm		
Injection volume	20 µL		
UV detection	3D Field, λ = 200 – 600 nm		
Wavelength (UV-VIS 1, 2)	210 nm, 225 nm		
Temperature	30 °C		
Flow rate	0.5 mL/min		
Mobile phases	A: water		
	B: acetonitrile with 0.1% formic acid		
Gradient program	Time	%A	%B
	0 min	90	10
	5 min	90	10
	20 min	10	90
	30 min	0	100
	32 min	90	10
	45 min	90	10

Applying the analytical method described above, tebuconazole eluted at a retention time of 26.3 min, 1,2,4-triazole at 5.3 min and pyraclostrobin at a retention time of 27.8 min (UV detection). Retention times of the different substances indicate that separation and differentiation of the components can be achieved by the described method.

Pyraclostrobin was calibrated in a concentration range between 0.1 µg/mL and 10 µg/mL.

4.2.6 LC-MS/MS method

The analytical method, which was depeloped to analyse the test substances pyraclostrobin and ethofumesate by LC-MS/MS, is summarised in Table 98.

Table 98: LC-MS/MS system and method for analysis of pyraclostrobin and ethofumesate.

LC conditions	
Instrument	Waters Acquity UPLC (including Solvent Manager, Sample Manager, TUV Detector, Water 2489)
Analytical column	Acquity UPLC BEH C18 1.7 µm 2.1 mm x 100 mm
Flow rate	0.3 mL/min

LC conditions			
Column temperature	30 °C		
Injection volume	10 µL or 20 µL		
Injection mode	Partial loop with needle overfill		
Mobile phases	A: water with 0.1% formic acid		
	B: acetonitrile with 0.1% formic acid		
Run time	6.50 min (including a pre-run of 1 min for equilibration)		
Gradient program	Time	%A	%B
	0 min	90	5
	0.1 min	95	5
	1.5 min	0	100
	3.0 min	0	100
	3.1 min	95	5
	5.5 min	95	5
MS conditions			
Instrument	Waters MS TQD		
Software	Waters MassLynx V4.1 SCN805		
Dwell	0.061 sec or 0.106 sec		
Ionisation mode	Electrospray positive (ES+)		
Data type	MRM data		
Function type	MRM of 5 channels		
Mass transitions	Pyraclostrobin:	388.17 → 163.01 (quantifier) 388.17 → 164.01 (qualifier) 388.17 → 194.23 (qualifier)	
	Ethofumesate:	287.02 → 121.09 (quantifier) 287.02 → 161.08 (qualifier)	

Applying the analytical method described above, pyraclostrobin eluted at a retention time of 2.8 min and ethofumesate at a retention time of 2.6 min.

Pyraclostrobin was calibrated in a concentration range of 0.025 ng/mL - 25 ng/mL or 0.05 ng/mL – 10 ng/mL and ethofumesate in a concentration range of 5 ng/mL – 500 ng/mL or 5 ng/mL – 150 ng/mL. Calibration solutions were prepared in acetonitrile/ultrapure water (50:50, v:v) with 0.1% formic acid.

4.2.7 Development of soil extraction methods

4.2.7.1 Soil extraction method for ^{14}C-tebuconazole and pyraclostrobin

An extraction method was developed for the test substances ^{14}C-tebuconazole and pyraclostrobin using the soils RefeSol 02A and RefeSol 04-A and (radio-)HPLC analysis (see Table 97). The method was also checked with soil LUFA 2.1 and with both test substances in one soil sample.

^{14}C-tebuconazole

The method development started for tebuconazole using the radiolabelled test substance: 50 g soil (dry weight basis) of RefeSol 02A and RefeSol 04-A were weighed into glass centrifuge tubes. 48.01 kBq of ^{14}C-labelled tebuconazole corresponding to 8.32 µg (corresponding to an application rate of 166 µg/kg or 125 g a.s./ha) were added in acetonitrile to each vessel. To extract the test substance, the soil samples were extracted 3 times with 100 mL acetonitrile by shaking on a horizontal shaker followed by treatment in an ultrasonic bath for 10 minutes. For RefeSol 02A a fourth extraction step (with 100 mL acetonitrile/water (80:20, v:v), 30 minutes on a horizontal shaker and 10 min in a ultrasonic bath) was carried out. Phase separation was achieved by centrifugation for 10 min at 2000 rpm using a Heraeus Multifuge 4KR. The organic supernatants of the extraction steps were decanted and analysed separately by liquid scintillation counting (LSC). Afterwards, the extracts of steps 1-3 were combined and radioassayed again.

This extraction procedure resulted in a recovery of applied radioactivity of 103.9% for RefeSol 02A (4 extraction steps) and of 101.2% for RefeSol 04-A (3 extraction steps).

In order to check the stability of the test substance tebuconazole throughout the extraction procedure (including storage of extracts for 1 day), radio-HPLC analysis of the extracts were carried out. For this purpose, aliquots of the extracts were evaporated by a gentle stream of nitrogen to residual volumes of about 0.25 mL which were then filled up to volumes of 1 mL using acetonitrile/water (50:50, v:v) with 0.1% formic acid. The solutions were mixed thoroughly and centrifuged for 5 minutes at 14000 rpm in an Eppendorf Centrifuge 5418. The recovery of radioactivity was analysed by LSC followed by HPLC measurements of the concentrated extracts (concentration factor about 3:1 or 4:1). The recovery of radioactivity during the concentration steps was between 91.5% and 106.4%. In the extracts exclusively radiolabelled tebuconazole was detected indicating that tebuconazole is stable during the extraction procedure.

4.2.7.1.1 Pyraclostrobin

Soil extraction method was also tested for pyraclostrobin: 50 g soil (dry weight basis) of RefeSol 02A and RefeSol 04-A were weighed into glass centrifuge tubes. 2.75 mg of non-labelled pyraclostrobin (corresponding to an application rate of 55 mg/kg or 41 kg a.s./ha) or 0.75 mg non-labelled pyraclostrobin (corresponding to an application rate of 15 mg/kg or 11 kg a.s./ha) were applied in acetonitrile to each vessel. The soil samples were extracted 3 times with 100 mL acetonitrile by shaking on a horizontal shaker followed by treatment in an ultrasonic bath for 10 min. Phase separation was achieved by centrifugation for 10 min at 2000 rpm using a Heraeus Multifuge 4 KR. The organic supernatants were decanted, combined and the volume of the combined extracts were determined. Aliquots of 1 mL were transferred into Eppendorf-tubes and were centrifuged for 5 min at 14000 rpm in an Eppendorf Centrifuge 5418. 0.5 mL of the supernatant was mixed with 0.5 mL ultrapure water and 1 µL formic acid in an HPLC vial and was analysed by HPLC.

The recovery of pyraclostrobin in extracts of both soils was between 99.3% and 99.7%.

4.2.7.1.2 Soil LUFA 2.1

Extraction efficiency was tested for soil LUFA 2.1 using ^{14}C-tebuconazole and pyraclostrobin separately: 50 g soil (dry weight basis) of LUFA 2.1 were weighed into glass centrifuge tubes. One replicate was applied with 50 kBq of ^{14}C-labelled tebuconazole (corresponding to 8.67 µg and an application rate of 173 µg/kg or 130 g a.s./ha), another replicate with 0.75 mg of non-labelled pyraclostrobin (corresponding to an application rate of 15 mg/kg or 11 kg a.s./ha). The soil samples were extracted 3 times with 100 mL acetonitrile by shaking on a horizontal shaker followed by treatment in an ultrasonic bath for 10 min. Phase separation was achieved by centrifugation for 10 min at 2000 rpm using a Heraeus Multifuge 4 KR. The organic supernatants were decanted, combined and the volume of the combined extracts were determined.

An aliquot of the combined extracts (30 mL) of replicate 1 (applied with tebuconazole) was evaporated by a gentle stream of nitrogen to a residual volume of about 0.25 mL which was then filled up to a volume of 1 mL using acetonitrile/water (50:50, v:v) with 0.1% formic acid. The solution was mixed thoroughly and centrifuged for 5 minutes at 14000 rpm in an Eppendorf Centrifuge 5418. The recovery of radioactivity was analysed by LSC followed by HPLC measurements of the concentrated extracts (concentration factor 3:1). The recovery of applied radioactivity after extraction based on LSC analysis was 99.6%. After the concentration steps, 113.9% of the radioactivity was detected in the concentrated extract. In the extract exclusively radiolabelled tebuconazole was found.

An aliquot of 1 mL of replicate 2 (applied with pyroclostrobin) was transferred into Eppendorf-tubes and was centrifuged for 5 min at 14000 rpm in an Eppendorf Centrifuge 5418. 0.5 mL of the supernatant was mixed with 0.5 mL ultrapure water and 1 µL formic acid in an HPLC vial and was analysed by HPLC. The recovery of Pyraclostrobin in the combined extract was 98.7%.

4.2.7.1.3 Extraction of ^{14}C-tebuconazole and pyraclostrobin present in one soil sample

The soil extraction method was adapted to use 3 x 50 mL acetonitrile as extractant instead of 3 x 100 mL acetonitrile. The extraction efficiency was tested for soils RefeSol 02A and RefeSol 04-A after application of both ^{14}C-tebuconazole and pyraclostrobin to each soil sample: 50 g soil (dry weight basis) of soils RefeSol 02A and RefeSol 04-A (two replicates each) were weighed into glass centrifuge tubes. The replicates were applied with 50 kBq of ^{14}C-labelled tebuconazole (corresponding to 8.67 µg and an application rate of 173 µg/kg or 130 g a.s./ha) and 0.5 mg of non-labelled pyraclostrobin (corresponding to an application rate of 10 mg/kg or 7.5 kg a.s./ha). The soil samples were extracted 3 times with 50 mL acetonitrile by shaking on a horizontal shaker followed by treatment in an ultrasonic bath for 10 min. Phase separation was achieved by centrifugation for 10 min at 2000 rpm using a Heraeus Multifuge 4 KR. The organic supernatants were decanted, combined and the volume of the combined extracts were determined. For ^{14}C-tebuconazole, soil RefeSol 02A needed to be extracted a fourth time with 50 mL acetonitrile by shaking on a horizontal shaker followed by treatment in an ultrasonic bath for 10 min. The supernatant was kept separately after centrifugation for 10 min at 2000 rpm using a Heraeus Multifuge 4 KR.

Analysis of extracts for ^{14}C-tebuconazole: An aliquot of the combined extracts (extraction steps 1-4 for RefeSol 02A and 1-3 for RefeSol 04-A, aliquots of about 18.5 mL each) was evaporated by a gentle stream of nitrogen to a residual volume of about 0.1 mL which was then filled up to a volume of 1 mL using acetonitrile/water (50:50, v:v) with 0.1% formic acid. The solution was mixed thoroughly and centrifuged for 5 minutes at 14000 rpm in an Eppendorf Centrifuge 5418. The recovery of radioactivity was analysed by LSC followed by radio-HPLC measurements of the concentrated extracts (concentration factor *ca.* 18:1). The recovery of applied radioactivity after

extraction based on LSC analysis was 109.1% (RefeSol 04-A) – 110.3% (RefeSol 02A) (mean values of two replicates). After the concentration steps, 99.7% - 101.4% of the radioactivity was detected in the concentrated extracts. Exclusively radiolabelled tebuconazole was found by radio-HPLC.

Analysis of extracts for pyraclostrobin: Aliquots of 1.4 mL of the extracts (extraction steps 1-3 for both soils) were transferred into Eppendorf-tubes and were centrifuged for 5 min at 14000 rpm in an Eppendorf Centrifuge 5418. 0.5 mL of the supernatant was mixed with 0.5 mL ultrapure water with 1% formic acid in an HPLC vial and was analysed by HPLC. The recovery of pyraclostrobin in the combined extracts was 96.1% (RefeSol 02A) – 98.4% (RefeSol 04-A).

4.2.7.2 Soil extraction method for pyraclostrobin

Since much lower application rates needed to be tested than used during method development, the extraction efficiency of the developed extraction method was tested for the test substance pyraclostrobin using the soils RefeSol 02A and LUFA 2.1 and LC-MS/MS analysis (see Table 98).

50 g soil (dry weight basis) of soils RefeSol 02A and LUFA 2.1 (two replicates each) were weighed into glass centrifuge tubes. The soil samples were applied with 8 µg of non-labelled pyraclostrobin (corresponding to an application rate of 160 µg/kg or 120 g a.s./ha). The soil samples were extracted 3 times with 50 mL acetonitrile by shaking on a horizontal shaker followed by treatment in an ultrasonic bath for 10 min. Phase separation was achieved by centrifugation for 10 min at 2000 rpm using a Heraeus Multifuge 4 KR. The organic supernatants were decanted, combined and the volume of the combined extracts were determined.

An aliquot of 1 mL of each extract was transferred into Eppendorf-tubes and was centrifuged for 5 min at 14000 rpm in an Eppendorf Centrifuge 5418. The supernatant was diluted with acetonitrile/ultrapure water (50:50, v:v) with 0.1% formic acid and analysed by LC-MS/MS. The recovery of pyraclostrobin in the combined extracts was 80.3% (RefeSol 02A) – 84.8% (LUFA 2.1) (mean value of two replicates).

4.2.7.3 Soil extraction method for ethofumesate

The extraction efficiency of the developed extraction method was tested for the test substance ethofumesate using the soil RefeSol 02A and LC-MS/MS analysis (see Table 98).

50 g soil (dry weight basis) of soil RefeSol 02A (two replicates) were weighed into glass centrifuge tubes. The soil samples were applied with 22.2 µg of non-labelled ethofumesate (corresponding to an application rate of 444 µg/kg or 333 g a.s./ha). The soil samples were extracted 3 times with 50 mL acetonitrile by shaking on a horizontal shaker followed by treatment in an ultrasonic bath for 10 min. Phase separation was achieved by centrifugation for 10 min at 2000 rpm using a Heraeus Multifuge 4 KR. The organic supernatants were decanted, combined and the volume of the combined extracts were determined.

An aliquot of 1 mL of each extract was transferred into Eppendorf-tubes and was centrifuged for 4 min at 14000 rpm in an Eppendorf Centrifuge 5418. The supernatant was diluted with acetonitrile/ultrapure water (50:50, v:v) with 0.1% formic acid and analysed by LC-MS/MS. The recovery of ethofumesate in the combined extracts was 97.1%.

4.2.8 Sample preparation

Samples of the soil were prepared by placing 50 g soil, based on dry weight, into glass vessels. Since the soil's water content was adjusted to about 45 % WHCmax, the actual water content had to be taken into account and respective amount of the soil was placed into the glass vessels. The soil samples were then pre-incubated under test conditions for 10 days.

To characterise the microbial biomass before sample preparation three samples of about 50 g (based on dry weight) soil used in the test set, were analysed using the substrate induced respiration method. For determination of microbial biomass during the study, subsamples of 50 g (based on dry weight) were weighed into vessels: treated vessels, non-treated vessels and vessels treated with the corresponding solvent were set up for the different soils and sampling times. The soil samples were then also pre-incubated under test conditions for 10 days.

4.2.9 Application rate and application

The application rates of the test substances were selected on the basis of normal agricultural practice recommended for specific uses and crops.

Conversion from an area basis into concentration according to the OECD test guideline 307 (OECD TG 307 2002) was performed following equation (1):

$$C_{soil}[mg/kg] = \frac{A[kg/ha] \times 10^6 [mg/kg]}{l[m] \times 10^4 [m^2/ha] \times d[kg_{soil}/m^3]} \qquad (1)$$

where

C_{soil} = initial concentration of test item in soil (dry weight)

A = recommended application rate following GAP

l = thickness of field soil layer (= 0.05 m)

d = dry bulk density of soil (= 1500 kg/m³)

The nominal application rates used during degradation experiments are summarized in Table 99.

Table 99: Nominal application rates of the test substances used in work package 4.

Name	Single application		Multiple application	
	[g/ha]	[mg/kg]	[g/ha]	[mg/kg]
Tebuconazole	675	0.900	-	-
Pyraclostrobin	500	0.667	2 x 250	2 x 0.333
Ethofumesate	600	0.800	2 x 300	2 x 0.400

Since the water solubility of the test substances is low, application was performed using an organic solution of the test item. Acetonitrile was used as organic solvent and each subsample received < 0.5 mL (< 1 %) of the solvent as required by the OECD-guideline 307 (2002). The respective volume of the application solution was pipetted onto the soil samples which were then mixed carefully.

To determine the actual application rate, the respective application volume of the application solutions was applied at least before and after the application of the soil samples to a known volume of solvent in a volumetric flask. These solutions ("application controls") were analysed to determine the accurate applied amount of test substance for the respective samples.

The preparation of the application solutions and the application of the test substances is described in detail in the sections of the individual test sets.

4.2.10 Incubation conditions

The incubation of the applied soil samples was carried out in a temperature-controlled room at a test temperature of 20 ± 2°C.

The incubation of the soil samples applied with radiolabelled Tebuconazole was carried out in a flow through system. A constant stream of water saturated air was passed over the soil samples in order to maintain aerobic conditions. The outgoing gas was bubbled through three absorption traps in sequence containing ethylene glycol, 0.5 N H_2SO_4 and 1 N NaOH.

For the non-labelled test substances, incubation was performed in flask-type glass vessels covered with aluminum foil. Gas exchange occurred by means of diffusion. Moisture content of the soils was checked frequently (every 7 ± 3 days) by weighing and re-adjusting to the initial moisture content with purified water, if necessary.

Additional samples were incubated for the determination of microbial biomass during the incubation period. Incubation of soil samples for biomass determination was carried out in glass vessels covered with aluminum foil.

4.2.11 Sampling

Sampling was performed after the following incubation times:

Single application: 0 d (immediately after application), 7 d, 14 d, 30 d, 60 d, 90 d and 120 d after application.

Multiple application: 0 d (immediately after first application), 7 d, 14 d, 21 d (at least immediately after second application), 28 d, 35 d, 60 d, 90 d and 120 d after application.

Minor changes of the sampling regime occurred to avoid sampling on weekends or bank holidays.

After sampling, the soil samples were extracted and worked-up immediately as described in section 4.2.7.

For biomass measurement, untreated samples were taken before pre-incubation and immediately after application (0 d). In addition, non-treated samples, samples treated with organic solvent and samples treated with the test substance (in duplicate) were taken at about 60 d (mid of incubation) and about 120 d after application (end of incubation). Minor changes of the sampling times occurred to avoid biomass measurements on weekends or bank holidays. Biomass measurements were performed by using the substrate induced respiration method as described in section 4.2.12.

4.2.12 Characterization of the microbiological status of the soils

The soil samples were analysed for their actual microbial biomass by using the substrate induced respiration method according to the DIN procedure DIN ISO 14240-1.

On the sampling dates, 2 parallel samples of each soil were spiked with glucose and the respiration activity was determined by means of a respiration monitor.

4.2.13 Sample processing

4.2.13.1 Soil extraction

The soil samples were transferred into glass centrifuge tubes and were extracted 3 times with 50 mL acetonitrile by shaking on a horizontal shaker followed by treatment in an ultrasonic bath for 10 minutes. Phase separation was achieved by centrifugation for 10 min at 2000 rpm using a

Heraeus Multifuge 4 KR. The organic supernatants were decanted, combined and the volume of the combined extracts was determined.

4.2.13.2 Preparation of soil extracts for analysis

In case of radiolabelled tebuconazole, appropriate aliquots of the acetonitrile extracts were concentrated before radio-HPLC analysis. Aliquots of acetonitrile extracts were evaporated to a residual volume of ca. 100 µL – 500 µL under a gentle stream of nitrogen. The volume of the residual solution was determined and the concentrated sample was filled up to 1 mL using acetonitrile:water (50:50, v:v) with 0.1% formic acid. The resulting solution was homogenised thoroughly and the total radioactivity was analysed by LSC before radio-HPLC analysis.

Extracts of non-labelled test substances were analysed by LC-MS/MS after dilution with acetonitrile:water (50:50, v:v) with 0.1% formic acid (1:2 -1:100 dilution).

4.2.13.3 Absorption traps

Using radiolabelled test substances, volatile compounds formed during the incubation period can be quantified. Trapping of the exhaust gases was carried out by solutions of ethylene glycol, sulfuric acid and sodium hydroxide (see section 4.2.10).

At each sampling time and every 7-21 days the absorption traps were sampled. Immediately after sampling the volume of each trapping solution was measured and total radioactivity in each solution was determined by LSC (see section 4.2.3). The pH-value of the NaOH-absorption traps was determined and amounted always to pH 14.

4.2.13.4 Quantification of non-extractable radioactivity

When radiolabelled Tebuconazole was used, the non-extractable radioactivity in the remaining soil after extraction was determined by combustion. The extracted soil samples were air dried in a fume hood and ground to a uniform consistency and then five replicates (200 - 500 mg each) of each soil sample were combusted using an Oxidizer. The resulting $^{14}CO_2$ was trapped in Oxysolve C 400 and afterwards quantified by LSC. For that purpose, each vial (volume: 20 ml) was measured for 5 minutes by means of a liquid scintillation analyser. The efficiency of oxidation was determined by combustion of quality control standards. Using the mass data and the LSC data, the radioactivity remaining in the soil after the extraction procedure described above was calculated for each sample.

4.2.14 Data evaluation

4.2.14.1 Amounts of test item

The amount of actually applied substance was calculated from the analytical results of the application control samples (see section 4.2.9). This amount of initial applied substance was set to 100% for all single applications. For multiple applications, the amount of actually applied test substance at the first application was set to 100% for the incubation period 0 days – 21 days. After the second application, the amounts of actually applied test substance of the two application events were summed up and set to 100% for the incubation period of 21 days – 120 days. The sum of the applied amounts at the two applications were used as 100%-value - and not the amounts actually present at the time point of the second application - in order to have a consistent basis for comparison of the different samples across the test sets.

The amounts of test substance were calculated as % of the actually applied amount and in mg/kg soil (dry weight basis).

4.2.14.2 Calculation of DT50/DT90-values

Based on the achieved data set, the calculation of rate constants and DT50/DT90 values of the test substances in soil was calculated by means of the computer software “CAKE” version 3.6 (Release) running on R version 4.1.1 (2021-08-10) according to FOCUS guidance (FOCUS 2006/2014) including the demonstration of the goodness of fit.

The DT50/DT90 values were calculated based on results of the parent compounds in % of initial applied test substance. The kinetics considered for all data were “Single First Order” (SFO), "Double First Order in Parallel" (DFOP) and “First Order Multi Compartment” (FOMC). In addition, the kinetic "hockey stick” (HS) was considered for the test sets with pyraclostrobin and ethofumesate for comparison purposes.

The selection of the best fit kinetic model was based on (FOCUS 2006/2014). One of the validity criterions of the selected kinetic model (together with the visual fit of the residuals) is the so called chi^2-value. In case chi^2 is > 15%, the respective kinetic model does not properly describe the substance behaviour and should not be selected according to FOCUS Guidance (2006/2014). In a first step, DT50/DT90 values are calculated using the SFO kinetic. If acceptable statistical results are obtained (chi^2 values < 15% and acceptable visual fit), SFO model is acceptable and chosen as best fit. Otherwise, calculations using FOMC kinetic are carried out and should be compared with SFO. In the case FOMC describes better the degradation curve, DFOP is run and the best fit is selected from the models.

Besides the acceptable statistical results (chi^2-values < 15%), the SFO kinetic model was selected also as a basis for comparison since it is essential for this study to compare the DT50-values of the different test set throughout the study and it is not recommendable to compare DT50-values based on different kinetics. For this reason and due to chi^2-values always < 15%, the SFO model was chosen for all experiments.

4.2.14.3 Mass balance and distribution of radioactivity to parent and metabolites

When radiolabelled tebuconazole was used, a mass balance could additionally be established for each soil sample and the amount of parent and metabolites, if present, could be determined.

For each sample applied with ^{14}C-tebuconazole a mass balance was performed by summing the radioactivity detected in the ethylene glycol, sulfuric acid and sodium hydroxide traps, in the organic soil extract plus the radioactivity detected as non-extractable radioactivity. In addition, this sum was compared with the total radioactivity which had initially been applied to the samples determined by application controls (refer to section 4.2.9). The amounts of radioactivity and its distribution in soil phase, volatile substances and non-extractable residues were calculated as % of initially applied radioactivity (AR).

The amount of test item and metabolites at each sampling time was calculated from determined radioactivity in the extract (LSC) in combination with the relative distribution of parent compound and metabolites (if present) in the extract analysed by means of radio-HPLC. The sum of each individual (parent or metabolite) gives the total amount of test item and metabolite at the respective sampling date as % of the initially applied radioactivity (AR) in each compartment.

4.3 Results of the degradation performance after single and multiple application of one test substance (STEP 1)

4.3.1 Degradation performance after single application of ^{14}C-tebuconazole

4.3.1.1 Preparation of the application solution and application

The target application rate for tebuconazole was 900 µg/kg corresponding to 45 µg/50g soil (dry weight). This means that 45 µg tebuconazole were applied per soil sample consisting of 50g soil. This amount was achieved by mixing 180 kBq ^{14}C-tebuconazole (=31.21 µg) and 13.79 µg non-labelled tebuconazole.

An appropriate aliquot of ^{14}C-tebuconazole stock solution in acetonitrile (928 µL) was mixed with non-labelled tebuconazole (522 µL of a stock solution in acetonitrile corresponding to 689.5 µg) and was added up to 10 mL with acetonitrile (= application solution). For analysis, 3 x 10 µL were mixed with 990 µL acetonitrile/water (50:50, v:v) with 0.1% formic acid and were analysed by HPLC and LSC (3 x 50 µL each).

The application volume was determined to be 199.3 µL in order to apply the nominal amount of 45 µg per one sample (50 g dry mass). Thus, each subsample received < 0.5 mL (< 1 %) of the solvent as required by the OECD-guideline 307. The respective volume of the application solution was pipetted onto the soil samples.

The radiochemical purity of ^{14}C-tebuconazole immediately before application was determined to be 100% by HPLC analysis. The specific radioactivity was measured by LSC and HPLC analysis and was found to be 4.403 MBq/mg.

The actual application rate was determined to be 179.5 kBq/sample for RefeSol 02A samples (except 0d-samples) and 180.5 kBq/sample (LUFA 2.1 and 0d-samples of RefeSol 02A) corresponding to 40.8 µg/sample (816 µg/kg) - 41.0 µg/sample (820 µg/kg) and 90.7% and 91.1% of target.

4.3.1.2 Characterisation of the microbial status of the soils

Biomass measurements of the two soils were performed by means of the substrate induced respiration method described in section 4.2.12. Principal events of soil preparation for this test set are summarised in Table 169. Microbial biomass measurement before incubation was conducted in untreated samples in triplicate. Microbiological status expressed as biomass in mg microbial carbon per kg soil was measured to be 392 mg C_{mic}/kg dry mass (RefeSol 02A) and 409 mg C_{mic}/kg dry mass (LUFA 2.1). Correlated to the organic carbon (C_{org}) content of the soils, this corresponded to a C_{mic}/C_{org} rate of 3.7% (RefeSol 02A) and 7.4 % (LUFA 2.1). The value indicated a normal microbial activity of the soil with values > 1% C_{mic} of C_{org}.

The microbial biomass status during the incubation was carried out in the beginning, in the mid and in the end of the aerobic incubation. Microbial biomass was determined in untreated samples, in samples treated with the organic solvent acetonitrile and in samples treated with tebuconazol (nominal 45 µg/50 g soil dry mass). Table 100 presents the results of biomass measurement of the soils during the incubation period. Microbiological status is expressed as biomass in mg microbial carbon per kg soil in the following table as mean of two replicates.

Table 100: Microbial biomass determined by means of substrate induced respiration method of degradation experiments after single application of tebuconazole (given as mean values of two replicates).

Soil type	Soil sample	Biomass [mg C_{mic}/kg dry mass]		
		0d	59d	120d
RefeSol 02A	Non-treated	625	242	217
	Treated with solvent	-	228	389
	Treated with tebuconazole	-	260	226
LUFA 2.1	Non-treated	586	77	60
	Treated with solvent	-	64	44
	Treated with tebuconazole	-	64	32

The biomass of both soils showed very high microbial activities at the beginning of incubation. However, the microbial biomass decreased to values of around 240 mg C_{mic}/kg dry mass (RefeSol 02A) and around 70 mg Cmic/kg dry mass (LUFA 2.1) after 59 days of incubation. Afterwards the biomass in RefeSol 02A remained relatively stable until the end of incubation after 120 days whereas biomass in LUFA 2.1 decreased slightly further on. A decreasing microbial activity at the end of the 120 days incubation time is commonly seen in soil batch tests – especially for sandy soils like LUFA 2.1.

The results of microbial biomass show the existence of an active microbial population throughout the incubation period. In addition, the results indicate that in RefeSol 02A amounts of biomass at test end were comparable in soil samples treated with the solvent acetonitrile or the test substance tebuconazole and the untreated control.

Since the results of soil LUFA 2.1 - especially the values after 120 days of incubation - were very low due to nutrient depletion, the data is regarded to be not reliable to estimate possible adverse effects on the microbial activity.

4.3.1.3 Distribution of radioactivity to compartments and mass balance

The distribution of the applied radioactivity (expressed as % AR) during the aerobic degradation of ^{14}C-tebuconazole in two soils is presented in Table 101 and Table 102. The tables show the distribution of the test substance found in organic extracts, in the traps for formed volatiles and as non-extractable residues (NER) determined by combustion analysis of the soils.

Table 101: Distribution of radioactivity in RefeSol 02A soil treated with 0.900 mg/kg of ^{14}C-tebuconazole by single application in % of applied radioactivity (% AR).

Sampling time	Soil extract [% AR]	Non-extractable residues (NER) [% AR]	Volatiles[1] [% AR]	Recovery [% AR]
0d-1	96.2	3.3	-	99.5
0d-2	97.1	3.5	-	100.6
7d-1	93.5	5.8	< 0.1	99.3
7d-2	95.3	5.8	< 0.1	101.1

Sampling time	Soil extract [% AR]	Non-extractable residues (NER) [% AR]	Volatiles[1] [% AR]	Recovery [% AR]
14d-1	91.5	7.4	0.2	99.1
14d-2	94.9	5.9	0.1	100.9
30d-1	90.5	9.9	0.2	100.6
30d-2	90.3	9.3	0.2	99.9
60d-1	85.8	14.5	0.2	100.6
60d-2	85.3	10.9	0.3	96.5
91d-1	92.4	13.2	0.3	95.9
91d-2	85.3	13.6	0.3	99.2
120d-1	80.2	18.6	0.3	99.1
120d-2	82.8	16.4	0.7	99.9

[1] Volatiles consisted exclusively of $^{14}CO_2$, no radioactivity was quantified in ethylene glycol or 0.5 N H_2SO_4 traps.

Table 102: Distribution of radioactivity in LUFA 2.1 soil treated with 0.900 mg/kg of ^{14}C-tebuconazole by single application in % of applied radioactivity (% AR) (given as mean values of two replicates).

Sampling time	Soil extract [% AR]	Non-extractable residues (NER) [% AR]	Volatiles[1] [% AR]	Recovery [% AR]
0d-1	99.1	2.6	-	101.7
0d-2	98.4	2.3	-	100.7
7d-1	97.9	3.5	< 0.1	101.4
7d-2	96.8	3.9	< 0.1	100.7
14d-1	96.3	4.2	< 0.1	100.5
14d-2	95.8	4.7	< 0.1	100.6
30d-1	89.3	8.7	< 0.1	98.1
30d-2	90.9	7.3	< 0.1	98.3
60d-1	89.3	8.8	< 0.1	98.3
60d-2	90.9	8.7	< 0.1	99.7
91d-1	76.1	16.1	< 0.1	91.4
91d-2	79.1	11.6	< 0.1	90.7
120d-1	78.2	22.8	< 0.1	101.1
120d-2	79.5	21.3	< 0.1	100.9

[1] Volatiles consisted exclusively of $^{14}CO_2$, no radioactivity was quantified in ethylene glycol or 0.5 N H_2SO_4 traps.

The results presented in Table 101 to Table 102 can be summarised as follows:

Recovery

The overall recoveries ranged between 90 and 110 % of initially applied radioactivity (AR) for all samples.

Volatiles:

Volatiles consisted exclusively of $^{14}CO_2$ which was trapped in 1 N sodium hydroxide. No radioactivity was found in the ethylene glycol traps and the 0.5 N sulfuric acid traps. Since only negligible or very low amounts of radioactivity were detected in the sodium hydroxide traps, it can be stated that mineralization was very low (< 1% AR).

Extractable radioactivity:

The amount of radioactivity extracted from soil by acetonitrile decreased in both soils from 96.2 % – 99.1% AR at the beginning of incubation to 78.2% – 82.8 % AR after 120 days.

Non-extractable radioactivity (NER):

Generally, the amount of non-extractable radioactivity (NER) increased continuously from 2.3 % - 3.5 % AR at day 0 to maximum values of 16.4 % (RefeSol 02A) – 22.8 % AR (LUFA 2.1) after 120 days of incubation.

4.3.1.4 Identification of extractable radioactivity

To determine the amount of unchanged parent compound and its metabolites by radio-HPLC, the soil extracts needed to be concentrated before analyses.

The concentration procedure can be described as follows: Aliquots of the soil extract (4-5 mL each) were transferred into pear shaped glass flasks and were evaporated by a gentle stream of nitrogen to a volume of about 150 µL or to dryness. The remaining volume/residue was added up to 1mL using acetonitrile/water (50:50, v:v) with 0.1% formic acid and was centrifuged for 5 minutes at 14000 rpm. The supernatant was transferred to an HPLC vial and was analysed by LSC and HPLC. Recovery of radioactivity after the concentration step was always between 98.9% and 107.0%.

The results presented in Table 103 and Table 104 show the amounts of parent compound and its metabolites as mean values of two replicates. The values are expressed in percent of the total initially applied radioactivity (AR).

Table 103: Tebuconazole and metabolites in the soil extract of RefeSol 02A (analysed by HPLC) as mean values of two replicates and given in percent of the applied radioactivity (% AR).

Radioactive fraction	Incubation time [d]						
	0	7	14	30	60	91	120
Tebuconazole (Ret. 26.3 min)	96.6	94.4	92.8	89.1	84.0	81.6	79.2
Unassigned Ret. 25.5 min	*	*	0.5	1.4	1.5	2.2	2.7

* not detectable

Table 104: Tebuconazole and metabolites in the soil extract of LUFA 2.1 (analysed by HPLC) as mean values of two replicates and given in percent of the applied radioactivity (% AR).

Radioactive fraction	Incubation time [d]						
	0	7	14	30	60	91	120
Tebuconazole (Ret. 26.3 min)	98.7	97.4	96.1	90.1	90.1	77.1	78.9
Unassigned Ret. 25.5 min	*	*	*	*	*	*	*

* not detectable

As can be seen in the result tables, the amount of parent compound in the soil extracts decreased slowly from maximum levels between 96.6 % - 98.7 % AR immediately after application to amounts in the range of 78.9 % - 79.2 % AR during 120 days of incubation. The degradation rate is relatively consistent in both soils. However, the formation of metabolites was detected exclusively in soil RefeSol 02A where a minor unassigned metabolite with the retention time of 25.5 minutes was found in amounts up to 2.7% AR after 120 days of incubation. A metabolite co-chromatographing with the reference substance 1,2,4-triazole (retention time of ca. 5.3 minutes) could not be detected.

4.3.1.5 Calculation of DT50/DT90 values of tebuconazole

The kinetic models considered for the analysis of tebuconazole were SFO (Single First Order), DFOP (Double First Order in Parallel) and FOMC (First Order Multi Compartment). The obtained data sets were analysed using the program CAKE. The results of the optimisation are presented in Table 105 - Table 106.

Table 105: Calculated DT50 and DT90 for tebuconazole after single application (0.900 mg/kg) in RefeSol 02A.

	chi^2 (%)	r^2 (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**1.09**	**0.9311**	**411**	**1370**	
FOMC	0.234	0.9653	5590	> 10000	> 10000
DFOP	0.2	0.9659	1240	4990	1620

Table 106: Calculated DT50 and DT90 for tebuconazole after single application (0.900 mg/kg) in LUFA 2.1.

	chi^2 (%)	r^2 (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**2.01**	**0.9102**	**336**	**1120**	
FOMC	2.13	0.9131	528	> 10000	3630
DFOP	2.34	0.9135	> 10000	> 10000	> 10000

As can be seen in Table 105 and Table 106, the DT50 and DT90 values for tebuconazole after single application were calculated much higher than 120 days (i.e. the period of incubation) in both soils. To evaluate if the degradation rate of tebuconazole is affected by the application rate and, thus, the degradation rate could be enhanced, a pre-test with different application rates of tebuconazole were carried out (see section 4.3.2).

4.3.2 Pretests with different application rates of ^{14}C-tebuconazole after single application

Since the results of the degradation experiments with ^{14}C-labelled tebuconazole after single application showed that degradation of tebuconazole in both soils was slow and resulted in high DT50 values (see section 4.3.1), a pretest was carried out to investigate if the application rate of 900 µg/kg dry weight may have had a negative effect on the degradation rate. For this purpose, soil samples of the two soils RefeSol 02A and LUFA 2.1 were applied with two target application rates (900 µg/kg dry weight and 350 µg/kg dry weight) and incubated for 30 days.

Soils were used after storage at 4 ± 3°C within the period of time of three months (from sampling until application) as requested by the OECD 307 guideline. Principal events of soil preparation are summarised in Table 170. The microbial activity of the soils was tested before storage and was found to be 280 mg C_{mic}/kg dry mass corresponding to 2.6% C_{mic}/C_{org} (RefeSol 02A) and 165 mg C_{mic}/kg dry mass corresponding to 3.0% C_{mic}/C_{org} (LUFA 2.1). No further biomass measurements were carried out during pretesting.

4.3.2.1 Preparation of the application solution and application

The target application rates for tebuconazole were 900 µg/kg corresponding to 45 µg/50g soil (dry weight) and 350 µg/kg corresponding to 17.5 µg/50 soil (dry weight). The amount of 45 µg per 50 g soil sample (application rate of 900 µg/kg) was achieved by mixing 180 kBq ^{14}C-tebuconazole (=31.21 µg) and 13.79 µg non-labelled tebuconazole. The amount of 17.5 µg per 50 g soil sample (application rate of 350 µg/kg) was achieved by using exclusively radiolabelled tebuconazole (100 kBq/sample corresponding to 17.3 µg/sample) without mixing it with non-labelled tebuconazole.

4.3.2.1.1 Nominal application rate of 900 µg/kg

An appropriate aliquot of ^{14}C-tebuconazole stock solution in acetonitrile (464 µL) was mixed with non-labelled tebuconazole (261 µL of a stock solution in acetonitrile corresponding to 344.8 µg) and was added up to 5 mL with acetonitrile (= application solution). For analysis, 3 x 10 µL were mixed with 990 µL acetonitrile/water (50:50, v:v) with 0.1% formic acid and were analysed by HPLC and LSC (3 x 50 µL each).

The application volume was determined to be 200.6 µL in order to apply the nominal amount of 45 µg per one sample (50 g dry mass). Thus, each subsample received < 0.5 mL (< 1 %) of the solvent as required by the OECD-guideline 307 and the application volume was comparable to the previous experiments. The respective volume of the application solution was pipetted onto the soil samples.

The radiochemical purity of ^{14}C-tebuconazole immediately before application was determined to be 98.9% by HPLC analysis. The specific radioactivity was measured by LSC and HPLC analysis and was found to be 4.422 MBq/mg.

The actual application rate was determined to be 182.1 kBq/sample for all samples corresponding to 41.2 µg/sample (824 µg/kg) and 86.3% of target.

4.3.2.1.2 Nominal application rate of 350 µg/kg

An appropriate aliquot of ^{14}C-tebuconazole stock solution in acetonitrile (300 µL, specific activity of 5.768 MBq/mg) was added up to 5 mL with acetonitrile (= application solution). For analysis, 3 x 10 µL were mixed with 990 µL acetonitrile/water (50:50, v:v) with 0.1% formic acid and were analysed by HPLC and LSC (3 x 25 µL each).

The application volume was determined to be 169 µL in order to apply the nominal amount of 17.5 µg per one sample (50 g dry mass). Thus, each subsample received < 0.5 mL (< 1 %) of the solvent as required by the OECD-guideline 307 and the application volume was comparable to the experiments with the application rate of 900 µg/kg. The respective volume of the application solution was pipetted onto the soil samples.

The actual application rate was determined to be 100.9 kBq/sample for all samples corresponding to 17.5 µg/sample (350 µg/kg) and 100.9% of target.

4.3.2.2 Distribution of radioactivity to compartments and mass balance

The distribution of the applied radioactivity (expressed as % AR) during the pretest with ^{14}C-tebuconazole and two soils is presented in Table 107 and Table 108. The tables show the distribution of the test substance found in organic extracts, in the traps for formed volatiles and as non-extractable residues (NER) determined by combustion analysis of the soils.

Table 107: Pretest: Distribution of radioactivity in RefeSol 02A soil treated with ^{14}C-tebuconazole by single application in % of applied radioactivity (% AR).

Sampling time	Soil extract [% AR]	Non-extractable residues (NER) [% AR]	Volatiles[1] [% AR]	Recovery [% AR]
Application rate of 0.900 mg/kg				
0d-1	94.9	2.0	-	96.9
0d-2	95.5	2.1	-	97.6
7d-1	73.1[2]	6.7	0.1	79.8[2]
7d-2	94.5	6.6	0.1	101.1
14d-1	92.3	8.2	0.1	100.6
14d-2	93.9	7.7	0.1	101.7
30d-1	83.9	10.7	0.2	94.8
30d-2	85.3	11.4	0.2	96.9
Application rate of 0.350 mg/kg				
0d-1	97.8	6.4	-	104.2
0d-2	95.6	6.9	-	102.5
7d-1	91.3	6.8	0.1	98.2
7d-2	94.9	7.0	0.1	102.0
14d-1	91.4	8.8	0.1	100.2
14d-2	90.4	9.6	0.1	100.1

Sampling time	Soil extract [% AR]	Non-extractable residues (NER) [% AR]	Volatiles[1] [% AR]	Recovery [% AR]
30d-1	86.0	11.7	0.2	97.9
30d-2	87.9	12.3	0.2	100.4

[1] *Volatiles consisted exclusively of $^{14}CO_2$, no radioactivity was quantified in ethylene glycol or 0.5 N H_2SO_4 traps.*

[2] *Sample 7d-1 (application rate of 900 µg/kg) is regarded as outsider*

Table 108: Pretest: Distribution of radioactivity in LUFA 2.1 soil treated with ^{14}C-tebuconazole by single application in % of applied radioactivity (% AR).

Sampling time	Soil extract [% AR]	Non-extractable residues (NER) [% AR]	Volatiles[1] [% AR]	Recovery [% AR]
Application rate of 0.900 mg/kg				
0d-1	97.7	1.6	-	99.3
0d-2	92.2	1.6	-	93.8
7d-1	96.4	3.6	<0.1	100.1
7d-2	97.0	3.7	0.1	100.8
14d-1	95.0	4.3	0.1	99.3
14d-2	95.7	4.4	0.1	100.2
30d-1	90.8	6.8	0.1	97.6
30d-2	90.4	6.8	0.1	97.3
Application rate of 0.350 mg/kg				
0d-1	99.1	5.2	-	104.2
0d-2	98.9	5.1	-	104.0
7d-1	95.4	4.0	0.1	99.4
7d-2	97.6	4.4	0.1	102.1
14d-1	91.6	4.1	0.1	95.8
14d-2	96.0	5.4	0.1	101.5
30d-1	95.0	6.0	0.1	101.1
30d-2	92.6	7.8	0.2	100.6

[1] Volatiles consisted exclusively of $^{14}CO_2$, no radioactivity was quantified in ethylene glycol or 0.5 N H_2SO_4 traps.

The results presented in Table 107 to Table 108 can be summarised as follows:

Recovery

The recovery of radioactivity was always determined to be in the range of 90 – 110% except for one replicate of soil RefeSol 02A (nominal application rate of 900 µg/kg, 7d-1). For this replicate, a lower amount of radioactivity was found in the soil extract compared to the second replicate of

the same sampling time and, consequently, the overall recovery was < 80% AR. For this reason, the RefeSol 02A sample 7d-1 (application rate 900 µg/kg) is regarded as outsider.

Volatiles:

Volatiles consisted exclusively of $^{14}CO_2$ which was trapped in 1 N sodium hydroxide. No radioactivity was found in the ethylene glycol traps and the 0.5 N sulfuric acid traps. Since only very low amounts of radioactivity were detected in the sodium hydroxide traps during the incubation time of 30 days, it can be stated that mineralisation was very low (< 0.5% AR).

Extractable radioactivity:

The amount of radioactivity extracted from soil using acetonitrile decreased in both soils and both application rates from 92.2 % – 99.1% AR at the beginning of incubation to 82.9% – 87.9% AR (RefeSol 02A) or 90.4% - 95.0% AR (LUFA 2.1) after 30 days.

Non-extractable radioactivity (NER):

Generally, the amount of non-extractable radioactivity (NER) increased continuously from 1.6% - 2.1% AR (application rate 900 µg/kg) and 5.1% - 6.9% AR (application rate 350 µg/kg) for both soils at day 0 to maximum values of 10.7% – 12.3% AR (RefeSol 02A, both application rates) or 6.0% - 7.8% AR (LUFA 2.1, both application rates) after 30 days of incubation.

4.3.2.3 Identification of extractable radioactivity

To determine the amount of unchanged parent compound and its metabolites by radio-HPLC, the soil extracts needed to be concentrated before analyses.

The concentration procedure can be described as follows: Aliquots of the soil extract (4 or 8 mL each) were transferred into pear shaped glass flasks and were evaporated by a gentle stream of nitrogen to a volume of about 100-200 µL or to dryness. The remaining volume/residue was added up to 1mL using acetonitrile/water (50:50, v:v) with 0.1% formic acid and was centrifuged for 5 minutes at 14000 rpm. The supernatant was transferred to an HPLC vial and was analysed by LSC and HPLC. Recovery of radioactivity after the concentration step was always between 92.2% and 111.6%.

The results presented in Table 109 and Table 110 show the amounts of parent compound and its metabolites for both nominal application rates and both soils as mean values of two replicates. The values are expressed in percent of the total initially applied radioactivity (AR).

Table 109: Pretest: tebuconazole and metabolites in the soil extract of RefeSol 02A (analyzed by HPLC) as mean values of two replicates and given in percent of the applied radioactivity (% AR).

Radioactive fraction	Incubation time [d]			
	0	7	14	30
Application rate of 0.900 mg/kg				
Tebuconazole (Ret. 26.3 min)	95.2	94.5[1]	91.9	82.8
Unassigned Ret. 25.5 min	*	*	1.2	1.8
Application rate of 0.350 mg/kg				
Tebuconazole (Ret. 26.3 min)	96.7	93.1	89.9	85.1
Unassigned Ret. 25.5 min	*	*	1.0	1.9

* not detectable

[1] The value is based on exclusively one replicate (7d-2)

Table 110: Pretest: tebuconazole and metabolites in the soil extract of LUFA 2.1 (analyzed by HPLC) as mean values of two replicates and given in percent of the applied radioactivity (% AR).

Radioactive fraction	Incubation time [d]			
	0	7	14	30
Application rate of 0.900 mg/kg				
Tebuconazole (Ret. 26.3 min)	94.9	96.7	94.4	88.9
Unassigned Ret. 25.5 min	*	*	0.9	1.6
Application rate of 0.350 mg/kg				
Tebuconazole (Ret. 26.3 min)	99.0	96.5	91.3	91.9
Unassigned Ret. 25.5 min	*	*	1.8	1.9

* not detectable

As can be seen in the result tables, the amount of parent compound in the soil extracts decreased slowly from maximum levels between 94.9% - 99.0% AR immediately after application to amounts in the range of 82.8% - 85.8% AR (RefeSol 02A) and 88.9% - 91.9% AR (LUFA 2.1) during 30 days of incubation. The degradation of tebuconazole seemed to be slightly faster in soil RefeSol 02A compared to LUFA 2.1. However, the degradation rate in a given soil was relatively consistent for both application rates.

In contrast to the previous experiments with ^{14}C-tebuconazole, the formation of an unassigned metabolite with the retention time of 25.5 minutes was detected in both soils in minor amounts (maximum of 1.6% - 1.9% AR at 30 days of incubation in both soils and for both application rates). The metabolite 1,2,4-triazole (retention time of ca. 5.3 minutes) could not be detected.

4.3.2.4 Calculation of DT50/DT90 values of tebuconazole

The kinetic models considered for the analysis of Tebuconazole were SFO (Single First Order), DFOP (Double First Order in Parallel) and FOMC (First Order Multi Compartment). The obtained

data sets were analysed using the program CAKE. The results of the optimisation are presented in Table 111 - Table 112.

Table 111: Pretest: Calculated DT50 and DT90 for tebuconazole after single application in RefeSol 02A.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
Application rate of 0.900 mg/kg					
SFO	**3.69**	**0.2169**	**190**	633	
FOMC	4.08	0.2768	> 10 000	> 10 000	> 10 000
DFOP	*n.d.*	0.2968	375	1350	420
Application rate of 0.350 mg/kg					
SFO	**13.0**	**0.06702**	**112**	373	
FOMC	14.6	0.1469	> 10000	> 10000	> 10000
DFOP	*n.d.*	0.1793	> 10000	> 10000	>10000

Table 112: Pretest: Calculated DT50 and DT90 for tebuconazole after single application in LUFA 2.1.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
Application rate of 0.900 mg/kg					
SFO	1.13	0.6749	275	913	
FOMC	1.41	0.6744	281	984	296
DFOP	n.d.	0.6749	275	913	275
Application rate of 0.350 mg/kg					
SFO	1.56	0.6253	112	373	
FOMC	1.41	0.7612	> 10000	> 10000	> 10000
DFOP	n.d.	0.7929	> 10000	> 10000	> 10000

DT50/DT90 values based on SFO kinetics indicated that degradation of tebuconazole may be slightly faster using an application rate of 350 µg/kg compared to an application rate of 900 µg/kg. However, the degradation rates of tebuconazole could not be enhanced significantly for the two soils in a way that DT50 and preferably also DT90 values could be achieved within the incubation time of 120 days. Therefore, it was decided to use another test substance for the degradation experiments in work package 4.

Non-labelled ethofumesate was selected as test substance in work package 4 instead of tebuconazole.

4.3.3 Degradation performance after single and multiple application of pyraclostrobin

4.3.3.1 Preparation of the application solution and application

The nominal application rate for the single application of pyraclostrobin was 667 µg/kg corresponding to 33.3 µg/50 g soil (dry weight). The nominal application rate for the multiple application rate of pyraclostrobin was 2 x 333 µg/kg (in sum also 667 µg/kg) corresponding to 2 x 16.65 µg/50 g soil (dry weight). The interval between the two applications was set to 21 days.

An application solution of pyraclostrobin for both single and multiple application was prepared by weighing 7.12 mg pyraclostrobin into a 50 mL volumetric flask which was then added up to 50 mL with acetonitrile. Hence, the application solution had a concentration of 7.12 mg/50 mL corresponding to 142.4 µg/mL. The application volume for the single application was calculated to be 234 µL per sample and the application volume for the multiple application to be 2 x 117 µL per sample. Thus, each subsample received < 0.5 mL (< 1 %) of the solvent as required by the OECD-guideline 307. The respective volume of the application solution was pipetted onto the soil samples.

The actual application rate after single application was determined to be 29.3 µg/sample (RefeSol 02A samples and 0d-samples of LUFA 2.1) and 30.3 µg/sample (LUFA 2.1 except 0d-samples) corresponding to 586 µg/kg - 606 µg/kg and 87.9% - 90.9% of the nominal application rate.

The amounts actually applied during the multiple applications of pyraclostrobin were also determined: During the first application 15.1 µg/sample (RefeSol 02A samples and 0d-samples of LUFA 2.1) and 15.6 µg/sample (LUFA 2.1 except 0d-samples) were applied to the soil samples. At the second application after 21 days, 14.2 µg/sample (RefeSol 02A samples and 0d-samples of LUFA 2.1) and 13.7 µg/sample (LUFA 2.1 except 0d-samples) were additionally applied. In sum, 29.2 – 29.3 µg/sample corresponding to 584 µg/kg – 586 µg/kg and 87.8% - 88.0% of nominal application rate were applied during the experiments with multiple application.

4.3.3.2 Characterisation of the microbial status of the soils

Biomass measurements of the two soils were performed by means of the substrate induced respiration method described in section 4.2.12. The principal events of soil preparation for this test set are summarised in Table 171. Microbial biomass measurement before incubation was determined in untreated samples in duplicate. Microbiological status expressed as biomass in mg microbial carbon per kg soil was measured to be 280 mg C_{mic}/kg dry mass (RefeSol 02A) and 165 mg C_{mic}/kg dry mass (LUFA 2.1). Correlated to the organic carbon (C_{org}) content of the soils, this was corresponding to a C_{mic}/C_{org} rate of 2.6% (RefeSol 02A) and 3.0% (LUFA 2.1). The value indicated a normal microbial activity of the soil with values > 1% C_{mic} of C_{org}.

The microbial biomass status during the incubation was carried out in the beginning, in the mid and in the end of the aerobic incubation. Microbial biomass was determined in untreated samples, in samples treated with the organic solvent acetonitrile and in samples treated with Pyraclostrobin (nominal 33.3 µg/50 g soil dry mass, single application). Table 113 presents the results of biomass measurement of the soils during the incubation period. Microbiological status is expressed as biomass in mg microbial carbon per kg soil in the following table as mean of two replicates.

Table 113: Microbial biomass determined by means of substrate induced respiration method of degradation experiments after single application of pyraclostrobin (given as mean values of two replicates).

Soil type	Soil sample	Biomass [mg C_{mic}/kg dry mass]		
		0d	58d	122d
RefeSol 02A	Non-treated	272	259	256
	Treated with solvent	-	194	189
	Treated with pyraclostrobin	-	202	149
LUFA 2.1	Non-treated	185	131	140
	Treated with solvent	-	99	46
	Treated with pyraclostrobin	-	93	40

The biomass of both soils showed microbial activities at the beginning of incubation comparable to those determined before the pre-equilibration indicating constant activity of the soil microorganisms. In addition, the microbial biomass remained relatively stable until the end of incubation (measured at day 122) in untreated samples of both soils. In soil samples treated with solvent as well as in samples treated with the test substance, the soil biomass decreased throughout the incubation period in both soils. This effect was more pronounced in the sandy soil LUFA 2.1 compared to RefeSol 02A. The observation that biomass in treated soil samples (either treated with solvent or with pyraclostrobin) decreased more during the incubation time than the non-treated samples can probably be explained by adverse effects of acetonitrile / the test substance on the soil microbial community or by an exhaustion after a previous enhancement of activity due to the utilisation of these compounds as substrates. However, a toxic effect on soil microbial community is usually observed immediately as an abrupt event without recoverage, but it is not expected to take place continuously. Therefore, the biomass data of treated soil samples rather suggest a depletion of nutrients.

However, the results of microbial biomass show the existence of an active microbial population throughout the incubation period.

4.3.3.3 Determination of pyraclostrobin amounts during aerobic degradation after single and multiple application

The amounts of pyraclostrobin during aerobic degradation after single and multiple application were determined in the soils RefeSol 02A and LUFA 2.1 by means of LC-MS/MS analysis.

Nominal and actual application rates for both single and multiple application of pyraclostrobin are described in section 4.3.3.1. Application interval during experiments with multiple application of the test substance was 21 days. The amounts of test item were calculated as % of the applied amount and in µg/kg soil (dry weight basis). Results of single application are presented in Table 114, results of multiple application in Table 115.

Table 114: Soil concentrations of pyraclostrobin after single application (0.677 mg/kg) expressed as µg/kg dry weight and % of applied test substance.

Sampling time	RefeSol 02A		LUFA 2.1	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
0d-1	531	90.5	567	96.6
0d-2	571	97.3	526	89.7
7d-1	432	73.7	482	79.6
7d-2	446	76.0	493	81.3
15d-1	290	49.3	386	63.6
15d-2	296	50.5	400	65.9
30d-1	177	30.2	287	47.4
30d-2	200	34.2	269	44.3
60d-1	130	22.1	185	30.6
60d-2	137	23.4	181	29.9
91d-1	116	19.8	173	28.5
91d-2	111	19.0	188	31.1
120d-1	93	15.8	154	25.5
120d-2	82	14.0	154	25.4

Table 115: Soil concentrations of pyraclostrobin after multiple application (2 x 0.333 mg/kg) expressed as µg/kg dry weight and % of applied test substance.

Sampling time	RefeSol 02A		LUFA 2.1	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
First application (0d)				
0d-1	269	89.2[1]	276	91.7[1]
0d-2	263	87.3[1]	280	92.8[1]
7d-1	196	65.1[1]	225	71.8[1]
7d-2	203	67.5[1]	234	74.8[1]
15d-1	123	40.8[1]	189	60.4[1]
15d-2	141	46.8[1]	174	55.8[1]
21d-1[3]	108	35.8[1]	166	53.2[1]
21d-2[3]	106	35.3[1]	167	53.5[1]
Second application (21d)				
21d-1	358	61.2[2]	427	71.7[2]

Sampling time	RefeSol 02A		LUFA 2.1	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
21d-2	356	61.0[2]	428	71.9[2]
28d-1	309	52.9[2]	368	62.7[2]
28d-2	301	51.5[2]	378	64.4[2]
35d-1	258	44.1[2]	311	53.1[2]
35d-2	268	45.9[2]	309	52.8[2]
60d-1	147	25.1[2]	198	33.9[2]
60d-2	142	24.3[2]	181	30.9[2]
91d-1	109	18.7[2]	167	28.5[2]
91d-2	114	19.4[2]	168	28.7[2]
120d-1	107	18.3[2]	171	29.1[2]
120d-2	100	17.1[2]	170	29.0[2]

1 values in % of applied amount of Pyraclostrobin during first application

2 values in % of applied amount of Pyraclostrobin as a sum of first and second application

3 calculated values (not measured), calculation: amount detected after the second application minus applied amount at 21d taking the extraction efficiency at 0d into account.

As can be seen in Table 114 and Table 115, amounts of Pyraclostrobin decreased continuously in the two soils RefeSol 02A and LUFA 2.1 resulting in 14.9% (RefeSol 02A) to 25.4% (LUFA 2.1) of the applied pyraclostrobin after single application and 17.7% (RefeSol 02A) to 38.7 % of the applied test substance (LUFA 2.1) after multiple applications (mean values) at the end of the incubation (120 days).

4.3.3.4 Calculation of DT50/DT90 values of pyraclostrobin after single and multiple application

The kinetic models considered for the analysis of pyraclostrobin were SFO (Single First Order), DFOP (Double First Order in Parallel) and FOMC (First Order Multi Compartment). The obtained data sets were analysed using the program CAKE. The results of the optimisation are presented in Table 116 - Table 119. Detailed CAKE results including HS kinetic model, plots and residuals are presented in Table 177- Table 182.

Table 116: Calculated DT50 and DT90 for pyraclostrobin after single application (0.667 mg/kg) in RefeSol 02A.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**14.7**	**0.9319**	**27.3**	**90.6**	
FOMC	6.32	0.9835	18.4	181	54.3
DFOP	4.64	0.9908	17.6	276	207

Table 117: Calculated DT50 and DT90 for pyraclostrobin after single application (0.667 mg/kg) in LUFA 2.1.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**10.8**	**0.9192**	**47.7**	**159**	
FOMC	4.83	0.9818	32.3	435	131
DFOP	2.94	0.9916	29.7	> 10000	> 10000

Table 118: Calculated DT50 and DT90 for pyraclostrobin after multiple application (2 x 0.333 mg/kg) in RefeSol 02A.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
after first application (0d – 21d)					
SFO	**1.62**	**0.9901**	**15.6**	**51.7**	
FOMC	2.03	0.9901	15.6	52	15.7
DFOP	n.d.	0.9901	15.6	51.7	15.6
after second application (21d – 120d)					
SFO	**8.16**	**0.9529**	**41.7**	**138**	
FOMC	4.72	0.9853	32.2	289	87.1
DFOP	3.3	0.9938	29.9	> 10000	> 10000

Table 119: Calculated DT50 and DT90 for pyraclostrobin after multiple application (2 x 0.333 mg/kg) in LUFA 2.1.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
after first application (0d – 21d)					
SFO	**2.23**	**0.9762**	**25.1**	**83.2**	
FOMC	1.19	0.9887	28.7	422	127
DFOP	n.d.	0.9897	32.2	> 10000	> 10000
after second application (21d – 120d)					
SFO	**10.1**	**0.8872**	**56.7**	**188**	
FOMC	5.8	0.9669	41.6	1070	323
DFOP	3.98	0.987	35.1	> 10000	> 10000

In this test set, the chi²-values of SFO kinetics were always < 15%, therefore, SFO kinetics were regarded as acceptable.

When the two soils RefeSol 02A and LUFA 2.1 are compared, DT50/DT90-values of pyraclostrobin in RefeSol 02A are generally lower than those in the sandy soil LUFA 2.1, i.e. in the experiments after single application as well as after both application time points of the multiple application test set.

The calculated DT50-values ranged from 27.3 days (RefeSol 02A) to 47.7 days (LUFA 2.1) after single application of pyraclostrobin.

Regarding the experiments with multiple application, DT50-values of 15.6 days after the first application and of 41.7 days after the second application were observed in RefeSol 02A. In soil LUFA 2.1, DT50-values of 25.1 days after the first application and of 56.7 days after the second application were calculated. Hence, the DT50-values of the multiple application encompassed the DT50-value calculated for the single application experiment. The DT50-value for the incubation phase of 0 days – 21 days indicated an increased degradation rate of pyraclostrobin when applied in a lower application rate. However, kinetic calculations after the first application are based on a limited number of results (four time points with two replicates) which represent a shortcoming for kinetic evaluation (six sampling times are regarded to be minimum for kinetic evaluation). In contrast, the incubation phase of 21 days – 120 days comprise six time points (with two replicates each) and a longer period of time. For this incubation period it has to be considered that the microbial community was degrading the test substance already for 21 days and may have changed its composition or activity during this period of time.

4.3.4 Degradation performance after single and multiple application of ethofumesate

4.3.4.1 Preparation of the application solution and application

The nominal application rate for the single application of ethofumesate was 800 µg/kg corresponding to 40 µg/50 g soil (dry weight). The nominal application rate for the multiple application rate of ethofumesate was 2 x 400 µg/kg (in sum also 800 µg/kg) corresponding to 2 x 20 µg/50 g soil (dry weight). The interval between the two applications was set to 21 days.

An application solution of ethofumesate for both single and multiple application was prepared by weighing 5.73 mg ethofumesate into a 25 mL volumetric flask which was then added up to 25 mL with acetonitrile. Taking the purity of 98.1% into account, the application solution had a concentration of 224.8 µg/mL. The application volume for the single application was calculated to be 177.7 µL per sample and the application volume for the multiple application to be 2 x 88.85 µL per sample. Thus, each subsample received < 0.5 mL (< 1 %) of the solvent as required by the OECD-guideline 307. The respective volume of the application solution was pipetted onto the soil samples.

The actual application rate after single application was determined to be 38.0 µg/sample corresponding to 759 µg/kg and 94.9% of the nominal application rate.

The amounts actually applied during the multiple applications of ethofumesate were also determined: During the first application 18.8 µg/sample were applied to the soil samples. At the second application after 21 days, 18.3 µg/sample were additionally applied. In sum, 37.1 µg/sample corresponding to 742 µg/kg and 92.8% of nominal application rate were applied during the experiments with multiple application.

The samples immediately after application (0 d samples) of both soils were repeated during this test set for both single and multiple application due to not plausible analytical results where a mistake during application could not be excluded. For this purpose, further soil samples were

applied with ethofumesate and the actual application rates were also determined. The actual application rate of repeated 0d-samples after single application was 38.2 µg/sample corresponding to 765 µg/kg and 95.6%. During the first application of the multiple application experiments, 18.6 µg/sample were actually applied to the repeated 0d-samples.

4.3.4.2 Characterisation of the microbial status of the soils

Biomass measurements of the two soils were performed by means of the substrate induced respiration method described in section 4.2.12. The principal events of soil preparation for this test set are summarised in Table 172. Microbial biomass measurement before incubation was determined in untreated samples in triplicate. Microbiological status expressed as biomass in mg microbial carbon per kg soil was measured to be 140 mg C_{mic}/kg dry mass (RefeSol 02A) and 272 mg C_{mic}/kg dry mass (LUFA 2.1). Correlated to the organic carbon (C_{org}) content of the soils, this was corresponding to a C_{mic}/C_{org} rate of 1.3% (RefeSol 02A) and 5.0% (LUFA 2.1). The value indicated a normal microbial activity of the soil with values > 1% C_{mic} of C_{org}.

The microbial biomass status during the incubation was carried out in the beginning, in the mid and in the end of the aerobic incubation. Table 120 presents the results of biomass measurement of the soils during the incubation period. Microbial biomass was determined in untreated samples, in samples treated with the organic solvent acetonitrile and in samples treated with ethofumesate (nominal 40 µg/50 g soil dry mass, single application). Microbiological status is expressed as biomass in mg microbial carbon per kg soil in the following table as mean of two replicates.

Table 120: Microbial biomass determined by means of substrate induced respiration method of degradation experiments after single application of ethofumesate (given as mean values of two replicates).

Soil type	Soil sample	Biomass [mg C_{mic}/kg dry mass]		
		0d	61d	119d
RefeSol 02A	Non-treated	178	196	173
	Treated with solvent	-	167	154
	Treated with ethofumesate	-	263	199
LUFA 2.1	Non-treated	242	218	108
	Treated with solvent	-	76	26
	Treated with ethofumesate	-	70	29

The biomass of both soils at the beginning of incubation showed similar microbial activities compared to results before the pre-equilibration phase. In addition, the microbial biomass remained relatively stable in soil RefeSol 02A throughout the incubation period of 120 days. No significant adverse effect on microbial activity by application of the solvent acetonitrile or the test substance ethofumesate was observed.

In soil LUFA 2.1, microbial biomass decreased continuously from values of around 240 mg C_{mic}/kg dry mass at the beginning of the incubation to 108 mg C_{mic}/kg dry mass in untreated samples and to 26-29 mg C_{mic}/kg dry mass in treated samples after 119 days of incubation indicating a very low microbial activity at test end. A decreasing microbial activity at the end of the 120 days incubation time is commonly seen in soil batch tests – especially for sandy soils like LUFA 2.1. The observation that biomass in treated soil samples (either treated with solvent or

with ethofumesate) decreased more during the incubation time compared to the non-treated samples can probably be explained by adverse effects of acetonitrile or the test substance on the soil microbial community. However, another possible reason for this observation– which is more likely to occur in this sandy soil – is that a first enhancement of the microbial activity due to the consumption of acetonitrile or ethofumesate as substrates resulted in microbial growth, increasing biomass and, after a certain incubation time, in depletion of nutrients and in a subsequent decreasing microbial biomass.

However, the results of microbial biomass show the existence of an active microbial population throughout the incubation period.

4.3.4.3 Determination of ethofumesate amounts during aerobic degradation after single and multiple application

The amounts of ethofumesate during aerobic degradation after single and multiple application were determined in the soils RefeSol 02A and LUFA 2.1 by means of LC-MS/MS analysis.

Nominal and actual application rates for both single and multiple application of ethofumesate are described in section 4.3.4.1. Application interval during experiments with multiple application of the test substance was 21 days. The amounts of test item were calculated as % of the applied amount and in µg/kg soil (dry weight basis). Results of single application are presented in Table 121, results of multiple application in Table 122.

Table 121: Soil concentrations of ethofumesate after single application (0.800 mg/kg) expressed as µg/kg dry weight and % of applied test substance.

Sampling time	RefeSol 02A		LUFA 2.1	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
0d-1	656	85.7	682	89.2
0d-2	701	91.6	625	81.7
7d-1	482	63.4	568	74.8
7d-2	508	66.9	571	75.2
14d-1	531	69.9	606	79.8
14d-2	524	69.1	642	84.6
29d-1	466	61.3	525	69.1
29d-2	414	54.5	617	81.3
60d-1	393	51.8	627	82.6
60d-2	390	51.4	572	75.4
91d-1	391	51.5	565	74.4
91d-2	354	46.7	610	80.3
120d-1	259	34.1	536	70.7
120d-2	285	37.5	517	68.1

Table 122: Soil concentrations of ethofumesate after multiple application (2 x 0.400 mg/kg) expressed as µg/kg dry weight and % of applied test substance.

Sampling time	RefeSol 02A		LUFA 2.1	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
First application (0d)				
0d-1	337	90.8[1]	302	81.1[1]
0d-2	299	80.5[1]	301	81.1[1]
7d-1	225	59.9[1]	283	75.2[1]
7d-2	235	62.5[1]	263	70.0[1]
14d-1	232	61.8[1]	310	82.5[1]
14d-2	228	60.7[1]	275	73.1[1]
21d-1	218	58.2[1]	273	72.6[1]
21d-2	197	52.5[1]	293	78.1[1]
Second application (21d)				
21d-1	487	65.6[2]	581	78.3[2]
21d-2	528	71.1[2]	574	77.4[2]
28d-1	515	69.3[2]	653	87.4[2]
28d-2	508	68.4[2]	625	84.1[2]
35d-1	491	66.2[2]	642	86.4[2]
35d-2	499	67.2[2]	631	84.9[2]
60d-1	374	50.3[2]	563	75.8[2]
60d-2	438	58.9[2]	541	72.9[2]
91d-1	348	46.8[2]	573	77.2[2]
91d-2	348	46.9[2]	556	74.9[2]
120d-1	299	40.3[2]	447	60.2[2]
120d-2	284	38.3[2]	407	54.8[2]

[1] values in % of applied amount of ethofumesate during first application

[2] values in % of applied amount of ethofumesate as a sum of first and second application

Table 121 and Table 122 show that the amounts of ethofumesate decreased continuously in the two soils RefeSol 02A and LUFA 2.1 resulting in 35.8% (RefeSol 02A) to 69.4% (LUFA 2.1) of the applied ethofumesate after single application and 39.3% (RefeSol 02A) to 65.7% of the applied test substance (LUFA 2.1) after multiple applications (mean values) at the end of the incubation (120 days).

4.3.4.4 Calculation of DT50/DT90 values of ethofumesate after single and multiple application

The kinetic models considered for the analysis of ethofumesate were SFO (Single First Order), DFOP (Double First Order in Parallel) and FOMC (First Order Multi Compartment). The obtained data sets were analysed using the program CAKE. The results of the optimisation are presented in Table 123 - Table 126. Detailed CAKE results including HS kinetic model, plots and residuals are shown in Table 183 - Table 188.

Table 123: Calculated DT50 and DT90 for ethofumesate after single application (0.800 mg/kg) in RefeSol 02A.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**8.42**	**0.8221**	**110**	**366**	
FOMC	6.32	0.9044	106	> 10 000	> 10 000
DFOP	4.55	0.9487	92.6	419	141

Table 124: Calculated DT50 and DT90 for ethofumesate after single application (0.800 mg/kg) in LUFA 2.1.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**3.51**	**0.2746**	**739**	**2450**	
FOMC	3.75	0.2809	> 10 000	> 10 000	> 10 000
DFOP	3.71	0.347	902	3200	989

Table 125: Calculated DT50 and DT90 for ethofumesate after multiple application (2 x 0.400 mg/kg) in RefeSol 02A.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
after first application (0d – 21d)					
SFO	**6.82**	**0.7504**	**31.8**	**106**	
FOMC	2.36	0.927	629	> 10 000	> 10 000
DFOP	n.d.	0.932	58.5	291	100
after second application (21d – 120d)					
SFO	**1.89**	**0.9523**	**117**	**389**	
FOMC	2.09	0.9523	118	401	121
DFOP	2.38	0.9523	117	389	117

Table 126: Calculated DT50 and DT90 for ethofumesate after multiple application (2 x 0.400 mg/kg) in LUFA 2.1.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
after first application (0d – 21d)					
SFO	**3.00**	**0.09756**	**304**	**1010**	
FOMC	2.56	0.3236	> 10 000	> 10 000	> 10 000
DFOP	n.d.	0.3394	> 10 000	> 10 000	> 10 000
after second application (21d – 120d)					
SFO	**5.35**	**0.6894**	**233**	**772**	
FOMC	5.90	0.6889	234	794	239
DFOP	6.73	0.6894	233	772	233

In this test set, the chi²-values of SFO kinetics were always < 15% and the visual fit was acceptable. Therefore, SFO kinetics was assumed for ethofumesate for both soils and single and multiple application.

When the two soils RefeSol 02A and LUFA 2.1 are compared, DT50/DT90-values of ethofumesate in RefeSol 02 are generally lower than those in the sandy soil LUFA 2.1, i.e. in the experiments after single application as well as after both application time points of the multiple application test set.

The calculated DT50-values ranged from 110 days (RefeSol 02A) to 739 days (LUFA 2.1) after single application of ethofumesate.

Regarding the experiments with multiple application, DT50-values of 31.8 days after the first application and of 117 days after the second application were observed in RefeSol 02A. The DT50-value after the second application is in the same range of the DT50-value calculated for the single application experiment with a comparable application rate and, hence, in good agreement. The DT50-value for the incubation phase of 0 days – 21 days indicated an increased degradation rate of ethofumesate when applied to RefeSol 02A in a lower application rate. However, kinetic calculations after the first application are based on a limited number of results (four time points with two replicates) which represent a shortcoming for kinetic evaluation (six sampling times are regarded to be minimum for kinetic evaluation). In contrast, the incubation phase of 21 days – 120 days comprise six time points (with two replicates each) and a longer period of time. For this incubation period it has to be taken into account that the microbial community was degrading the test substance already for 21 days and may have changed its composition or activity during this period of time.

For soil LUFA 2.1, DT50-values of 304 days (first application) and 233 days (second application) were calculated. Both DT50-values were considerably lower than the DT50-value determined in experiments after single application (739 days) indicating a faster degradation of ethofumesate when 2 x 300 g/ha is applied instead of 1 x 600 g/ha.

Comparing the calculated DT50-values of ethofumesate with the DT50-values of pyraclostrobin (see section 4.3.3.4), it is observed that ethofumesate is generally degraded slower than pyraclostrobin.

4.4 Results of the degradation performance after single and multiple application of two test substances (STEP 2)

4.4.1 Degradation performance after single and multiple application of pyraclostrobin and ethofumesate as a mixture

4.4.1.1 Preparation of the application solution and application

The nominal application rate for the single application of pyraclostrobin was 667 µg/kg corresponding to 33.3 µg/50 g soil (dry weight). 2 x 333 µg/kg (in sum also 667 µg/kg) corresponding to 2 x 16.65 µg/50 g soil (dry weight). The nominal application rate for the single application of ethofumesate was 800 µg/kg corresponding to 40 µg/50 g soil (dry weight). The nominal application rate for the multiple application rate of ethofumesate was 2 x 400 µg/kg (in sum also 800 µg/kg) corresponding to 2 x 20 µg/50 g soil (dry weight). The interval between the two applications was set to 21 days.

One application solution including both test substances pyraclostrobin and ethofumesate was prepared. For a stock solution of pyraclostrobin, 11.25 mg pyraclostrobin were wighed into a 10 mL volumetric flask which was then added up to 10 mL with acetonitrile. Taking the purity of 99.9% into account, 2.963 mL of the pyraclostrobin stock solution were transferred into a 20 mL volumetric flask. To prepare a stock solution of ethofumesate, 10.67 mg ethofumesate was weighed into a 10 mL volumetric flask which was topped up to mark with acetonitrile. Taking the purity of 98.1% into account, 3.822 mL of the ethofumesate stock solution were transferred into the same 20 mL volumetric flask where pyraclostrobin was already present. The flask was then added up to 20 mL with acetonitrile. The application solution had a concentration of 166 µg pyraclostrobin/mL and 200 µg ethofumesate/mL. The application volume was calculated to be 200 µL per sample for the single application and 2 x 100 µL per sample for the multiple application. Thus, each subsample received < 0.5 mL (< 1 %) of the solvent as required by the OECD-guideline 307. The respective volume of the application solution was pipetted onto the soil samples.

The actual application rate after single application was determined to be 34.6 µg pyraclostrobin/sample corresponding to 692 µg/kg and 103.9% of nominal concentration as well as 35.8 µg ethofumesate/sample corresponding to 716 µg/kg and 89.5% of nominal concentration.

The amounts actually applied during the multiple applications of pyraclostrobin were also determined: During the first application 16.6 µg/sample were applied to the soil samples. At the second application after 21 days, 15.8 µg/sample were additionally applied. In sum, 32.5 µg/sample corresponding to 649 µg/kg and 97.3% of nominal were applied during the experiments with multiple application. Regarding ethofumesate, the following amounts were actually applied during the multiple applications: During the first application 17.1 µg/sample were applied to the soil samples. At the second application after 21 days, 18.4 µg/sample were additionally applied. In sum, 35.6 µg/sample corresponding to 711 µg/kg and 88.9% of the nominal concentration were applied.

4.4.1.2 Characterisation of the microbial status of the soil

Biomass measurements of the soil RefeSol 02A were performed by means of the substrate induced respiration method described in section 4.2.12. The principal events of soil preparation for this test set are summarised in Table 173. Microbial biomass measurement before incubation was determined in untreated samples in triplicate. Microbiological status expressed as biomass in mg microbial carbon per kg soil was measured to be 140 mg C_{mic}/kg dry mass corresponding

to a C_{mic}/C_{org} rate of 1.3%. The value indicated a normal microbial activity of the soil with values > 1% C_{mic} of C_{org}.

The microbial biomass status during the incubation was carried out in the beginning, in the mid and in the end of the aerobic incubation. Table 127 presents the results of biomass measurement of the soil during the incubation period. Microbial biomass was determined in untreated samples, in samples treated with the organic solvent acetonitrile and in samples treated with both pyraclostrobin and ethofumesate as mixture (nominal 33.3 µg pyraclostrobin/50 g soil dry mass and 40 µg ethofumesate/50 g soil, single application). Microbiological status is expressed as biomass in mg microbial carbon per kg soil in the following table as mean of two replicates.

Table 127: Microbial biomass determined by means of substrate induced respiration method of degradation experiments after single application of both pyraclostrobin and ethofumesate as a mixture (given as mean values of two replicates).

Soil type	Soil sample	Biomass [mg C_{mic}/kg dry mass]		
		0d	61d	119d
RefeSol 02A	Non-treated	250	175	169
	Treated with solvent	-	180	133
	Treated with pyraclostrobin and ethofumesate	-	226	147

The microbial biomass remained relatively stable in soil RefeSol 02A throughout the incubation period of 120 days. No significant adverse effect on microbial activity by application of the solvent acetonitrile or the test substances pyraclostrobin and ethofumesate was observed. The results of the microbial biomass measurements show the existence of an active microbial population throughout the incubation period.

4.4.1.3 Determination of pyraclostrobin and ethofumesate amounts during aerobic degradation after single application

The amounts of pyraclostrobin and ethofumesate during aerobic degradation after single application were determined in the soil RefeSol 02A by means of LC-MS/MS analysis.

Nominal and actual application rates for single application of pyraclostrobin and ethofumesate as a mixture are described in section 4.4.1.1. The amounts of test item were calculated as % of the applied amount and in µg/kg soil (dry weight basis). Results of pyraclostrobin and ethofumesate which were applied at the same time point and were present in the soil samples are presented in Table 128.

Table 128: Soil concentrations of pyraclostrobin and ethofumesate after single application as a mixture expressed as µg/kg dry weight and % of applied test substance; applicationn rates were 0.677 mg/kg for pyracolostrobin and 0.800 mg/kg for ethofumesate.

Sampling time	Pyraclostrobin		Ethofumesate	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
0d-1	639	92.4	619	86.5
0d-2	636	91.9	603	84.3

Sampling time	Pyraclostrobin		Ethofumesate	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
7d-1	473	68.3	652	91.0
7d-2	540	78.0	563	78.6
14d-1	410	59.2	588	82.1
14d-2	406	58.7	561	78.4
29d-1	293	42.3	499	69.7
29d-2	276	40.0	521	72.8
60d-1	152	21.9	418	58.4
60d-2	155	22.3	429	60.0
90d-1	121	17.5	321	44.8
90d-2	120	17.3	385	53.8
120d-1	68	9.9	345	48.2
120d-2	81	11.7	335	46.8

Table 128 shows that amounts of both pyraclostrobin and ethofumesate decreased continuously in the soil RefeSol 02A resulting in 10.8% (pyraclostrobin) and 47.5% (ethofumesate) of the applied test substances after single application (mean values) at the end of the incubation (120 days).

4.4.1.4 Determination of pyraclostrobin and ethofumesate amounts during aerobic degradation after multiple application

The amounts of pyraclostrobin and ethofumesate during aerobic degradation after multiple application were determined in the soil RefeSol 02A by means of LC-MS/MS analysis.

Nominal and actual application rates for multiple application of pyraclostrobin and ethofumesate as a mixture are described in section 4.4.1.1. The amounts of test item were calculated as % of the applied amount and in µg/kg soil (dry weight basis). Results of pyraclostrobin and ethofumesate which were applied two times contemporaneously as a mixture are presented in Table 129.

Table 129: Soil concentrations of pyraclostrobin and ethofumesate after multiple application as a mixture expressed as µg/kg dry weight and % of applied test substance; application rates were 2 x 0.333 mg/kg for pyraclostrobin and 2 x 0.400 mg/kg for ethofumesate.

Sampling time	Pyraclostrobin		Ethofumesate	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
First application of pyraclostrobin and ethofumesate (0d)				
0d-1	310	93.4[1]	337	98.5[1]
0d-2	305	91.7[1]	333	97.4[1]
7d-1	224	67.5[1]	309	90.2[1]

Sampling time	Pyraclostrobin		Ethofumesate	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
7d-2	231	69.4[1]	298	87.2[1]
15d-1	193	58.0[1]	264	77.1[1]
15d-2	190	57.2[1]	281	82.0[1]
21d-1	138	41.5[1]	216	63.2[1]
21d-2	139	41.9[1]	219	64.1[1]
Second application of pyraclostrobin and ethofumesate (21d)				
21d-1	450	69.3[2]	495	69.5[2]
21d-2	465	71.6[2]	531	74.6[2]
28d-1	395	60.8[2]	542	76.2[2]
28d-2	411	63.3[2]	478	67.2[2]
35d-1	304	46.8[2]	421	59.2[2]
35d-2	311	47.9[2]	442	62.2[2]
60d-1	140	21.6[2]	320	45.0[2]
60d-2	138	21.2[2]	347	48.8[2]
90d-1	77	11.8[2]	269	37.8[2]
90d-2	75	11.5[2]	271	38.1[2]
120d-1	45	6.9[2]	314	44.1[2]
120d-2	47	7.2[2]	294	41.4[2]

[1] *values in % of applied amount of pyraclostrobin or ethofumesate during first application*

[2] *values in % of applied amount of pyraclostrobin and ethofumesate as a sum of the first and second application*

As can be seen in Table 129, amounts of both pyraclostrobin and ethofumesate decreased continuously in soil RefeSol 02A resulting in 7.0% (pyraclostrobin) and 42.8% (ethofumesate) of the applied test substance after multiple applications (mean values) at the end of the incubation (120 days).

4.4.1.5 Calculation of DT50/DT90 values of pyraclostrobin and ethofumesate as a mixture after single and multiple application

The kinetic models considered for the analysis of pyraclostrobin and ethofumesate were SFO (Single First Order), DFOP (Double First Order in Parallel) and FOMC (First Order Multi Compartment). The obtained data sets were analysed using the program CAKE. The results of the optimisation are presented in Table 130 - Table 133. Detailed CAKE results including HS kinetic model, plots and residuals are shown in Table 189 - Table 194.

Table 130: Calculated DT50 and DT90 for pyraclostrobin after single application of both test substances as a mixture in RefeSol 02A (substances applied together); nominal application rates were 0.677 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**7.46**	**0.9773**	**30.5**	**101**	
FOMC	1.65	0.9946	23.7	144	43.3
DFOP	1.69	0.9947	23.5	141	68.7

Table 131: Calculated DT50 and DT90 for ethofumesate after single application of both test substances as a mixture in RefeSol 02A (substances applied together); application rates were 0.667 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**2.55**	**0.9396**	**123**	**408**	
FOMC	2.09	0.948	130	1230	371
DFOP	2.1	0.95	137	> 10 000	> 10 000

Table 132: Calculated DT50 and DT90 for pyraclostrobin after multiple application of both test substances in RefeSol 02A (substances applied together); application rates were 2 x 0.333 mg/kg for pyraclostrobin and 2 x 0.400 mg/kg for ethofumesate.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
after first application of pyraclostrobin and ethofumesate (0d – 21d)					
SFO	**2.42**	**0.9878**	**18.9**	**62.9**	
FOMC	2.98	0.9882	19	74.2	22.3
DFOP	n.d.	0.9905	19.1	66.9	20.6
after second application of pyraclostrobin and ethofumesate (21d – 120d)					
SFO	**4.66**	**0.992**	**24.8**	**82.4**	
FOMC	4.79	0.9925	23.7	87.7	26.4
DFOP	5.00	0.9936	23.3	92.6	> 10 000

Table 133: Calculated DT50 and DT90 for ethofumesate after multiple application of both test substances in RefeSol 02A (substances applied together); application rates were 2 x 0.333 mg/kg for pyraclostrobin and 2 x 0.400 mg/kg for ethofumesate.

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
after first application of pyraclostrobin and ethofumesate (0d – 21d)					
SFO	**0.178**	**0.9863**	**46.5**	**155**	
FOMC	0.227	0.9863	46.6	156	47
DFOP	n.d.	0.9863	46.5	155	46.6
after second application of pyraclostrobin and ethofumesate (21d – 120d)					
SFO	**7.54**	**0.8265**	**96.8**	**322**	
FOMC	5.86	0.898	104	7820	2360
DFOP	5.48	0.9216	> 10 000	> 10 000	> 10 000

In this test set, the chi²-values of SFO kinetics were always < 15% and the visual fit was acceptable. Therefore, SFO kinetics was assumed for both pyraclostrobin and ethofumesate after single and multiple application as a mixture.

The calculated DT50-values ranged from 30.5 days (pyraclostrobin) to 123 days (ethofumesate) after single application of a mixture of these two substances.

Regarding the experiments with multiple application, DT50-values of 18.9 days after the first application and of 24.8 days after the second application were observed for pyraclostrobin in the mixture. Both DT50-values are lower compared to the DT50-value calculated for the tests with single application (30.5 days). For ethofumesate, DT50-values of 46.5 days (first application) and 96.8 days (second application) were calculated. These DT50-values were also lower than the DT50-value determined in experiments after single application (123 days). The results indicated that pyraclostrobin and ethofumesate could be degraded slightly faster when applied at a lower rate.

To compare the observed DT50-values of pyraclostrobin and ethofumesat applied in a mixture with the DT50-values determined when the test substances were applied individually, the DT50-values are summarised in Table 134.

Table 134: DT50-values (based on SFO) of pyraclostrobin and ethofumesate in RefeSol 02A after single and multiple application as individual substance or within a mixture; application rates were 0.667 mg/kg (pyraclostrobin) and 0.800 mg/kg (ethofumesate) for single application and 2 x 0.333 mg/kg (pyraclostrobin) and 2 x 0.400 mg/kg (ethofumesate) for multiple application.

Test substance	Nominal application rate	Test set	DT50-values (SFO) [d]	
			Applied individually (STEP 1)	Applied within a mixture
Pyraclostrobin	0.667 mg/kg (pyraclostrobin)	single application	27.3	30.5

Test substance	Nominal application	Test set	DT50-values (SFO) [d]	
	0.800 mg/kg (ethofumesate)			
	2 x 0.333 mg/kg (pyraclostrobin) 2 x 0.400 mg/kg (ethofumesate)	multiple application, 0d-21d	15.6	18.9
		multiple application 21d-120d	41.7	24.8
Ethofumesate	0.667 mg/kg (pyraclostrobin) 0.800 mg/kg (ethofumesate)	single application	110	123
	2 x 0.333 mg/kg (pyraclostrobin) 2 x 0.400 mg/kg (ethofumesate)	multiple application, 0d-21d	31.8	46.5
		multiple application 21d-120d	117	96.8

As can be seen in Table 134, the respective DT50-values of pyraclostrobin and ethofumesate after single application as a mixture were in the same range of the corresponding DT50-values after application as single components. The DT50-values of both test substances were slightly higher when applied as a mixture, however, statistical evaluation of the significance was not possible due to the limited number of data and therefore the determined DT50-values were regarded as comparable.

With regard to the multiple application setups, slightly higher DT50-values were observed for the first application (period of 0d-21d) of both pyraclostrobin and ethofumesate applied as individual components compared to the DT50-values for application as a mixture. In contrast, lower DT50-values were found for the second application (period of 21d – 120d) of both substances when applied as individual components compared to the DT50-values as a mixture (DT50 values of 41.7 days for pyraclostrobin and 117 days for ethofumesate when applied as individual components compared DT50-values of 24.8 days for pyraclostrobin and 96.8 days for ethofumesate when applied as a mixture).

4.4.2 Degradation performance after time-delayed single application of pyraclostrobin and ethofumesate

4.4.2.1 Preparation of the application solution and application

The nominal application rate for the single application of pyraclostrobin was 667 µg/kg corresponding to 33.3 µg/50 g soil (dry weight). The nominal application rate for the single application of ethofumesate was 800 µg/kg corresponding to 40 µg/50 g soil (dry weight). The test substances were applied separately at two different time points. The interval between the two application dates was set to 21 days.

Two separate application solutions were prepared – one application solution for pyraclostrobin and a second application solution for ethofumesate. For a stock solution of pyraclostrobin, 11.25 mg pyraclostrobin were weighed into a 10 mL volumetric flask which was then added up to 10 mL with acetonitrile. Taking the purity of 99.9% into account, 2.963 mL of the pyraclostrobin stock solution were transferred into a 20 mL volumetric flask. Acetonitrile was then added to the flask up to 20 mL. The application solution had a concentration of 166.5 µg pyraclostrobin/mL and the application volume was calculated to be 200 µL per sample.

To prepare a stock solution of ethofumesate, 10.36 mg ethofumesate was weighed into a 10 mL volumetric flask which was topped up to mark with acetonitrile. Taking the purity of 98.1% into account, 3.936 mL of the ethofumesate stock solution were transferred into a (separate) 20 mL volumetric flask which was then added up to 20 mL with acetonitrile. The application solution had a concentration of 200 µg ethofumesate/mL. The application volume was calculated to be 200 µL per sample.

Applying 200 µL of the pyraclostrobin application solution and - at a second time point - 200 µL of the ethofumesate application solution, each subsample received < 0.5 mL (< 1 %) of the solvent as required by the OECD-guideline 307. The respective volume of the application solution was pipetted onto the soil samples.

The actual application rates after single application of pyraclostrobin was determined to be 33.5 µg/sample (first application time point) and 33.7 µg/sample (second application time point) corresponding to 671 - 674 µg/kg and 100.7 – 101.2% of nominal concentration. The actual application rates after single application of ethofumesate was determined to be 35.6 µg/sample (first application time point) and 33.3 µg/sample (second application time point) corresponding to 666 - 713 µg/kg and 83.3 – 89.1% of nominal concentration.

4.4.2.2 Characterisation of the microbial status of the soil

Biomass measurements of the soil RefeSol 02A were performed by means of the substrate induced respiration method described in section 4.2.12. The principal events of soil preparation for this test set are summarised in Table 174. Microbial biomass measurement before incubation was determined in untreated samples in duplicate. Microbiological status expressed as biomass in mg microbial carbon per kg soil was measured to be 196 mg C_{mic}/kg dry mass corresponding to a C_{mic}/C_{org} rate of 1.9%. The value indicated a normal microbial activity of the soil with values > 1% C_{mic} of C_{org}.

The microbial biomass status during the incubation was carried out in the beginning, in the mid and in the end of the aerobic incubation. Table 135 presents the results of biomass measurement of the soil during the incubation period. Microbial biomass was determined in untreated samples, in samples treated with the organic solvent acetonitrile and in samples treated with pyraclostrobin and ethofumesate at different time points (nominal 33.3 µg pyraclostrobin/50 g soil dry mass and 40 µg ethofumesate/50 g soil, single application). Microbiological status is expressed as biomass in mg microbial carbon per kg soil in the following table as mean of two replicates.

Table 135: Microbial biomass determined by means of substrate induced respiration method of degradation experiments after single application of ethofumesate at 0 days and pyraclostrobin at 21 days (given as mean values of two replicates).

Soil type	Soil sample	Biomass [mg C_{mic}/kg dry mass]		
		0d	61d	119d
RefeSol 02A	Non-treated	168	210	234
	Treated with solvent	-	336	117
	Treated with ethofumesate (first) and pyraclostrobin (second)	-	362	164

The microbial biomass remained relatively stable in soil RefeSol 02A throughout the incubation period of 120 days in non-treated samples. In both solvent-treated samples as well as in samples

treated with the test substances, the microbial biomass was increased (336-362 mg Cmic/kg) compared to the non-treated samples (210 mg Cmic/kg) in the mid of the incubation period at 61 days. This result indicated that the soil microorganisms are enhanced by the degradation of both solvent and test substances. Afterwards, microbial biomass in solvent treated samples and in samples treated with test substances decreased to lower values (117-164 mg Cmic/kg) compared to the non-treated samples (234 mg Cmic/kg) at the end of incubation at 119 days. The observation that biomass in treated soil samples (either treated with solvent or with ethofumesate) decreased more during the incubation time compared to the non-treated samples can be explained by depletion of nutrients after the biomass enhancement during the mid of incubation time due to the consumption of acetonitrile, ethofumesate and pyraclostrobin as substrates.

However, the results of the microbial biomass measurements show the existence of an active microbial population throughout the incubation period.

4.4.2.3 Determination of pyraclostrobin and ethofumesate amounts during aerobic degradation after time-delayed single application

The amounts of pyraclostrobin and ethofumesate during aerobic degradation after single application at different time points were determined in the soil RefeSol 02A by means of LC-MS/MS analysis.

Nominal and actual application rates for single application of pyraclostrobin and ethofumesate are described in section 4.4.2.1. The amounts of test item were calculated as % of the applied amount and in µg/kg soil (dry weight basis). Results of pyraclostrobin and ethofumesate which were applied separately at two different time points (0 days and 21 days) are presented in Table 136 and Table 137.

Table 136: Soil concentrations of pyraclostrobin (0d) and ethofumesate (21d) after time-delayed single application expressed as µg/kg dry weight and % of applied test substance; application rates were 0.667 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate.

Sampling time	Pyraclostrobin		Ethofumesate	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
Application of pyraclostrobin (0d)				
0d-1	580	86.5		
0d-2	594	88.6		
7d-1	480	71.6		
7d-2	476	70.9		
14d-1	378	56.3		
14d-2	381	56.8		
Application of ethofumesate (21d)				
21d-1	291	43.3	640	96.0
21d-2	280	41.8	655	98.3
28d-1	237	35.3	452	67.8

Sampling time	Pyraclostrobin		Ethofumesate	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
28d-2	244	36.4	527	79.1
35d-1	219	32.7	518	77.8
35d-2	209	31.2	530	79.5
60d-1	137	20.4	485	72.8
60d-2	139	20.8	474	71.1
90d-1	84	12.6	433	65.0
90d-2	90	13.4	434	65.1
120d-1	82	12.2	371	55.7
120d-2	72	10.7	385	57.8

Table 137: Soil concentrations of ethofumesate (0d) and pyraclostrobin (21d) after time-delayed single application expressed as µg/kg dry weight and % of applied test substance; application rates were 0.667 mg/kg for pyraclostrobin and 0.400 mg/kg for ethofumesate.

Sampling time	Pyraclostrobin		Ethofumesate	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
Application of ethofumesate (0d)				
0d-1			647	90.8
0d-2			636	89.3
7d-1			542	76.1
7d-2			561	78.8
14d-1			585	82.0
14d-2			553	77.6
Application of pyraclostrobin (21d)				
21d-1	598	88.7	487	68.3
21d-2	625	92.8	454	63.7
28d-1	493	73.1	361	50.7
28d-2	493	73.2	402	56.3
35d-1	447	66.3	412	57.8
35d-2	443	66.2	429	60.2
60d-1	306	45.4	363	51.0
60d-2	262	38.8	368	51.7
90d-1	193	28.6	329	46.2

Sampling time	Pyraclostrobin		Ethofumesate	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
90d-2	192	28.5	318	44.7
120d-1	160	23.8	318	44.6
120d-2	137	20.3	317	44.5

As can be seen in Table 136, amounts of both pyraclostrobin (applied at day 0) and ethofumesate (applied at day 21) decreased continuously in soil RefeSol 02A resulting in 11.7% (pyraclostrobin) and 56.7% (ethofumesate) of the applied test substance after separate single applications (mean values) at the end of the incubation (120 days). For ethofumesate, it has to be considered that it was applied with a time delay of 21 days. Hence, minimum amounts of ethofumesate at the end of the incubation phase of 120 days cannot be compared with those when ethofumesate was applied at 0 days.

As can be seen in Table 137, amounts of both ethofumesate (applied at day 0) and pyraclostrobin (applied at day 21) decreased continuously in soil RefeSol 02A resulting in 22.0% (pyraclostrobin) and 44.6% (ethofumesate) of the applied test substance after separate single applications (mean values) at the end of the incubation (120 days). For pyraclostrobin, it has to be considered that incubation phase lasted only 99 days instead of 120 days.

4.4.2.4 Calculation of DT50/DT90 values of pyraclostrobin and ethofumesate after single application at different time points

The kinetic models considered for the analysis of pyraclostrobin and ethofumesate were SFO (Single First Order), DFOP (Double First Order in Parallel) and FOMC (First Order Multi Compartment). The obtained data sets were analysed using the program CAKE. The results of the optimisation are presented in Table 138 - Table 141. Detailed CAKE results including HS kinetic model, plots and residuals are shown in Table 195 - Table 198.

Table 138: Calculated DT50 and DT90 for pyraclostrobin (0d) after time-delayed single application of both test substances; application rates were 0.667 mg/kg for pyraclostrobin (0d) and 0.800 mg/kg ethofumesate (21d).

	chi^2 (%)	r^2 (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**8.42**	**0.9767**	**25.7**	**85.4**	
FOMC	3.49	0.9947	21.4	127	38.3
DFOP	2.83	0.9966	21.5	166	150

Table 139: Calculated DT50 and DT90 for ethofumesate (21d) after time-delayed single application of both test substances; application rates were 0.667 mg/kg for pyraclostrobin (0d) and 0.800 mg/kg for ethofumesate (21d).

	chi^2 (%)	r^2 (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**6.69**	**0.7257**	**159**	**528**	
FOMC	4.74	0.8654	1320	> 10 000	> 10 000

	chi²	r²	DT50	DT90	DT50 slow rate / DT90/3.32
DFOP	3.08	0.9316	160	689	228

Table 140: Calculated DT50 and DT90 for ethofumesate (0d) after time-delayed single application of both test substances; application rates were 0.667 mg/kg for pyraclostrobin (21d) and 0.800 mg/kg for ethofumesate (0d).

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**8.74**	**0.7924**	**103**	**341**	
FOMC	5.56	0.9174	93	> 10 000	4340
DFOP	5.44	0.9289	102	> 10 000	> 10 000

Table 141: Calculated DT50 and DT90 for pyraclostrobin (21d) after time-delayed single application of both test substances; application rates were 0.667 mg/kg for pyraclostrobin (21d) and 0.800 mg/kg for ethofumesate (0d).

	chi² (%)	r² (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**5.27**	**0.9754**	**42.7**	**142**	
FOMC	1.94	0.9928	34.3	258	77.5
DFOP	2.33	0.9925	34.3	193	75

In this test set, the chi²-values of SFO kinetics were always < 15% and the visual fit was acceptable. Therefore, SFO kinetics was assumed for both pyraclostrobin and ethofumesate after single application at two time points.

The calculated DT50-values ranged from 25.7 days – 42.7 days(pyraclostrobin) and 103 days – 159 days (ethofumesate) after single application at two different time points. For both pyraclostrobin and ethofumesate, the DT50-values are lower when the test substance is applied at the beginning of incubation (day 0) compared to the DT50-values obtained when the substance is applied after 21 days and a further substance is already present in the soil. In a soil batch test, the microbial activity is usually decreasing throughout the incubation time due to nutrient depletion. However, the microbial biomass measurements of this test set indicated the existence of an active microbial population throughout the complete incubation period with considerably high biomass amounts at 61 days (see Table 135). So a lack of microbial activity seems to be unlikely for these results.

To compare the observed DT50-values of pyraclostrobin and ethofumesat applied at two time points with the DT50-values determined when the test substances were applied individually, the DT50-values are summarised in Table 142.

Table 142: DT50-values (based on SFO) of pyraclostrobin and ethofumesate in RefeSol 02A after single application as individual substances or as a time-delayed mixture; application rates were 0.667 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate.

Test substance	Test set	DT50-values (SFO) [d]		
		Individually applied (STEP 1)	Substance applied at day 0 (further substance present from day 21)	Substance applied at day 21 (further substance present from day 0)
Pyraclostrobin	single application (at 0d)	27.3[1]	25.7	
Pyraclostrobin	single application (at 21d)			42.7
Ethofumesate	single application (at 0d)	110[1]	103	
Ethofumesate	single application (at 21d)			159

[1] *Single application of the individual component was performed at 0d (application rate stated above)*

The DT50-value of pyraclostrobin in the time-delayed test after single application at day 0 (25.7 days) was in the same range of the corresponding DT50-value after application as single component (27.3 days). This means that the addition of ethofumesate after 21 days of incubation did not affect the further degradation rate of pyraclostrobin significantly.

In contrast, the DT50-value is considerably higher when pyraclostrobin is applied after 21 days and ethofumesate is already present in the soil (42.7 days) indicating that the presence of another substance in soil could possibly have a negative effect on degradation of the substance applied subsequently. However, it has to be considered that the single application of the individual component (during STEP 1) was carried out at day 0 and not at day 21 which may have an effect on the microbial activity which usually decreases during the incubation time and, hence, slows down the degradation rate. For comparison, pyraclostrobin exclusively applied for the second time during the multiple application test set (STEP 1, 2 x half application rate) had a DT50-value of 41.7 days. Therefore, it cannot be proved unambiguously that the negative effect on pyraclostrobin degradation is due to the presence of ethofumesate in soil. Further research should be conducted.

Regarding ethofumesate, the DT50-value in the time-delayed test after single application at day 0 (103 days) was also in a comparable range to the DT50-value after application of the single component (110 days) indicating that the degradation of the firstly applied substance was not affected by the later application of a further substance. As seen for pyraclostrobin, a higher DT50-value was determined when ethofumesate is applied after 21 days to soil where pyraclostrobin is already present (159 days). A lack of microbial activity is regarded to be unlikely for being the reason of these results since ethofumesate applied for the second time during the multiple application test set (STEP 1, 2 x half application rate) had a DT50-value of 117 days. Thus, the results indicated that the presence of another substance could have had an effect on the degradation of the substance applied afterwards.

Results of pyraclostrobin and ethofumesate indicate that a test substance applied to soil where a second test substance is still present from earlier application may have a negative impact on the degradation of the freshly applied substance. However, the degradation of the substance applied first seemed not to be affected by a later applied second substance.

4.5 Results of the degradation performance after single application of pyraclostrobin or ethofumesate in a soil with an existing multiple contamination (STEP 3)

4.5.1 Preparation of the application solution and application

The nominal application rate for the single application of pyraclostrobin was 667 µg/kg corresponding to 33.3 µg/50 g soil (dry weight). The nominal application rate for the single application of ethofumesate was 800 µg/kg corresponding to 40 µg/50 g soil (dry weight). The test substances were applied separately to the soil with an existing multiple contamination (see section 4.2.1, Table 175) in one single application.

Hence, two separate application solutions were prepared – one application solution for pyraclostrobin and a second application solution for ethofumesate. For a stock solution of pyraclostrobin, 10.14 mg pyraclostrobin were weighed into a 10 mL volumetric flask which was then added up to 10 mL with acetonitrile. Taking the purity of 99.9% into account, 1.644 mL of the pyraclostrobin stock solution were transferred into a 10 mL volumetric flask. Acetonitrile was then added to the flask up to 10 mL. The application solution had a concentration of 166.5 µg pyraclostrobin/mL and the application volume was calculated to be 200 µL per sample.

For ethofumesate, 1.968 mL of the ethofumesate stock solution (10.36 mg ethofumesate with a purity of 98.1% in 10 mL acetonitrile) were transferred into a (separate) 10 mL volumetric flask which was then added up to 10 mL with acetonitrile. The application solution had a concentration of 200 µg ethofumesate/mL. The application volume was calculated to be 200 µL per sample.

Applying 200 µL of the pyraclostrobin or ethofumesate application solution, each subsample received < 0.5 mL (< 1 %) of the solvent as required by the OECD-guideline 307. The respective volume of the application solution was pipetted onto the soil samples.

The actual application rate after single application of pyraclostrobin was determined to be 34.4 µg/sample corresponding to 688 µg/kg and 103.4% of nominal concentration. The actual application rate of ethofumesate was determined to be 34.2 µg/sample corresponding to 684 µg/kg and 85.5% of nominal concentration.

4.5.2 Characterisation of the microbial status of the soil

Biomass measurements of the soil freshly sampled in the field at the sampling site of RefeSol 02A were performed by means of the substrate induced respiration method described in section 4.2.12. The principal events of soil preparation for this test set are summarised in Table 176. Microbial biomass measurement before incubation was determined in untreated samples in duplicate. Microbiological status expressed as biomass in mg microbial carbon per kg soil was measured to be 428 mg C_{mic}/kg dry mass corresponding to a C_{mic}/C_{org} rate of ca. 4.0%. The value indicated a normal microbial activity of the soil with values > 1% C_{mic} of C_{org}.

The microbial biomass status during the incubation was carried out in the beginning, in the mid and in the end of the aerobic incubation. Table 143 presents the results of biomass measurement of the soil during the incubation period. Microbial biomass was determined in untreated samples, in samples treated with the organic solvent acetonitrile and in samples treated with pyraclostrobin or ethofumesate (nominal 33.3 µg pyraclostrobin/50 g soil dry mass or 40 µg ethofumesate/50 g soil, single application). Microbiological status is expressed as biomass in mg microbial carbon per kg soil in the following table as mean of two replicates.

Table 143: Microbial biomass determined by means of substrate induced respiration method of degradation experiments after single application of ethofumesate or pyraclostrobin to a soil with existing multiple contamination (given as mean values of two replicates).

Soil type	Soil sample	Biomass [mg C_{mic}/kg dry mass]		
		0d	61d	119d
Soil with existing multiple contamination (RefeSol 02A)	Non-treated	448	372	467
	Treated with solvent	-	372	443
	Treated with ethofumesate	-	365	451
	Treated with pyraclostrobin	-	442	428

The microbial biomass remained relatively stable in soil throughout the incubation period of 120 days. No significant adverse effect on microbial activity by application of the solvent acetonitrile or the test substances pyraclostrobin and ethofumesate was observed. The results of the microbial biomass measurements show the existence of an active microbial population throughout the incubation period.

4.5.3 Determination of pyraclostrobin and ethofumesate amounts during aerobic degradation after single application to a soil with existing multiple contamination

The amounts of pyraclostrobin and ethofumesate during aerobic degradation after single application to a soil with existing multiple contamination were determined by means of LC-MS/MS analysis.

Nominal and actual application rates for single application of pyraclostrobin and ethofumesate are described in section 4.4.2.1. The amounts of test item were calculated as % of the applied amount and in µg/kg soil (dry weight basis). Results of pyraclostrobin and ethofumesate which were applied separately are presented in Table 144.

Table 144: Soil concentrations of pyraclostrobin and ethofumesate after single application to a soil with existing multiple contamination expressed as µg/kg dry weight and % of applied test substance; nominal application rates were 0.667 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate.

Sampling time	Pyraclostrobin		Ethofumesate	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
0d-1	609	88.4	586	85.8
0d-2	586	85.1	561	82.1
7d-1	402	58.4	543	79.4
7d-2	410	59.6	537	78.6
15d-1	370	53.7	475	69.5
15d-2	343	49.8	479	70.1
30d-1	194	28.1	422	61.7
30d-2	179	26.1	409	59.8

Sampling time	Pyraclostrobin		Ethofumesate	
	[µg/kg dry weight]	[%]	[µg/kg dry weight]	[%]
60d-1	84	12.3	338	49.5
60d-2	88	12.7	151	22.0
90d-1	47	6.9	111	16.2
90d-2	45	6.6	234	34.2
120d-1	39	5.7	127	18.6
120d-2	30	4.4	147	21.4

As can be seen in Table 144, amounts of both pyraclostrobin and ethofumesate decreased continuously in the soil with existing multiple contamination resulting in 5.0% (pyraclostrobin) and 20.0% (ethofumesate) of the applied test substance (mean values) at the end of the incubation (120 days). For ethofumesate, relatively high variations were observed for the two replicates at the sampling times 60 days and 90 days. However, the mean values of the two replicates were still reasonable for a degradation curve. It is possible in degradation experiments that the microbial community in the different soil replicates may diverge and differ then - especially at longer incubation times.

4.5.4 Calculation of DT50/DT90 values of pyraclostrobin and ethofumesate after single application to a soil with existing multiple contamination

The kinetic models considered for the analysis of pyraclostrobin and ethofumesate were SFO (Single First Order), DFOP (Double First Order in Parallel) and FOMC (First Order Multi Compartment). The obtained data sets were analysed using the program CAKE. The results of the optimisation are presented in Table 145 - Table 146. For detailed CAKE results including HS kinetic model, plots and residuals refer to Table 199- Table 200.

Table 145: Calculated DT50 and DT90 for pyraclostrobin after single application to a soil with existing multiple contamination; nominal application rate was 0.667 mg/kg.

	chi^2 (%)	r^2 (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**8.1**	**0.9855**	**20**	**66.3**	
FOMC	6.44	0.99	17.5	80.3	24.2
DFOP	7.21	0.9897	17.7	80.5	49.1

Table 146: Calculated DT50 and DT90 for ethofumesate after single application to a soil with existing contamination; nominal application rate was 0.800 mg/kg.

	chi^2 (%)	r^2 (-)	DT50 (d)	DT90 (d)	DT50 slow rate / DT90/3.32 (d)
SFO	**3.01**	**0.9308**	**53.3**	**177**	
FOMC	3.24	0.9308	53	179	53.9

	chi²	r²	DT50	DT90	DT50 slow rate / DT90/3.32
DFOP	3.53	0.931	52.4	193	> 10000

In this test set, the chi²-values of SFO kinetics were always < 15% and the visual fit was acceptable. Therefore, SFO kinetics was assumed for both pyraclostrobin and ethofumesate after single application to a soil with existing multiple contamination.

The calculated DT50-values ranged from 20 days (pyraclostrobin) to 53.3 days (ethofumesate) after single application. The microbial biomass measurements during this test set indicated the existence of an active microbial population throughout the complete incubation period with considerably high biomass amounts (see Table 143) compared to other test sets.

To compare the observed DT50-values of pyraclostrobin and ethofumesate applied to a soil with an existing multiple contamination with the DT50-values determined in RefeSol 02A after single application (STEP 1), the DT50-values are summarised in Table 147.

Table 147: DT50-values (based on SFO) of pyraclostrobin and ethofumesate after single application to a soil with existing multiple contamination; application rates were 0.667 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate.

Test substance	Test set	DT50-values (SFO) [d]	
		Individually applied to RefeSol 02A (STEP 1)	Applied to a soil with existing multiple contamination
Pyraclostrobin	single application (at 0d)	27.3	20
Ethofumesate	single application (at 0d)	110	53.3

As can be seen in Table 147, the DT50-value of pyraclostrobin after single application to a soil with existing contamination (20 days) was slightly lower compared to the corresponding DT50-value after application to soil RefeSol 02A (27.3 days). This means that the presence of other applied pesticides applied before did not affect the degradation rate of pyraclostrobin considerably. Regarding ethofumesate, the DT50-value after single application to a soil with existing contamination (53.3 days) was considerably lower compared to the DT50-value after application to RefeSol 02A without additional contamination (110 days).

When comparing the amounts of microbial biomass of this test set with other test sets with single application, the biomass amounts were continuously high (in the range of 365 – 467 mg Cmic/kg, see Table 143) whereas in other test sets amounts in the range of 117 – 362 mg Cmic/kg (see Table 120, Table 127 and Table 135) were determined. Therefore, it is not completely clear if the observed enhancing effect on the degradation rate of pyraclostrobin and ethofumesate is due to the existing contaminants in the soil (as additional content of organic carbon or other nutrients like nitrogen etc.) or due to higher amounts of microbial biomass or both. The outdoor soil was treated with several fungicides, herbicides and growth regulators during the agricultural use of the last 2 years (see Table 175). However, pyraclostrobin and ethofumesate were not applied in the last years. At the date of soil sampling, the crop winter wheat was growing on the field. Just 8 days before, several fungicides were applied (see Table 175) including kresoxim-methyl, a strobilurin fungicide as pyraclostrobin. Therefore, an adaptation of the soil microbial community to the test substances, especially pyraclostrobin, may have occurred. Differences in general soil characteristics should not have had an impact on the degradation rate since the soil with the existing contamination was (freshly) sampled at the

original sampling site for RefeSol 02A meaning that the soil characteristics should not vary significantly.

Results of this STEP 3 test set indicate that existing contaminants additionally present in the soil did not affect the degradation rates of both pyraclostrobin and ethofumesate in soil adversely and, in contrast, might have enhanced the soil degradation (at least in tests with ethofumesate).

4.6 Conclusions

4.6.1 Discussion of the used methods – data and kinetic evaluations

Introducing non-standard parameters like multiple applications at different time points or application of two different substances in a standard degradation test increased the complexity of the test setting during this work package. For this reason, the following evaluation proceedings were used to allow comparability of the results in all test sets as far as possible.

For both single and multiple application tests, the total applied amount of test substance was set to 100% and the measured concentrations of the test substance at different sampling times were expressed as % of applied substance. These values were the basis for the kinetic evaluations. For the tests with multiple applications, the applied amount at 0 days (half of application rate) was set to 100% after the first application. After the second application of the substance (21 days), the sum of the test substance amount applied at the first application and at the second application was set to 100% (2 x half of the application rate). This procedure should provide a direct visual comparability of the incubation period of 21 d – 120 d with each other but also with the single applications performed within this project. Alternatively, also other approaches could have been used like e.g. taking the amount of test substance actually present at day 0 and day 21 into account and setting this to 100% in order to consider the extraction efficiency of the substances and the degradation occurred within the first 21 days leading to more realistic values. In this case, the %-values of the different settings could not be directly compared with each other, but this alternative approach would not alter the resulting DT50-value significantly.

Another aspect is the choice of the kinetics for the derivation of DT50 values. To ensure comparability of the DT50-values throughout the test sets, SFO kinetic were chosen for all tests as these were statistically acceptable (chi^2 < 15%). Nevertheless, visual evaluations indicated in some cases that the more complex models, e.g. the first-order multi compartment and the biphasic models, would be more appropriate to explain the degradation of the substance. The decision scheme, which is present in FOCUS 2014 (Chapter 7) and which is employed in regulatory praxis for the selection of trigger and modelling endpoints, would probably have the consequence, that other kinetic models (but also different models for each test) should be chosen but this would not allow comparability between the test sets performed within this project. Nevertheless, further evaluations comparing other kinetic models (e.g. pseudo SFO derived from the FOMC kinetic model) would be valuable.

4.6.2 Effect of single and multiple applications of individual substances (STEP 1)

DT50-values were calculated based on the generated data for the degradation of the individual substances pyraclostrobin and ethofumesate in the soils RefeSol 02A and LUFA 2.1 after single and multiple applications. The nominal application rates for single application were 0.667 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate for single application. For multiple applications, two times the half application rate, i.e. 2 x 0.333 mg/kg for pyraclostrobin and 2 x 0.400 mg/kg for ethofumesate, were applied. Hence, in total the same amount of test substance was applied during the two test sets. In addition, the same soil charges for the test

sets with single and multiple application were used. By this way, differences in soil microbial biomass between the two test sets can be excluded. The DT50-values are summarised in Table 148.

Table 148: Summarised DT50-values (SFO) of pyraclostrobin and ethofumesate after single and multiple application as individual substance; nominal application rates were 0.667 mg/kg (pyraclostrobin) and 0.800 mg/kg (ethofumesate) for single application and 2 x 0.333 mg/kg (pyraclostrobin) and 2 x 0.400 mg/kg (ethofumesate) for multiple application.

Test substance	Soil	DT50-values [d]		
		Single application	Multiple application (1st application 0d-21d)	Multiple application (2nd application 21d-120d)
Pyraclostrobin	RefeSol 02A	27.3	15.6	41.7
Ethofumesate	RefeSol 02A	110	31.8	117
Pyraclostrobin	LUFA 2.1	47.7	25.1	56.7
Ethofumesate	LUFA 2.1	739	304	233

Generally, a slower degradation of both substances was observed in soil LUFA 2.1 compared to RefeSol 02A. LUFA 2.1 is a sandy soil (87.5% sand) with a low organic carbon content (0.55%) whereas RefeSol 02A is a silty soil (2.3% sand) with an organic carbon content of 1.04%. Sandy soils usually cannot provide many nutrients and, therefore, microbial biomass is lower. Soil degradation tests are batch tests, therefore, microbial biomass especially in sandy soils usually decreases towards the end of incubation time of 120 days since optimal nutrient conditions cannot be maintained throughout the whole incubation period.

Comparing the calculated DT50-values of ethofumesate with the DT50-values of pyraclostrobin, it can be observed that ethofumesate is generally degraded slower than pyraclostrobin in both soils.

When comparing the DT50-values of the single application with the DT50-values of multiple applications, the DT50-value of the first application was generally lower for both substances and soils indicating that lower application rates (in this test set 0.5 x application rate) can be degraded faster compared to higher application rates (1 x application rate). For this reason, soil degradation tests are usually carried out with the highest application rate per year (according to the GAP) in order to simulate worst-case conditions. However, when a substance is applied repeatedly (in this test set 2 x half rate with an interval of 21 days), the differences in DT50-values before and after the second application (faster degradation before the second application and slower rates afterwards) were probably not only due to the difference in the application rate but also due to an intrinsic effect of the batch test design of soil degradation studies. Since the microbial biomass may decrease during the incubation period due to nutrient depletion, the microbial community may be not as active at e.g. 21 days of incubation as at the start of the test and, thus, degradation may slow down. When the applications were not carried out at the same microbial conditions, the DT50-values were not exactly comparable to each other. In addition, kinetic calculations of the first incubation phase of 0 days – 21 days are based on a limited number of results (four time points with two replicates) which may result in uncertainties for kinetic evaluations. In contrast, the incubation phase of 21 days – 120 days comprise six time points and a longer period of time. However, results indicated for pyraclostrobin, as a relatively

good degradable substance, that there was no adaptation of the microbial community to degrade pyraclostrobin faster after repeated application.

In case of ethofumesate, as a more slowly degradable substance compared to pyraclostrobin, repeated application resulted in a slightly higher DT50-value of the second application than that of the single application in soil RefeSol 02A, but the increase was within a 10% range and, therefore, the DT50 values are regarded as comparably high. In soil LUFA 2.1, the DT50-value of the second application was lower than the DT50-value of the single application and even lower than the DT50 of the first application. This result indicated that in soil LUFA 2.1 an adaptation of the microorganisms in this soil took place to degrade ethofumesate faster – even at low nutrient conditions.

In conclusion, the DT50-values of pyraclostrobin and ethofumesate in both soils after multiple application were generally lower after the first application for both soils and test substances and in a comparable range or higher after the second application exept for ethofumesate in soil LUFA 2.1 where an enhancement of degradation was observed (lower DT50-value). These results indicated that the presence of previously applied test substance may have a negative effect on the degradation in some cases.

4.6.3 Effect of single and multiple applications of substances in a mixture (STEP 2 and STEP 3)

DT50-values were calculated based on the generated data for the degradation of the substances pyraclostrobin and ethofumesate in different mixtures in soil RefeSol 02A (STEP 2). Both single and multiple applications were carried out when pyraclostrobin and ethofumesate were applied contemporaneously in each soil sample. In a further test set (also STEP 2), pyraclostrobin and ethofumesate were applied individually but time-delayed into one soil sample, and in a last step (STEP 3) pyraclostrobin and ethofumesate were applied separately to soil with an existing multiple contamination. The nominal application rates for single application were – as for STEP 1 - 0.667 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate for single application. For multiple applications, again two times the half application rate, i.e. 2 x 0.333 mg/kg for pyraclostrobin and 2 x 0.400 mg/kg for ethofumesate, were applied. Hence, in total the same amount of test substance was applied during the two test sets. The DT50-values are summarised in Table 134, Table 142 and Table 147. The results are schematically summarised in Table 149.

Table 149: Effect of application of pyraclostrobin and ethofumesate as a mixture compared to application as individual substances in soil RefeSol 02A; nominal application rates were 0.667 mg/kg (pyraclostrobin) and 0.800 mg/kg (ethofumesate) for single application and 2 x 0.333 mg/kg (pyraclostrobin) and 2 x 0.400 mg/kg (ethofumesate) for multiple application.

Test substance	Treatment	DT50 (d) and respective increase or decrease of DT50-values compared to individual component application		
		Single application	Multiple application (1st application 0d-21d)	Multiple application (2nd application 21d-120d)
Pyraclostrobin	Individual component	**27.3**	**15.6**	**41.6**
Ethofumesate	Individual component	**110**	**31.8**	**117**
Pyraclostrobin	contemporaneous applications	**30.5 (+)**	**18.9 (+)**	**24.8 -**

Test substance	Treatment	DT50 (d) and respective increase or decrease of DT50-values compared to individual component application		
Ethofumesate	contemporaneous applications	**123 (+)**	**46.5 +**	**96.8 (-)**
Pyraclostrobin	Time-delayed single application	**25.7 (-)**	(pyraclostrobin at 0d, ethofumesate at 21d)	
Pyraclostrobin	Time delayed single application	**42.7 (+)**	(ethofumesate at 0d, pyraclostrobin at 21d)	
Ethofumesate	Time delayed single application	**103 (-)**	(ethofumesate at 0d, pyraclostrobin at 21d)	
Ethofumesate	Time delayed single application	**159 +**	(pyraclostrobin at 0d, ethofumesate at 21d)	
Pyraclostrobin	Single application to soil with existing contamination	**20 (-)**		
Ethofumesate	Single application to soil with existing contamination	**53.3 -**		

\+ increase (higher DT50-values compared to the application of the respective individual substance)
(+) DT50-values in a comparable range but slightly higher
decrease (lower DT50-values compared to the application of the respective individual substance)
(-) DT50-values in a comparable range but slightly lower

As can be seen in Table 149, both increase and decrease of DT50-values can be observed when pyraclostrobin and ethofumesate are applied as a mixture compared to the application as an individual substance.

Regarding pyraclostrobin, the presence of ethofumesate (within a mixture or already present on the soil) did not have a consistent effect on degradation of pyraclostrobin. In the case pyraclostrobin was applied within a mixture with ethofumesate as single application, the calculated DT50-values were slightly higher compared to the single application of exclusively pyraclostrobin. Statistical evaluation, though, was not possible and, therefore, the calculated DT50-values of the two test sets were regarded as comparably high. When pyraclostrobin is applied together with ethofumesate in a multiple application test set, the DT50-value of pyraclostrobin after the first application (incubation time of 0d-21d) was slightly higher (18.9 days versus 15.6 days) indicating that the presence of ethofumesate might have a negative effect on the degradation of pyraclostrobin. However, the DT50-value of pyraclostrobin after the second application (incubation time of 21d-120d) was considerably lower (24.8 days) when applied in a mixture with ethofumesate compared to the multiple application as individual substance (47.1 days). This result showed an enhancing effect of ethofumesate on pyraclostrobin degradation when concurrently applied.

In the case of a time-delayed application, ethofumesate did not show a considerable effect on pyraclostrobin degradation when applied as second substance at 21 days of incubation. In contrast, the DT50-value of pyraclostrobin (42.7 days) was higher when applied as second substance (at 21 days) and ethofumesate was already present in the soil compared to the single application as individual component (27.3 days), but this retarded degradation of pyraclostrobin could not be attributed definitively to the presence of ethofumesate (see section 4.4.2.4 for details).

In addition, existing contaminants additionally present in the soil did not affect the degradation rate of pyraclostrobin in soil adversely.

For ethofumesate, a similar pattern can be observed as for pyraclostrobin. In fact, slightly increased DT50-values were observed when ethofumesate was applied in a mixture with pyraclostrobin during single application which was regarded – as for pyraclostrobin - as comparably high since a statistical evaluation was not possible. After the first application of the multiple application of both substances (incubation time of 0d-21d), the DT50-value of ethofumesate was considerably increased indicating that the presence of pyraclostrobin had a negative impact on the degradation rate of ethofumesate. However, this effect could not be observed for the second application of the two substances within the multiple application test (incubation period of 21d-120d). In fact, degradation of ethofumesate was faster when it was applied together with pyraclostrobin at 21 days (96.8 days compared to 117 days when applied as individual substance).

Another case where the DT50-value of ethofumesate increased and, hence, degradation was slower, was when pyraclostrobin was applied first and ethofumesate was applied time-delayed after 21 days. This effect was also observed vice versa for pyraclostrobin when applied time-delayed as second substance but could not be attributed unambiguously to ethofumesate. However, in case of ethofumesate as second substance, the results showed clearly that the reason for the increased DT50-value of ethofumesate should have been the presence of pyraclostrobin already in the soil. A possible interpretation of this result could be that the substance applied first to the soil might lead to the development of a microbial community being more specialised to use the first substance as a substrate resulting in a different microbial composition and, hence, a selected degradation activity of the soil microbial community to the first substrate. As a result, the time-delayed application of a second substance might have to induce a conversion of the microbial community which might need more time.

As for pyraclostrobin, existing contaminants additionally present in the soil did not affect the degradation rate of ethofumesate in soil adversely and, in contrast, might have enhanced the soil degradation.

The results of the tests indicated that an effect of the different mixtures of pyraclostrobin and ethofumesate on the soil degradation was observed during the study, but that this effect could be negative or positive depending on different factors. Results still demonstrate that the determination of degradation rates from individual components and single application testing does not reflect the actual degradation rates in all cases that would occur in field after normal agricultural praxis, indicating a deviation between endpoints used in the regulatory practise and what actually happens to PPP applied in the field.

5 Conclusions and recommendations

5.1 Effects on function and structure of soil microorganisms

Chapter 2 focused on assessing the risks posed by plant protection products, biocides and veterinary pharmaceuticals for soil microorganisms with the aim to compare five additional test methods to the current approach based on OECD TG 216 (N-transformation): ISO 15685 on potential nitrification, MicroResp™ on basal and substrate induced respiration, ISO 20130 on enzymatic activity, ISO 10832 on the spore germination of AMF and the fingerprinting method ARISA on the microbial community structure.

Based on six test substances and three soils the following main results were obtained:

- The N-Transformation test (OECD 216) was often less sensitive and the lowest observed effect concentration (LOEC) of alternative methods was found to be up to a factor of 100 lower.
- The potential nitrification test (ISO 15685) showed no relevant differences to the currently used N-Transformation test (OECD 216), although if in a sandy soil (LUFA 2.1) the effects were stronger than in the N-Transformation limit test (OECD 216).
- The microbial respiration test (MicroRespTM) showed the lowest variability of controls and the lowest minimum detectable differences. However, it often did not provide the lowest LOEC values and therefore was therefore not the most sensitive test system.
- The enzymatic activity test (ISO 20130) most often gave the lowest LOEC and was therefore the most sensitive test method, sometimes indicating long-term effects (no recovery to deviations < 25 % within 84 days). It was often the only method for functional endpoints that gave the lowest LOEC.
- The ARISA data evaluated using ordination plots revealed often gave the same low estimated LOECs as the enzymatic activity test (ISO 20130), but was less often the only approach resulting in the lowest LOEC. However, due to the aim of testing six test items in three soils, only a limited number of test concentrations and replicates could be tested and statistical testing was not always possible.
- The Spore gemination test on Arbuscular mycorrhiza fungi (AMF) (ISO 10832), conducted with the species *Funneliformis mosseae* was tested in one natural soil. Test results were never among the methods with the lowest LOECs. The results of the project indicate that the existing guideline for spore germination testing of AMF (ISO 10832) needs to be revised if natural soils are used as test substrate. Following adaptations should be considered:
  - The establishment of other AMF species (e.g. *Rhizophagus irregularis*), which could be used in the spore germination test and which may be a more sensitive species, should be considered.
  - For *F. mosseae*, the maximum water holding capacity of the soil has to be reduced from 90%, as indicated in the guideline, to about 50%, if natural soils are used as test substrate. However, this value probably may need to be adjusted depending on the characteristics of the natural soil intended to be used for testing.

- Soils used for regulatory testing with a pH below 5 (e.g.; Lufa 2.1) were not suitable for testing *F. mosseae*. The soil pH of natural test soils should be above pH 5.
- Increasing the temperature improves the spore germination of *F. mosseae*. Using the upper end of the permitted range of 24 ± 2 °C according to ISO 10832 improved the spore germination in RefeSol 02A.

- Of the three natural soils tested, RefeSol 02A, the soil with the lowest sand content, the highest pH value, but a lower organic carbon content compared to e.g. RefeSol 04A or Lufa 2.1, showed the lowest effects for the five tested methods due to the application of the exemplarily used six active substances.

The framework of ecotoxicological testing of microorganisms differs in many respects from other areas in environmental risk assessment, such as soil organisms, birds and mammals, or aquatic organisms. Test systems contain intact communities of soil microorganisms and deliver either information on their functional (as described in the above section 2.1) or on their structural state. This poses a fundamental aspect of contextualizing them in a tiered ERA approach. The ranking criterion *complexity of a test system* drops out.

From these results the following preliminary conclusions are drawn:

- Microbial respiration (MicroRespTM) and potential nitrification (ISO 15685) were less sensitive than the other tested, functional endpoints. Enzymatic activity (ISO 20130) was the most sensitive test method indicating long-term effects where N-transformation (OECD 216) was not. Therefore, it is proposed to complement OECD 216 by ISO 20130 in the first step of the risk assessment to cover a broad set of soil microbial functions.
- Spore gemination of AMF (ISO 10832) was more sensitive than N-Transformation (OECD 216) but less sensitive than enzymatic activity (ISO 20130). Thus, a test with AMF is also proposed for lower tier testing. A revision of the method is required in advance of its implementation within the risk assessment framework.
- Soil type affects the test results. LUFA 2.1 was not always the soil resulting in the lowest endpoints.
- The ARISA method was the only technique tested in the project specifically addressing microbial community structure and, by extension, the biodiversity. While ARISA revealed impacts on biodiversity, the limited available data do not allow for definitive conclusions regarding the long-term implications of these changes. Therefore, its recommendation as a routine test should be further evaluated and supported by additional data, studies and agreements for evaluation of results and thresholds needs to be set for the derivation of endpoints. Moreover, during the three-year project duration, the costs of microbiome metabarcoding and metagenomics have significantly decreased, making these techniques more feasible for routine use. Now, for a slightly higher cost in relation to the costs for ARISA, these methods offer greater taxonomic and functional resolution compared to ARISA. However, the turnaround time and the lack of standardization in sequencing methods as well as test evaluation remain limitations that need to be addressed in future.

In the current risk assessment for plant protection products, biocides and veterinary pharmaceuticals the microbial community in soils and its ecosystem services are considered to be not at risk, if effects of the maximum PEC in-field on the Nitrogen transformation (OECD TG 216) above 25 % are restricted to less than 100 days. Based on the results obtained here and considering the recommendations of the EFSA PPR panel (2017), it is recommended to

supplement the Tier 1 assessment by an additional test on bacterial function and a test covering effects on arbuscular mycorrhizal fungi. Due to its higher sensitivity found for the substances tested within the project, the ISO 20130 is preferred over MicroResp™. In contrast to EFSA PPR panel (2017) we do not consider additional functional tests (providing additional endpoints) to be a useful Tier 2 option because indicated risk by e. g. OECD 216 is not refined by testing other functions. Instead, refined exposure could be tested with additional soils, e.g. natural soils sampled in agricultural fields as refinement option. At Tier 3 and 4 artificial communities in the laboratory or natural communities in field test (with known structural composition, e.g. by microbiome sequencing) can be tested. The latter ones would serve as the reference tier for calibrating or validating lower tiers. Without a comparison with a reference tier it is not clear whether the current assessment (based only on OECD TG 216) is sufficiently protective.

5.2 Antibiotic resistance

Using soil-relevant bacteria for Minimum Inhibitory Concentration (MIC) determination is an important step in understanding the effectiveness of antimicrobial agents in environments that are ecologically relevant. MIC variability among the four tested bacterial strains spanned up to two orders of magnitude, highlighting differences in sensitivity across species. No single strain was the most sensitive; however, *Acidovorax facilis* demonstrated the *lowest MICs* for two of the four antibiotics, whereas while *Arthrobacter* sp. was never the most sensitive. This variability underscores the importance of using multiple strains to capture the range of responses in soil bacteria.

The minimum MICs observed for the four antibiotics tested were at least two orders of magnitude lower than those for the two other substances, $CuSO_4$ and TWEEN 20. The lowest MIC found was 0.06 mg/L for chlortetracycline hydrochloride. However, due to missing data on K_{OC} or PEC_{soil}, risk quotients could not be calculated for most substances, except for tiamulin fumarate. For this antibiotic, the MIC indicated a higher risk than the standard ecotoxicological tests (RQ > 100), suggesting that traditional test may underestimate the environmental impact of some antibiotics on soil microorganisms. Generally, these results suggest that the tested soil bacteria have higher MIC values compared to clinical strains, indicating lower susceptibility or greater resistance in soil environments.

Conclusions: The species sensitivity distribution (SSD) analysis showed that one strain, *Acidovorax facilis,* was more sensitive to one of the antibiotics (Chlortetracycline) than the clinically relevant strains. This finding emphasizes the need for ecologically relevant testing in environmental risk assessments of antimicrobial agents, as soil bacteria may exhibit unique resistance profiles compared to clinical isolates.

5.3 Degradation of PPP in soil

Actually, the degradability of active substances used in plant protection products is estimated in laboratory tests with standardized soils and one maximum application rate. In chapter 4, it was analysed whether multiple applications (STEP 1), binary mixtures (STEP 2) and background contamination (STEP 3) affect the degradation time (expressed as DT50 values).

Soil degradation tests based on OECD 307 were carried out using the two test substances pyraclostrobin (fungicide) and ethofumesate (herbicide) in two soils RefeSol 02A (silt loam, STEP 1-2) and LUFA 2.1 (sand, STEP 1) as well as in a soil with a field background contaminantion (STEP 3) freshly sampled at the original sampling location of RefeSol 02A. Two test concentrations for both substances were chosen and were consistent throughout the degradation tests. One test concentration level reflected the maximum application rate for the

two active substances (0.667 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate) being used for single applications. The second test concentration level was half the maximum application rate and was applied twice for multiple applications (2 x 0.333 mg/kg for pyraclostrobin and 2 x 0.400 mg/kg for ethofumesate) and, hence, resulting in total in the same concentration level used for single applications. The following main results were obtained:

- Degradation times of both substances applied as single application were higher in LUFA 2.1. compared to degradation times in Refesol 02A, depending on the characteristic of the tested substance as well as used test soil. DT50-values of pyraclostrobin were about twice as high in Lufa 2.1 compared to DT50-values observed in Refesol 02A, whereas DT50-values of ethofumesate were about seven times higher in LUFA 2.1 compared to Refesol 02A. Observed differences in degradation times between different representative soils within this project are in line with the current state of knowledge. Even if existing differences in degradation times between soils are well known, they are not fully reflected by the current risk assessment scheme for microorganisms exposed to chemicals. For example, the current risk assessment framework for microorganisms exposed to pesticides does not include any assessment factor for the comparison of ecotoxicological effects with the predicted environmental concentration, which is currently based on the worst case degradation time of the tested soils. According to the EU Regulation (No. 283/2013) setting out the data requirements for active substances, studies on the rate of aerobic degradation of an active substance should be reported for at least four different soils. The soils used for the derivation of the DegT50 and DegT90 are not defined and can be chosen freely by applicants. Testing of a 'worst case soil' is not required due to the current framework. The new guidance document for predicting environmental concentrations in soil (EFSA PPR Panel 2017) changes this approach: in future, geometric mean values of the tested soils are used for the derivation of the degradation time endpoint for PECsoil calculations. To ensure a sufficient protective risk assessment framework, worst-case soils should be included in experiments observing the degradation time and assessment factors for soil microorganisms exposed to pesticides should be adapted to reflect the differences in half-lifes of active substances occurring in different soils. Further research is needed to derive an adapted assessment factor as the work conducted within this project can only be seen as precursor study.

- Single applications of the two active substances pyraclostrobin and ethofumesat individually applied to both soils were compared to multiple applications (i.e. two successive applications) of the same substance with an interval of 21 days. The effect of multiple application on the degradation half-life did not show a uniform picture: DT50-values of pyraclostrobin were higher in both soils after the second application, whereas the DT50-values for ethofumesat were comparable in soil Refesol 02A but were lower in soil Lufa 2.1 after multiple application. These results indicated that the presence of the test substance may have a negative effect on its further degradation when applied for a second time.

- The effect of binary mixtures on the soil degradation of pyraclostrobin and ethofumesate was investigated using single and multiple applications of both substances at the same time as well as single applications of both substances individually but time-delayed into the same soil (RefeSol 02A). The results showed that the presence of a second substance (within a mixture or already present on the soil) did not have a consistent effect on degradation of the given substance in the binary mixture. However, a similar pattern could be observed for pyraclostrobin and ethofumesate. For single application, degradation times of pyraclostrobin and ethofumesat were slightly prolonged, if applied as mixture, but the

difference was small so it was regarded as comparably high since a statistical evaluation was not possible. For multiple application of the mixture of both substances, degradation of both substances was slower after the first applicaiton (0 – 21d), but degradation of both substances was faster after the second application (21 – 120d). For time-delayed applications, the application of a substance after 21 days with a second substance already present in soil led to an increase of the half-life of – at least - ethofumesate. This effect could indicate that the microbial community probably adapt to the use of the substance firstly present in the soil and the addition of a second substance may lead to another adaptation phase which also affects the microorganisms already degrading the first substance.

- Obtained DT50-values for soil degradation tests using single applications of pyraclostrobin and ethofumesate individually applied to a soil with background contaminations due to normal agricultural praxis were lower compared to the results for soil degradation tests using single applicatons of pyraclostrobin and ethofumesat in untreated soil RefeSol 02A.

Soil degradation studies according to OECD 307 are usually carried out to determine degradation rates of active substances which are used for the estimation of the predicted environmental concentration of an intended use of chemicals like plant protection products. Usually, degradation tests are performed with a set of different soils and the respective worst-case value of the tested soils is used for the estimation of the predicted environmental concentration in soil which is compared to effect values for test organisms like microorganisms within the risk assessment of chemicals.

Currently, the risk assessment for chemicals like plant protection products is performed for single substances based on the intended use of one plant production product. However, up to 25 plant protection products, containing up to 4 active subsances, are applied within one growing season (Knillmann et al. 2021). A recent monitoring study conducted with agricultural soils of 10 European countries confirms that the application of spray series leads to the presence of mixtures with up to 21 active substances in one soil sample (Knuth et al. 2024). However, the occurance of multiple residues in agricultural soils is not reflected by the current risk assessment schemes for chemicals.

The results of the MICROSOIL project show that multiple application of the same substance as well as the presence of another substance may have an effect on the degradation time of the tested substances: Both positive and negative effects on the degradation rate of the test substances used in this study were observed. However, a negative impact on the degradation rate was found, for instance, for Pyraclostrobin when applied for a second time during multiple application testing or for ethofumesate when applied to a soil where a second substance (here: pyraclostrobin) was already present.

The results of this project are specific for the used test substances pyrclostrobin and ethofumesate as well as for the tested soils RefeSol 02A and Lufa 2.1. Further research, reflecting real application patterns of plant protection products as well as fertilizing practices including residues of biocides, industry chemicals and pharmaceuticals is needed to determine the actual and correct degradation time of substances which are used for the estimation of the predicted environmental concentration of substances. The estimation of predicted environmental concentrations of actives substances should acknowledge the presence of a variety of soils in Europe as well as the occurrence of multiple applications and mixture residues in agricultural used soils in order to ensure a sufficient protection of the environment.

5.4 Outlook

The MICROSOIL project underlines the necessity of the European commission's mandate to update the Guidance document on terrestrial ecotoxicology (EFSA, 2025). Witin this project, an updated risk assessment scheme is suggested for the risk assessment for in-soil microorganisms, exposed to plant protection products. The currently used test for nitrification (OECD 216) is not sensitive, if compared to other functional tests, especially the test for enzymatic activity (ISO 20130). The latter should be integrated in the first-tier risk assessment for in-soil microorganisms, as well a standard test on AMF, as effects soil fungi, specifically arbuscular mycorrhiza fungi, is not considered in the actual risk assessment at all. In relation to the risk assessment for plant protection products and in accordance to EFSA PPR panel (2017), a tiered approach is suggested for the revision of the risk assessment scheme including refined exposure, additional soils, e.g. natural soils sampled in agricultural fields as well as additional AMF species as refinement option. Effects on communities of microorgisms (e.g.; by ARISA or microbiome sequencing) should be included, if the first-tier risk assessment failed.

Other regulatory frameworks, like the risk assessment scheme for veterinary pharmaceuticals, might also need an update, if effects on in-soil microorganisms are evaluated. The outcome of this projects shows effects of pharmaceuticals on the antibiotic restistance of environmentally relevant bacterial stains, which is not covered by the current risk assessment scheme, at the moment.

Moreover, the estimation of the predicted environmental concentrations should acknowledge the occurance of different soil types in the agricultural landscape as well as multiple applications and spray series to allow a precise and realistic risk assessment for in-soil microorganisms, exposed to chemicals.

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A Appendix 1 - Literature search (work package 1)

Table 150: Summary of used terms and the resulting hits within the literature search.

Focus	Term	Year span	Result/Hits per year span[1]
General terms	Soil structure and function	2000 – 2021 2010 – 2021	~ 151.000 109.000
	Soil microorganisms function structure	2000 – 2021	~ 27.000
	Soil quality assessment	2000 – 2021 2010 – 2021	~ 125.000 ~ 76.000
	Ecosystem services soil microorganisms	2000 – 2021 2010 – 2021	~ 125.000 ~ 76.000
Methods	Soil structural test methods	2000 – 2021 2010 – 2021	~ 55.600 27.200
	Soil microbial community	2000 – 2021 2010 – 2021	~ 77.000 ~ 42.300
	Soil microbial community shift	2000 – 2021	~ 17.200
	Soil function test methods	2000 – 2021 2010 – 2021	~ 88.600 49.900
	Microbial diversity functional methods soil	2000 – 2021	~ 35.100
	ISO soil quality methods	2000 – 2021	11.900
	Soil microbial microorganism assay	2000 – 2021	~ 16.500
	Microplate assay soil microorganisms	2000 – 2021	~ 893
	Mycorrhiza test methods / assay	2000 – 2021	~ 2.360
	Soil ecology test method / assay	2000 – 2021	~ 13.100
	Effect soil enzymes assay test method	2000 – 2021	~ 19.200
Effect studies	Effect pesticides soil microbes/ soil microorganisms/ soil / mycorrhiza / fungi	2010 – 2021	~ 7.040 – 17.500
	Effect antibiotics soil microbes / soil microorganisms / soil / mycorrhiza / fungi	2010 – 2021	~ 487 – 4.930
	Effect biocides soil microbes / soil microorganisms / soil / mycorrhiza / fungi	2010 – 2021	~ 2.340 – 10.600
	Effect herbicides soil microbes / soil microorganisms / soil / mycorrhiza / fungi	2010 – 2021	~ 3.210 – 9.970
	Effect fungicides soil microbes / soil microorganisms / soil / mycorrhiza / fungi	2010 – 2021	~ 3.210 – 9.770
	Effect insecticides soil microbes / soil microorganisms / soil / mycorrhiza / fungi	2010 – 2021	~ 1.620 – 10.200
	Effect mycorrhiza soil	2000 – 2021	~ 14.300

[1] The range between years was adjusted for individual searches due to the high number of hits.

Table 151: Summary of available methods found in the literature search.

Method/Guideline	Full name	Source	Reference number in chapter 1.1.3
OECD 217	Test No. 217: Soil Microorganisms: Carbon Transformation Test	OECD Library	I
ISO 14240-1/2	Determination of soil microbial biomass – Part 1: substrate-induced respiration method Part 2: fumigation-extraction method	Römbke et al. (2018)	II + III
ISO 16072	Laboratory methods for determination of microbial soil respiration	Römbke et al. (2018)	IV
ISO 17155	Determination of abundance and activity of soil microflora using respiration curves.	Römbke et al. (2018)	V
Biolog®	Microbial Community Analysis with EcoPlates	Garland and Mills (1991), Imfeld and Vuilleumier (2012), Blagodatskaya and Kuzyakov (2013), Brandt et al. (2015); https://www.biolog.com	1
MicroResp™	MicroResp™ is a unique microplate-based respiration system that enables the user to analyse up to 96 soil, sediment or water samples and test a range of carbon sources and/or replicates in a small compact space.	Campbell et al. (2003), Chapman et al. (2007), Imfeld and Vuilleumier (2012), Blagodatskaya and Kuzyakov (2013), Creamer et al. (2015), Brandt et al. (2015), Hund.Rinke et al. (2019); https://www.microresp.com	2
Fe(III) reduction test	Microbial inhibition by pharmaceutical antibiotics in different soils--dose-response relations determined with the iron(III) reduction test	Thiele-Bruhn (2005); Brandt et al. (2015)	3
OECD 216	Test No. 216: Soil Microorganisms: Nitrogen Transformation Test	OECD library	i
ISO 14238	Determination of N mineralization and nitrification in soils and the influence of chemicals on these processes	Römbke et al. (2018)	ii

Method/Guideline	Full name	Source	Reference number in chapter 1.1.3
ISO 15685	Soil quality — Determination of potential nitrification and inhibition of nitrification — Rapid test by ammonium oxidation	Brandt et al. (2015), Römbke et al. (2018) and Thiele-Bruhn et al. (2020)	4
ISO 20131-1	Soil quality — Easy laboratory assessments of soil denitrification, a process source of N_2O emissions — Part 1: Soil denitrifying enzymes activities	Römbke et al. (2018)	5
ISO 20131-2	Soil quality — Easy laboratory assessments of soil denitrification, a process source of N_2O emissions — Part 2: Assessment of the capacity of soils to reduce N_2O	Römbke et al. (2018)	6
ISO 20130	Soil quality — Measurement of enzyme activity patterns in soil samples using colorimetric substrates in micro-well plates. Determination of : arylamidase, arylsulfatase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-glucosaminidase, acid, alkaline and global phosphatases, urease	Römbke et al. (2018)	7
ISO 22939	Soil quality — Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates. Determination of : arylsulfatase, α −glucosidase, β -glucosidase, Cellubisidase, β -Xylosidase, phosphodiesterase (PDE), chitinase, phosphomonoesterase (PME), leucine-aminopeptidase, Alanine-aminopeptidase	Rhia et al. (2014), Giacometti et al. (2014), Brandt et al. (2015), Römbke et al. (2018)	8
ISO 18187	Soil quality — Contact test for solid samples using the	Brandt et al. (2015)	9

Method/Guideline	Full name	Source	Reference number in chapter 1.1.3
	dehydrogenase activity of *Arthrobacter globiformis*		
ISO 23753-1	Soil quality — Determination of dehydrogenases activity in soils — Part 1: Method using triphenyltetrazolium chloride (TTC)	Römbke et al. (2018)	10
ISO 23753-2	Soil quality — Determination of dehydrogenases activity in soils — Part 2: Method using iodotetrazolium chloride (INT)	Brandt et al. (2015), Römbke et al. (2018)	11
Urease	Urease activity in soils	Lloyd and Sheaffe (1973); May and Douglas (1976); Bodenbiologische Arbeitsmethoden 2. Auflage	12
2,2'-azinobis-(-3 ethylbenzothiazoline-6-sulfononic acid) diammonium salt (ABTS) assay	ABTS assay of phenol oxidase activitiy in soil	Floch et al. (2007)	13
Peroxidase	Measuring phenol oxidase and peroxidase activities with pyrogallol, l-dopa, and abts: Effect of assay conditions and soil type.	Sinsabaugh (2010), Bach et al. (2013)	14
Fluorescein diacetate assay (FDA)	Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (fda) in a range of soils.	Adam and Duncan (2001), Rhia et al. (2014), Bodenbiologische Arbeitsmethoden 2. Auflage	15
ISO 10832	Soil quality — Effects of pollutants on mycorrhizal fungi — Spore germination test	Römbke et al. (2018), Mallmann et al. (2018), Hannula et al. (2016)	16
Laccase	Laccase activity in soils: Considerations for the measurement of enzyme activity.	Eichlerova et al. (2012), Thiele-Bruhn et al. (2020)	17
ISO 17601	Soil quality — Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil	Römbke et al. (2018)	18

Method/Guideline	Full name	Source	Reference number in chapter 1.1.3
ISO 29843-1/2	Soil quality — Determination of soil microbial diversity — Part 1: Method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis Soil quality — Determination of soil microbial diversity — Part 2: Method by phospholipid fatty acid analysis (PLFA) using the simple PLFA extraction method	Blagodatskaya and Kuzyakov (2013), Brandt et al. (2015), Römbke et al. (2018),	19
DGGE	New and current microbiological tools for ecosystem ecologists: Towards a goal of linking structure and function.	Drenovsky et al. (2008); Imfeld and Vuilleumier (2012), Blagodatskaya and Kuzyakov (2013)	20
T-RFLP	T-RFLP analysis.	Liesack and Dunfield (2004), Drenovsky et al. (2008); Imfeld and Vuilleumier (2012), Blagodatskaya and Kuzyakov (2013)	21
ARISA	New and current microbiological tools for ecosystem ecologists: Towards a goal of linking structure and function.	Drenovsky et al. (2008)	22
OECD 56	Guidance document on the breakdown of organic matter in litter bags	Römbke et al. (2018)	23

B Appendix 2 - Exemplary testing of methods (work package 2)

Table 152: Results of the spore germination test with ethofumesate after 14 days of incubation at 25.5 °C and with a WHC_{max} of 50 %. Mean: arithmetic mean; Std.Dev.: standard deviation; CV: coefficient of variation

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
0	1	30	29	96.7
	2	30	22	73.3
	3	30	28	93.3
	4	30	25	83.3
	5	29	28	96.6
	6	29	24	82.8
			Mean	87.7
			Std.Dev.	9.38
			CV [%]	10.7
2	1	30	25	83.3
	2	29	26	89.7
	3	29	25	86.2
	4	29	23	79.3
	5	30	23	76.7
	6	30	23	76.7
			Mean	82.0
			Std.Dev.	5.33
			CV [%]	6.51
			Inhibition [%]	7.92
20	1	30	26	86.7
	2	30	24	80.0
	3	29	15	51.7
	4	30	23	76.7
	5	30	17	56.7

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
	6	29	22	75.9
			Mean	71.3
			Std.Dev.	13.9
			CV [%]	19.4
			Inhibition [%]	17.8

Table 153: Results of the spore germination test with tebuconazole after 14 days of incubation at 25.5 °C and with a WHC_{max} of 50 %. Mean: arithmetic mean; Std.Dev.: standard deviation; CV: coefficient of variation

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
0	1	30	28	93.3
	2	29	26	89.7
	3	29	24	82.8
	4	29	21	72.4
	5	30	25	83.3
	6	29	25	86.2
			Mean	84.6
			Std.Dev.	7.18
			CV [%]	8.49
1	1	28	25	89.3
	2	30	23	76.7
	3	29	22	75.9
	4	29	19	65.5
	5	30	24	80.0
	6	30	26	86.7
			Mean	79.0
			Std.Dev.	8.52

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
			CV [%]	10.8
			Inhibition [%]	6.31
10	1	30	24	80.0
	2	30	25	83.3
	3	26	21	80.8
	4	30	21	70.0
	5	30	22	73.3
	6	29	26	89.7
			Mean	79.5
			Std.Dev.	7.04
			CV [%]	8.86
			Inhibition [%]	5.91

Table 154: Results of the spore germination test with pyraclostrobin after 14 days of incubation at 25.5 °C and with a WHC_{max} of 50 %. Mean: arithmetic mean; Std.Dev.: standard deviation; CV: coefficient of variation

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
0	1	30	24	80.0
	2	30	24	80.0
	3	30	26	86.7
	4	30	21	70.0
	5	30	21	70.0
	6	28	21	75.0
			Mean	76.9
			Std.Dev.	6.53
			CV [%]	8.49
3	1	29	22	75.9

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
	2	29	28	96.6
	3	27	16	59.3
	4	30	23	76.7
	5	28	21	75.0
	6	30	24	80.0
			Mean	77.2
			Std.Dev.	11.9
			CV [%]	15.4
			Inhibition [%]	-1.55
30	1	30	20	66.7
	2	29	23	79.3
	3	30	23	76.7
	4	29	14	48.3
	5	29	15	51.7
	6	29	17	58.6
			Mean	63.5
			Std.Dev.	12.9
			CV [%]	20.3
			Inhibition [%]	13.6

Table 155: Results of the spore germination test with propamocarb hydrochloride after 14 days of incubation at 25.5 °C and with a WHC_{max} of 50 %. Mean: arithmetic mean; Std.Dev.: standard deviation; CV: coefficient of variation

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
0	1	30	27	90.0
	2	29	25	86.2
	3	29	28	96.6

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
	4	29	25	86.2
	5	30	27	90.0
	6	30	24	80.0
			Mean	88.2
			Std.Dev.	5.50
			CV [%]	6.24
3	1	29	23	79.3
	2	29	17	58.6
	3	30	23	76.7
	4	30	26	86.7
	5	30	27	90.0
	6	30	25	83.3
			Mean	79.1
			Std.Dev.	11.1
			CV [%]	14.1
			Inhibition [%]	9.99
30	1	30	25	83.3
	2	30	28	93.3
	3	30	23	76.7
	4	30	25	83.3
	5	30	27	90.0
	6	28	21	75.0
			Mean	83.6
			Std.Dev.	7.18
			CV [%]	8.59
			Inhibition [%]	5.38

Table 156: Results of the spore germination test with Tiamulin hydrogen fumarate after 14 days of incubation at 25.5 °C and with a WHC_{max} of 50 %. Mean: arithmetic mean; Std.Dev.: standard deviation; CV: coefficient of variation

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
0	1	26	25	96.2
	2	29	26	89.7
	3	25	24	96.0
	4	26	24	92.3
	5	25	23	92.0
	6	26	20	76.9
			Mean	90.5
			Std.Dev.	7.11
			CV [%]	7.86
0.36	1	27	26	96.3
	2	30	29	96.7
	3	28	25	89.3
	4	29	27	93.1
	5	29	28	96.6
	6	18	11 c	61.1
			Mean	94.4
			Std.Dev.	3.21
			CV [%]	3.40
			Inhibition [%]	-5.23
3.6	1	32*	18	56.3
	2	30	21	70.0
	3	30	19	63.3
	4	28	17	60.7
	5	27	20	74.1
	6	28	22	78.6

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
			Mean	67.2
			Std.Dev.	8.50
			CV [%]	12.7
			Inhibition [%]	29.9

*Note: More than the 30 spores were found, which can be explained by a static charge causing the spores to jump on the filter when separating them with a cannula. Spores at the edge of a filter can quickly be overlooked and too many spores may have been used. In the calculations, the 32 spores were set as 100 % and should not be considered in further calculations.

Table 157: Results of the 1st spore germination test with DDAC after 14 days of incubation at 25.5 °C and with a WHC_{max} of 50 %. Mean: arithmetic mean; Std.Dev.: standard deviation; CV: coefficient of variation

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
0	1	29	24	82.8
	2	29	23	79.3
	3	30	25	83.3
	4	30	23	76.7
	5	28	24	85.7
	6	27	19	70.4
			Mean	79.7
			Std.Dev.	5.57
			CV [%]	6.99
3	1	22	14	63.6
	2	30	22	73.3
	3	28	12	42.9
	4	29	21	72.4
	5	29	16	55.2
	6	28	21	75.0
			Mean	63.7

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
			Std.Dev.	12.7
			CV [%]	19.9
			Inhibition [%]	16.1
300	1	27	20	74.1
	2	30	25	83.3
	3	29	18	62.1
	4	29	24	82.8
	5	29	25	86.2
	6	28	19	67.9
			Mean	76.0
			Std.Dev.	9.67
			CV [%]	12.7
			Inhibition [%]	3.64

Table 158: Results of the 2nd spore germination test with DDAC after 14 days of incubation at 25.5 °C and with a WHC_{max} of 50 %. Mean: arithmetic mean; Std.Dev.: standard deviation; CV: coefficient of variation

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
0	1	29	24	82.8
	2	30	17	56.7
	3	30	22	73.3
	4	27	20	74.1
	5	30	15	50.0
	6	27	19	70.4
			Mean	67.9
			Std.Dev.	12.2
			CV [%]	17.9

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
3	1	30	16	53.3
	2	26	11	42.3
	3	30	20	66.7
	4	26	21	80.8
	5	30	19	63.3
	6	30	20	66.7
			Mean	62.2
			Std.Dev.	13.1
			CV [%]	21.1
			Inhibition [%]	6.26
300	1	25	17	68.0
	2	30	22	73.3
	3	30	19	63.3
	4	30	13	43.3
	5	29	19	65.5
	6	29	24	82.8
			Mean	66.0
			Std.Dev.	13.1
			CV [%]	19.9
			Inhibition [%]	2.02

Table 159: Results of the spore germination test with tebuconazole after 14 days of incubation at 25.5 °C and with a WHC_{max} of 50 % using RefeSol 04A. Mean: arithmetic mean; Std.Dev.: standard deviation.

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
0	1	14	0	0.00
	2	25	0	0.00

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
	3	30	3	10.0
	4	16	2	12.5
	5	23	1	4.35
	6	18	2	11.1
			Mean	6.33
			Std.Dev.	5.63
			CV [%]	89.0
1	1	18	8	44.4
	2	14	1	7.14
	3	13	1	7.69
	4	27	1	3.70
	5	26	0	0.00
	6	30	0	0.00
			Mean	10.5
			Std.Dev.	17.0
			CV [%]	162
10	1	30	4	13.3
	2	30	0	0.00
	3	28	1	3.57
	4	31	3	9.68
	5	28	2	7.14
	6	27	9	33.3
			Mean	11.2
			Std.Dev.	11.8
			CV [%]	106

Table 160: Results of the spore germination test with pyraclostrobin after 14 days of incubation at 25.5 °C and with a WHC_{max} of 50 % using RefeSol 04A. Mean: arithmetic mean; Std.Dev.: standard deviation.

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
0	1	14	0	0.00
	2	25	0	0.00
	3	30	3	10.0
	4	16	2	12.5
	5	23	1	4.35
	6	18	2	11.1
			Mean	13.6
			Std.Dev.	11.0
			CV [%]	89.0
3	1	28	4	14.3
	2	28	2	7.14
	3	22	2	9.09
	4	24	5	20.8
	5	29	2	6.90
	6	28	1	3.57
			Mean	10.3
			Std.Dev.	6.25
			CV [%]	60.6
30	1	29	10	34.5
	2	28	8	28.6
	3	29	3	10.3
	4	25	6	24.0
	5	18	3	16.7
	6	18	4	22.2
			Mean	22.7

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
			Std.Dev.	8.54
			CV [%]	37.6

Table 161: Results of the spore germination test with tiamulin hydrogen fumarate after 14 days of incubation at 25.5 °C and with a WHC_{max} of 50 % using RefeSol 04A. Mean: arithmetic mean; Std.Dev.: standard deviation.

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
0	1	30	0	0.00
	2	30	5	16.7
	3	29	4	13.8
	4	27	1	3.70
	5	30	5	16.7
	6	29	9	31.0
			Mean	13.6
			Std.Dev.	11.0
			CV [%]	80.7
0.36	1	30	4	13.3
	2	29	5	17.2
	3	30	2	6.67
	4	30	9	30.0
	5	30	8	26.7
	6	27	9	33.3
			Mean	21.2
			Std.Dev.	10.4
			CV [%]	49.2
3.6	1	29	2	6.90
	2	28	9	32.1

Nominal concentration [mg a.s./kg dw soil]	Replicate number	Number of recovered spores	Number of germinated spores	Germination rate [%]
	3	30	0	0.00
	4	28	7	25.0
	5	28	4	14.3
	6	30	2	6.67
			Mean	14.2
			Std.Dev.	12.3
			CV [%]	86.5

C Appendix 3 - Calculation for MIC_{soil} from MIC_{water} using equilibrium partitioning (work package 3)

MIC_{water} values can be transformed into MSCs for soil using the equilibrium partitioning (EqP) method as used under the water framework directive (EC, 2018) and REACH (ECHA, 2008, ECHA, 2017) and also applied for the calculation of PECs for sediment in phase II CVMP guideline (EMEA/CVMP/ERA/418282/2005-Rev.1).

In this approach it is presumed that the interaction of the antimicrobial and the bacterium will take place in the pore water where also the AMR processes occur. The pMSC as determined for surface water also applies for pore water. From the pMSC for (pore)water and the K_{oc}, the associated concentration for soil can be calculated and used for the derivation of the pMSCsoil. This is considered not to introduce an additional uncertainty as otherwise the similar (reverse) calculations would be applied for calculating an $PEC_{surface}$ water from the PEC_{soil}.

Table 162: Calculation of MIC_{soil} based on MIC_{water} values

Equations and parameters	Chlortetra-cylin min	Chlortetra-cylin geomean	Colistin min	Colistin geomean	Neomycin min	Neomycin geomean	Tiamulin min	Tiamulin geomean	Explanation and units
$pMSC_{soil} = \frac{K_{soil-water}}{RHO_{soil}} \cdot pMSC_{surface\ water} \cdot 1000 \cdot CONV_{soil}$									
MSCsoil							**9.6**	**613**	predicted Minimal Selective Concentration for soil [µg/kg dw]
Ksoil-water	0.2	0.2	0.2	0.2	0.2	0.2	16.28	16.28	Soil-water partitioning coefficient [m3/m3] see equation 2
RHOsoil	1700	1700	1700	1700	1700	1700	1700	1700	Bulk density of wet soil [1700 kg ww/m3]
pMSCsurface water	0.06	1.2	0.5	13	4	10	1	64	predicted Minimal selective concentration for surface water [µg/L]
CONVsoil	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13	Conversion factor for soil concentrations: wwt to dwt [kgwwt/kgdwt] see equation
Conversion factor	1000	1000	1000	1000	1000	1000	1000	1000	Conversion factor for litre to m3 [1000 L/m3]
$K_{soil-water} = Fair_{soil} \times K_{air-water} + Fwater_{soil} + Fsolid_{soil} \times \frac{Kp_{soil}}{1000} \times RHOsolid$									
Ksoil-water	2.00E-01	2.00E-01	2.00E-01	2.00E-01	2.00E-01	2.00E-01	1.63E+01	1.63E+01	Soil-water partitioning coefficient [m3/m3]
Kair-water	1.00E-09	1.00E-09	1.00E-09	1.00E-09	1.00E-09	1.00E-09	1.00E-09	1.00E-09	Air-water partitioning coefficient [m3/m3] see equation 5
Fairsoil	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	Volume fraction of air in soil [0.2 m3/m3]
Fwatersoil	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	Volume fraction of water in soil [0.2 m3/m3]
Fsolidsoil	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	Volume fraction of solids in soil [0.6 m3/m3]
Kpsoil	0	0	0	0	0	0	10.72	10.72	Partition coefficient solids and water in soil (v/w) [L/kg dw] see equation 4
Conversion factor	1000	1000	1000	1000	1000	1000	1000	1000	Conversion factor for litre to m3 [1000 L/m3]
RHOsolid	2500	2500	2500	2500	2500	2500	2500	2500	Bulk density of solids [2500 kg dw/m3]
$CONV_{soil} = \frac{RHO_{soil}}{Fsolid_{soil} \times RHOsolid}$									
CONVsoil	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13	Conversion factor for soil concentrations: ww to dw [kg ww/kg dw] see equation 3
RHOsoil	1700	1700	1700	1700	1700	1700	1700	1700	Bulk density of wet soil [1700 kg ww/m3]
Fsolidsoil	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	Volume fraction of solids in soil [0.6 m3/m3]
RHOsolid	2500	2500	2500	2500	2500	2500	2500	2500	Bulk density of solids [2500 kg dw/m3]
$Kp_{soil} = K_{oc} \times Foc_{soil}$									
Kpsoil	0	0	0	0	0	0	10.72	10.72	Partition coefficient solids and water in soil (v/w) [L/kg dw]
Koc							536	536	Organic carbon partitioning coefficient [L/kg oc]
Focsoil	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	Weight fraction organic carbon in sediment [0.02 kg oc/kg dw]
Koc range							536- 7312	536- 7312	
$K_{air-water} = \frac{H}{R \times TEMP}$									
Kair-water	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	Air-water partitioning coefficient [m3/m3]
H									Henry's law constant [Pa.m3/mol]
R	8.314	8.314	8.314	8.314	8.314	8.314	8.314	8.314	gas constant [8.314 Pa.m3/mol.K]
Temp	285	285	285	285	285	285	285	285	environmental temperature [285 K]
Kair-water	1.00E-09	1.00E-09	1.00E-09	1.00E-09	1.00E-09	1.00E-09	1.00E-09	1.00E-09	Kair-water has very limited influence and is proposed to be set at 10-10 m3/m3

Orange fields, necesary input data. Input data were only found foir Tiamulin (BVL 2018)

Table 163: Chlortetracyclin - MIC database*.

Species	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	Distributions	Observations
Actinobacillus pleuropneumoniae	0	0	0	0	0	0	0	28	27	1	4	0	0	0	0	0	0	0	0	3	60
Mannheimia haemolytica	0	0	0	0	0	0	0	3	38	19	14	1	5	1	0	2	0	0	0	4	83
Pasteurella multocida	0	0	0	0	0	0	2	20	21	4	7	3	2	1	0	0	0	0	0	3	60
Staphylococcus aureus	0	0	0	0	0	0	13	60	28	0	1	0	1	1	1	0	0	0	0	5	105
Staphylococcus pseudintermedius	0	0	0	0	1	16	49	24	1	0	0	0	0	9	0	0	0	0	0	5	100
Streptococcus equi subspecies equi	0	0	0	0	0	0	48	38	27	5	1	1	0	0	0	0	0	0	0	8	120
Streptococcus equi subspecies zooepidemicus	0	0	0	0	0	0	0	19	62	18	10	14	5	2	0	0	0	0	0	9	130
Pseudomonas gessardii							0	0	0	10	0	0	0								10
Arthrobacter sp.									0	0	0	0	10	0	0	0					10
Acidovorax facilis					0	10	0	0	0	0	0	0									10
Lactiplantibacillus plantarum						0	0	0	0	0	0	10	0	0	0	0					10

* lilac-coloured wells indicate MIC determination in the context of this project, not included in the EUCAST database

Table 164: Colistin - MIC database*.

Species	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	Distributions	Observations
Achromobacter xylosoxidans	0	0	0	0	0	0	0	0	2	7	33	34	7	5	0	0	0	0	0	2	88
Acinetobacter baumannii	0	0	0	0	0	0	0	0	805	1451	539	54	0	29	1	0	0	0	0	4	2879
Acinetobacter pittii	0	0	0	0	0	0	0	0	36	79	11	2	0	0	0	0	0	0	0	2	128
Aeromonas caviae	0	0	0	0	0	0	0	9	19	7	0	0	0	0	0	0	0	0	0	1	35
Aeromonas hydrophila	0	0	0	0	0	0	0	1	2	12	1	2	0	2	1	34	0	0	0	1	55
Aeromonas veronii	0	0	0	0	0	0	0	2	8	8	4	0	1	0	0	2	0	0	0	1	25
Citrobacter freundii	0	0	0	0	0	0	1	20	3	0	0	0	0	0	0	0	0	0	0	1	24
Enterobacter cloacae	0	0	0	0	0	0	16	255	398	76	15	6	9	17	23	8	22	2	2	6	849
Escherichia coli	0	0	0	0	0	2	231	2058	2747	845	56	16	14	5	8	2	30	0	0	13	6014
Escherichia coli ATCC 25922	0	0	0	0	0	0	0	12	38	42	2	0	0	0	0	0	0	0	0	2	94
Escherichia coli NCTC 13846	0	0	0	0	0	0	0	0	0	0	11	89	22	0	0	0	0	0	0	2	122
Klebsiella aerogenes	0	0	0	0	0	4	4	53	138	44	10	3	3	3	0	0	4	0	0	5	266

Species	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	Distributions	Observations
Klebsiella oxytoca	0	0	0	0	0	16	10	143	406	192	22	6	1	10	2	1	2	0	1	6	812
Klebsiella pneumoniae	0	0	0	0	0	0	24	453	938	281	32	18	19	42	19	5	9	0	1	8	1841
Klebsiella pneumoniae ATCC 700603	0	0	0	0	0	0	0	9	31	31	3	0	0	0	0	0	0	0	0	2	74
Pseudomonas aeruginosa	0	0	0	0	0	4	15	90	1507	9217	7911	400	38	58	14	4	12	0	0	21	19270
Pseudomonas aeruginosa ATCC 27853	0	0	0	0	0	0	0	0	6	52	47	0	0	0	0	0	0	0	0	2	105
Pseudomonas putida group	0	0	0	0	0	0	0	2	9	22	11	0	0	0	0	0	0	0	0	2	44
Salmonella Dublin	0	0	0	0	0	0	0	0	0	52	78	259	133	2	2	0	0	0	0	4	526
Stenotrophomonas maltophilia	0	0	0	0	0	0	0	13	123	228	232	255	282	358	50	17	5	0	0	5	1563
Pseudomonas gessardii					0	0	0	0	5	5	0	0									10
Arthrobacter sp.											0	0	0	0	0	0	0	0			10
Acidovorax facilis											0	0	0	0	0	8	2	0			10
Lactiplantibacillus plantarum												0	0	0	10	0	0	0	0		10

* Lilac-coloured wells indicate MIC determination in the context of this project, not included in the EUCAST database

Table 165: Neomycin - MIC database*.

Species	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	Distributions	Observations
Actinobacillus pleuropneumoniae	0	0	0	0	0	0	0	0	0	0	0	0	0	5	89	72	0	0	0	5	166
Bordetella bronchiseptica	0	0	0	0	0	0	0	0	0	0	2	51	23	0	0	4	0	0	0	2	80
Campylobacter coli	0	0	0	0	0	0	0	0	194	566	118	9	1	4	3	0	0	0	0	8	895
Enterococcus faecalis	0	0	0	0	0	0	0	0	0	0	1	2	7	29	87	189	203	2	90	9	610
Enterococcus faecium	0	0	0	0	0	0	0	0	0	0	9	106	300	296	152	46	8	0	5	8	922
Enterococcus hirae	0	0	0	0	0	0	0	0	0	0	15	42	42	53	40	41	6	0	1	5	240
Escherichia coli	0	0	0	0	0	0	0	1	4	1475	1421	741	94	17	91	61	21	20	0	15	3946
Haemophilus (Glaessarella) parasuis	0	0	0	0	0	3	0	1	0	4	11	21	6	0	0	1	0	0	0	1	47
Klebsiella oxytoca	0	0	0	0	0	0	0	0	2	19	6	0	0	0	0	0	0	0	0	1	27
Klebsiella pneumoniae	0	0	0	0	0	0	0	0	51	129	8	0	1	7	7	7	0	0	0	3	210
Klebsiella variicola	0	0	0	0	0	0	0	0	1	20	1	0	0	0	0	0	0	0	0	1	22
Mannheimia haemolytica	0	0	0	0	0	0	0	2	0	3	20	207	261	19	24	4	1	0	0	12	541

Species	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	Distributions	Observations
Pasteurella multocida	0	0	0	0	0	0	0	0	1	2	9	110	161	19	7	19	2	0	0	7	330
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0	1	3	5	29	44	28	7	4	0	0	0	4	121
Salmonella enterica	0	0	0	0	0	0	0	0	0	12	363	53	3	1	0	0	0	0	0	1	432
Staphylococcus aureus	0	0	0	0	0	0	10	102	156	45	3	0	0	0	0	0	0	0	0	3	316
Staphylococcus hyicus	0	0	0	0	0	2	23	64	35	8	4	0	0	0	0	0	0	0	0	5	136
Staphylococcus pseudintermedius	0	0	0	0	0	1	45	136	22	3	91	0	0	0	0	0	0	0	0	6	298
Yersinia enterocolitica	0	0	0	0	0	0	0	25	40	84	1	0	1	0	0	0	0	0	0	1	151
Pseudomonas gessardii							0	0	0	0	0	0	10	0							10
Arthrobacter sp.												0	0	0	0	0	0	0	0		10
Acidovorax facilis											0	0	0	0	10	0	0	0			10
Lactiplantibacillus plantarum							0	0	0	10	0	0	0	0							10

* Lilac-coloured wells indicate MIC determination in the context of this project, not included in the EUCAST database

Table 166: Tiamulin - MIC database*.

Species	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	Distributions	Observations
Actinobacillus pleuropneumoniae	0	0	0	0	0	0	0	0	0	0	0	4	54	78	0	0	0	0	0	4	136
Mannheimia haemolytica	0	0	0	0	0	0	0	4	0	5	1	12	111	96	15	0	0	0	0	7	244
Pasteurella multocida	0	0	0	0	0	0	0	2	1	0	4	11	44	105	100	16	0	0	0	7	283
Staphylococcus aureus	0	0	0	0	0	0	0	2	54	57	3	0	2	1	0	5	0	0	0	4	124
Staphylococcus hyicus	0	0	0	0	0	0	4	8	96	133	5	4	7	9	6	47	0	0	0	6	319
Streptococcus suis	0	0	0	0	0	0	0	3	1	9	23	26	1	0	0	1	0	0	0	2	64
Pseudomonas gessardii												0	0	0	0	0	0	0	10		10
Arthrobacter sp.												0	0	0	0	0	0	0	10		10
Acidovorax facilis					0	0	0	0	0	10	0	0									10
Lactiplantibacillus plantarum												0	0	0	0	10	0	0	0		10

* Lilac-coloured wells indicate MIC determination in the context of this project, not included in the EUCAST database

D Appendix 4 - Additional data concerning soil degradation tests (work package 4)

Table 167: History of fertilizer and pesticide use of soil RefeSol 02A for the year of the first sampling for soil degradation tests (December 2022) and previous four years.

Year	Pesticides	Fertiliser
2024	No pesticides	No fertilisation
2023	No pesticides	No fertilisation
2022	No pesticides	No fertilisation
2021	No pesticides	Liming, 1500 kg CaO/ha (04.02.2021)
2020	No pesticides	50 kg N/ha (07.04.2020)
2019	No pesticides	50 kg N/ha (02.04.2019)
2018	No pesticides	No fertilisation

Table 168: History of fertilizer and pesticide use of soil LUFA 2.1 for the year of the first sampling for soil degradation tests (December 2022) and previous four years.

Year	Pesticides	Fertiliser	Plants
2024	No pesticides	No fertilisation	Uncultivated
2023	No pesticides	No fertilisation	Uncultivated
2022	No pesticides	No fertilisation	Uncultivated
2021	No pesticides	No fertilisation	Uncultivated
2020	No pesticides	No fertilisation	Uncultivated
2019	No pesticides	No fertilisation	Uncultivated
2018	No pesticides	No fertilisation	Uncultivated

Table 169: Principal events of soil preparation for the test set with ^{14}C-tebuconazole, single application (STEP 1).

Soil preparation	Date
Soil collection LUFA 2.1 Charge F2.1 4822	01 December 2022
Soil collection RefeSol 02A Charge IME 02A-02	07 December 2022
Sieving of soil RefeSol 02A ≤ 2 mm	19 December 2022
Adjusting the water content of RefeSol 02A to 40 – 60 % WHC_{max}	20 December 2022
Re-determination of the water content of RefeSol 02A	20 December 2022
Receipt of soil LUFA 2.1	21 December 2022
Adjusting the water content of LUFA 2.1 to 40 – 60 % WHC_{max}	21 December 2022

Soil preparation	Date
Re-determination of the water content of LUFA 2.1	22 December 2022
Storage of soils RefeSol 02A - LUFA 2.1 in unsealed containers at ca. 8 °C	22 December 2022 – 13 January 2023
Determination of the actual microbial biomass of all soils	05 January 2023
Start pre-incubation	13 January 2023
Application of soil samples	23 January 2023

Table 170: Principal events of soil preparation for the pretest set with ^{14}C-tebuconazole at two application rates, single application (STEP 2).

Soil preparation	Date
Soil collection LUFA 2.1 Charge F2.1 1123	15 March 2023
Receipt of soil LUFA 2.1	27 March 2023
Adjusting the water content of LUFA 2.1 to 40 – 60 % WHC_{max}	27 March 2023
Soil collection RefeSol 02A Charge IME 02A-02	27 March 2023
Sieving of soil RefeSol 02A ≤ 2 mm	28 March 2023
Re-determination of the water content of LUFA 2.1	28 March 2023
Adjusting the water content of RefeSol 02A to 40 – 60 % WHC_{max}	29 March 2023
Re-determination of the water content of RefeSol 02A	29 March 2023
Determination of the actual microbial biomass of all soils	30 March 2023
Storage of soils RefeSol 02A - LUFA 2.1 in unsealed containers at ca. 8 °C	30 March 2023 – 26 May 2023
Start pre-incubation	26 May 2023
Application of soil samples	05 June 2023

Table 171: Principal events of soil preparation for the test set with pyraclostrobin, single and multiple application (STEP 1).

Soil preparation	Date
Soil collection LUFA 2.1 Charge F2.1 1123	15 March 2023
Receipt of soil LUFA 2.1	27 March 2023
Adjusting the water content of LUFA 2.1 to 40 – 60 % WHC_{max}	27 March 2023
Soil collection RefeSol 02A Charge IME 02A-02	27 March 2023
Sieving of soil RefeSol 02A ≤ 2 mm	28 March 2023
Re-determination of the water content of LUFA 2.1	28 March 2023
Adjusting the water content of RefeSol 02A to 40 – 60 % WHC_{max}	29 March 2023

Soil preparation	Date
Re-determination of the water content of RefeSol 02A	29 March 2023
Determination of the actual microbial biomass of all soils	30 March 2023
Storage of soils RefeSol 02A - LUFA 2.1 in unsealed containers at ca. 8 °C	30 March 2023 – 07 April 2023
Start pre-incubation	07 April 2023
Application of soil samples	17 April 2023

Table 172: Principal events of soil preparation for the test set with ethofumesate, single and multiple application (STEP 1).

Soil preparation	Date
Soil collection LUFA 2.1 Charge F2.1 4523	06 November 2023
Receipt of soil LUFA 2.1	10 November 2023
Soil collection RefeSol 02A Charge IME 02A-02	13 November 2023
Adjusting the water content of LUFA 2.1 to 40 – 60 % WHC_{max}	14 November 2023
Sieving of soil RefeSol 02A ≤ 2 mm	24 November 2023
Adjusting the water content of RefeSol 02A to 40 – 60 % WHC_{max}	29 November 2023
Re-determination of the water content of LUFA 2.1	30 November 2023
Re-determination of the water content of RefeSol 02A	01 December 2023
Determination of the actual microbial biomass of all soils	04 December 2023
Storage of soils RefeSol 02A - LUFA 2.1 in unsealed containers at ca. 8 °C	04 December 2023 – 11 December 2023
Start pre-incubation	11 December 2023
Application of soil samples	21 December 2023

Table 173: Principal events of soil preparation for the test set with pyraclostrobin and ethofumesate as a mixture, single and multiple application (STEP 2).

Soil preparation	Date
Soil collection RefeSol 02A Charge IME 02A-02	13 November 2023
Sieving of soil RefeSol 02A ≤ 2 mm	24 November 2023
Adjusting the water content of RefeSol 02A to 40 – 60 % WHC_{max}	29 November 2023
Re-determination of the water content of RefeSol 02A	01 December 2023
Determination of the actual microbial biomass of soil RefeSol 02A	04 December 2023
Storage of soil RefeSol 02A in unsealed containers at ca. 8 °C	04 December 2023 – 29 January 2024
Start pre-incubation	29 January 2024

Soil preparation	Date
Application of soil samples	08 February 2024

Table 174: Principal events of soil preparation for the test set with pyraclostrobin and ethofumesate, time-delayed single application at different time points (STEP 2).

Soil preparation	Date
Soil collection RefeSol 02A Charge IME 02A-02	15 February 2024
Sieving of soil RefeSol 02A ≤ 2 mm	20 February 2024
Adjusting the water content of RefeSol 02A to 40 – 60 % WHC_{max}	21 February 2024
Re-determination of the water content of RefeSol 02A	21 February 2024
Determination of the actual microbial biomass of soil RefeSol 02A	22 February 2024
Storage of soil RefeSol 02A in unsealed containers at ca. 8 °C	22 February 2024 – 26 February 2024
Start pre-incubation	26 February 2024
Application of soil samples	07 March 2024

Table 175: History of fertilizer and pesticide use of an agricultural soil for the year of sampling for soil degradation tests (April 16, 2024) and previous four years (STEP 3).

Year	Pesticides	Fertiliser
2024	08.04.2024: Empartis (0.88 L/ha, Boscalid, Kresoxim-methyl) Helocur 250 EW (0.3 L/ha, Tebuconazole) Initial Pro (0.88 L/ha, Proquinazid, Prothioconazole) CCC 720 (1 L/ha, Chlormequat chloride) Moxa (0.22 L/ha, Trinexapac)	No fertilisation
2024	21.03.2024: Moddus Start (0.15 L/ha, Trinexapac) Stabilan 720 (1 L/ha, Chlormequat chloride)	No fertilisation
2023	08.10.2023: Sunfire (0.5 L/ha, Flufenacet) Boxer (2.5 L/ha, Prosulfocarb)	No fertilisation
2023	07.10.2023: Celest (as seed treatment of winter wheat seeds, Fludioxonil)	No fertilisation
2023	16.06.2023: Motivell Forte (0.66 L/ha, Nicosulfuron) Vivendi 100 (1 L/ha, Clopyralid)	No fertilisation
2023	25.05.2023: Basilico (0.8 L/ha, Mesotrione) Gardo Gold (2.45 L/ha, S-Metolachlor, Terbuthylazine)	No fertilisation
2023	03.05.2023: Korit (as seed treatment of maize, Ziram)	No fertilisation
2023	05.04.2023: Profi 360 TF (5 L/ha, Glyphosate)	1.05 kg N/ha, 1.2 kg S/ha (05.04.2023)

Year	Pesticides	Fertiliser
2022	No pesticides	No fertilisation
2021	No pesticides	No fertilisation
2020	No pesticides	No fertilisation

Table 176: Principal events of soil preparation for the test set with pyraclostrobin and ethofumesate, single application to a soil with existing contamination (STEP 3).

Soil preparation	Date
Soil collection in the field (Soest)	16 April 2024
Sieving ≤ 2 mm	18 April 2024
Adjusting the water content to 40 – 60 % WHC_{max}	23 April 2024
Re-determination of the water content	23 April 2024
Determination of the actual microbial biomass	25 April 2024
Storage of soil in unsealed containers at ca. 8 °C	No storage
Start pre-incubation	26 April 2024
Application of soil samples	06 May 2024

Table 177: Summary of kinetic fits for pyraclostrobin after single application (0.667 mg/kg) in RefeSol 02A (STEP 1).

Parent: pyraclostrobin (single application)						
Soil	**Model**	**Chi² error**	**r^2**	**Parameter**	**DT50 [d]**	**DT90 [d]**
RefeSol 02A	SFO	**14.7**	**0.9319**	M_0: 87.25 k: 0.02543	**27.3**	**90.6**
	FOMC	6.32	0.9835	M_0: 95.62 α: 0.931 β: 16.62	18.4	181
	DFOP	4.64	0.9908	M_0: 95.53 k1: 0.05944 k2: 0.003347 g: 0.7485	17.6	276
	HS	3.56	0.9932	M_0: 95.16 k1: 0.04039 k2: 0.008542 tb: 26.67	17.2	170

Kinetic plots and residuals

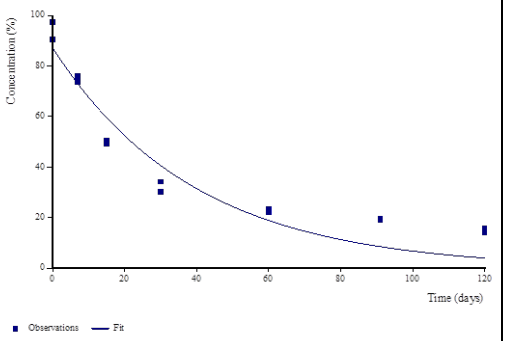
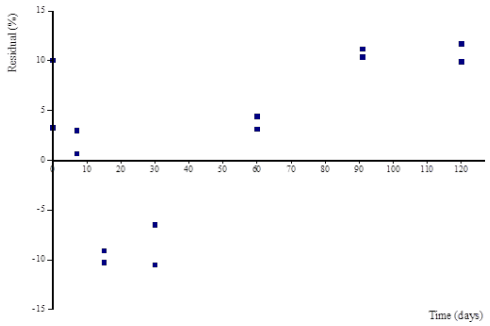
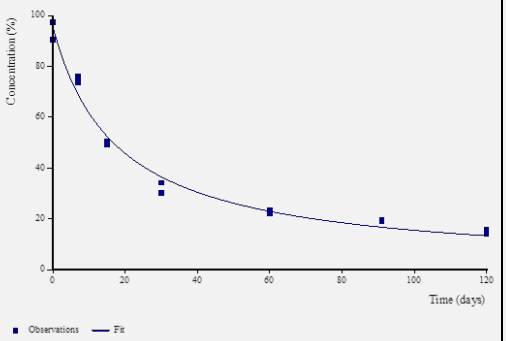
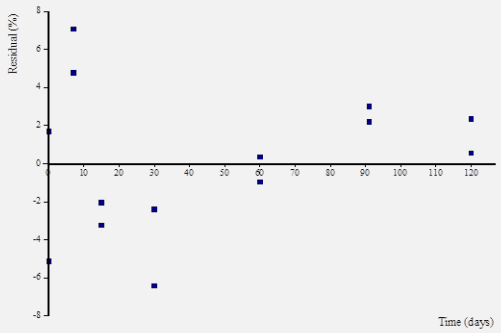
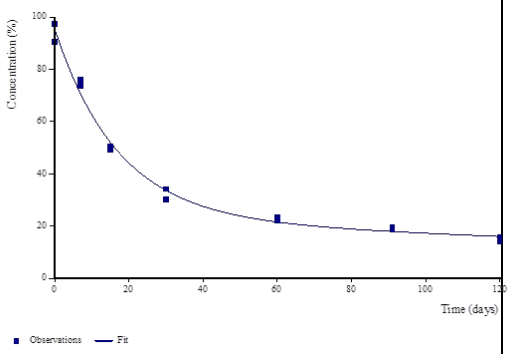
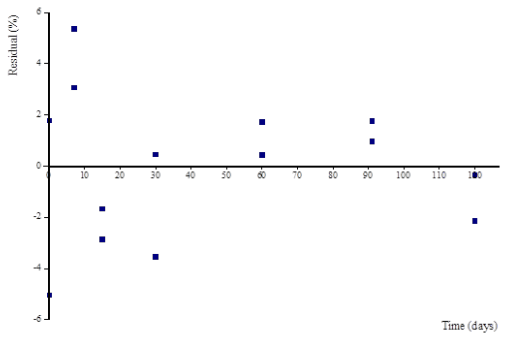
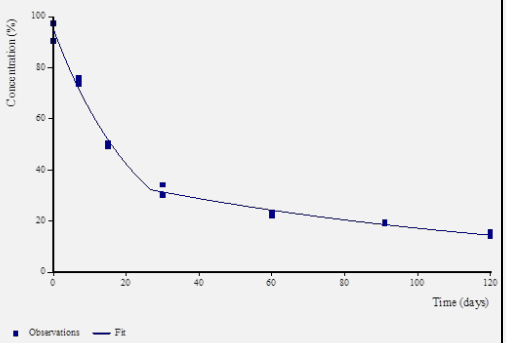
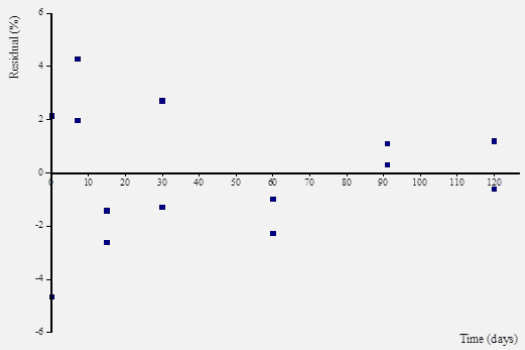
Parent: pyraclostrobin (single application)		
SFO		
FOMC		
DFOP		
HS		

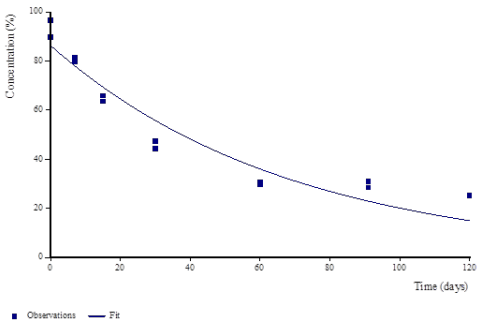
Table 178: Summary of kinetic fits for pyraclostrobin after single application (0.667 mg/kg) in LUFA 2.1 (STEP 1).

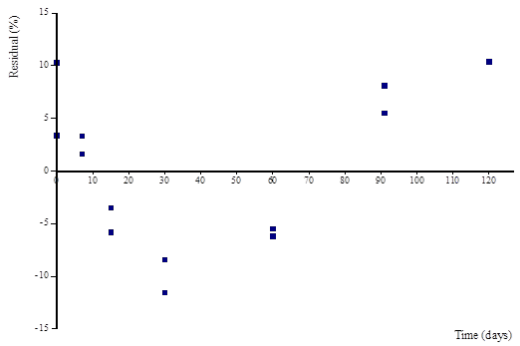
Parent: pyraclostrobin (single application)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**

Parent: pyraclostrobin (single application)						
LUFA 2.1	SFO	**10.8**	**0.9192**	M_0: 86.33 k: 0.01452	**47.7**	**159**
	FOMC	4.83	0.9818	M_0: 94.98 α: 0.7518 β: 21.32	32.3	435
	DFOP	2.94	0.9916	M_0: 94.68 k1: 0.03869 k2: 2.33E-011 g: 0.7322	29.7	> 10000
	HS	1.55	0.9947	M_0: 93.66 k1: 0.02385 k2: 0.002713 tb: 44.8	29.1	500

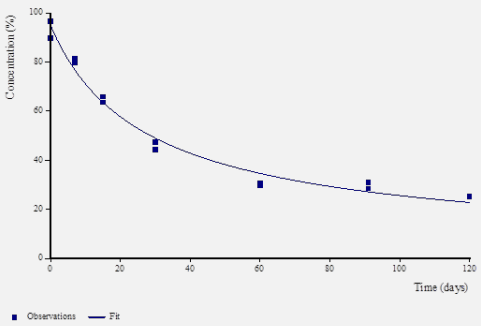
Kinetic plots and residuals

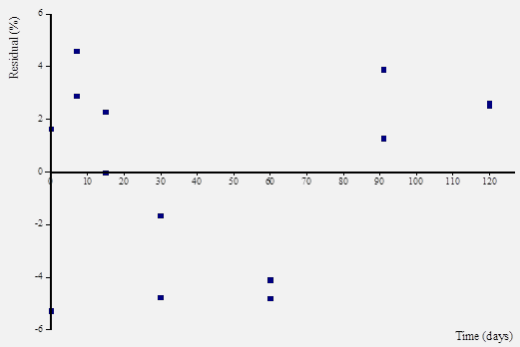
SFO



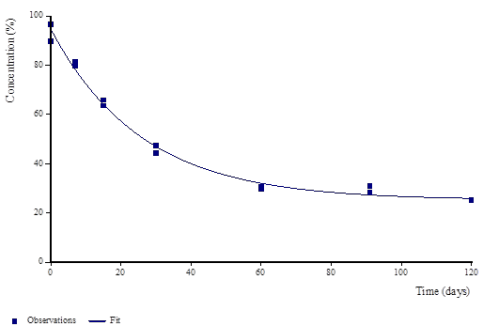


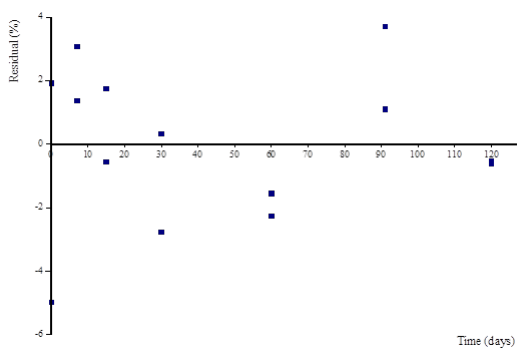
FOMC





DFOP





Parent: pyraclostrobin (single application)

HS

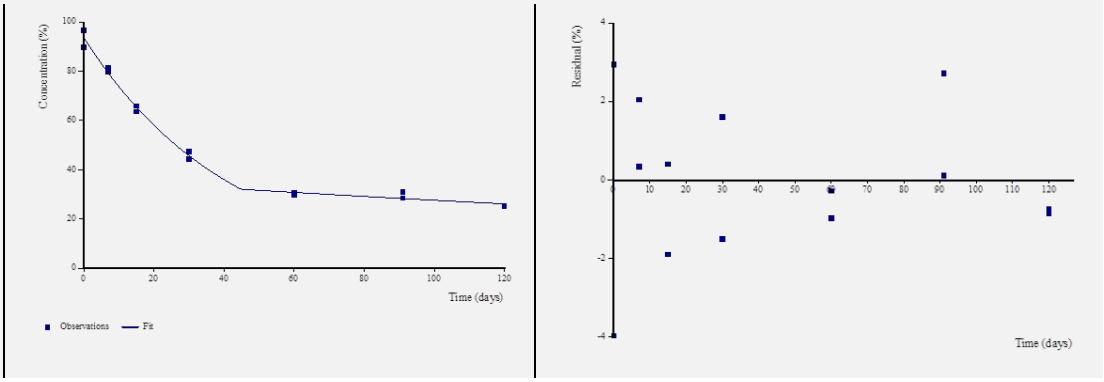
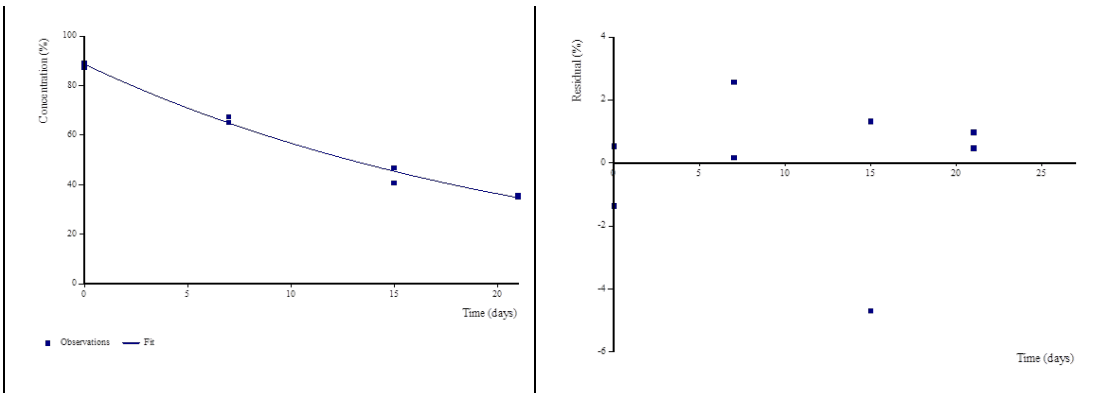


Table 179: Summary of kinetic fits for pyraclostrobin after multiple application (2 x 0.333 mg/kg) in RefeSol 02A – first application (0d – 21d) (STEP 1).

Parent: pyraclostrobin (multiple application – first application 0d – 21d)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
RefeSol 02A	SFO	**1.62**	**0.9901**	M_0: 88.67 k: 0.0445	**15.6**	**51.7**
	FOMC	2.03	0.9901	M_0: 88.68 α: 147.5 β: 3.30E+003	15.6	52.0
	DFOP	*n.d.*	0.9901	M_0: 88.67 k1: 0.04451 k2: 0.0445 g: 0.4238	15.6	51.7
	HS	*n.d.*	0.9907	M_0: 88.89 k1: 0.04551 k2: 0.003674 tb: 20.06	15.2	398

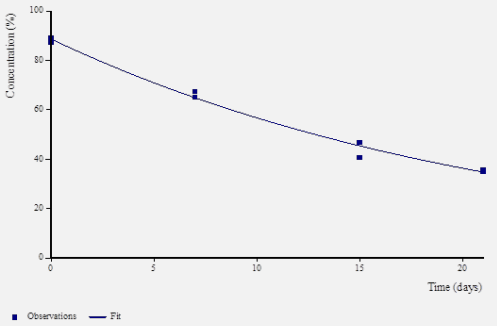
Kinetic plots and residuals

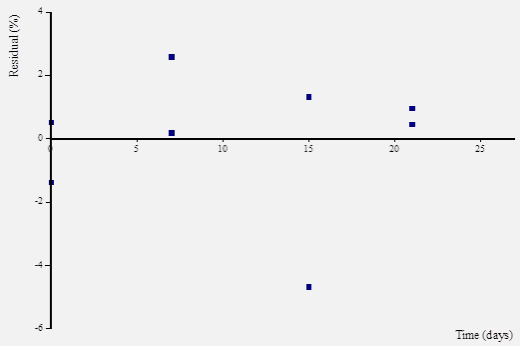
SFO



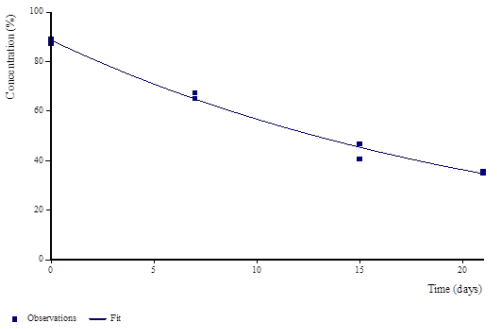
Parent: pyraclostrobin (multiple application – first application 0d – 21d)

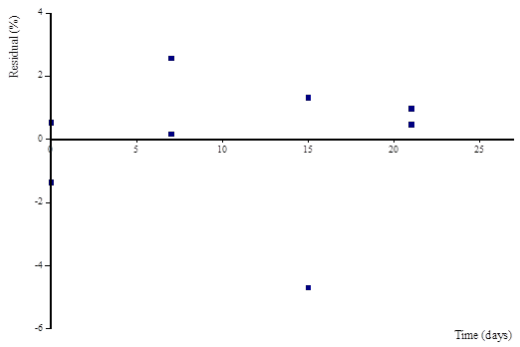
FOMC



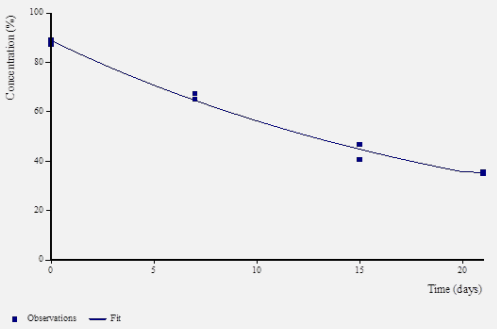


DFOP





HS



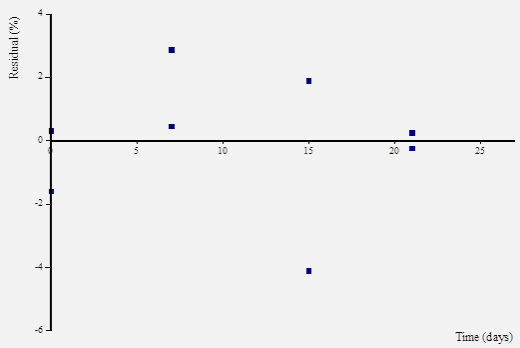


Table 180: Summary of kinetic fits for pyraclostrobin after multiple application (2 x 0.333 mg/kg) in RefeSol 02A – second application (21d – 120d) (STEP 1).

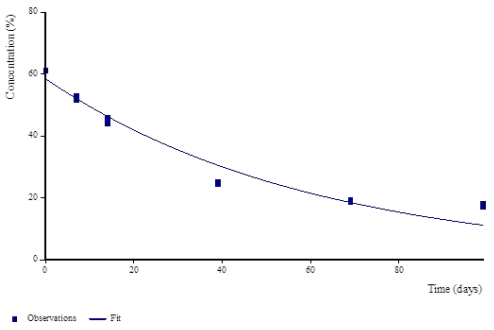
Parent: pyraclostrobin (multiple application – second application 21d – 120d)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
RefeSol 02A	SFO	**8.16**	**0.9529**	M_0: 58.49 k: 0.01664	**41.7**	**138**
	FOMC	4.72	0.9853	M_0: 62.11 α: 1.001 β: 32.22	32.2	289
	DFOP	3.3	0.9938	M_0: 62.11 k1: 0.03675 k2: 3.32E-015 g: 0.7494	29.9	> 10000

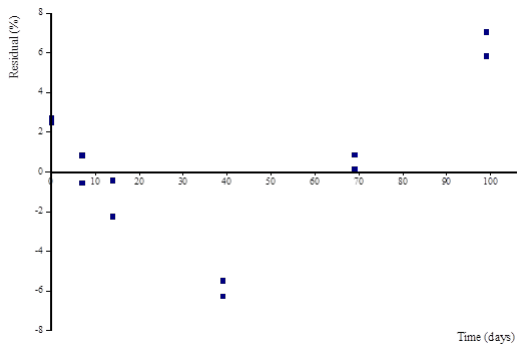
Parent: pyraclostrobin (multiple application – second application 21d – 120d)

	HS	0.753	0.9986	M_0: 61.33 k1: 0.02298 k2: 0.00245 tb: 48.73	30.2	532

Kinetic plots and residuals

SFO





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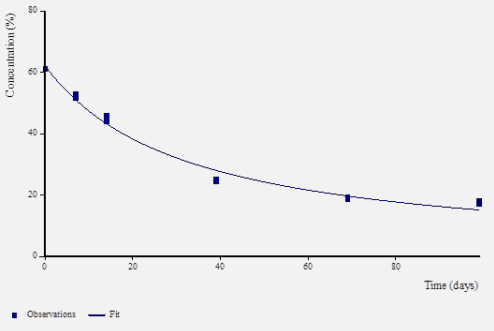

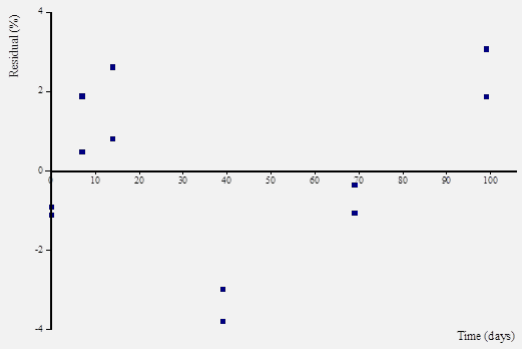

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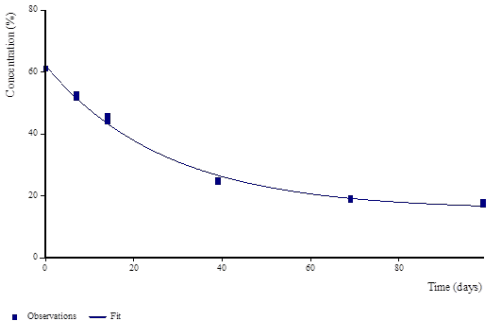
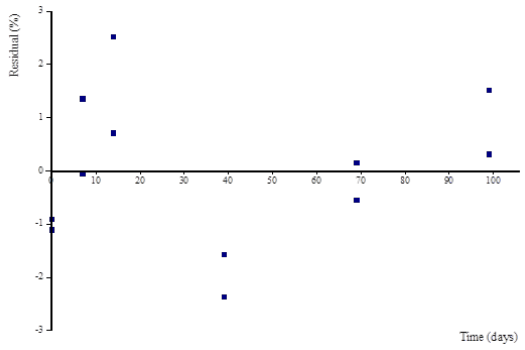
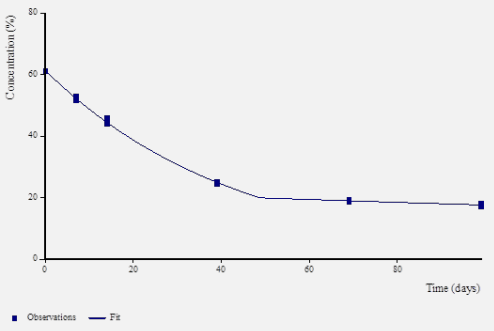




HS



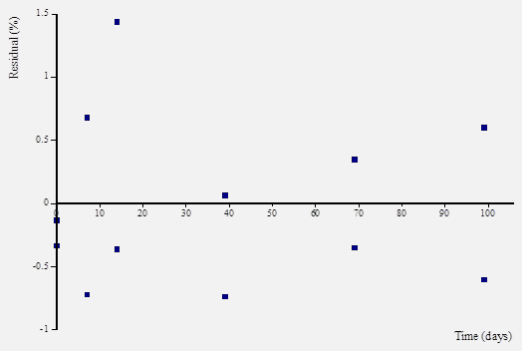
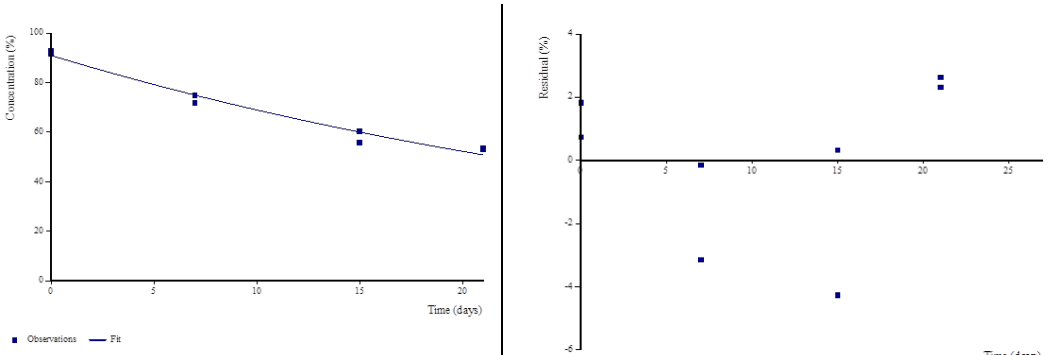


Table 181: Summary of kinetic fits for pyraclostrobin after multiple application (2 x 0.333 mg/kg) in LUFA 2.1 – first application (0d – 21d) (STEP 1).

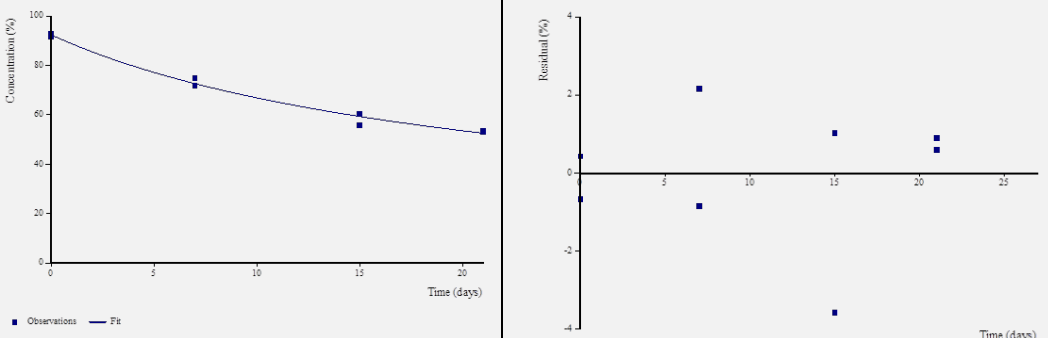
Parent: pyraclostrobin (multiple application – first application 0d – 21d)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
	SFO	**2.23**	**0.9762**	M_0: 90.97 k: 0.02767	**25.1**	**83.2**
	FOMC	1.19	0.9887	M_0: 92.37 α: 0.7144 β: 17.51	28.7	422
LUFA 2.1	DFOP	*n.d.*	0.9897	M_0: 92.41 k1: 0.06828 k2: 5.61E-010 g: 0.5624	32.2	> 10000
	HS	*n.d.*	0.9915	M_0: 92.25 k1: 0.03285 k2: 0.01422 tb: 13.37	31.2	145

Kinetic plots and residuals

SFO

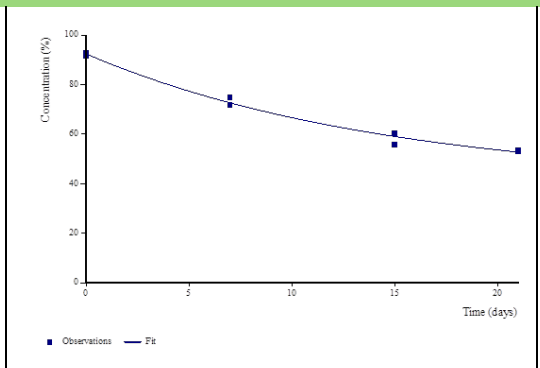


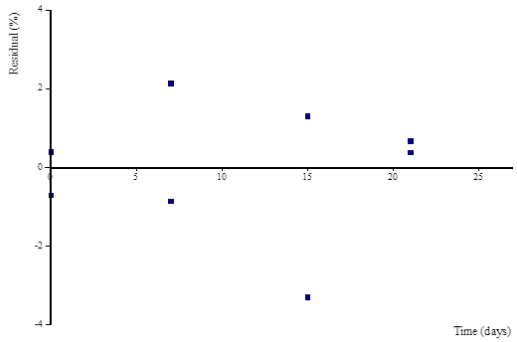
FOMC



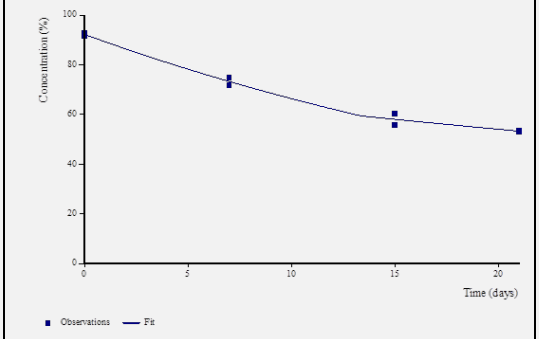
Parent: pyraclostrobin (multiple application – first application 0d – 21d)

DFOP





HS



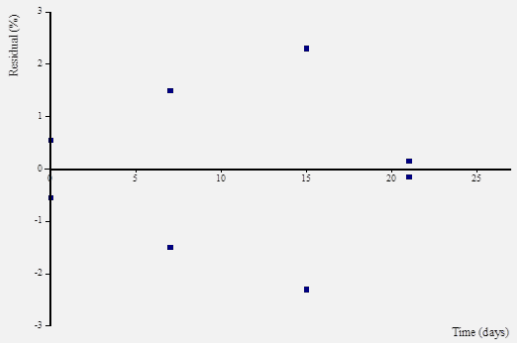


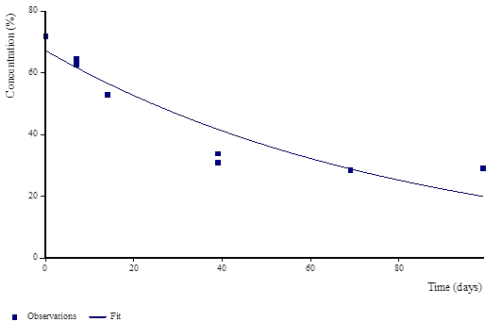
Table 182: Summary of kinetic fits for pyraclostrobin after multiple application (2 x 0.333 mg/kg) in LUFA 2.1 – second application (21d – 120d) (STEP 1).

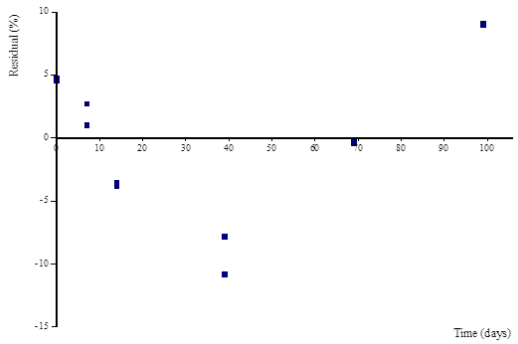
Parent: pyraclostrobin (multiple application – second application 21d – 120d)						
Soil	Model	Chi² error	r^2	Parameter	DT50 [d]	DT90 [d]
	SFO	**10.1**	**0.8872**	M_0: 67.19 k: 0.01222	**56.7**	**188**
	FOMC	5.8	0.9669	M_0: 73.29 α: 0.5488 β: 16.39	41.6	1070
LUFA 2.1	DFOP	3.98	0.987	M_0: 73.53 k1: 0.04388 k2: 1.53E-011 g: 0.6361	35.1	> 10000
	HS	2.01	0.9954	M_0: 72.33 k1: 0.02132 k2: 0.001905 tb: 38.6	32.5	815

Kinetic plots and residuals

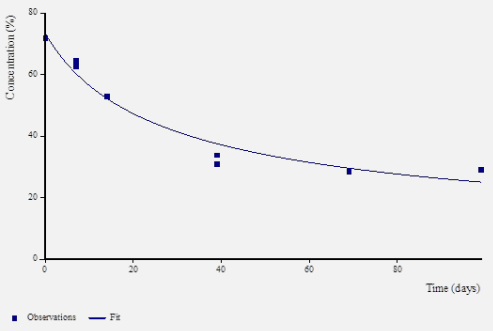
Parent: pyraclostrobin (multiple application – second application 21d – 120d)

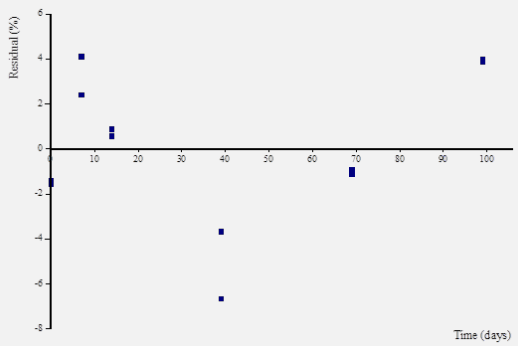
SFO



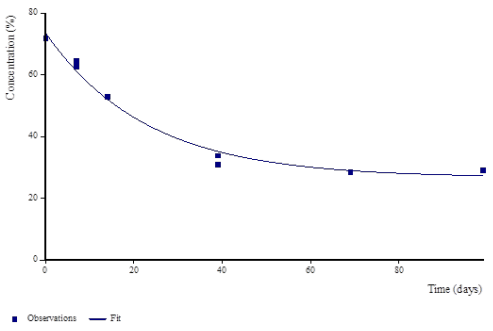


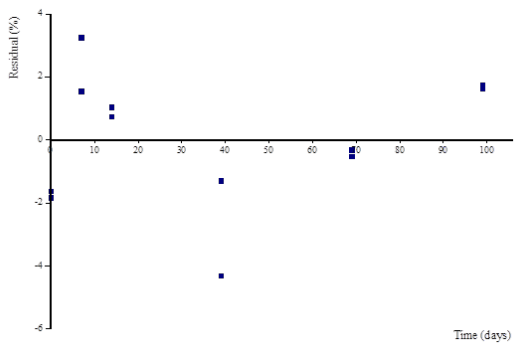
FOMC



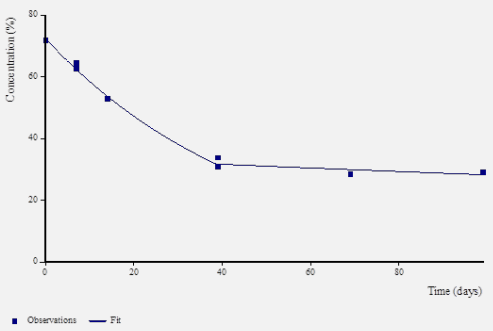


DFOP





HS



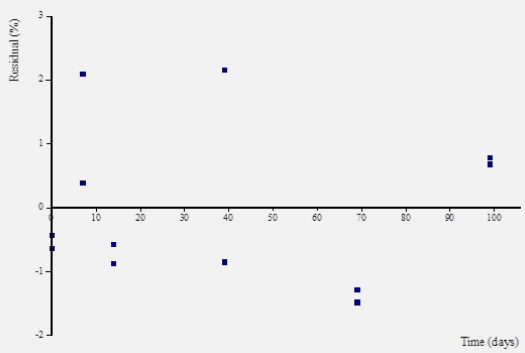
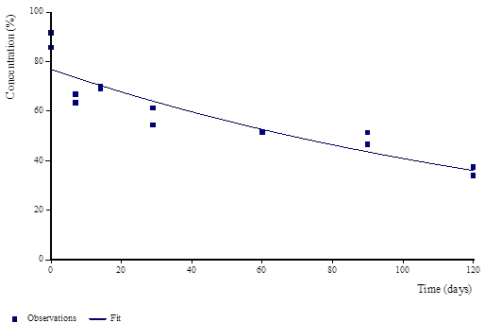


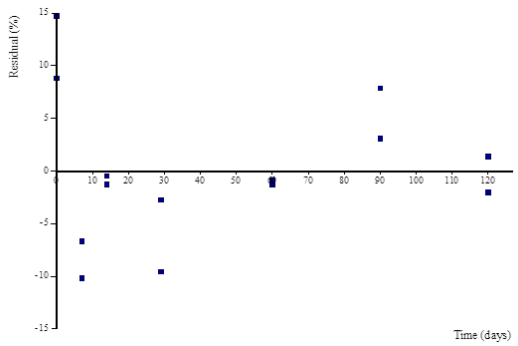
Table 183: Summary of kinetic fits for ethofumesate after single application (0.800 mg/kg) in RefeSol 02A (STEP 1).

Parent: ethofumesate (single application)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
RefeSol 02A	SFO	**8.42**	**0.8221**	M_0: 76.87 k: 0.006294	**110**	**366**
	FOMC	6.32	0.9044	M_0: 87.56 α: 0.2201 β: 4.728	106	> 10 000
	DFOP	4.55	0.9487	M_0: 88.65 k1: 2.722 k2: 0.004927 g: 0.2111	92.6	419
	HS	4.55	0.9487	M_0: 88.65 k1: 0.06412 k2: 0.004927 tb: 4.005	926	419

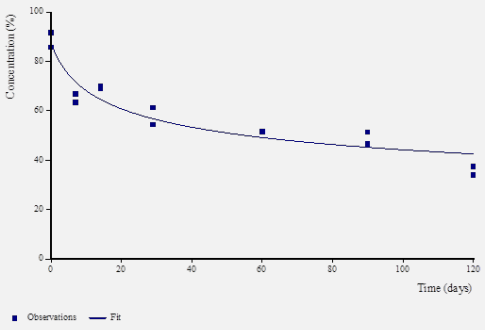
Kinetic plots and residuals

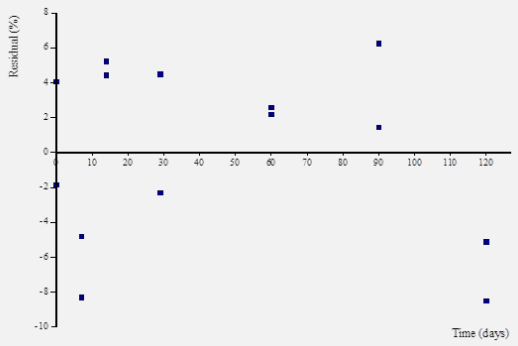
SFO





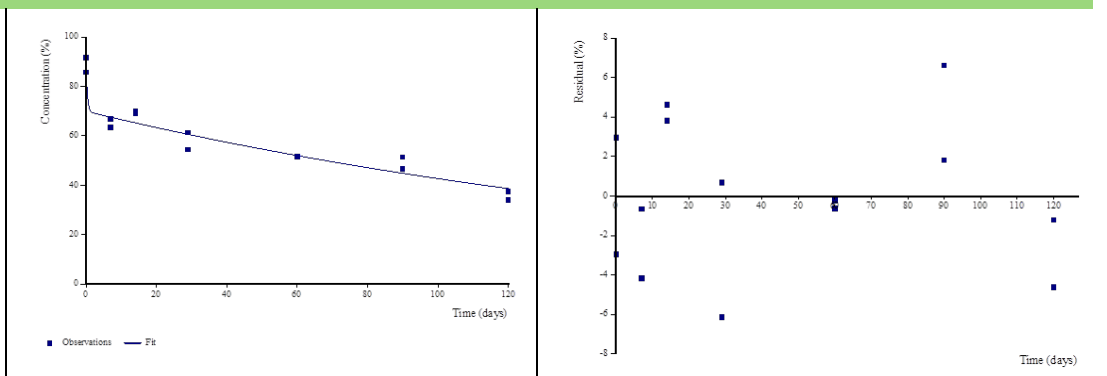
FOMC





Parent: ethofumesate (single application)

DFOP



HS

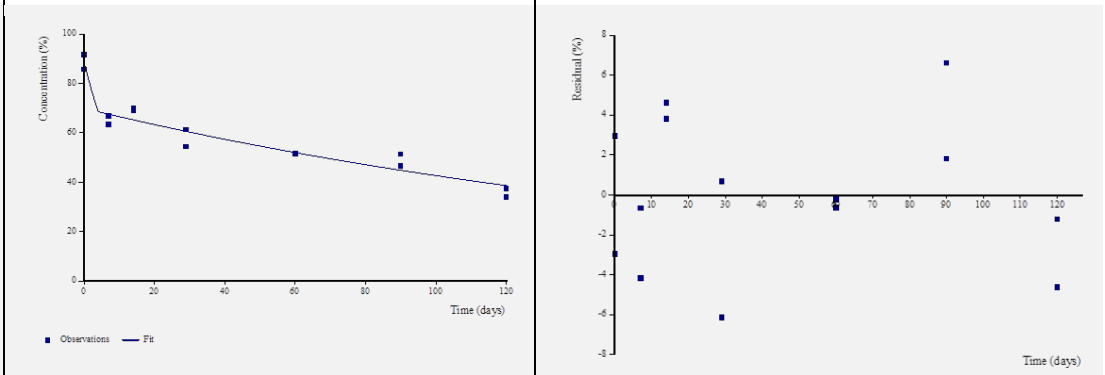


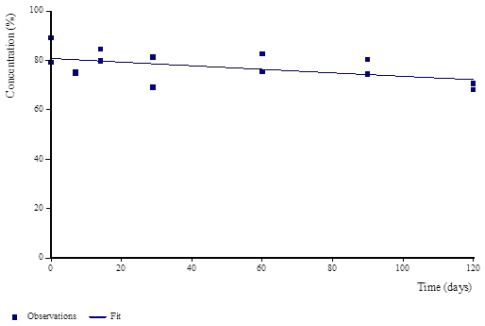
Table 184: Summary of kinetic fits for ethofumesate after single application (0.800 mg/kg) in LUFA 2.1 (STEP 1).

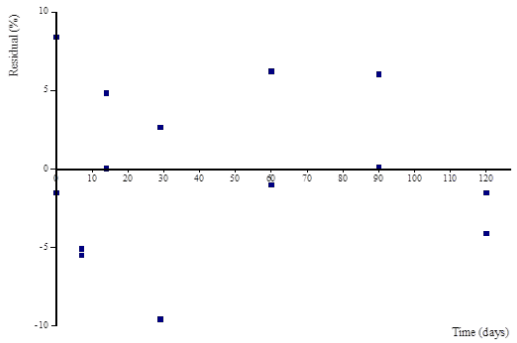
Parent: ethofumesate (single application)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
	SFO	**3.51**	**0.2746**	M_0: 80.82 k: 9.38E-004	**739**	**2450**
	FOMC	3.75	0.2809	M_0: 84.2 α: 0.01993 β: 0.2625	> 10 000	> 10 000
LUFA 2.1	DFOP	3.71	0.347	M_0: 84.25 k1: 2.56 k2: 7.01E-004 g: 0.05954	902	3200
	HS	3.71	0.347	M_0: 84.25 k1: 0.03892 k2: 7.01E-004 tb: 1.606	902	3200

Kinetic plots and residuals

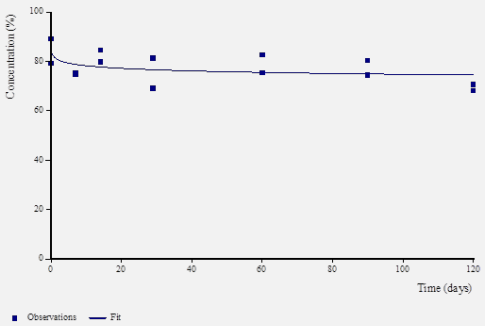
Parent: ethofumesate (single application)

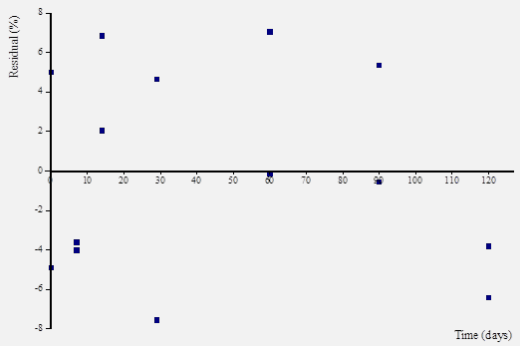
SFO



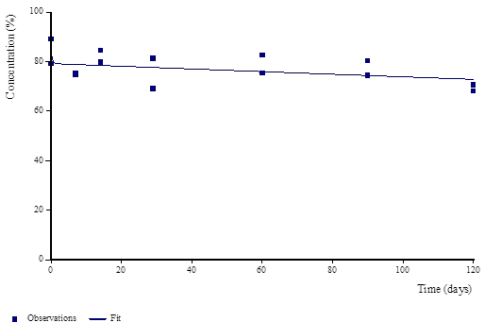


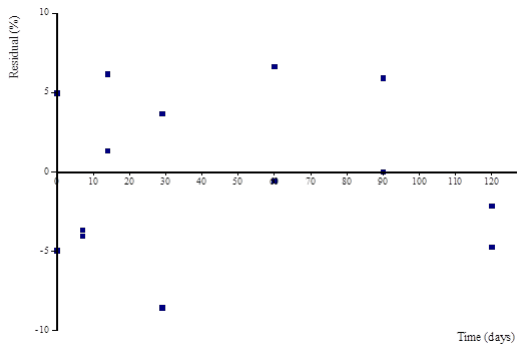
FOMC



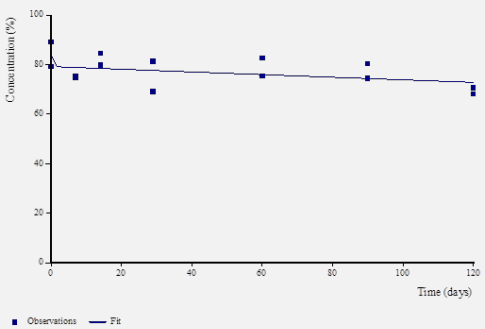


DFOP





HS



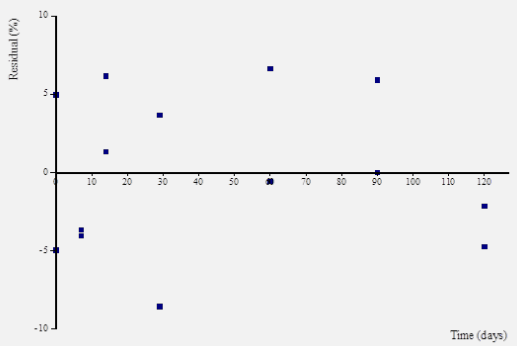
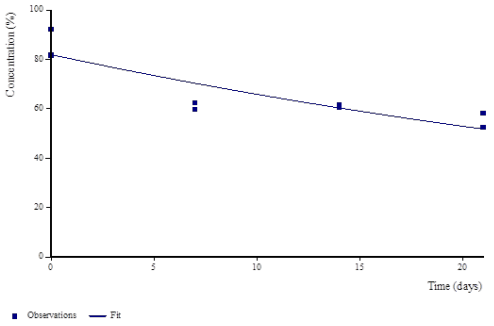


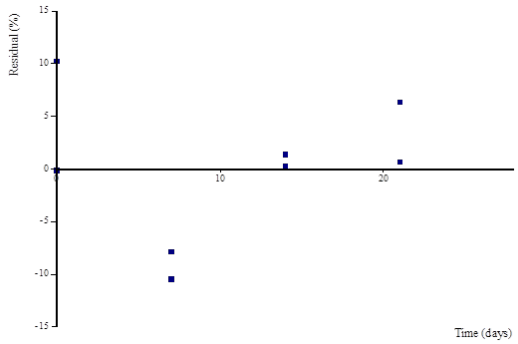
Table 185: Summary of kinetic fits for ethofumesate after multiple application (2 x 0.400 mg/kg) in RefeSol 02A – first application (0d – 21d) (STEP 1).

Parent: ethofumesate (multiple application – first application 0d – 21d)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
RefeSol 02A	SFO	**6.82**	**0.7504**	M_0: 81.96 k: 0.0218	**31.8**	**106**
	FOMC	2.36	0.927	M_0: 87 α: 0.07931 β: 0.1007	629	> 10 000
	DFOP	*n.d.*	0.932	M_0: 87 k1: 2.686 k2: 0.006934 g: 0.2499	58.5	291
	HS	*n.d.*	0.932	M_0: 87 k1: 0.04806 k2: 0.006934 tb: 6.992	58.5	291

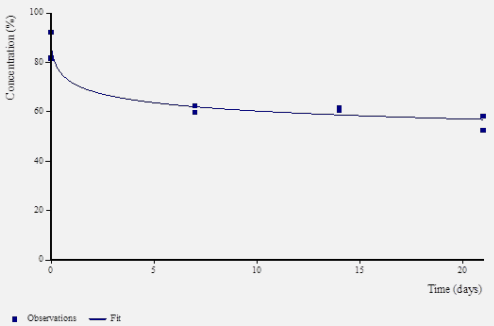
Kinetic plots and residuals

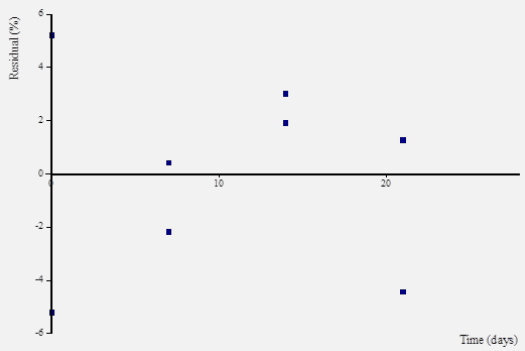
SFO





FOMC





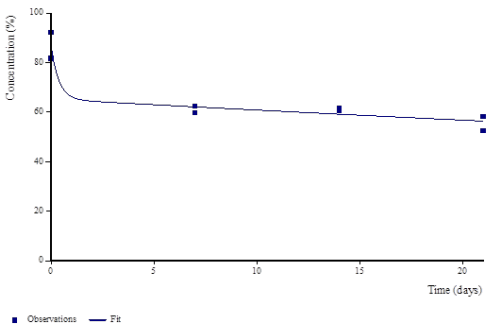
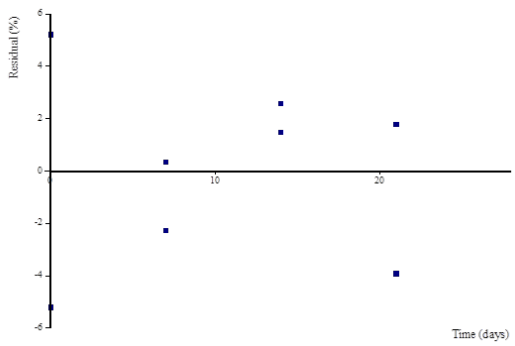
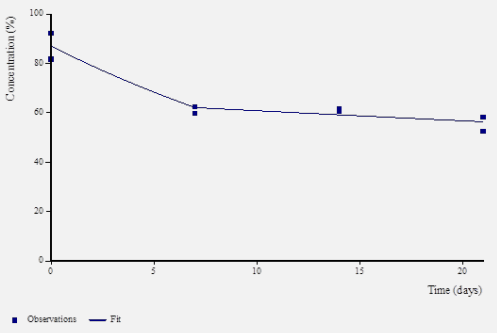
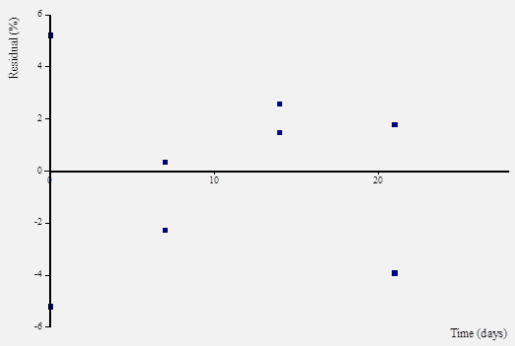
Parent: ethofumesate (multiple application – first application 0d – 21d)		
DFOP		
HS		

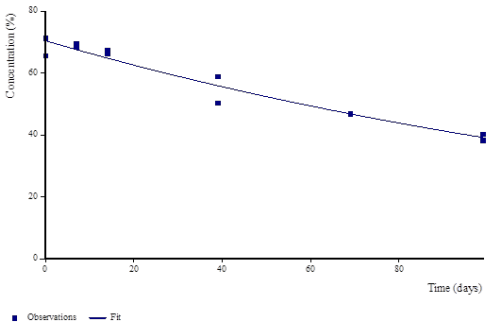
Table 186: Summary of kinetic fits for ethofumesate after multiple application (2 x 0.400 mg/kg) in RefeSol 02A – second application (21d – 120d) (STEP 1).

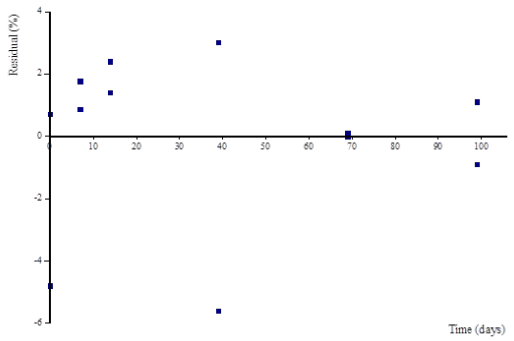
Parent: ethofumesate (multiple application – second application 21d – 120d)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
RefeSol 02A	SFO	**1.89**	**0.9523**	M_0: 70.4 k: 0.005913	**117**	**389**
	FOMC	2.09	0.9523	M_0: 70.42 α: 30.55 β: 5.12E+003	118	401
	DFOP	2.38	0.9523	M_0: 70.4 k1: 0.005953 k2: 0.005912 g: 0.01894	117	389
	HS	1.45	0.961	M_0: 68.6 k1: 3.12E-010 k2: 0.006226 tb: 7.156	119	377

Kinetic plots and residuals

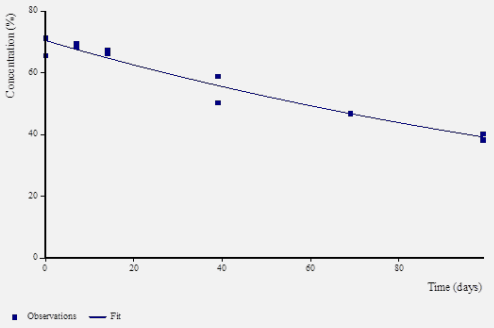
Parent: ethofumesate (multiple application – second application 21d – 120d)

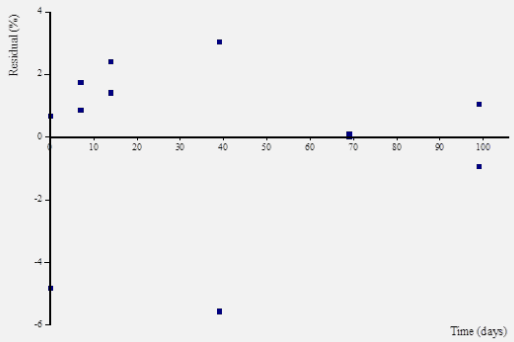
SFO



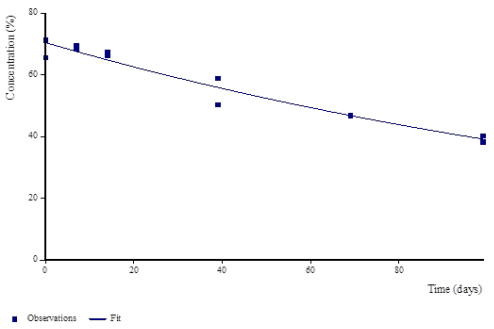


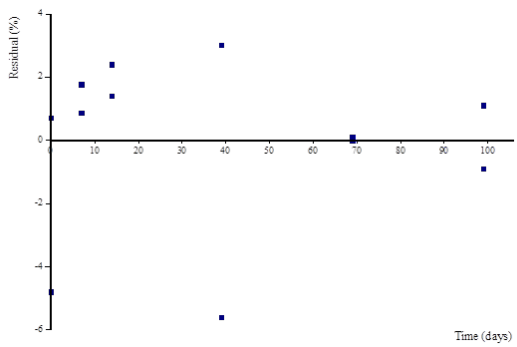
FOMC



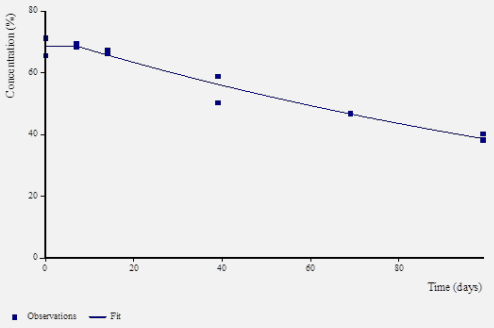


DFOP





HS



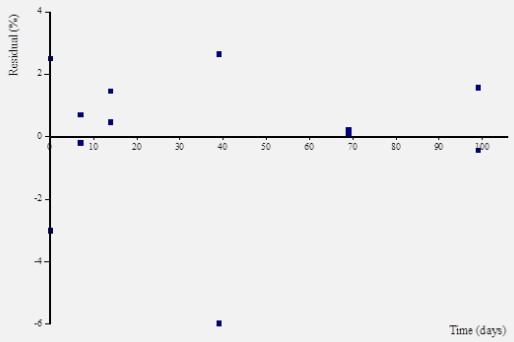
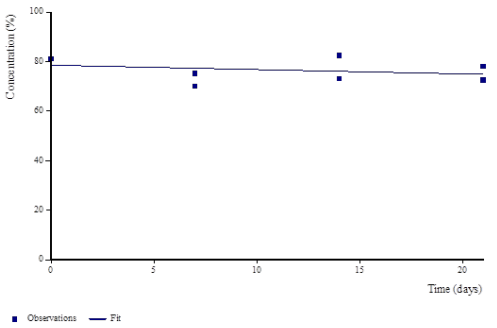


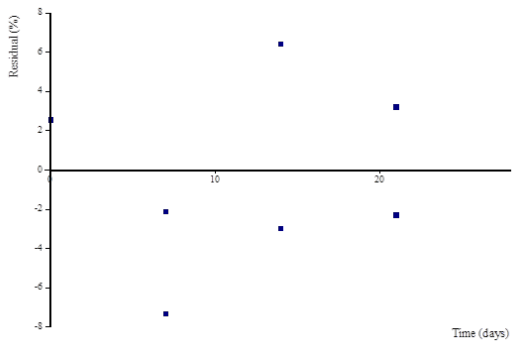
Table 187: Summary of kinetic fits for ethofumesate after multiple application (2 x 0.400 mg/kg) in LUFA 2.1 – first application (0d – 21d) (STEP 1).

Parent: ethofumesate (multiple application – first application 0d – 21d)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
LUFA 2.1	SFO	**3.00**	**0.09756**	M_0: 78.56 k: 0.00228	**304**	**1010**
	FOMC	2.56	0.3236	M_0: 81.15 α: 0.004119 β: 1.41E-007	> 10 000	> 10 000
	DFOP	*n.d.*	0.3394	M_0: 81.1 k1: 1.32 k2: 0 g: 0.07214	> 10 000	> 10 000
	HS	*n.d.*	0.3394	M_0: 81.1 k1: 0.01512 k2: 0 tb: 4.951	> 10 000	> 10 000

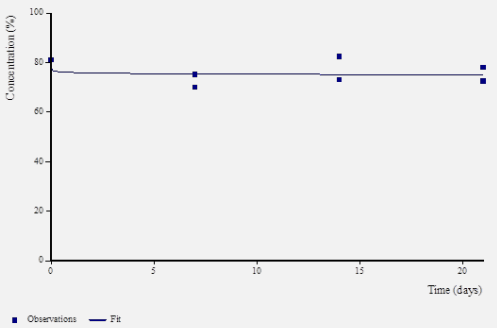
Kinetic plots and residuals

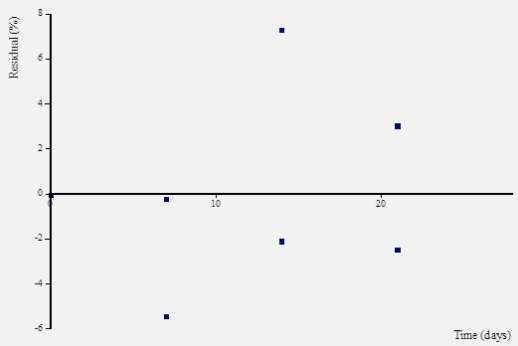
SFO





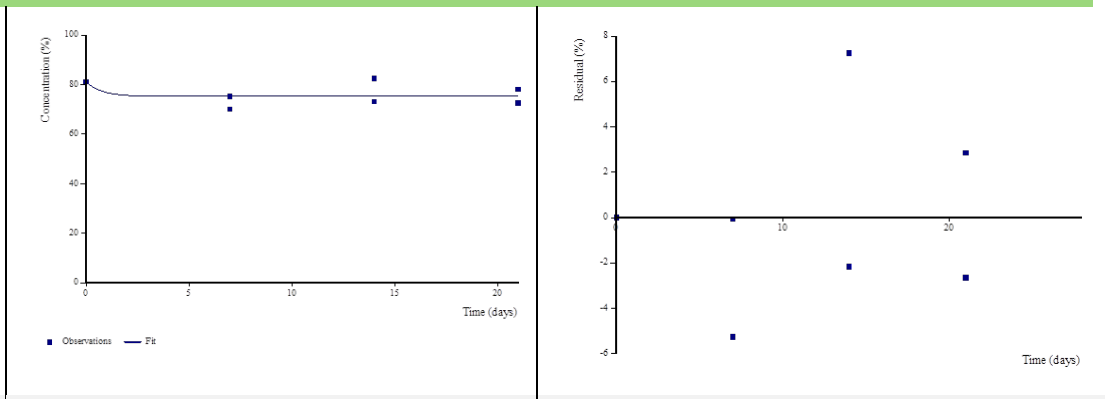
FOMC





Parent: ethofumesate (multiple application – first application 0d – 21d)

DFOP



HS

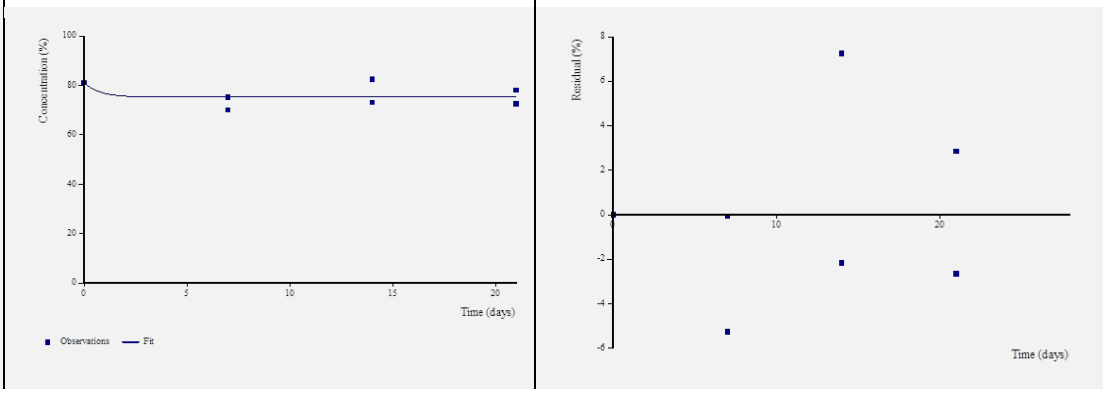


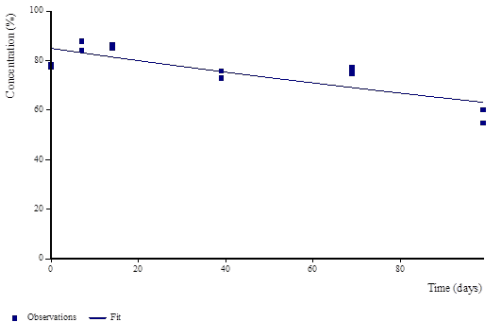
Table 188: Summary of kinetic fits for ethofumesate after multiple application (2 x 0.400 mg/kg) in LUFA 2.1 – second application (21d – 120d) (STEP 1).

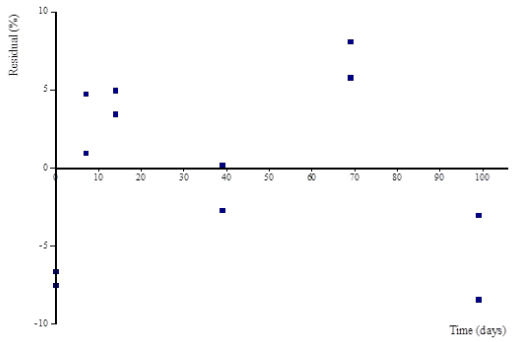
Parent: ethofumesate (multiple application – second application 21d – 120d)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
LUFA 2.1	SFO	**5.35**	**0.6894**	M_0: 84.92 k: 0.002982	**233**	**772**
	FOMC	5.90	0.6889	M_0: 84.93 α: 37.08 β: 1.24E+004	234	794
	DFOP	6.73	0.6894	M_0: 84.92 k1: 0.002986 k2: 0.002982 g: 0.005871	233	772
	HS	5.81	0.7616	M_0: 83.17 k1: 1.02E-012 k2: 0.003863 tb: 20.69	200	617

Kinetic plots and residuals

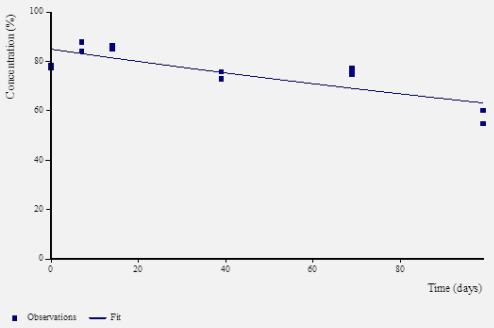
Parent: ethofumesate (multiple application – second application 21d – 120d)

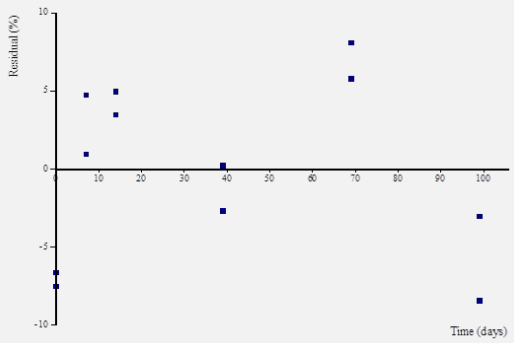
SFO





FOMC





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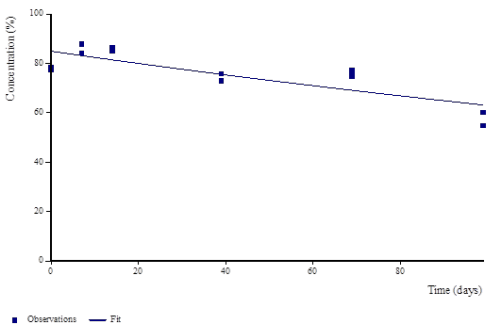
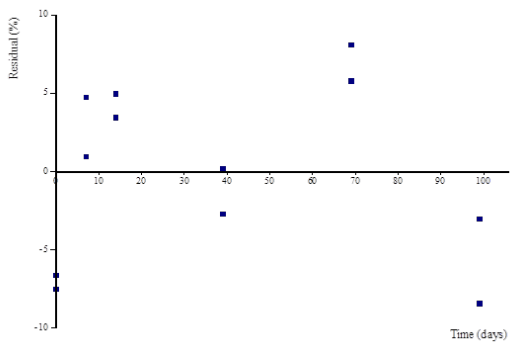
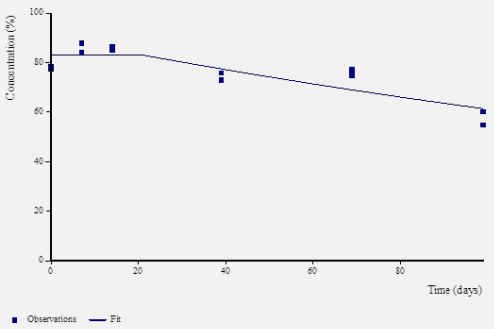




HS



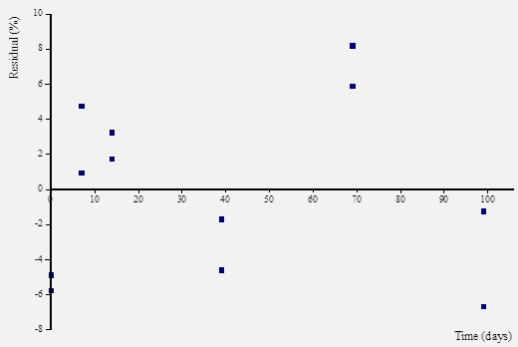
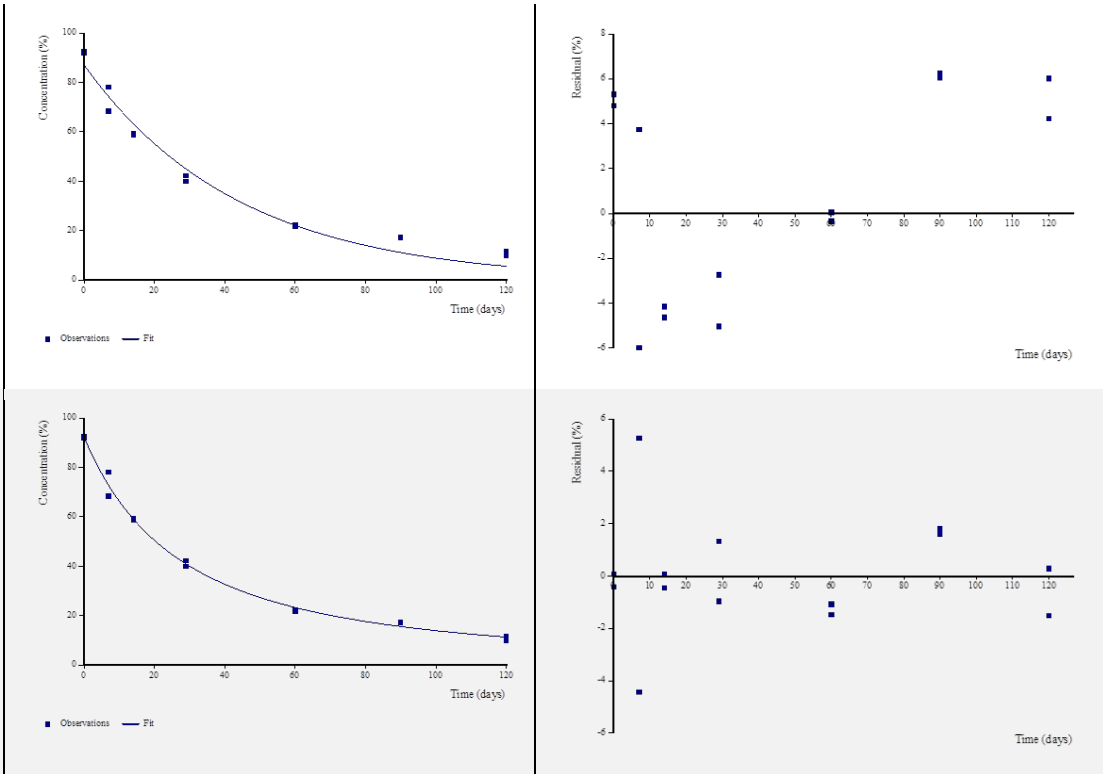


Table 189: Summary of kinetic fits for pyraclostrobin after single application of two test substances as a mixture in RefeSol 02A; nominal application rates were 0.677 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate (STEP 2).

Parent: pyraclostrobin (single application – applied as a mixture of pyraclostrobin and ethofumesate)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
RefeSol 02A	SFO	**7.46**	**0.9773**	M_0: 87.09 k: 0.02275	**30.5**	**101**
	FOMC	1.65	0.9946	M_0: 92.32 α: 1.549 β: 42.05	23.7	144
	DFOP	1.69	0.9947	M_0: 92.12 k1: 0.05186 k2: 0.01009 g: 0.5848	23.5	141
	HS	2.68	0.9935	M_0: 90.87 k1: 0.02885 k2: 0.01101 tb: 41.14	24	143

Kinetic plots and residuals

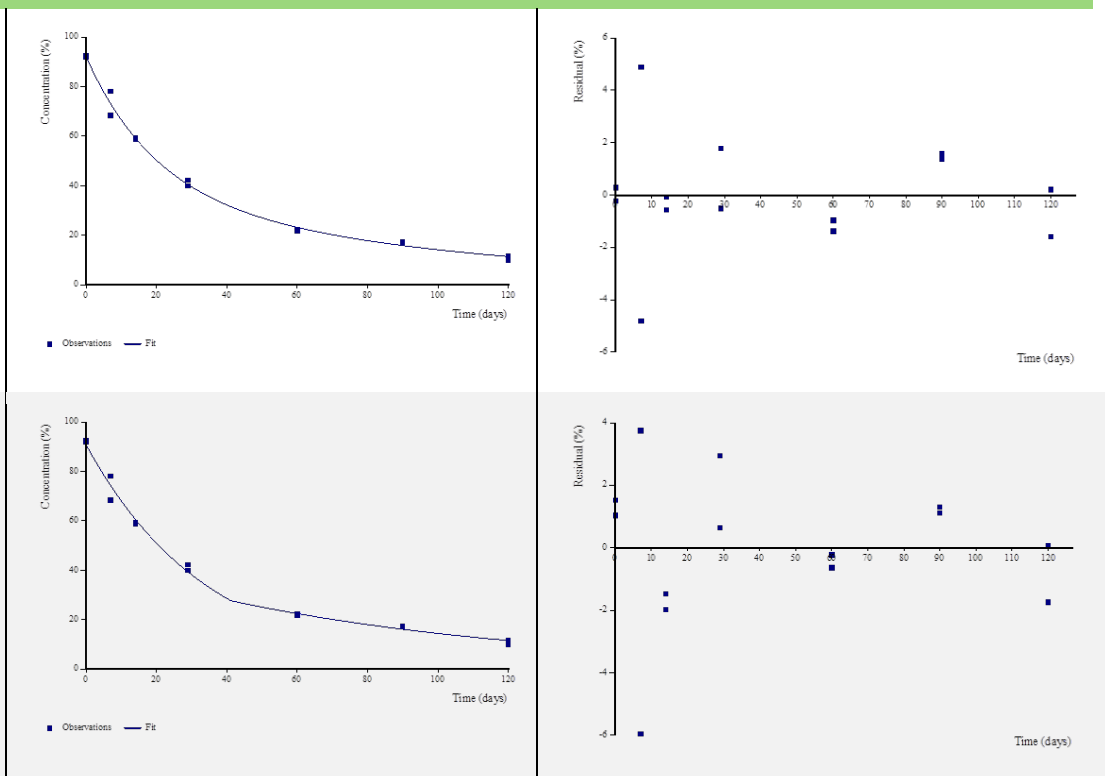
SFO



FOMC

Parent: pyraclostrobin (single application – applied as a mixture of pyraclostrobin and ethofumesate)

DFOP



HS

Table 190: Summary of kinetic fits for ethofumesate after single application of two test substances as a mixture in RefeSol 02A; nominal application rates were 0.677 mg/kg for pyraclostrobin and 0.800 mg/kg for ethofumesate (STEP 2).

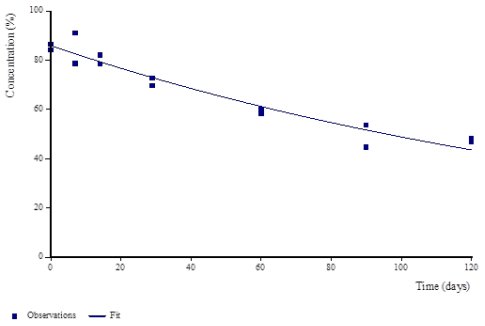
Parent: ethofumesate (single application – applied as a mixture of pyraclostrobin and ethofumesate)

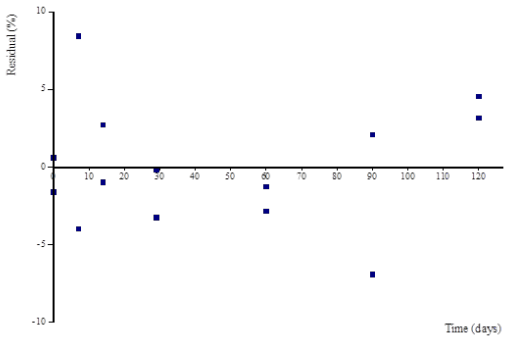
Soil	Model	Chi² error	r²	Parameter	DT50 [d]	DT90 [d]
RefeSol 02A	SFO	**2.55**	**0.9396**	M_0: 85.92 k: 0.005641	**123**	**408**
	FOMC	2.09	0.948	M_0: 87.51 α: 0.9591 β: 122.8	130	1230
	DFOP	2.1	0.95	M_0: 87.6 k1: 0.01259 k2: 6.86E-013 g: 0.6092	137	> 10 000
	HS	1.35	0.9557	M_0: 86.95 k1: 0.006417 k2: 0.00124 tb: 88.05	191	1490

Kinetic plots and residuals

Parent: ethofumesate (single application – applied as a mixture of pyraclostrobin and ethofumesate)

SFO





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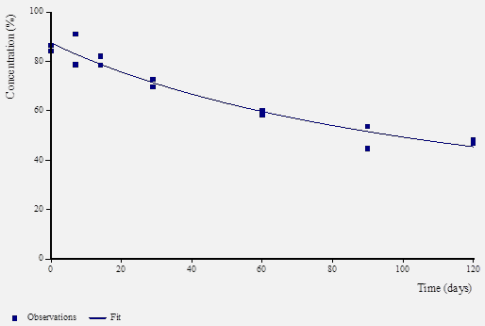
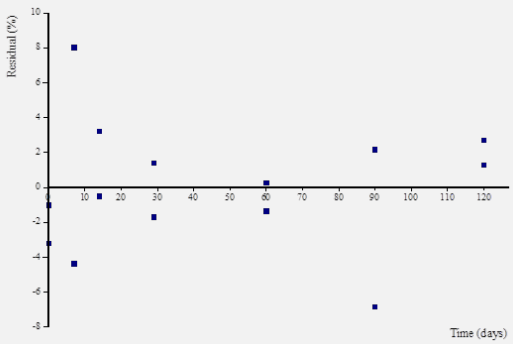
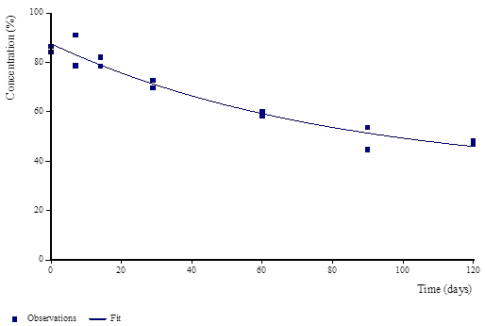
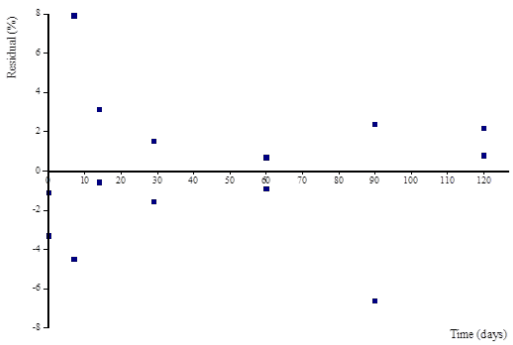


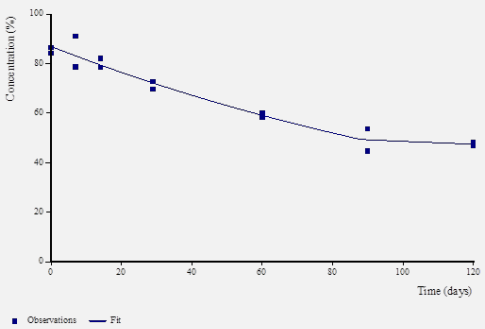


DFOP





HS



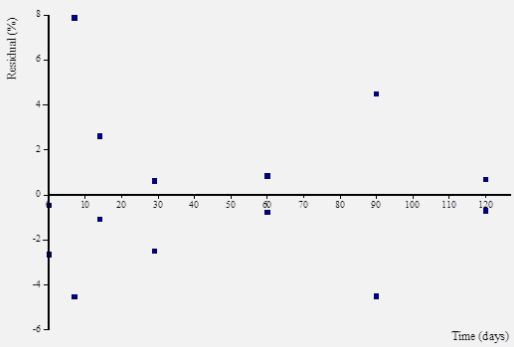


Table 191: Summary of kinetic fits for pyraclostrobin after multiple application of both test substances in RefeSol 02A; application rates were 2 x 0.333 mg/kg for

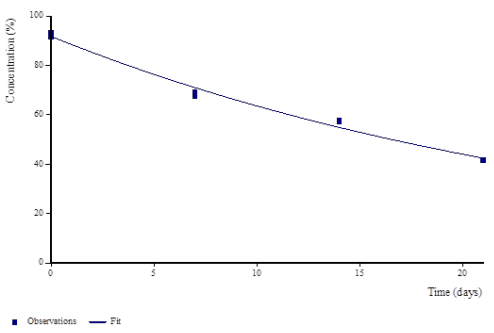
pyraclostrobin and 2 x 0.400 mg/kg for ethofumesate – first application (0d – 21d) (STEP 2).

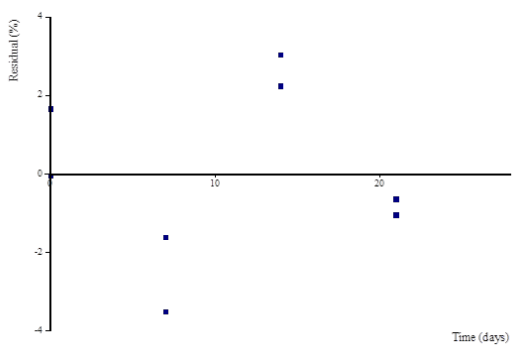
Parent: pyraclostrobin (multiple application – applied as a mixture of pyraclostrobin and ethofumesate, first application 0d – 21d)

Soil	Model	Chi² error	r²	Parameter	DT50 [d]	DT90 [d]
RefeSol 02A	SFO	**2.42**	**0.9878**	M_0: 91.75 k: 0.0366	**18.9**	**62.9**
	FOMC	2.98	0.9882	M_0: 92.05 α: 5.21 β: 133.5	19	74.2
	DFOP	*n.d.*	0.9905	M_0: 92.55 k1: 2.684 k2: 0.03365 g: 0.04949	19.1	66.9
	HS	*n.d.*	0.9905	M_0: 92.55 k1: 0.04132 k2: 0.03365 tb: 6.617	19.1	66.9

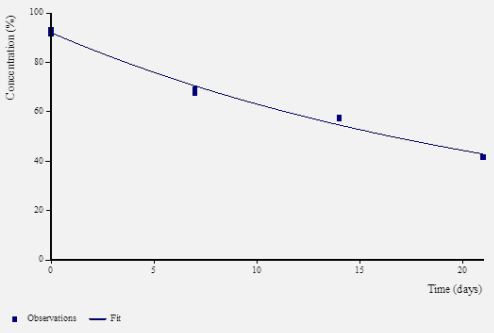
Kinetic plots and residuals

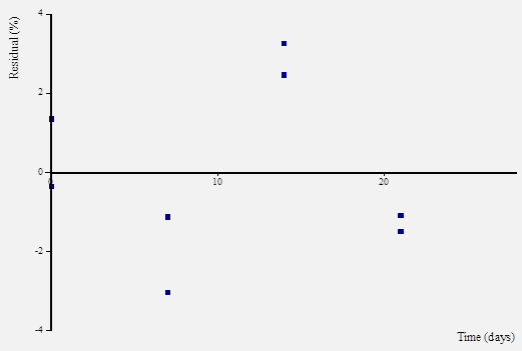
SFO





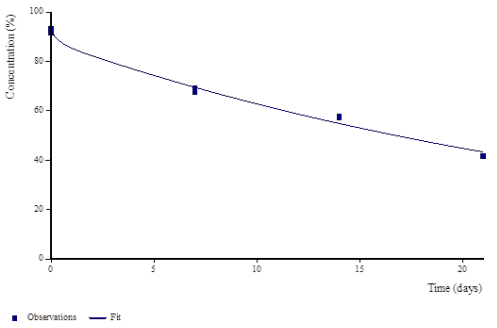
FOMC

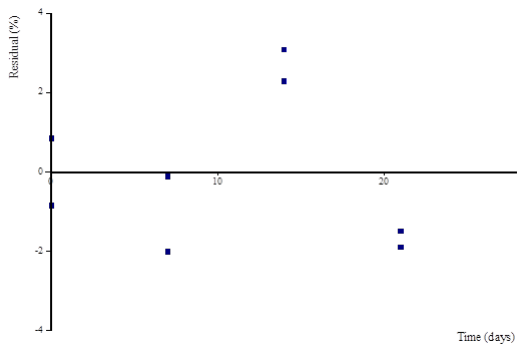




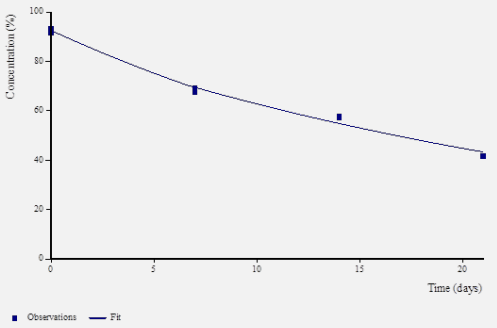
Parent: pyraclostrobin (multiple application – applied as a mixture of pyraclostrobin and ethofumesate, first application 0d – 21d)

DFOP





HS



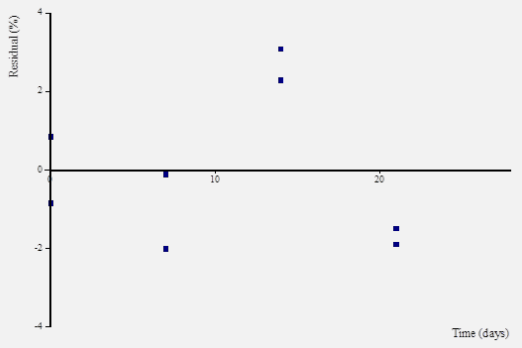


Table 192: Summary of kinetic fits for pyraclostrobin after multiple application of both test substances in RefeSol 02A; application rates were 2 x 0.333 mg/kg for pyraclostrobin and 2 x 0.400 mg/kg for ethofumesate – second application (21d – 120d) (STEP 2).

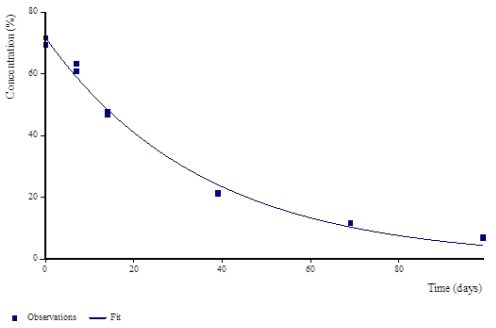
Parent: pyraclostrobin (multiple application – applied as a mixture of pyraclostrobin and ethofumesate, second application 21d – 120d)

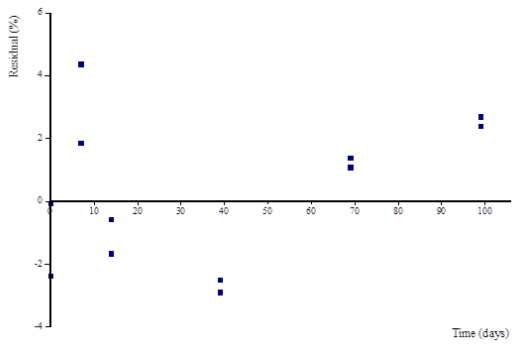
Soil	Model	Chi² error	r²	Parameter	DT50 [d]	DT90 [d]
RefeSol 02A	SFO	**4.66**	**0.992**	M_0: 71.68 k: 0.02795	**24.8**	**82.4**
	FOMC	4.79	0.9925	M_0: 72.37 α: 7.736 β: 253	23.7	87.7
	DFOP	5.00	0.9936	M_0: 72.52 k1: 0.03216 k2: 5.24E-012 g: 0.9482	23.3	92.6
	HS	4.49	0.9947	M_0: 72.46 k1: 0.02983 k2: 0.01674 tb: 51.37	23.2	97.4

Kinetic plots and residuals

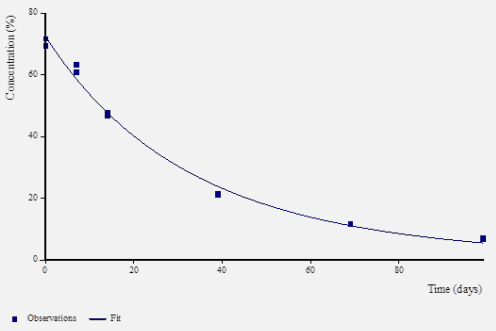
Parent: pyraclostrobin (multiple application – applied as a mixture of pyraclostrobin and ethofumesate, second application 21d – 120d)

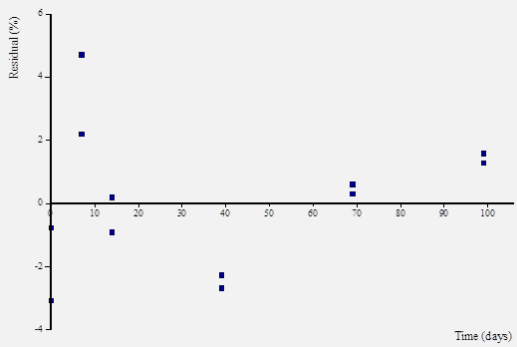
SFO



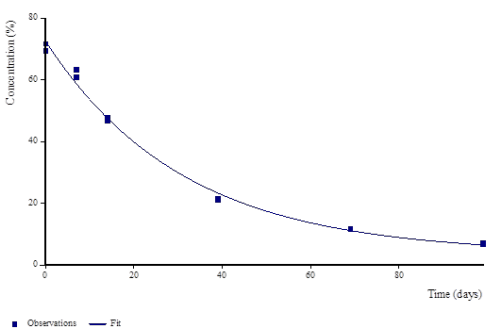


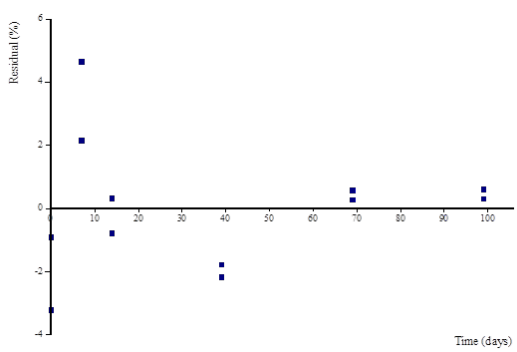
FOMC



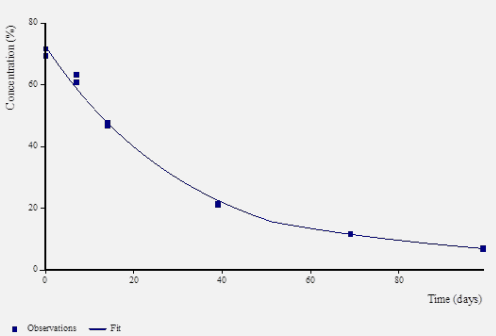


DFOP





HS



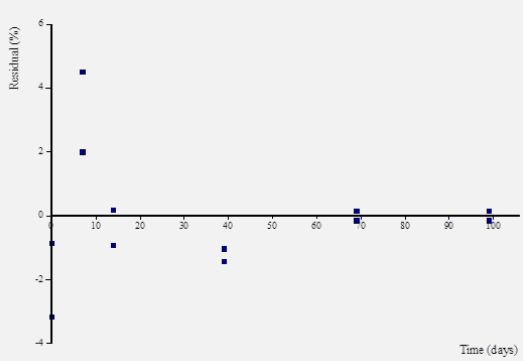


Table 193: Summary of kinetic fits for ethofumesate after multiple application of both test substances in RefeSol 02A; application rates were 2 x 0.333 mg/kg for

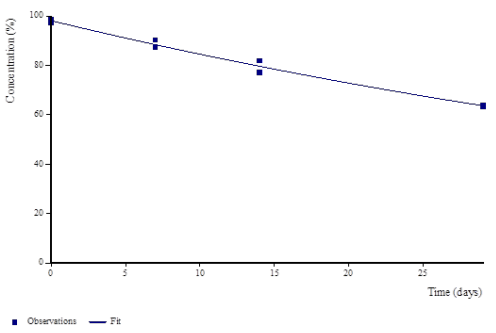
pyraclostrobin and 2 x 0.400 mg/kg for ethofumesate – first application (0d – 21d) (STEP 2).

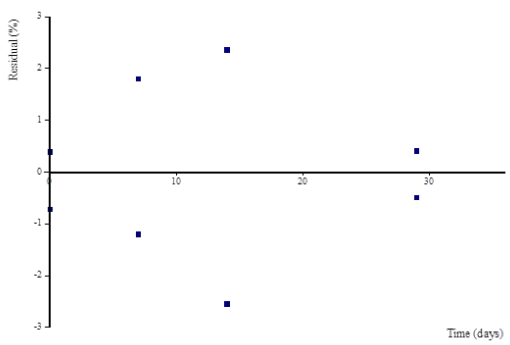
Parent: ethofumesate (multiple application – applied as a mixture of pyraclostrobin and ethofumesate, first application 0d – 21d)

Soil	Model	Chi² error	r²	Parameter	DT50 [d]	DT90 [d]
RefeSol 02A	SFO	**0.178**	**0.9863**	M_0: 98.12 k: 0.0149	**46.5**	**155**
	FOMC	0.227	0.9863	M_0: 98.13 α: 98.41 β: 6.59E+003	46.6	156
	DFOP	*n.d.*	0.9863	M_0: 98.12 k1: 0.01496 k2: 0.01489 g: 0.1456	46.5	155
	HS	*n.d.*	0.9865	M_0: 72.46 k1: 0.02983 k2: 0.01674 tb: 51.37	46.3	153

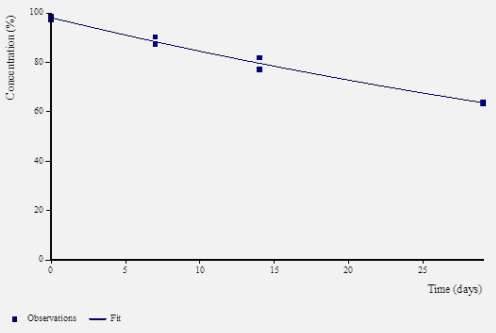
Kinetic plots and residuals

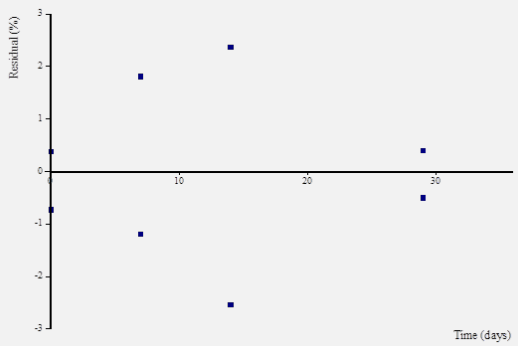
SFO





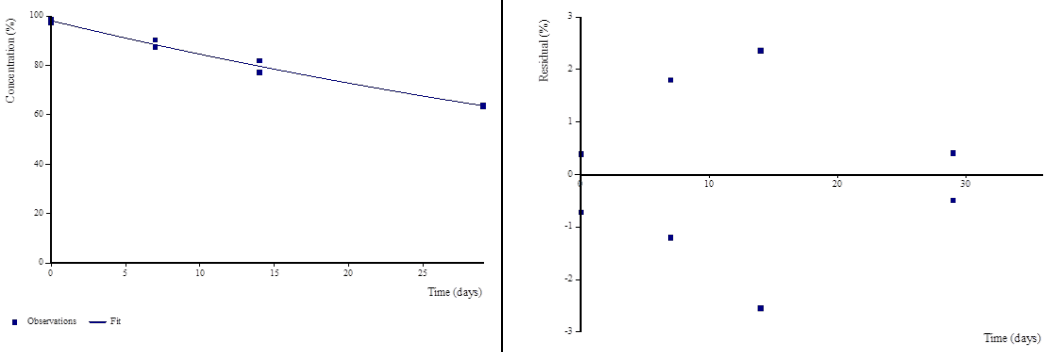
FOMC





Parent: ethofumesate (multiple application – applied as a mixture of pyraclostrobin and ethofumesate, first application 0d – 21d)

DFOP



HS

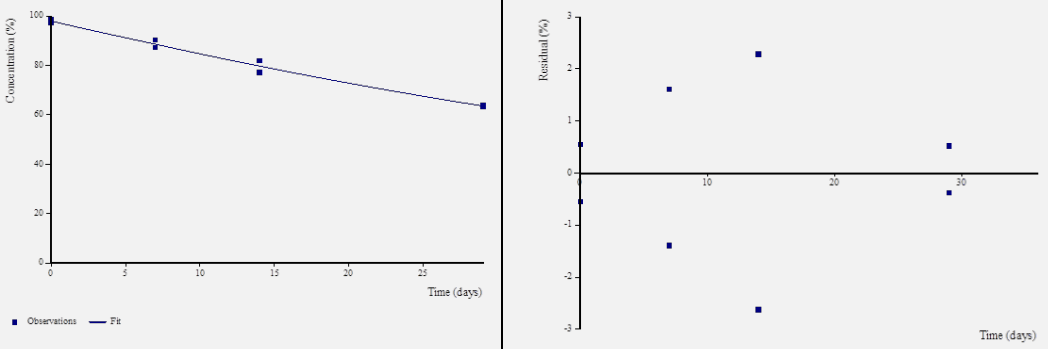


Table 194: Summary of kinetic fits for ethofumesate after multiple application of both test substances in RefeSol 02A; application rates were 2 x 0.333 mg/kg for pyraclostrobin and 2 x 0.400 mg/kg for ethofumesate – second application (21d – 120d) (STEP 2).

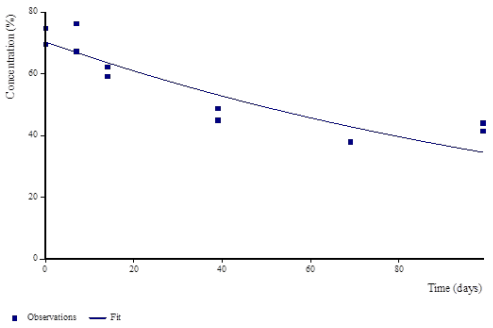
Parent: ethofumesate (multiple application – applied as a mixture of pyraclostrobin and ethofumesate, second application 21d – 120d)

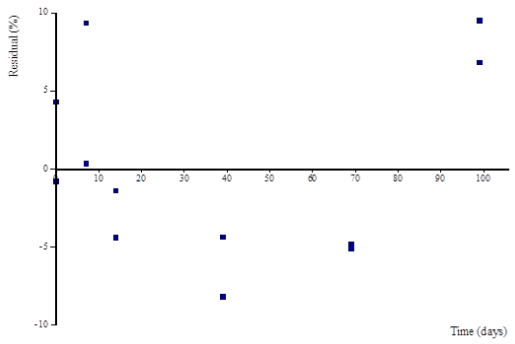
Soil	Model	Chi² error	r²	Parameter	DT50 [d]	DT90 [d]
RefeSol 02A	SFO	**7.54**	**0.8265**	M_0: 70.3 k: 0.007161	**96.8**	**322**
	FOMC	5.86	0.898	M_0: 74.5 α: 0.3889 β: 21.05	104	7820
	DFOP	5.48	0.9216	M_0: 74.99 k1: 0.03431 k2: 1.47E-010 g: 0.4904	> 10 000	> 10 000
	HS	4.18	0.9417	M_0: 72.46 k1: 0.02983 k2: 0.01674 tb: 51.37	> 10 000	> 10 000

Kinetic plots and residuals

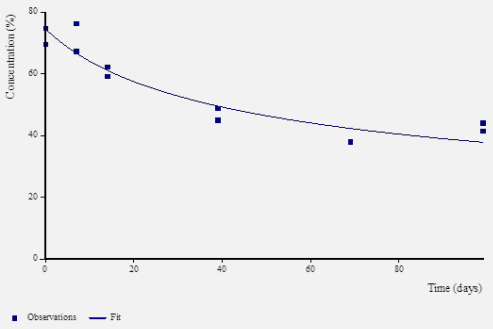
Parent: ethofumesate (multiple application – applied as a mixture of pyraclostrobin and ethofumesate, second application 21d – 120d)

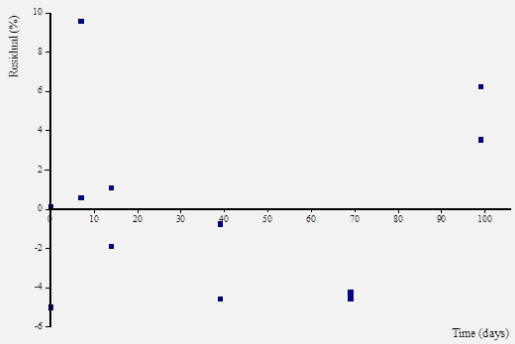
SFO



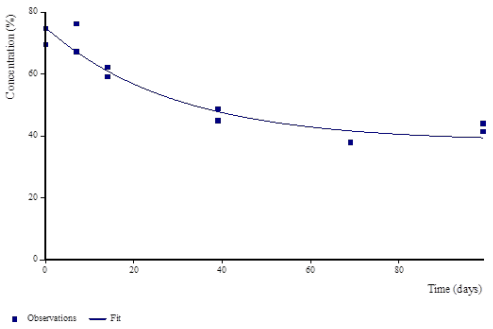


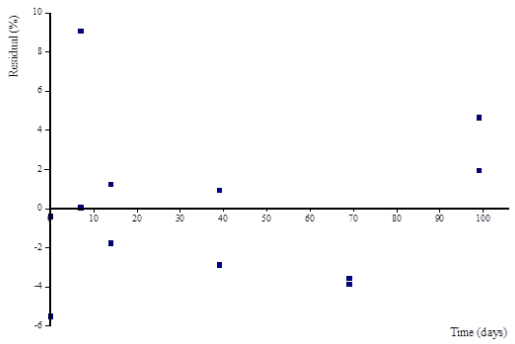
FOMC



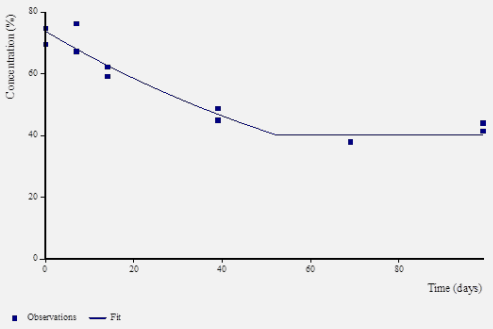


DFOP





HS



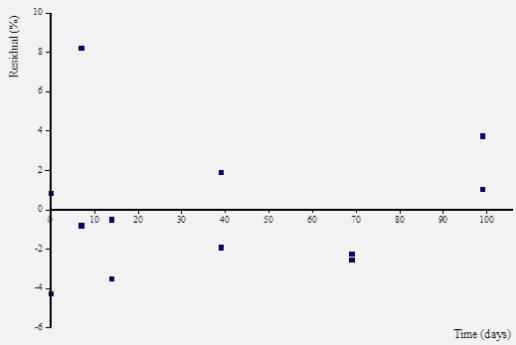
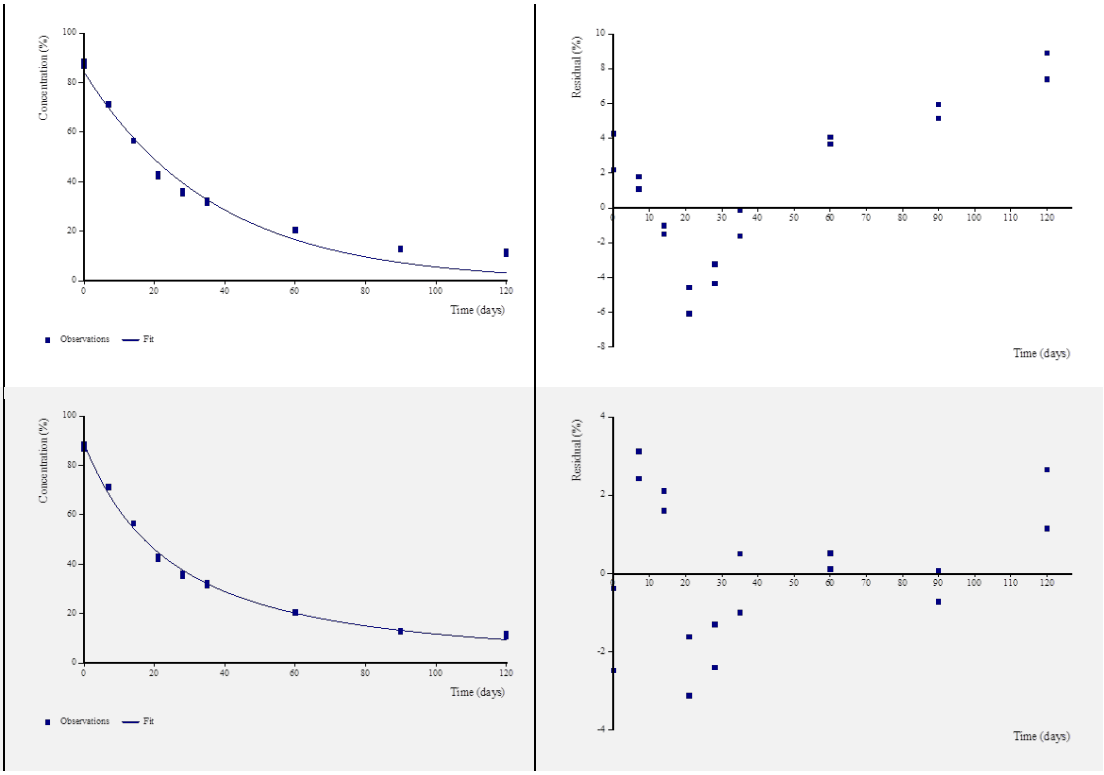


Table 195: Summary of kinetic fits for pyraclostrobin (0d) after single application of both test substances at two time points; application rates were 0.667 mg/kg for pyraclostrobin (0d) and 0.800 mg/kg ethofumesate (21d) (STEP 2).

Parent: pyraclostrobin (single application at 0d – in the presence of ethofumesate from 21d onwards)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
RefeSol 02A	SFO	**8.42**	**0.9767**	M_0: 84.32 k: 0.02697	**25.7**	**85.4**
	FOMC	3.49	0.9947	M_0: 88.98 α: 1.59 β: 39.11	21.4	127
	DFOP	2.83	0.9966	M_0: 88.5 k1: 0.04395 k2: 0.004622 g: 0.7855	21.5	166
	HS	2.75	0.9968	M_0: 88.22 k1: 0.03273 k2: 0.01417 tb: 29.4	21.2	124

Kinetic plots and residuals

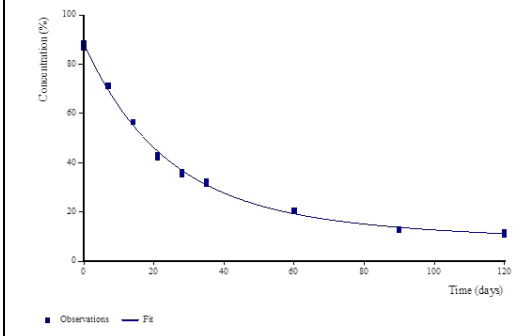
SFO

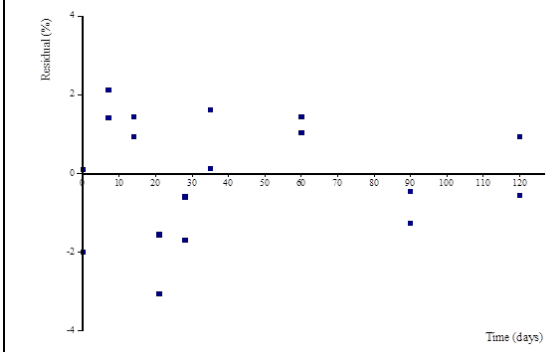


FOMC

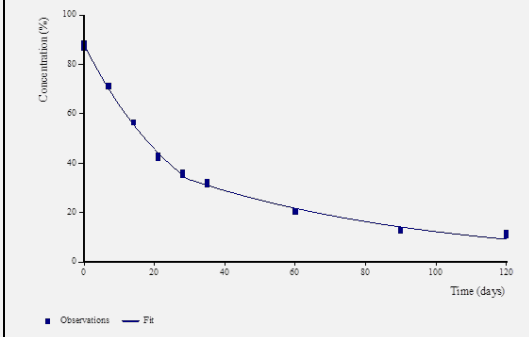
Parent: pyraclostrobin (single application at 0d – in the presence of ethofumesate from 21d onwards)

DFOP





HS



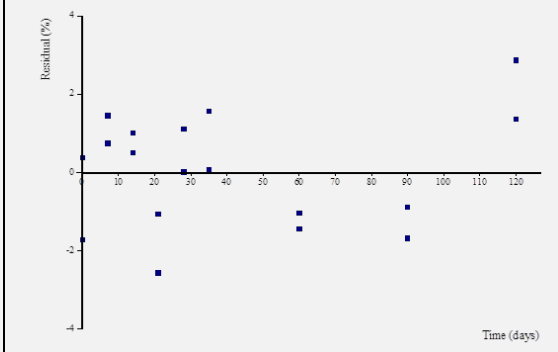


Table 196: Summary of kinetic fits for ethofumesate (21d) after single application of both test substances at two time points; application rates were 0.667 mg/kg for pyraclostrobin (0d) and 0.800 mg/kg ethofumesate (21d) (STEP 2).

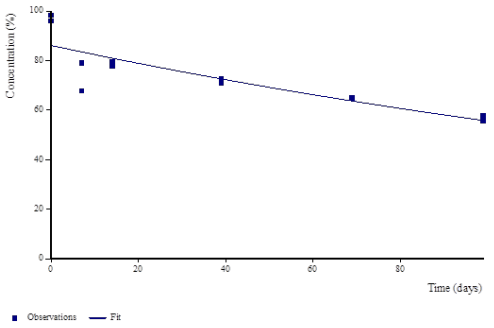
Parent: ethofumesate (single application at 21d – in the presence of pyraclostrobin from 0d onwards)

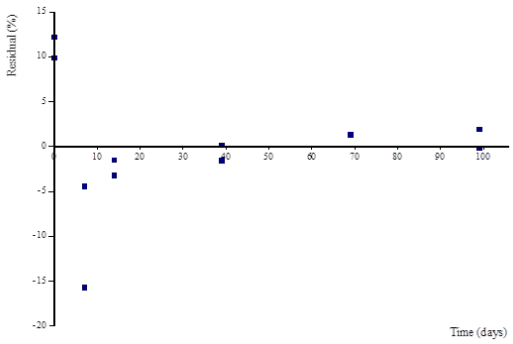
Soil	Model	Chi² error	r²	Parameter	DT50 [d]	DT90 [d]
RefeSol 02A	**SFO**	**6.69**	**0.7257**	M_0: 86.11 k: 0.004364	**159**	**528**
	FOMC	4.74	0.8654	M_0: 96.93 α: 0.09453 β: 0.8646	1320	> 10 000
	DFOP	3.08	0.9316	M_0: 97.15 k1: 2.825 k2: 0.003042 g: 0.1864	160	689
	HS	3.08	0.9316	M_0: 97.15 k1: 0.06064 k2: 0.003042 tb: 3.581	160	689

Kinetic plots and residuals

Parent: ethofumesate (single application at 21d – in the presence of pyraclostrobin from 0d onwards)

SFO





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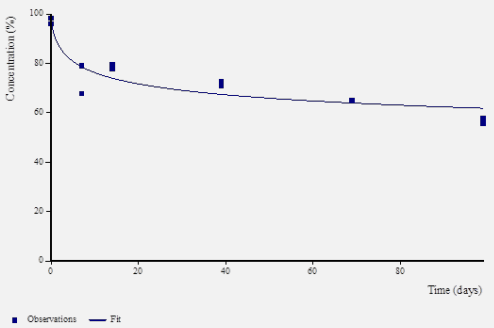
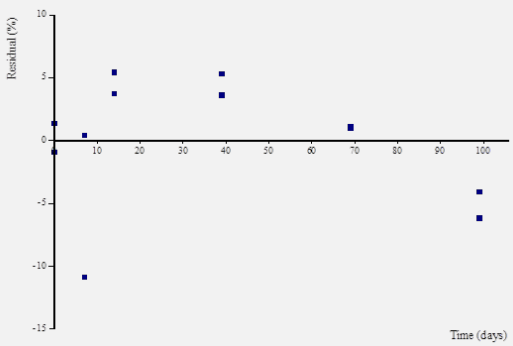
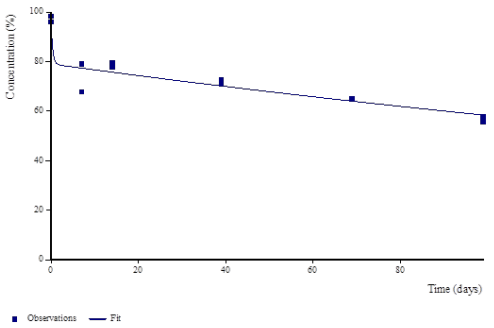
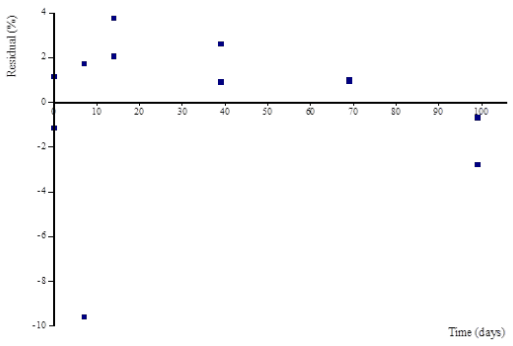


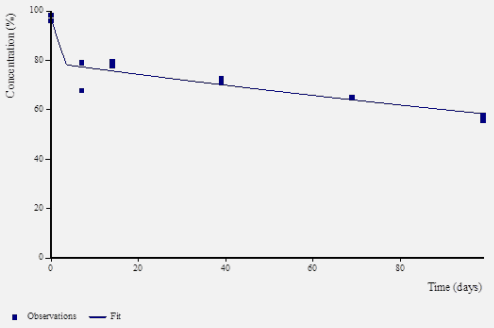


DFOP





HS



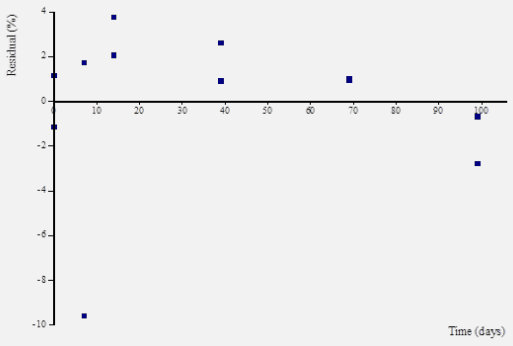
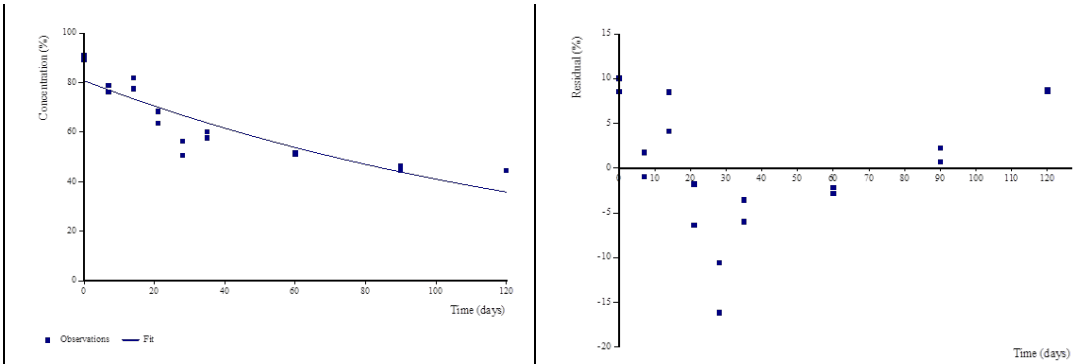


Table 197: Summary of kinetic fits for ethofumesate (0d) after single application of both test substances at two time points; application rates were 0.667 mg/kg for pyraclostrobin (21d) and 0.800 mg/kg ethofumesate (0d) (STEP 2).

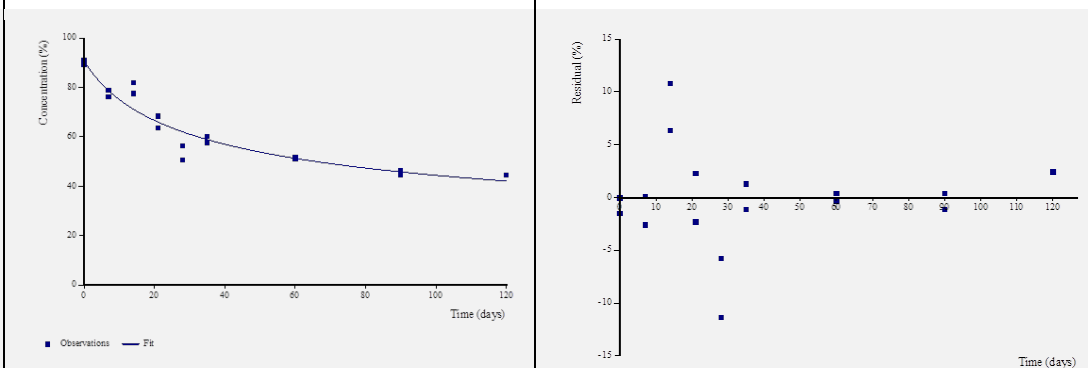
Parent: ethofumesate (single application at 0d – in the presence of pyraclostrobin from 21d onwards)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
RefeSol 02A	SFO	**8.74**	**0.7924**	M_0: 80.78 k: 0.006756	**103**	**341**
	FOMC	5.56	0.9174	M_0: 90.83 α: 0.3273 β: 12.72	93	> 10 000
	DFOP	5.44	0.9289	M_0: 90.43 k1: 0.0356 k2: 6.87E-020 g: 0.5138	102	> 10 000
	HS	5.29	0.9321	M_0: 89.42 k1: 0.01401 k2: 0.002449 tb: 36.52	111	768

Kinetic plots and residuals

SFO

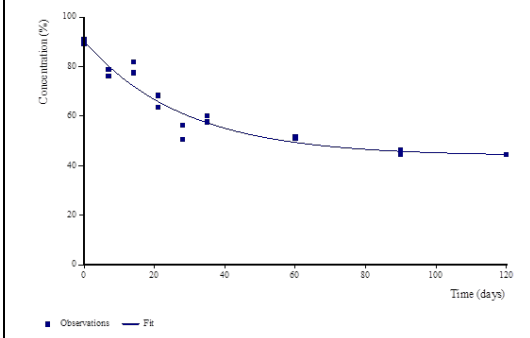


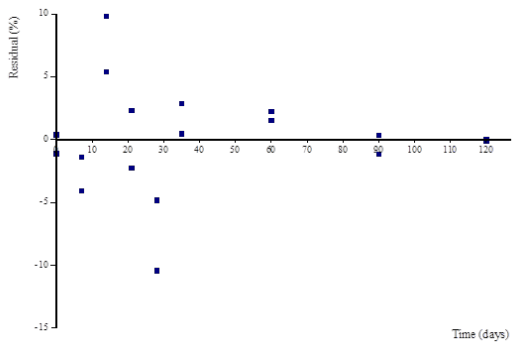
FOMC



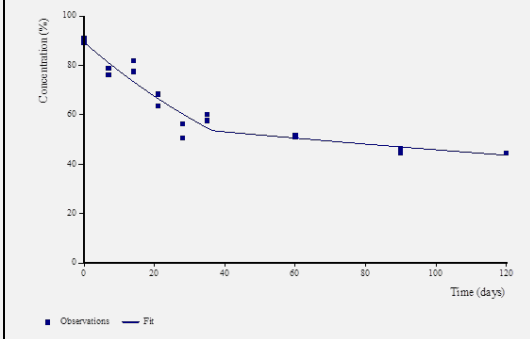
Parent: ethofumesate (single application at 0d – in the presence of pyraclostrobin from 21d onwards)

DFOP





HS



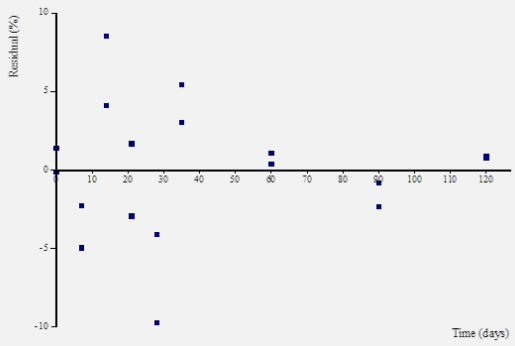


Table 198: Summary of kinetic fits for pyraclostrobin (21d) after single application of both test substances at two time points; application rates were 0.667 mg/kg for pyraclostrobin (21d) and 0.800 mg/kg ethofumesate (0d) (STEP 2).

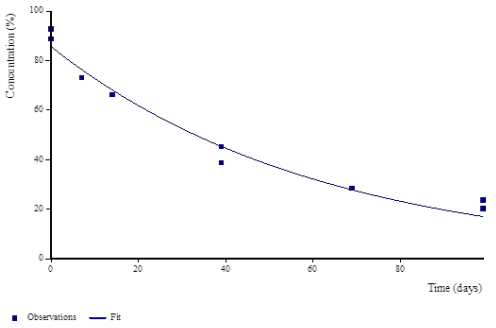
Parent: pyraclostrobin (single application at 21d – in the presence of ethofumesate from 0d onwards)

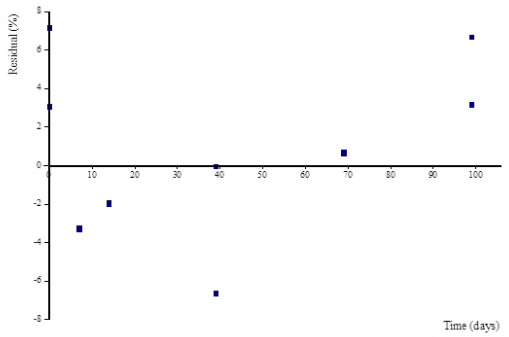
Soil	Model	Chi² error	r²	Parameter	DT50 [d]	DT90 [d]
RefeSol 02A	**SFO**	**5.27**	**0.9754**	M_0: 85.66 k: 0.01625	**42.7**	**142**
	FOMC	1.94	0.9928	M_0: 90.06 α: 1.187 β: 43.19	34.3	258
	DFOP	2.33	0.9925	M_0: 90 k1: 0.0525 k2: 0.009245 g: 0.4055	34.3	193
	HS	2.93	0.9911	M_0: 89.53 k1: 0.02336 k2: 0.01125 tb: 26.91	32.7	176

Kinetic plots and residuals

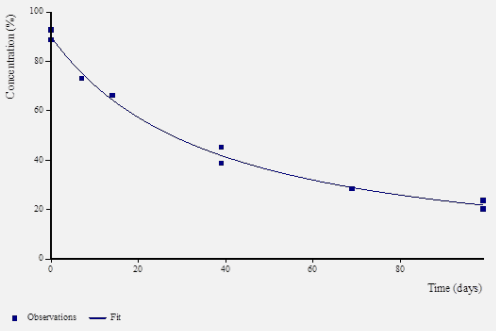
Parent: pyraclostrobin (single application at 21d – in the presence of ethofumesate from 0d onwards)

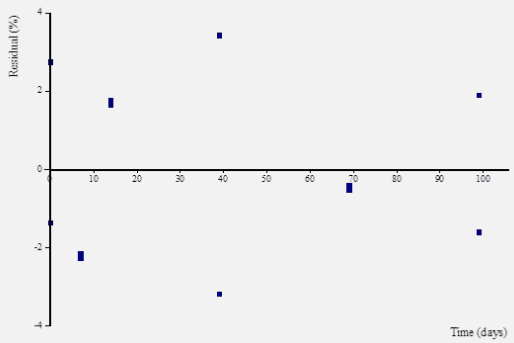
SFO



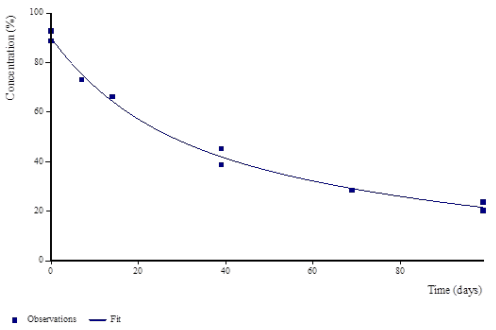


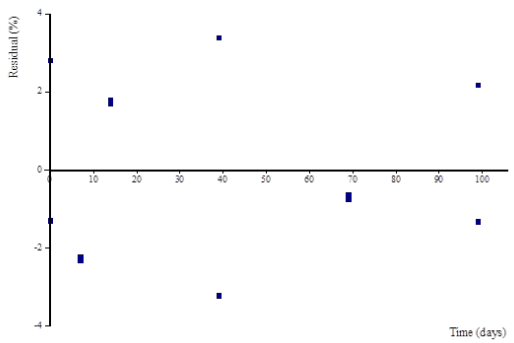
FOMC



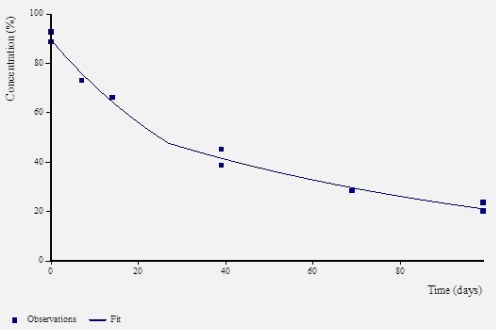


DFOP





HS



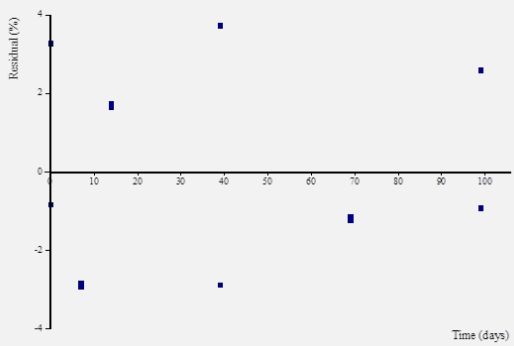
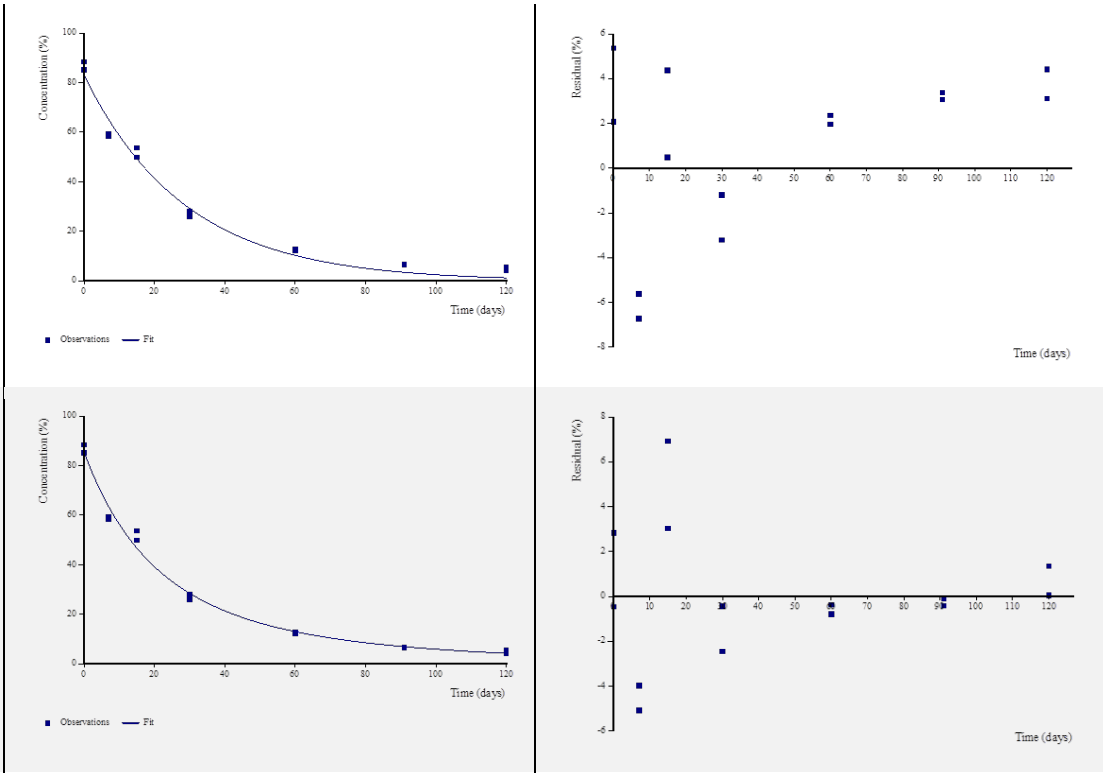


Table 199: Summary of kinetic fits for pyraclostrobin after single application to a soil with existing multiple contamination; nominal application rate was 0.667 mg/kg (STEP 3).

Parent: pyraclostrobin (single application)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
Soil with conta-mina-tions	SFO	**8.1**	**0.9855**	M_0: 83.04 k: 0.03472	**20**	**66.3**
	FOMC	6.44	0.99	M_0: 85.56 α: 2.722 β: 60.38	17.5	80.3
	DFOP	7.21	0.9897	M_0: 85.17 k1: 0.05278 k2: 0.01412 g: 0.7204	17.7	80.5
	HS	7.47	0.9891	M_0: 84.39 k1: 0.03772 k2: 0.01664 tb: 43.9	18.4	82.8

Kinetic plots and residuals

SFO



FOMC

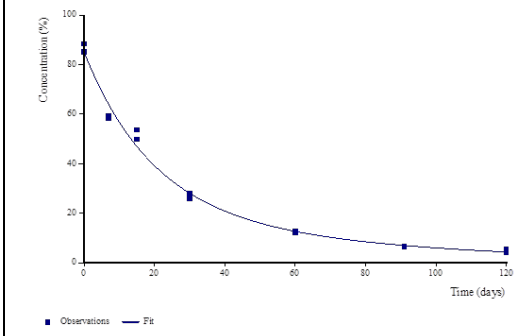
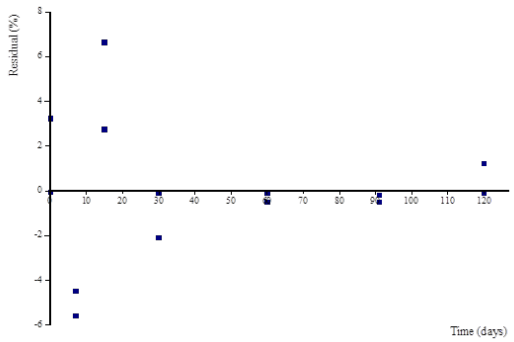
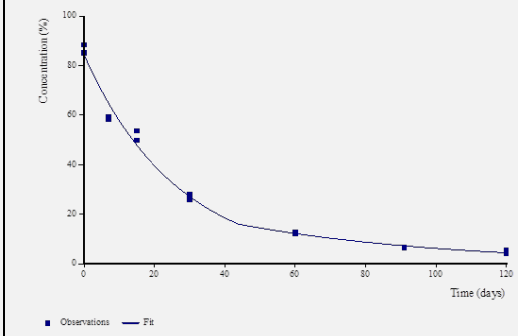
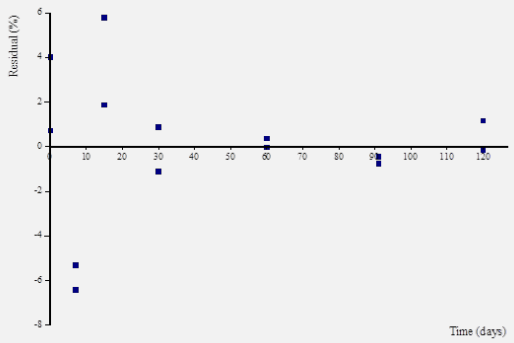
Parent: pyraclostrobin (single application)		
DFOP		
HS		

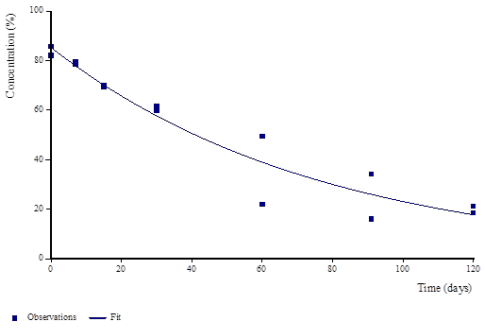
Table 200: Summary of kinetic fits for ethofumesate after single application to a soil with existing multiple contamination; nominal application rate was 0.800 mg/kg (STEP 3).

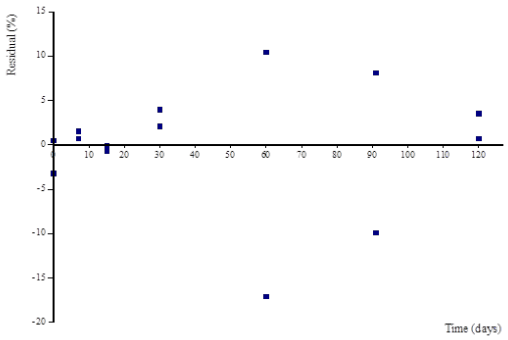
Parent: ethofumesate (single application)						
Soil	**Model**	**Chi² error**	**r²**	**Parameter**	**DT50 [d]**	**DT90 [d]**
Soil with contamina-tions	SFO	**3.01**	**0.9308**	M_0: 85.32 k: 0.01302	**53.3**	**177**
	FOMC	3.24	0.9308	M_0: 85.42 α: 47.76 β: 3.63E+003	53	179
	DFOP	3.53	0.931	M_0: 85.62 k1: 0.01392 k2: 2.41E-009 g: 0.966	52.4	193
	HS	3.18	0.9322	M_0: 85.71 k1: 0.01341 k2: 0.007975 tb: 91.61	51.7	226

Kinetic plots and residuals

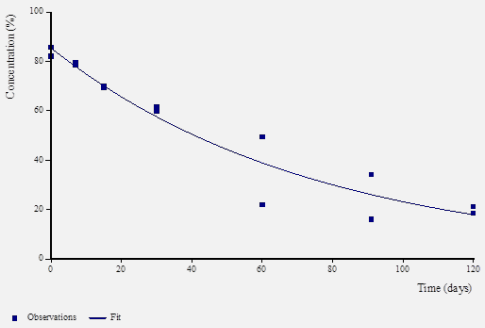
Parent: ethofumesate (single application)

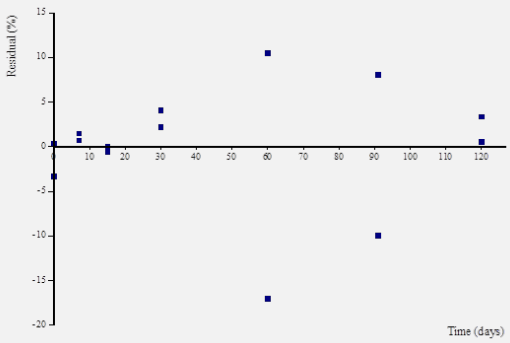
SFO



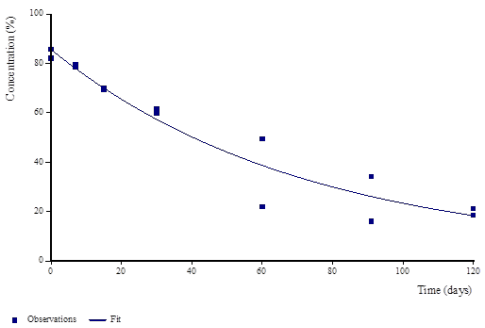


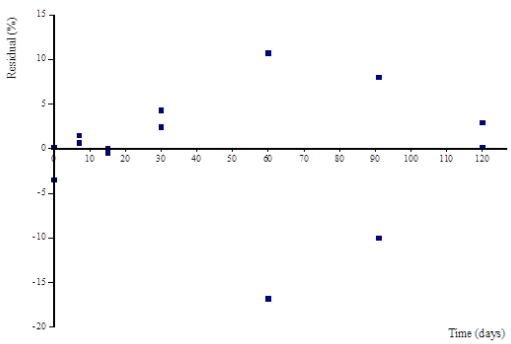
FOMC



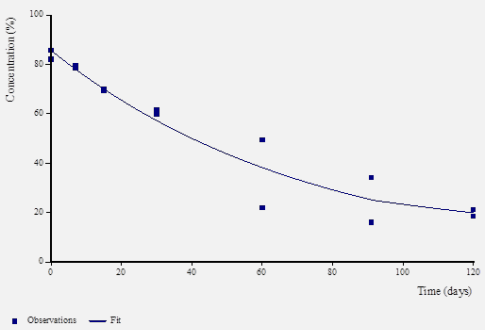


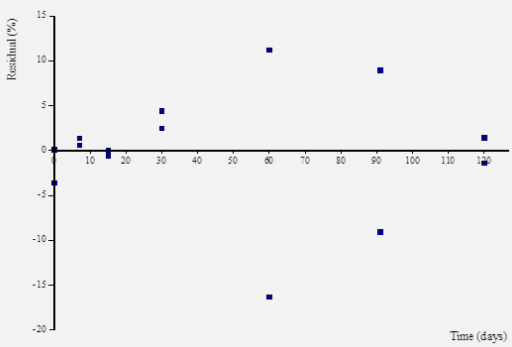
DFOP





HS





E Appendix 5 - Project-related database of experimental results (work package 5)

The technical implementation comprises a workflow that first defines a relational 'database's structure, takes the raw data input, preprocesses the data, when necessary, sends the data to a database backend and finally allows for showing the data in any frontend software.

As a first step, a relational database structure for ecotoxicological effect studies has been determined and laid down using a single MS Excel® workbook. The same workbook has been later used as a template for data input. The structure and procedures of filling the database are currently tested and could be adapted in future versions. Data fields (variables) could be discarded if not considered valuable or could be added if essentially lacking. The Excel workbook holds eight *tables* with the basic information needed to store the data from the ecotoxicological experiments using microorganisms. The variables holding primary keys have the prefix "pk", and foreign key fields in related tables that could join with the primary keys have the prefix "fk".

24. Studies table

The studies table holds information on the prevailing conditions of the study execution. Thirteen fields are to be filled by the processor of the study, most important the unique identifier (UID) of the study that serves as the primary key. The contents of the *studies* table are explained in more detail in Table 201. The table entry "*pk_study_id*" is used as a foreign key picklist in the *measurements* table and could be joined by this variable for further data analysis.

Table 201: Data fields of the *studies* table of the MICROSOIL ecotoxicological effects database.

Data field	Explanation
pk_study_id	Unique Identifier of the study, primary key
study_code	Internal code for recognising the study in the files of the laboratory
study_desciption	The general purpose of the study
study_design	It could be "limit", "dose-response", or "compromise" depending on the guideline
experimental_run_replicate	In case the experiment was run several times with identical prevailing conditions
study_duration	The duration of the study, from the start of the experimental period until the termination of the experiment, must be differentiated from the exposure period
study_duration_unit	Study duration should be given in days for consistency, and it could be any internationally acknowledged unit of duration
data_processed_by	The person that has been processing the data should be given in case later enquiries would be necessary
data_processed_date	The date when the data of the study was (started to) being entered into the database
exposure_duration	The time from exposing the test system until the end of the exposure period. In soil systems, mainly identical to termination of the experiment

Data field	Explanation
exposure_duration_unit	Exposure duration should be given in days for consistency, and it could be any internationally acknowledged unit of duration
exposure_type	Exposure could be put into effect by mixing the test substance into the soil or spraying the soil surface
note	Any noticeable remarks and peculiarities in the study execution

25. Test items table

The test items table informs on the identity, type of activity (mode-of-action) and physico-chemical properties of the test item, i.e. the active ingredient tested in an ecotoxicological study. It holds twenty-five variables, but most information could be queried from external databases, given that unambiguous identification by CAS or IUPAC names is possible. The field "*pk_test_item_id*" is used as a foreign key picklist in the *measurements* table for joining.

Table 202: Data fields of the *test items* table of the MICROSOIL ecotoxicological effects database.

Data field	Explanation
pk_test_item_id	Unique Identifier of the test item
test_item_name	Internal or trade name of the test item, often the name of a PPP
type_test_item	Could be "active ingredient", "plant protection product", "biocidal product", or "industrial chemical"; the list is freely expandable
cas_no	Unique identifier "CAS registry number" of the active ingredient from the Chemical Abstracts Service; in case a formulated product contains more than one active ingredient, the constituents must be separated by "&"-symbols for later parsing
iupac_name	Unique identifier "IUPAC name " of the active ingredient following the nomenclature of the International Union of Pure and Applied Chemistry; in case a formulated product contains more than one active ingredient, the constituents must be separated by "&"-symbols for later parsing
common_name	The common name of the active ingredient(s) are not necessarily unique and unambiguous but well-known
batch	The batch number of the supplier of the chemicals tested
acid"onstant	"Acidity constant", a measure of the strength of an acid in a solution
activity	Mainly deductible for pesticides could be insecticide, fungicide, nematicide, herbicide, acaricide (e.g., queried from https://pesticidecompendium.bcpc.org)
modeofaction	The mode of action involves in more detail the mechanism of toxic action (mainly for pesticides); nomenclature defined by the Fungicide Resistance Action Committee, Insecticide Resistance Action Committee, Herbicide Resistance Action Committee
atmorateconst	Atmospheric OH rate constant
charge	The total (or net) charge of a molecule (https://pubchemdocs.ncbi.nlm.nih.gov/pug-rest)
boilpoint	Boiling point

Data field	Explanation
hdonorcount	Number of hydrogen-bond donors in the structure (https://pubchemdocs.ncbi.nlm.nih.gov/pug-rest)
hacceptcount	Number of hydrogen-bond acceptors in the structure (https://pubchemdocs.ncbi.nlm.nih.gov/pug-rest)
fatsol	Solubility of the active substance in fat
henconst	Henry constant, the proportion of the ai concentration in the aqueous phase compared to the gas phase
logkow	Octanol-water partition coefficient
meltpoint	Melting point of the chemical substance
molweight	Molecular weight of the chemical substance
redens	Relative density of the chemical substance compared to water
vaporpress	Vapour pressure of the chemical substance
watersol	Water solubility of the chemical substance
dissconst	Acid dissociation rate constant pKA of the chemical substance
note	Any noticeable remarks and peculiarities in the study execution

26. Methods table

The *methods* table holds the list of methods used, preferably referring to an international guideline. The table contains three variables. The list entries of the primary key "*pk_method_id*" serve as a picklist for the measurements table and are joined with it by using the foreign key for data analysis.

Table 203: Data fields of the *methods* table of the MICROSOIL ecotoxicological effects database.

Data field	Explanation
pk_method_id	Unique Identifier of the method
guideline_reference	The international guideline used could also be an internal method or an adapted guideline
notes	Any noticeable remarks and peculiarities in the study execution

27. measurement_endpoints table

The *measurement endpoints* table comprises the list of possible measurements combined with the transformed substrates (in functional test methods) and the measured degradation products. The table entry "*pk_measurement_endpoint_id*" serves as a picklist in the *measurements* table (Table 208); the tables can be joined for further data analysis. The table holds seven variables, described in Table 204.

Table 204: Data fields of the *measurement endpoints* table of the MICROSOIL ecotoxicological effects database.

Data field	Explanation
pk_measurement_endpoint_id	Unique Identifier of the measurement endpoint
measurement_endpoint	If applicable, the measurement endpoint describes the measurement taken in connection with the substrate for enzyme activities. If not applicable, any other unambiguous description of the final measurement used for deducing statistical endpoints
substrate	The substrate that was added for measuring the degradation or enzymatic ability of a community of microorganisms
measurement	Indicates what was measured, could be, e.g. nitrification, substrate-induced respiration, enzyme activity, or any other unambiguous description of the measurement
measured_degradation_product	The amount of the measured degradation product as the proportion between the amount of the substrate and product per time (rate)
measured_degradation_product_unit	The unit corresponds to the measured degradation product and is often given as A rate.
note	Any noticeable remarks and peculiarities in the study execution

28. biological_entity table

The *biological entity* is either a test organism (e.g., a single species of mycorrhizal fungi), an assemblage of well-characterised populations of bacteria or a microbial community from natural sources. The variable "*pk_biological_entity_id*" builds the picklist of the "*fk_biological_entity_id*" in the *measurements* table and thus could be used for more information on the tested organisms for further analysis and filtering of results. The table holds five variables so far described in Table 205.

Table 205: Data fields of the *biological entity* table of the MICROSOIL ecotoxicological effects database.

Data field	Explanation
pk_biological_entity_id	Unique Identifier of the *biological entity*
entity_description	Name of the test species, an indication of the composition of an assemblage of different populations or the description of a microbial community as far as characterised in Detail.
entity_storage	The storage conditions indicate if a microbial community could have evolved and changed over time or if certain species could have been selected due to unfavourable storage circumstances.
entity_origin	The origin of an inoculum could be a natural or laboratory resource
entity_date_sampling	The date of sampling could be compared to, e.g., the start of the experiment and shed light on unexpected results
note	Any noticeable remarks and peculiarities in the study execution

29. soil_types table

The *soil types* table collects information on classifiers of the test soil exposed to the chemical test substance. Here, no information on the soil used for inoculum extraction is stored. The variable "*pk_soil_type_id*" builds the picklist of the "*fk_soil_type_id*" in the *measurements* table and thus could be used for more information on the tested soil types for further analysis and filtering of results. The table holds twelve variables so far described in Table 206.

Table 206: Data fields of the *soil types* table of the MICROSOIL ecotoxicological effects database.

Data field	Explanation
pk_soil_type_id	Unique Identifier of the *soil type*
soil_name	An informative name could be for internal use only or serve as a reference to a specific soil from the literature
texture_sand	The percentage of sand in the test soil; adds up to 100 per cent, with silt and clay percentages
texture_silt	The percentage of silt in the test soil; adds up to 100 per cent, with sand and clay percentages
texture_clay	The percentage of clay in the test soil; adds up to 100 per cent, with sand and silt percentages
texture_type	The texture type is derived in each case from a texture triangle; its form depends on the texture type classification system used (see below), given as, e.g., "silty clay" or "clayey sand"
texture_type_classification_system	There are numerous classification systems worldwide, the German system, the USDA triangle, and the HYPRES texture triangle of the European soil map. It should be given in a standardised form for comparability
soil_type	The soil type is the result of soil genesis and is difficult to determine, also depending on the classification system used (see below)
soil_type_classification_system	The taxonomies of soils differ from region to region worldwide. Maps and specifications are fixed by the European Joint Research Centre
corg_perc	The organic carbon weight percentage
cmic_perc	The microbial carbon weight percentage
whc	Water holding capacity
cec	Cation exchange capacity
ph	pH value
note	Any noticeable remarks and peculiarities in the study execution

30. treatments table

The *treatments* table collects information on the treatment levels of chemical substances used for the exposure of the test systems. The variable "*pk_soil_type*" builds the picklist of the "*fk_treatment_id*" in the *measurements* table and thus could be used for more information on the treatment levels for further analysis and for deriving statistical endpoints. The table holds seven variables so far described in Table 207.

Table 207: Data fields of the *treatments* table of the MICROSOIL ecotoxicological effects database.

Data field	Explanation
pk_treatment_id	Unique Identifier of the *treatment*
fk_study_id	The treatments are specific for each study, and thus, the *study_id* must be picked from a list that originates from the *study* table
fk_test_item_id	The test items are specific for each study, and thus, the *test_item_id* must be picked from a list that originates from the *study* table
treatment_level_code	Integer number, 0 indicating control level, 1 and higher numbers indication ascendingly ordered treatment levels of the test item
soil_concentration	The numerical value of the concentration of the active ingredient in the soil
soil_concentration_unit	The unit of the concentration of the active ingredient in the test soil; needs agreement on relevant soil depth and soil density and should preferably be given as mg ai/kg soil dry weight
note	Any noticeable remarks and peculiarities in the study execution

31. measurements table

The *measurements* table is the central table holding the specific experimental results that are used subsequently for the computation of derived endpoints (see Table 209). Multiple foreign keys point to picklist table entries described above. The table holds fourteen variables described in detail in Table 208 below.

Table 208: Data fields of the *measurements* table of the MICROSOIL ecotoxicological effects database.

Data field	Explanation
pk_measurement_id	Unique Identifier of the *measurement*
fk_study_id	Picklist entry of the unique identifier of the *studies* table
fk_test_item_id	Picklist entry of the unique identifier of the *test item* table
fk_method_id	Picklist entry of the unique identifier of the *method* table
fk_measurement_endpoint_id	Picklist entry of the unique identifier of the *measurement endpoint* table
fk_biological_entity_id	Picklist entry of the unique identifier of the *biological entity* table
fk_soil_type_id	Picklist entry of the unique identifier of the *soil_type* table
fk_treatment_id	Picklist entry of the unique identifier of the *treatment* table
treatment_replicate	String variable indicating the experimental replicate of the respective treatment level
value	The measured value of the measurement endpoint for the biological entity in the respective study

Data field	Explanation
measurement_time	The date after the start of the exposure period when the reported measurement was taken
measurement_time_unit	The unit of the date when the measurement was taken, preferably in days
note	Any noticeable remarks and peculiarities in the study execution

32. derived_endpoints table

The derived endpoints table is meant to contain all the statistical endpoints that were computed for the single experimental results. The table could be queried for risk characterisation (in conjunction with appropriate indicators of exposure (of the test or generically modelled soils) and used for comparisons and evaluation between experimental or statistical methods. Multiple foreign keys point to picklist table entries described above. The table holds fourteen variables described in detail in Table 209 below.

Table 209: Data fields of the *derived endpoints* table of the MICROSOIL ecotoxicological effects database.

Data field	Explanation
pk_derived_endpoint_id	Unique Identifier of the *derived endpoint*
fk_study_id	Picklist entry of the unique identifier of the *studies* table
fk_test_item_id	Picklist entry of the unique identifier of the *test item* table
fk_method_id	Picklist entry of the unique identifier of the *method* table
fk_measurement_endpoint_id	Picklist entry of the unique identifier of the *measurement endpoint* table
fk_biological_entity_id	Picklist entry of the unique identifier of the *biological entity* table
fk_soil_type_id	Picklist entry of the unique identifier of the *soil_type* table
statistical_endpoint	The statistical endpoint is the result of a statistical evaluation of the experimental results (a model, a method) and the convention on the relevance for the assessment (the lowest significantly effective soil concentration, the concentration causing 50 % of an effect); usually a NOEC or EC_x
evaluation_method	The statistical method applied (t-Test, Probit regression)
measurement_time	The date after the start of the exposure period when the reported measurement was taken
measurement_time_unit	The unit of the date when the measurement was taken, preferably in days
note	Any noticeable remarks and peculiarities in the study execution

33. lookup_units table

In cases when the original table lacks specification of the relevant units of a parameter, the lookup table can be consulted and joined using the variable name given in Table 210. This lack of knowledge is mainly given to the physico-chemical properties of the *test items* table.

Table 210: Data fields of the *lookup units* table of the MICROSOIL ecotoxicological effects database.

Data field	Explanation
pk_variable_id	Unique Identifier of a variable
unit	Unit of the measurement for the variable
note	Any noticeable remarks and peculiarities in the study execution

Table 211: Data fields of the *lookup units* table of the MICROSOIL ecotoxicological effects database.

Data field	Explanation
pk_degradation_id	Unique Identifier of the *derived endpoint*
fk_study_id	Picklist entry of the unique identifier of the *studies* table
fk_test_item_id	Picklist entry of the unique identifier of the *test item* table
fk_method_id	Picklist entry of the unique identifier of the *method* table
fk_measurement_endpoint_id	Picklist entry of the unique identifier of the *measurement endpoint* table
fk_biological_entity_id	Picklist entry of the unique identifier of the *biological entity* table
fk_soiltype_id	Picklist entry of the unique identifier of the *soil_type* table
fk_treatment_id	Picklist entry of the unique identifier of the *treatments* table
test_item_name	
application_type	
application_rate_nominal	
application_rate_nominal_unit	
application_interval	
application_interval_unit	
soil_name	
experimental_run	
kinetics	
chi_squared	
r_squared	
dt50	
dt50_unit	

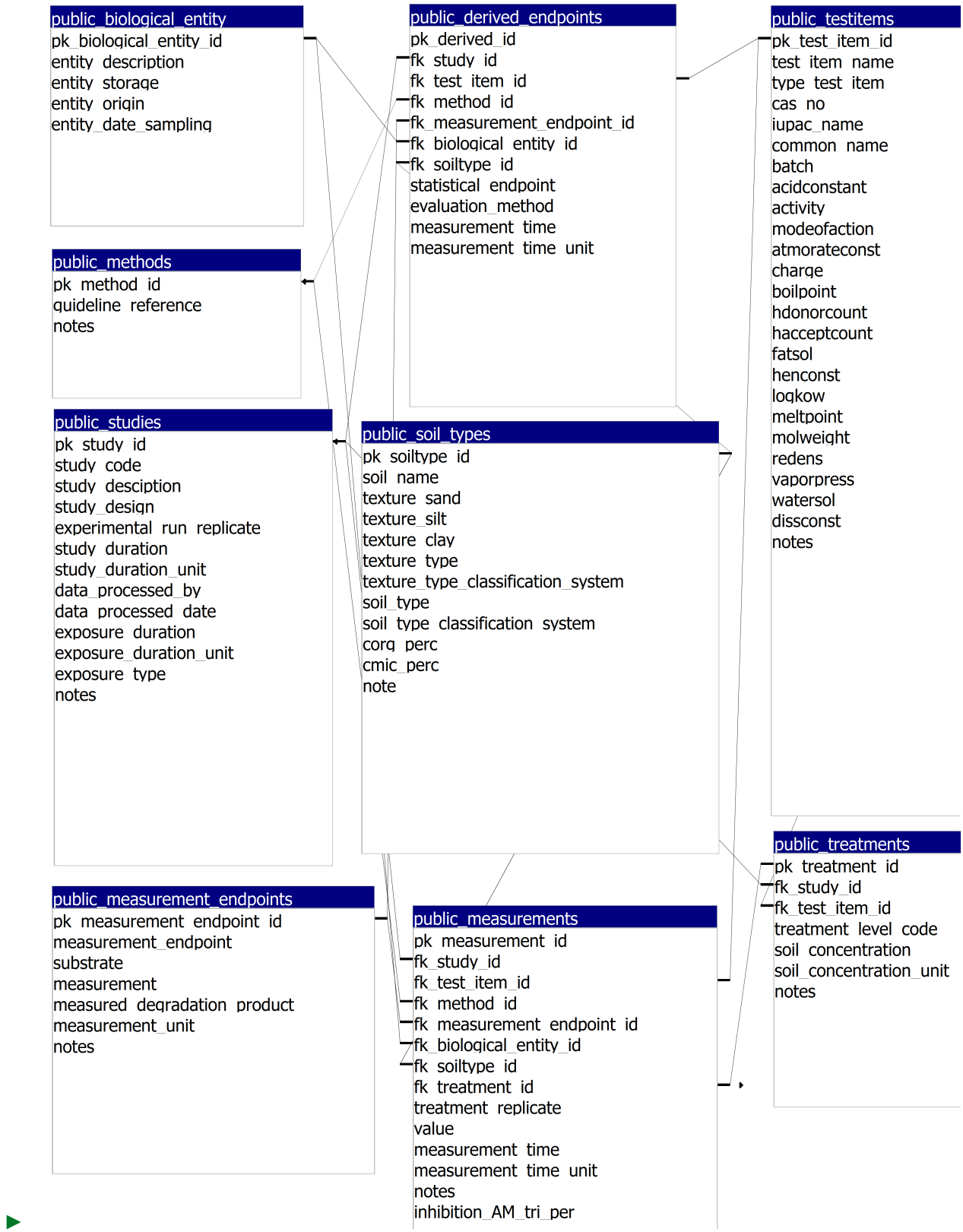
Data field	Explanation
dt50_operator	
dt90	
dt90_unit	
dt90_operator	
fitparam_1	
fitparam_2	
fitparam_3	
dt50_1	
dt50_1_unit	
dt50_1_operator	
dt50_2	
dt50_2_unit	
dt50_2_operator	
lower_90cl_k1	
upper_90cl_k1	
lower_95clk1	
upper_95_cl_k1	
lower_90cl_k2	
upper_90cl_k2	
lower_95_cl_k2	
upper_95cl_k2	
lower_90cl_ gtb	
upper_90cl_gtb	
lower_95cl_gtb	
upper_95cl_gtb	

The import of the raw data from MS Excel tables is realized using the *R* programming language (Version R: 4.1.1 (202–0“-10) -- "Ki”k Things", R Core Team, 2021 and the Integrated Development Environment IDE *R-Studio* (version RStudio 2021.“9.0+351 "Gho”t Orchid" Release (077589bcad3467ae79f318afe8641a1899a51606, 2021-09-20) for Windows, RStudio Team, 2021 by the package *openxlsx* (https://joshuasturm.github.io/openxlsx/articles/Formatting).

A PostgreSQL database (version 16) has been running for the time being at *localhost* but could be transferred to any server-based installation of Postgres if desired. The readily pre-processed

data tables are sent via the *dbWriteTable* function of the package *DBI* (R Special Interest Group on Databases, 2021). Primary and foreign keys are directly added from within the R-environment via the *rpostgis* package and its valuable function *dbAddKey*. Further database configuration can be executed by the *pgAdmin* backend (pgAdmin 4 version 5.7). Optionally, an MS Access® frontend view can be used via an ODBC connection to the Postgres server. The relationships between the tables and the matching data fields (primary and foreign keys) can then be quickly reviewed by the Access relationship view (see an example in Figure 85).

Figure 85: Relationships between the tables of the MICROSOIL ecotoxicological effect database from MS Access frontend view of the PostgreSQL database.



E.1 Import and pre-processing of ARISA data

- Description of the binning algorithm

  - Regarding the script, in the Ramette (Ramette 2009) publication the WScutoff was determined experimentally but 2 is an accepted value of imprecision which could be used in R-scripts used for the MICROSOIL project. There are also scripts which allow an automatic calculation of a series of WS values (e.g., 0.5, 1, 2, 3, 4, and 5 bp).

  - r-code: *WScutoff <- 2* #This is the window size cutoff, i.e. the tolerance window in which you group the peaks into one.

  - R-code: *shiftsize <- 0.2* #The distance between two consecutive binning frames is defined as the shift (Sh) value.

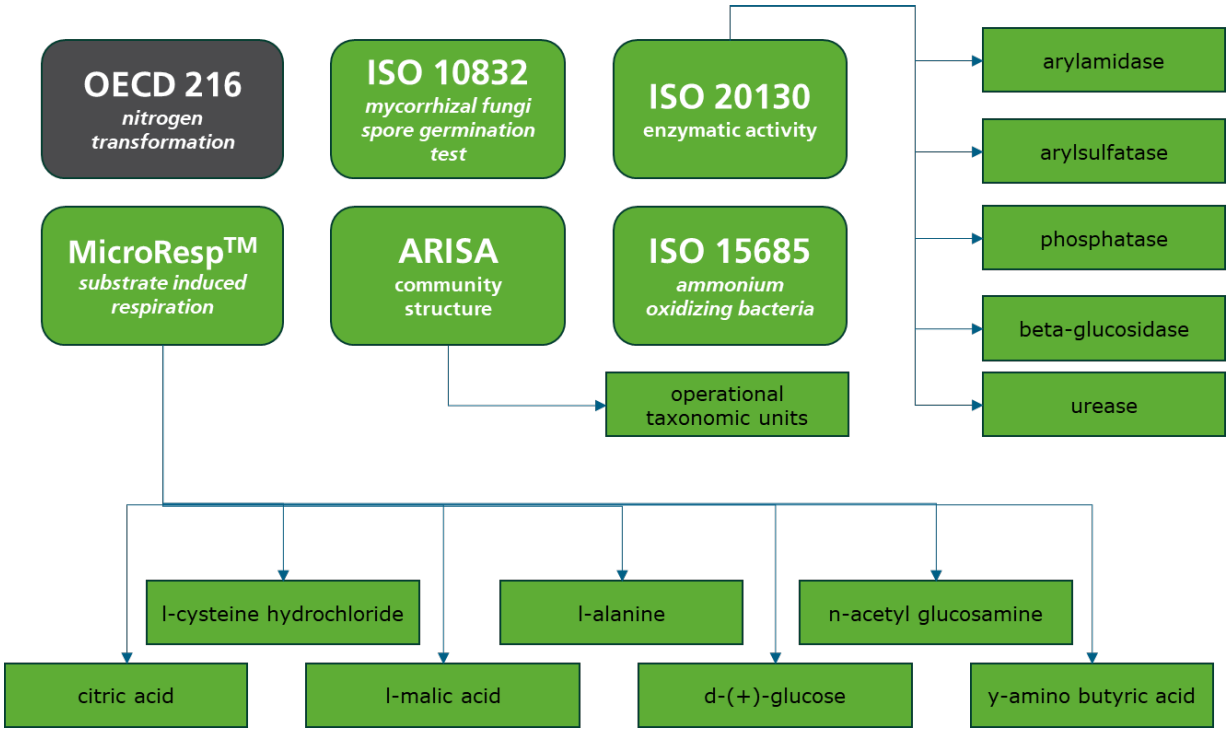
    A shifting window size binning strategy was used since it offers the possibility to optimally aligned electrophoretic profiles and to deal with different window starting positions. The binning frame that offers the highest similarity among samples is identified out of all binning frames starting at a given position. The distance between two consecutive binning frames is defined as the shift (Sh) value. In the original description of the binning approach, only integer values were considered as *true* values to which peaks needed to be assigned to by taking the appropriate technical inaccuracy, resulting in Sh 1 bp. This would mean that the two bin frames (WS 2) would start, in our case, at 100 and at 101, respectively. Due to dye migration discrepancies, however, the actual, true (but unknown) size value may be different from integer values (i.e., a decimal value could be also representing a “true” size) and may change over the range of sizes being examined due to sequence-specific migration discrepancies. For this reason, Sh values must also be variable and a good, but the computing-demanding value may be 0.1 bp.

  - The *relative abundance* was added within the binning scripts based on the absolute vales (peak area). It should be considered which is the best reference group for relativisation (now: *batch* + *soil_type* + *substance*)

E.2 Import and pre-processing of transformation and inhibition data

Figure 86 gives an overview of the methods to describe effects on the transformation ability of soil microorganims of different substrates applied within the MICROSOIL project, which were subjected to data import into the project-related database.

Figure 86: Overview of test methods and measurement parameters.



Source: own illustration, Darwin Statistics

Experimental results are available for the following five study types:

1. ISO_20130 --> enzymatic activity, five enzymes
2. MicroResp --> substrate induced respiration, 8 substrates
3. ISO_15685 --> nitrite production rate, 1 endpoint
4. OECD_216 --> nitraAte production, 1 endpoint
5. ISO10832 --> germination rates of fungal spores, 1 soil

E.3 Import and pre-processing of substance degradation data

The data was imported from a single Excel-table into a separate database table.

E.4 Sending data to the database

For *derived_endpoints* data (transformation and inhibition data)

- Start with all empty tables in the PostgreSQL database --> run script *03.1*, chapters *03.1.7*, either *03.2.1* [*measurements*] or *03.2.2* [*derived_endpoints*] for building empty tables for treatments, measurements, and *derived_endpoints*. Only run the chapters once! Check with pgAdmin if it worked.
- Run the script *02_wrangle_rawdata* as the source.
- Run selected chapters of script *03.2*. Send *treatments* (chapter *04.7*), *derived_endpoints* (*04.9*) to the database.
- Change input parameters and run the script 02_wrangle_rawdata as source again.