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**THESE DE DOCTORAT DE L'ETABLISSEMENT UNIVERSITE BOURGOGNE
FRANCHE-COMTE**

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Ecole doctorale n°554

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Doctorat de Sciences agronomiques

Par

DROCCO Camilla

Assessment of the toxicity of pesticide mixtures on soil microorganisms

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Title: Assessment of the toxicity of pesticide mixtures on soil microorganisms

Keywords: soil microbes, free-living nematodes, pesticide mixtures, ammonia oxidizers, environmental risk assessment

Abstract: Within the ARISTO project, this thesis addresses the need to advance the Environmental Risk Assessment (ERA) of pesticides for soil microbes. Given the magnitude of pesticide applications annually and the possible accumulation of active ingredients and their main residues in soil, there is an urgent need to develop an accurate and effective risk assessment strategy. Particular attention is directed towards ammonia oxidizers, crucial contributors to the soil nitrogen cycling. While soil microbial responses to pesticides are relatively well studied, there is a significant knowledge gap regarding the effects on free-living nematodes, organisms closely interacting with the microbial community. This thesis aims also to explore this gap by investigating the responses of both soil microbes and free-living nematodes communities to pesticide mixtures, through a lab-to-field experimental approach.

Molecular techniques such as qPCR and next generation sequencing were employed to study changes in microbial community due to pesticide mixture application, while impacts on free-living nematodes were recorded with the traditional taxonomical identification. Our findings suggest that pesticide mixtures do not have a stronger impact on the microbial and nematode communities than that produced by each of the pesticide studied separately. Overall, molecular techniques targeting microbes and taxonomical identification of nematode provide comprehensive information on the impact of pesticide mixture on the key players of the soil food web, that could be of interest to be implemented in a further revision of the current pesticides EU regulation.

This thesis was carried out at the national Research institute for Agriculture, Food and the Environment (INRAE Bourgogne Franche-Comté), Dijon, France, and at ECT Oekotoxikologie GmbH, Flörsheim am Main, Germany.

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*“Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza”*

Dante, Inferno XXVI

*"You were not to live as brute,
but to follow virtue and knowledge"*

Dante, Inferno XXVI

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Chapter 1

GENERAL INTRODUCTION

1.1 Pesticides use in Europe

Plant protection products (PPPs) are commonly sprayed in conventional agriculture and as an ultimate solution in integrated pest management (IPM) to ensure an adequate production of food and fibers. Without PPPs application, about 78% of the fruits will be lost for pest damage, along with 54% of vegetables and 32% of cereals (Zhang, 2018). According to Eurostat, 355'175 tons of PPPs were sold in 2021 in the European market: 44% of them were fungicides and bactericides, 34% were herbicides, haulm destructors and moss killers, and the rest (14%) insecticides and acaricides. As compared to 2011, overall the EU PPPs market remained stable, despite their declining sales in 12 of 17 European countries (EUROSTAT, 2023).

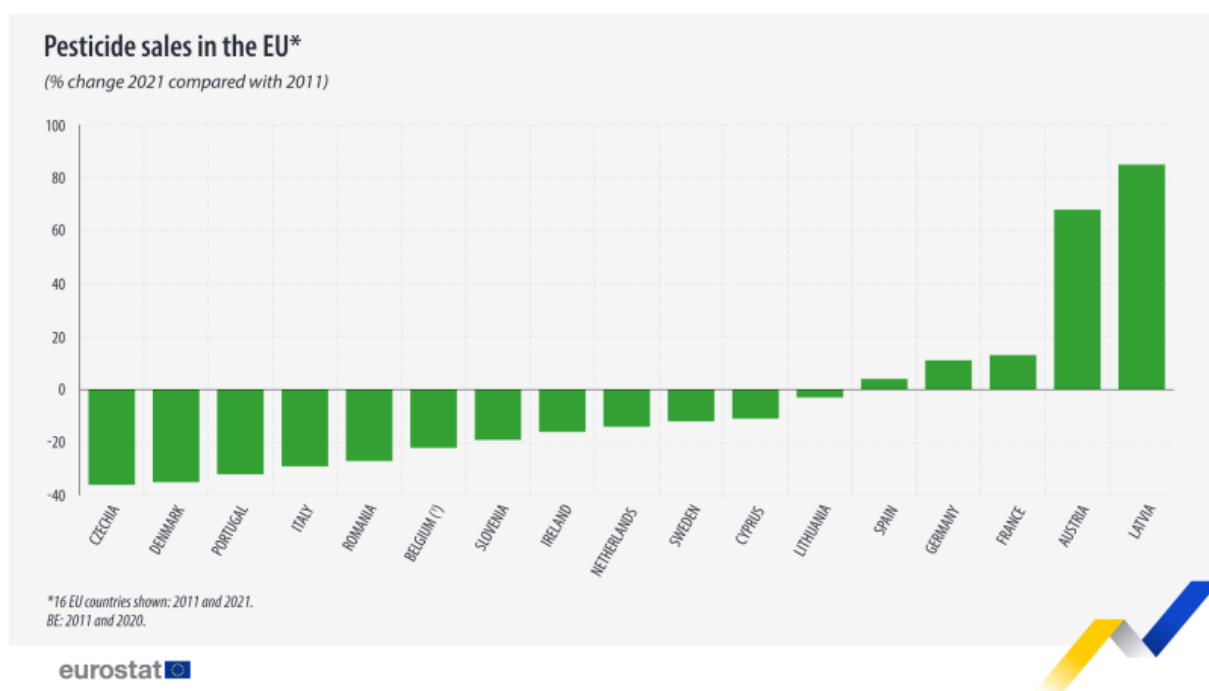


Figure 1. Pesticide sales in Europe expressed as % of change in 2021 compared to 2011 (EUROSTAT, 2023).

Over the past years, the European Commission have put a lot of effort in mitigating the hazardous effects deriving from PPPs use. The Directive 2009/128/EC aims to achieve the sustainable use of pesticides by implementing

environmentally friendly alternatives like IPM and biological control. The recently introduced Farm to Fork strategy, part of the European Green Deal, push for the 50% reduction of use and risk of chemical pesticides by 2030 by adopting non-chemical pest control tools. The need to decrease the quantity of PPPs sprayed is linked to their adverse effects on the environment and on human health that have become increasingly evident in the past years (Carvalho, 2017; Damalas and Koutroubas, 2016; Mahmood et al., 2016; Wimalawansa and Wimalawansa, 2014). Some researchers have highlighted that the cost on human health due to chronic exposure to PPPs might have exceeded the benefits (Bourguet and Guillemaud, 2016). As a reaction, the demand and market of more sustainable pest control strategies as biopesticides is increasing. Up to now, there is not an univocal definition of biopesticides in Europe (Villaverde et al., 2013), but the US EPA (United States Environmental Protection Agency) classify them as any product deriving from natural materials, but also nature identical (biosimilar), with pesticidal activity (Marrone, 2019). They are marketed as non-persistent in the environment and less toxic towards non-target organisms. Biopesticides are also claimed to be target specific, reducing the problem of resistant pests (Nawaz et al., 2016), and because of their short re-entry interval (Lengai et al., 2020), they can be also applied towards the end of the crop growth season for late treatment.

1.2 Biotic and abiotic processes regulating PPPs fate in the environment

Of the quantity of PPPs sprayed during the crop season, only a tiny fraction of them reaches the target pest. The rest (ranging from 30 up to 90%) is dispersed in the different compartments of the environment by spray drift, volatilization and accumulation in the soil (Rodriguez Eugenio et al., 2018), leading to the contamination of water, air and soil resources where they potentially impact non-target organisms. The fate of PPPs in the environment depends on many factors among which soil properties, climatic factor but, mainly on the characteristics of

the active ingredient (a.i) that they contain (Gavrilescu, 2005). Indeed, the physio-chemical characteristics of the a.i play a major role in driving its behavior and degradation rate in the environment: highly hydrophilic a.i are most likely to be lost through leaching, with subsequent pollution of groundwater (Bernardes et al., 2015). Volatile a.i have a greater chance to be degraded by volatilization. The potential environmental risks of an a.i is not only linked to its degradation rate (Gavrilescu, 2005), but also to the amount of sprayed product (Bernardes et al., 2015) and to the characteristics of the transformation products (TPs) that they produce (Fenner et al., 2013), that in some cases can be more persistent or dangerous than the parent compound.

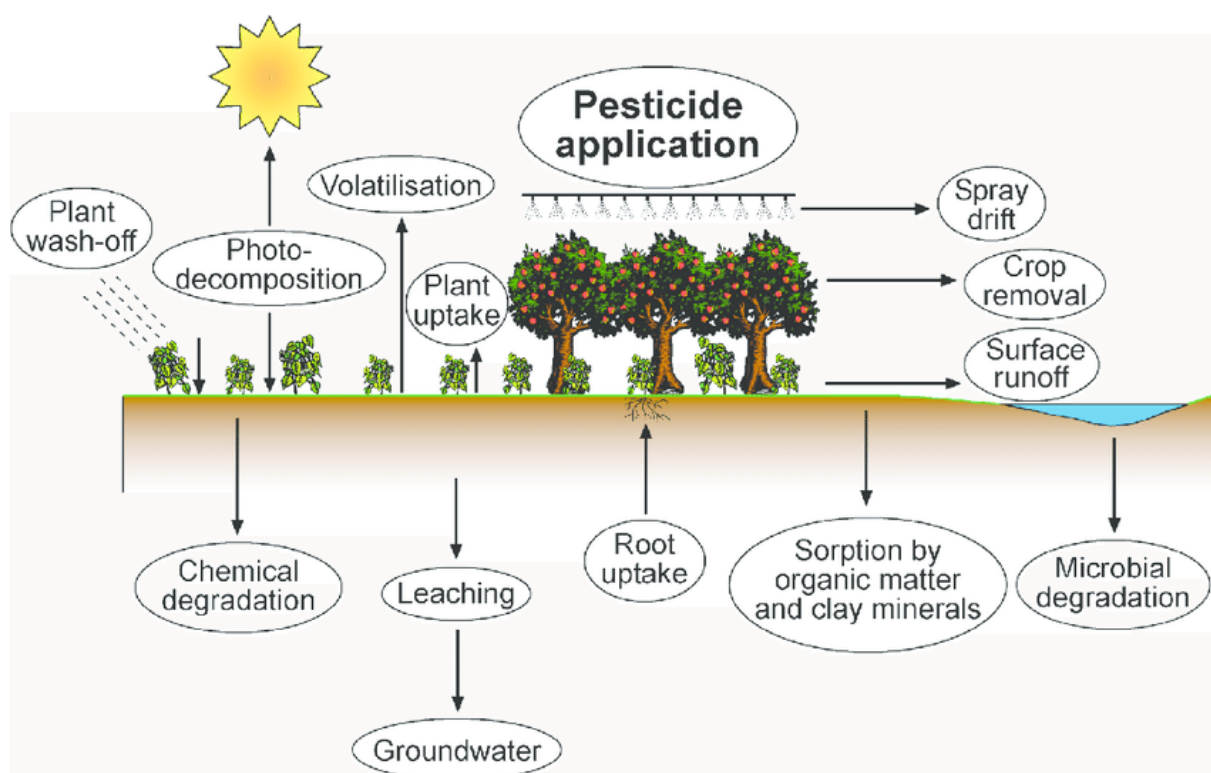


Figure 2. The environmental fate of pesticides (Sarmah et al., 2004). Following pesticide application, there are several pathways by which the pesticide can be lost. Initially, it may be lost directly due to spray drift. Once in the environment, the pesticide can be washed off and subsequently leach into groundwater or move via surface runoff. Additionally, it may be absorbed by organic matter or taken up by plants. Chemical or photo degradation, as well as utilization by microbes for metabolism, are also important processes affecting the fate of the pesticide.

1.3 Environmental contaminations with cocktails of PPPs residues

During the crop growth cycle, more than one PPP is normally sprayed. The applied products can reach the soil, where they undergo biotic and abiotic transformations, resulting in a mixture of residues of a.i and their TPs accumulating in soil. This is the normality in fields conducted under conventional agricultural practices: it is hard to find arable soils free of pesticide residues or contaminated with only one residue as most of them have being contaminated with a range of pesticide residues (Froger et al., 2023; Geissen et al., 2021; Tang and Maggi, 2021). The exposure of non-target organism to this residue mixture can lead to a potentially infinite combination of additive, antagonistic or synergistic effects, that have been rarely addressed by the scientific community. Cedergreen (2014) reviewed the available literature on chemical mixtures, mainly dominated by aquatic studies, concluding that only specific groups of a.i. are involved in synergies: cholinesterase inhibitors and azole fungicides. More research is anyway needed to better understand the impact of cocktail of residues, especially in the soil compartment. Also, the current environmental risk assessment (ERA) of pesticides does not address mixture of residues deriving from different sources (Weisner et al., 2021), while it evaluates mixture of a.i. in a single formulation. The debate over residues mixture has been going on for years and models as “concentration addition” or “independent actions” were applied to predict pesticide mixture toxicity (Cedergreen et al., 2008). Further steps forward on the mixtures problem have been taken with the publication of the “Guidance on harmonized methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals” (EFSA Scientific Committee et al., 2019). This document describes different approaches for the assessment of chemical mixture toxicity for human, animals and the environment: the whole mixture, in which the mixture is considered as a unique entity and evaluated in the same way as a single pesticide, and the component-based approach, for which the mixture is evaluated based on the individual components

and their interaction. The whole mixture approach is particularly useful when the composition of the mixture is only partially understood (*e. g.* oil not fully chemically characterized), while the component-based approach is used for fully defined mixtures (*e. g.* pesticide residues). In both cases, the evaluation of mixture toxicity is performed with a tiered approach starting from the problem formulation and exposure assessment, then hazard assessment and finally risk characterization (Cattaneo et al., 2023). All the information produced have to be summarized in a reporting table. This guidance is very important as it gives definitions of different mixtures (intentional, unintentional and coincidental), acknowledge their problem, and describes a new approach for their toxicity estimation.

1.4 The current a priori ERA of pesticide and related problems

There are currently 390 substances entering in the composition of PPPs that are available on the European market (European Commission, n.d). The approval and subsequent placing in the European market of one of these a.i. is directed by the Regulation 1107/2009 (*Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC*, 2009), which repeals the Council Directives 79/117/EEC and 91/414/EEC. The regulation sets the requirements needed for the *a priori* approval of a substance at the EU level: it must carry a beneficial effect to the crop, and not have any unacceptable effect on human and animal health, and on the environment. To assess so, the substance must undergo a tiered system approach, where any potential risk must be further investigated in a more realistic assumption (higher tier). The required ecotoxicological studies are performed on different trophic levels of the aquatic (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013) and terrestrial compartments (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017) following OECD guidelines. The tested species are: *Oncorhynchus mykiss*, *Daphnia magna*,

Pseudokirchneriella subcapitata, *Lemna*, *Eisenia fetida*, *Folsomia candida*, *Hypoaspis aculeifer*. The ecotoxicological evaluation for the soil compartment is performed on only one microbial endpoint: the Nitrogen Transformation Test (OECD, 2000), which estimates the nitrate concentration after 28 days of incubation in soil exposed or not to the a.i. (acceptable effect is set at the threshold of $\leq 25\%$ effect). This test does not consider long-term effect on N transformation if the difference between the control and the lower treatment is $\leq 25\%$. Considering the number of processes to which soil microbes take part (investigated later in this thesis) it is not clear how considering only this test, one can get a clear picture of the ecotoxicological risk of an a.i. on microbes and on all ecosystem services they provide. In response to this, the EFSA recently published a scientific opinion (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017) where they acknowledged the data gap for soil microbes during the ERA, but they still consider the N transformation as a good indicator to assess pesticide ecotoxicity. Furthermore, due to the lack of standardization and the difficulty of linking it to functional data, the widely used DNA-based molecular tools are still not considered as valid candidates in the ERA of pesticides on soil microbial communities. In fact, there are already projects employing standardized protocols for soil DNA analysis, such as the Earth Microbiome Project (Earth Microbiome Project, n.d.), which stands as one of the pioneering initiatives aiming to standardize the characterization of the soil microbiome on a global scale. Also, Karpouzas et al. (2022) reviewed the already standardized DNA based tests available, and stressed on the importance of implementing them in the risk assessment. They also highlighted the importance of having more standardized amplicon sequencing and ecotoxicological analytical methods, to better describe the pesticide toxicity on important microbial guilds (*i. e.* ammonia oxidizers and arbuscular mycorrhiza fungi), for which the N-transformation test fails to detect negative pesticide effects (Karpouzas et al., 2022; Pedrinho et al., 2024). Amplicon sequencing gives a complete picture of

the bacteria, fungal and eukaryotic communities in soils, providing a great number of information on their status and potentially reducing the problem of functional redundancy deriving from functional endpoints analysis (Langer et al., 2015). The Microbiome Stress Project (Microbiome Stress Project, n.d.) uses the procedures described in the Earth Microbiome Project to study and identify microbial taxa sensible to specific stressors. This instance serves as a virtuous example of the judicious application of standardized protocols for the surveillance of microbial community under stressed dynamics. As such, there exists potential for their incorporation into the ERA for pesticides as recently proposed by (Debode et al., 2024) who proposed a roadmap for the integration of environmental microbiomes in risk assessments.

In the absence of a soil protection directive in the EU, there is no obligation to monitor the fate and impact of PPPs after their release on the market and their application to crops (*a posteriori*). As such, little is known of the effect of PPPs once they have been applied to crops and dispersed in the environment. Despite the strict legislations introduced to minimize the risks associated with pesticide use, the literature predominantly highlights adverse effects on humans, animals, and the environment. It is evident that only *a priori* ERA of pesticide is not enough for understating the impact of these molecules on the non-target organisms. Projects as described in Froger et al. (2023) and (Silva et al., 2019) to monitor the occurrence of pesticides in French and European soils, or the LUCAS (Land Use and Coverage Area frame Survey) Soil Pesticides tool (Orgiazzi et al., 2022) are the first attempts highlighting the widespread occurrence of pesticide residues in arable soils, and the prolonged persistence of the a.i. and their TPs (i.e. DDT that was banned between the 70s and 80s in Europe is still one of the most frequently detected pesticides (Silva et al., 2019)). These studies were not addressing the impact of observed pesticide residues on in soil living organisms and supported ecological functions.

1.5 Soil microbes and free-living nematodes contribute to soil health

Soil is a key contributor of ecosystem services and is critically important to human life. Without it, humans would not be able to produce food, fibers, or building material (Brevik et al., 2018). Soil supports also more “hidden” ecosystemic services: it takes part to nutrient cycle, water purification, disease suppression, pollutant decontamination, and much more (Brevik et al., 2018; Pulleman et al., 2012). Many studies addressed the importance of having healthy soils for an efficient provision of these services, which is directly linked to its biodiversity (Cardinale et al., 2012; Pulleman et al., 2012; Wall et al., 2015). Indeed, each component of the soil ecosystem contributes to one or more ecological functions (Setälä et al., 2005), which are influenced by the activity of other soil compartments and by external disturbances. In general, soil inhabitants can be classified in three functional groups according to (Kibblewhite et al., 2008; Pulleman et al., 2012): the “decomposers” and “nutrient transformers”, mainly microorganisms that contribute to nutrient cycling and organic matter decomposition; the “biocontrollers”, like nematodes, that graze on microbes, feed on plants or other organisms; the “ecosystem engineers” which contribute to soil structure, as earthworms.

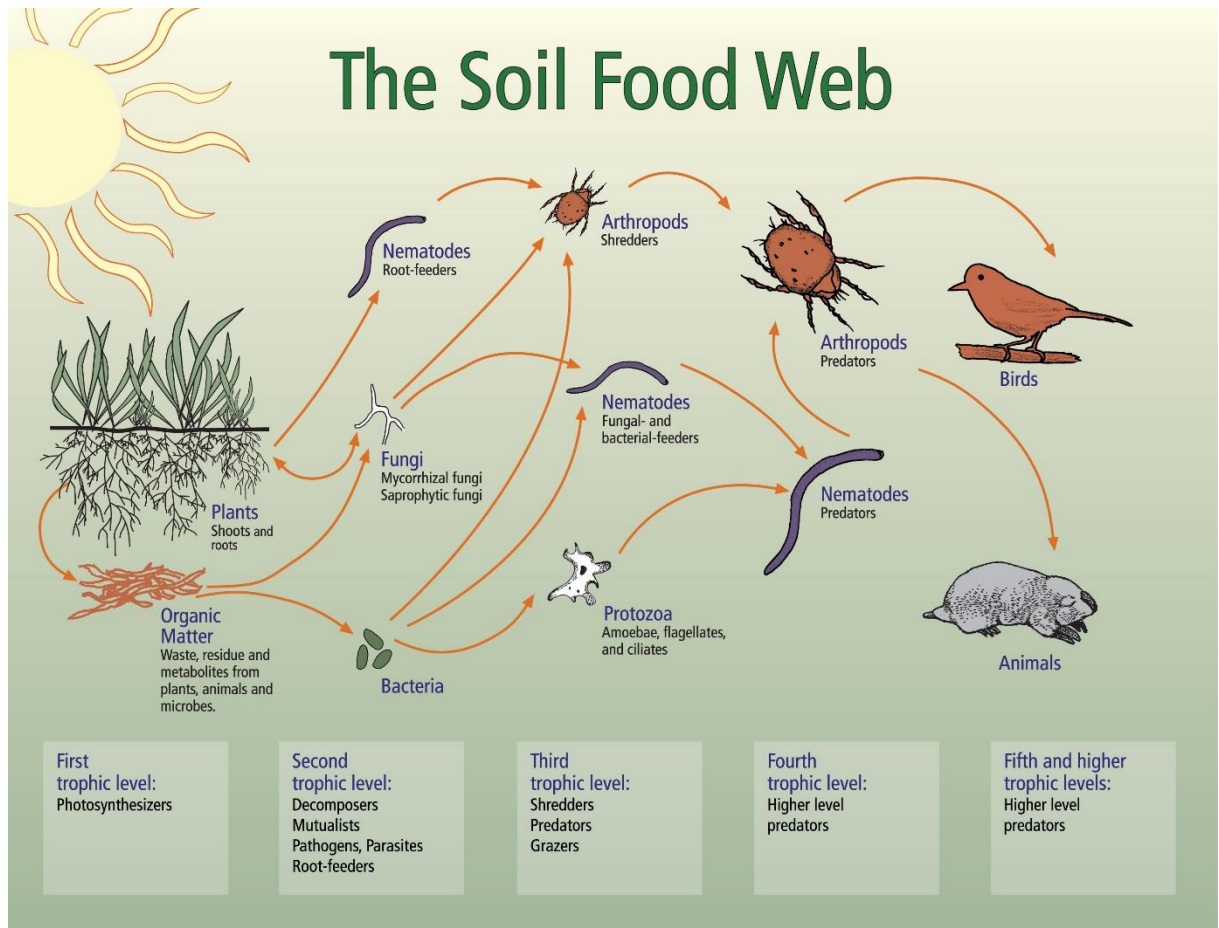


Figure 3. Graphic representation of the soil food web.

The microbes are a wide group of organisms that includes bacteria, fungi, archaea, protozoa and viruses. In soil, they take part to the most fundamental roles, contributing to organic matter decomposition releasing nutrients in the food web (Whitman et al., 1998), but also establishing mutualistic symbiosis with plants where they provide crucial nutrients like phosphorous and nitrogen (Nielsen and Winding, 2002). It can be stated that all organisms rely on microbial process to survive (Singh, 2015).

The nitrogen cycle is entirely dependent on microorganisms. The N_2 in the atmosphere is not available for direct use of the plants, and only the nitrogen fixing bacteria can fix it and transform it in ammonia which is transported into plants, used then as N source to build amino acids and proteins (Moebius-Clune

et al., 2016). The ammonia oxidizing bacteria and archaea take part to the nitrification converting the NH_4^+ derived from organic nitrogen into NO_2^- (Lehtovirta-Morley, 2018), while the newly discovered comammox can transform ammonia directly into NO_3^- (Koch et al., 2019). The inverse process from NO_2^- to N_2 is performed by the denitrifiers or anammox bacteria (Kuypers et al., 2018). The N-cycle involves a huge number of different microbial taxa, that are all important to maintain its functioning. Molecular analysis like real time quantitative-PCR (qPCR) are particularly useful for investigating the size of the functional communities, especially for what concerns the N cycle microbial guilds. The quantification of the sequences of genes coding enzymes involved in the nitrogen cycle like the ammonia monooxygenase (*amoA*), which plays a pivotal role in converting ammonia to nitrate, provides insights into the abundance of the microbial guild harboring this gene in soil and indirectly, its potential activity level (Petersen et al., 2012). More comprehensive studies of the soil microbiome diversity and composition can be achieved with next-generation DNA sequencing analysis of the 16S rRNA genes and of the Internal Transcribed Spacer 2 (ITS2) region of the rRNA gene for the analysis of prokaryotes and fungi, respectively. These amplicon-based metabarcoding analyses provide a variety of information relating to the diversity and composition of microbial communities. The information can be summarized in different indices that describe:

- **the alpha diversity**: an indication of the within-sample diversity, which is estimated with the:
 - **Observed Species (OS)**: a measure of the sample's richness based on the count of the different taxa observed within a sample.
 - **Shannon Index (H')**: an estimator of a sample's diversity based on its species number and the evenness of their abundance. The minimum value is 0, meaning that the diversity is 0 as there is

only one taxon in the sample. There is no upper limit, but the higher, the more similar the relative abundance of each taxa are.

- **Simpson Index (D):** the probability that two individuals in a sample belong to the same taxa. It ranges from 0 to 1, where 0 indicates infinite diversity and 1 no diversity.
- **Phylogenetic Diversity (PD):** measure of biodiversity based on a phylogenetic tree (Faith, 1992). This indicator of diversity is based on the sum of all the branches of the phylogenetic tree representing the different taxa in a sample.
- **the beta diversity:** indication of the similarity/dissimilarity between samples based on distance matrices (Bray Curtis, Weighted or Unweighted Unifrac). The more distant two samples are, the more different their microbial composition is.

Nematodes are the most abundant metazoan on earth (Bongers and Ferris, 1999). They are ubiquitous and can be found in all ecosystems that can offer a carbon source, playing key roles in organic matter decomposition and nutrients cycle (Yeates and Bongers, 1999; Zhang et al., 2017), and contributing to disease suppression (Freckman and Caswell, 1985). Free-living nematodes studies can be easily performed with the identification at the family level. They can then be grouped based on their feeding behavior: plant, fungal and bacterial feeders, omnivores and carnivores. Being at the center of the soil food web (Neher, 2010), they are considered as a good indicator of the soil web dynamics (Bongers and Ferris, 1999; Pulleman et al., 2012): typically, the bacterivore abundance is higher when the microbial abundance is also higher, while for example, fungal feeders develop faster in acid condition or heavy metal contamination (Bongers and Bongers, 1998). Due to their permeable cuticula and low mobility, they are sensitive to pollutants (Bongers, 1990) and more investigations are needed to assess the effects of pollutants on their diversity.

Nematode assemblages can also be characterized based on their life strategy (the r-K strategy), assigning a number from 1 to 5 along the colonizer-persister (c-p) range of values (Bongers, 1990). Typical r-strategist are the “colonizers”, nematodes that have a short life-cycle, produce a large number of eggs and are resistant to disturbances. They are normally very abundant, especially in food-enriched conditions, when microbial growth is boosted (Ferris et al., 2001). Typical colonizers belong to the Rhabditidae and Panagrolaimidae families with low c-p values. The K-strategies are the persisters, nematodes with a long-life cycle and low reproduction rate. They typically occur in low abundance and exhibit high sensitivity to disturbances, that is why they are mostly found in stable and mature environments. They correspond to higher c-p values as compared to the colonizers.

The c-p scaling has been revolutionary in nematode community studies, from which different indices were derived and are now used for the description of nematode assemblages:

- **Maturity index (MI):** defined as “the weighted mean c-p value of the individuals in a representative soil sample” (Bongers and Ferris, 1999). It is a measure of the environmental conditions according to the nematode community composition. A low MI value (<2) suggests high abundance of opportunistic nematodes (c-p value 1 or 2), and occurs in nutrient-enriched systems or in polluted environments, as nematode species displaying low c-p values are usually more tolerant to stress (Odum, 1995), while high MI values (>3) are typical of undisturbed, stable and structured systems (Bongers and Ferris, 1999; Du Preez et al., 2022).
- **Enrichment index (EI):** this index is based on the “expected responsiveness of the opportunistic non-herbivorous to food resource enrichment” (Ferris et al., 2001). It is based on the abundance of primary consumers, the c-p 1 bacterivores and c-p 2 fungivores, as a response to

food availability. The EI can be classified as low (0-30), intermediate (30-60) and high (60-100) (Du Preez et al., 2022) and reflects the food availability.

- **Structure index (SI):** “represents an aggregate of longevity, body size and disruption-sensitivity of functional guilds” (Ferris et al., 2001). It describes the increasing complexity of the food web in a maturing system, characterized by c-p 3-5 nematodes. It gives also an indication of the level of disturbance to which the nematode community is subjected. It can be classified as low (0-30), intermediate (30-60) and high (60-100) (Du Preez et al., 2022). Low values suggest disturbed soils, while high values correspond to a structured environment.
- **Channel index (CI):** “indicates the predominant decomposition pathway” (Ferris et al., 2001). It is a measure of the contribution of the fungivore to the decomposition. Low values (<50) are typical of bacterial decomposition pattern, while high values (>50) of fungal decomposition (Du Preez et al., 2022).
- **Maturity index 2-5 (MI 2.5):** an indication of environmental pollution, which exclude cp1 nematodes in order to not confuse eutrophication-induced stress with pollution-induced stress (Korthals et al., 1996).
- **Plant parasite index (PPI):** can be defined as a MI only for the plant-feeding nematodes. It decreases in case of enriched nutrient condition (Bongers, 1990).

The SI and EI can be combined together (Fig. 4) and provide a good picture of the food web condition, classified as disturbed (quadrat A), maturing (quadrat B), structured (quadrat C) or degraded (quadrat D) (Ferris et al., 2001).

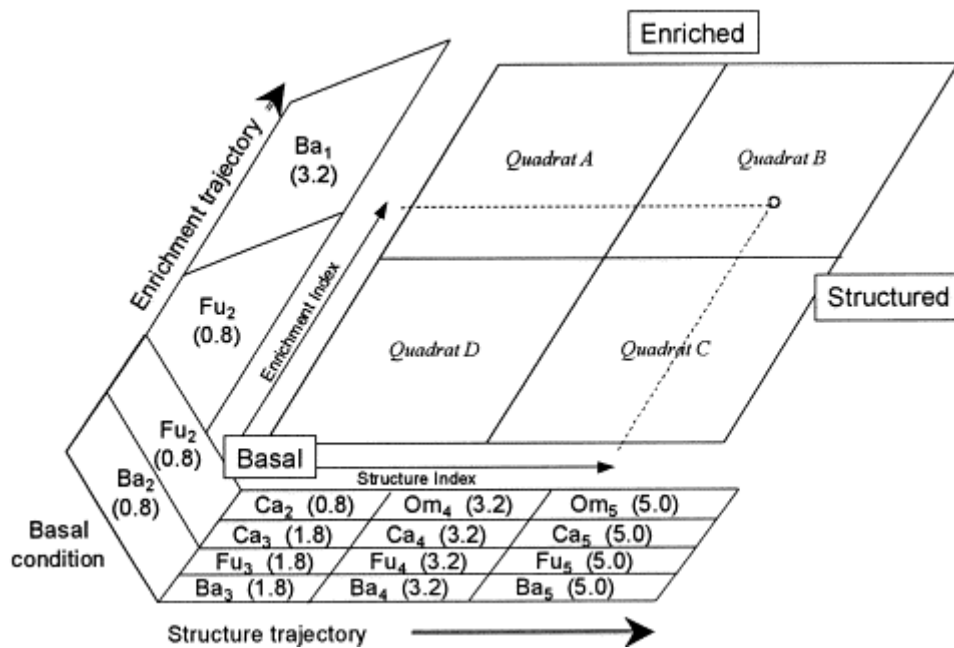


Figure 4. The characterization of the food web condition based on the structure and enrichment trajectories (Ferris et al., 2001)

Given the importance of free-living nematodes in soil ecosystem services provision and their sensitivity to pollutants, it is notable how little research has been carried out on them so far (Grabau et al., 2020; Höss et al., 2022; Waldo et al., 2019). Additionally, nematodes are not included in the current ERA of pesticides, but they were considered by EFSA as a specific protection goal to be monitored (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017).

1.6 The effect of pesticides on soil microbes and free-living nematodes

Soil microbes and free-living nematodes are exposed to PPPs and their residues after the application on the crop. Extensive research has already been carried out on the effect of pesticides on soil microbes. Puglisi (2012) examined the available literature on terrestrial ecotoxicology, finding that the microbial community structure (considering studies on the community composition and/or diversity) was the parameter mostly impacted by pesticide application, *i. e.* in 87%

of the studies by herbicides and in 95% by insecticides and fungicides. Frequently, the community could not recover completely, returning to its initial composition, as exposure to a.i can lead to shift of microbial community composition and diversity loss towards resistant microbial strains, that might be able to use these molecules as energy substrate (el Fantroussi et al., 1999; Ramírez-Saad et al., 2000). In the long term, this might result into poor efficacy of a.i, that are fast degraded by the resistant microbial community with consequent problems for plant protection (Gallego et al., 2019). The exposition to a.i can also inhibit microbial activity (*i.e.* dehydrogenase activity, microbial respiration an different soil enzyme) and decrease their abundance (Dungan et al., 2003; Gomez et al., 2009; Karpouzas et al., 2014). Always according to Puglisi (2012), microbial biomass and activity (*i.e.* enzymatic activity, respiration, substrate degradation, etc...) were impacted in 23% and 27% of the studies by fungicides, in 22% and 32% by herbicides and in 15% and 20% by insecticide, respectively. When shifts in microbial community composition coincide with diversity decline, it poses challenges for ecosystem stability and functioning (Philippot et al., 2013), which might not be clear considering only one process, like the N transformation, because of functional redundancy (Allison and Martiny, 2008), and the multitude of other important function that microbes carry out. (Wagg et al., 2014) explored different soil community complexities' impact on ecosystem function, and concluded that the microbial community diversity decline has a major impact on nutrient cycles. This study further supports the thesis that soil communities shift is deeply connected to shifts in functions.

Among all the microbial functional groups, the ammonia oxidizing microorganisms have been described to be particularly sensitive to pesticide application, thus being a good indicator of their toxicity (Karas et al., 2018; Karpouzas et al., 2022). The main reasons include their ecological relevance, their well understood ecology and consistent sensitivity to pesticides. Nevertheless,

already standardized tools are available for their quantification, which can be linked to functional aspects (Karpouzas et al., 2022). Another study by Sim et al. (2022) investigated the effect of 20 PPPs on some N cycle parameters, which also concluded that ammonia oxidizing microorganisms are susceptible to PPPs application. Interestingly, this became clear after the analysis of the bacterial community composition, and not by only considering the abundance of these N cycle microbial guilds. These changes in community composition were correlated with measurements of functional activities (potential nitrification or extracellular enzymatic activities) and were impacted by some of the analyzed PPPs. This emphasizes why also evaluating the size of the nitrifying community would be a good tool for establishing toxicity of PPPs, as it can predict effects at the functional level as well. Up to now, it still needs to be clarified the impact of pesticides on the recently discovered comammox, capable of conducting the entire nitrification process within a single cell. Limited research has been conducted on them to date.

Compared to microbes, less comprehensive ecotoxicological researches have been carried out on soil free-living soil nematodes so far. As any disturbance, pesticides alter the nematode community leading generally to shifts towards a community resistant to pollution and characterized by families with short life cycle that reproduce a large number of eggs, and negatively impacting the slow growing nematodes. Being highly disturbed, agricultural soils are normally characterized by an “enriched” nematode community, with great abundance of c-p1 bacterivores and c-p2 fungivores (Lazarova et al., 2021) that take advantage of the increased bacterial abundance after tillage or organic matter input. This is also a common response after pesticide application: Ettema and Bongers (1993) characterized the nematode community for 60 weeks after fumigation. The first detected taxa after treatments were Rhabditina, *Acrobeloides* (Fam. Cephalobidae), *Acrobeles* (Fam. Cephalobidae) and *Aphelenchoides* (Fam.

Aphelenchoididae), all typical “colonizers” taxa. Only later (44-53 weeks after treatment application) the “persisters” become more abundant. On the opposite, Höss et al. (2022) observed an increasing bacterivore population, especially *Acrobeloides*, 28 and 56 days after application of high dose of the fungicide mancozeb, in conjunction with a drastic decline of omnivores and carnivores, suggesting a delayed effect of the product on the nematode community. These different responses are mainly due to the differences in community composition, one originated from an already pesticide contaminated soil (Ettema and Bongers, 1993), and the other from a undisturbed natural grassland (Höss et al., 2022). (Waldo et al., 2019) investigated the effect of several nematicides on non-target free-living nematodes, finding out that the most impacting nematicide, fluopyram, induce negative short- and long-term effects to both “colonizers” and “persisters” at the recommended dose.

Further studies are required to fully understand the impact of pesticide mixtures on both soil microbes and free-living nematode communities. According to existing literature, the only available paper on the effect of pesticide mixtures on free-living nematodes was conducted by Yardirn and Edwards (1998) who explored the effect of the sequential application of pesticides in an agricultural field. Their findings suggested that such sequential applications inhibited bacterivores while stimulating plant parasites. Most of the research on pesticide mixture effect has focused on reference nematode *Caenorhabditis elegans* (Huang et al., 2023, 2022; Martin et al., 2009; Svendsen et al., 2010). In regards to soil microbes, more researches have been carried out (Baćmaga et al., 2015; Bonfleur et al., 2015; Joly et al., 2012; Schuster and Schröder, 1990a; Zhang et al., 2023), yet it remains insufficient compared to single pesticide studies for a comprehensive understanding of the effects of pesticide cocktails on the soil microbial community and on the N cycle microbial guilds. Additionally, no study up to now have simultaneously investigated microbial communities and free-

living nematodes responses to pesticide application. This might highlight some important, yet unexplored, pesticide effect on a deeper layer of the soil food web.

1.7 The effect of global change and compounded disturbances on soil microbes

Climate change and extreme climatic events are of major concerns nowadays. Their effects are becoming increasingly evident, affecting all ecosystems on Earth. Less evident to human eyes might be the effects on the soil microbial community, which can react by changing its community composition (Barnard et al., 2013; Calderón et al., 2018; Jurburg et al., 2017a; Meisner et al., 2018; Mooshammer et al., 2017), causing problems for ecosystem functioning and resilience to future perturbations. Indeed, the loss of biodiversity affects nutrients cycling, which normally rely on a huge biodiversity for its functioning. The N cycle has been described to be sensitive to global change (Calderón et al., 2018; Mooshammer et al., 2017; Sahrawat, 2008; Szukics et al., 2010). The perturbation of any of its steps, and especially that of nitrification, poses serious challenges for nitrogen supply in the soil, leading to the disruption of nitrogen availability in soil.

Normally, ecosystems are exposed to more than a single disturbance overtime. This phenomenon is known as ‘compounded’ disturbance. The effect of the initial disturbance may have legacy effect and consequently alter the ability of the microbial community to cope with the following ones. The responses to the following stress are variable (Vinebrooke et al., 2004): the exposure to multiple perturbations of the same nature might lead to microbial selection and high community resilience to that specific stress (Calderón et al., 2018). If the subsequent perturbation is of a different nature, the disturbed community might be more resistant to the following stress because of community priming (Rillig et al., 2015), or more sensitive (Calderón et al., 2018) because of negative species co-tolerance (Vinebrooke et al., 2004). Up to now, little research has investigated

the compounded effect of environmental disturbance and pesticide application. *I.e.*, the application of the insecticide oxyfluorfen and the fungicide chlpyrifos to a community subjected to drought induced a higher effect on the microbial community and enzymatic activity compared to watered soil treated with pesticides (Franco-Andreu et al., 2016). Also, Pesaro et al. (2004) concluded that the degradation of the fungicide metalaxyl-M and of the insecticide lufenuron were lower in soil subjected to drought. These findings underscore the increasing challenges faced by microbial communities in recovering from multiple stresses, particularly in the context of climate change characterized by an increasing number of extreme climatic events. These events exert significant impacts on microbial communities, further complicating their ability to recover when coupled with environmental pollutants.

1.8 My PhD thesis in the context of a Marie-Curie project

My PhD thesis is part of a broader Marie-Curie project named “ARISTO” (<https://aristo.bio.uth.gr/>) funded by the European Commission under the MSCA – ITN – EID – H2020 funding scheme (grant agreement No 956496). The aim of the project is to develop tools, methods and procedures to assess in the most comprehensive and robust way the toxicity of pesticides on soil microorganisms, and contribute to filling microbial data gap for a more sustainable regulatory framework in the environmental risk assessment of pesticides (ARISTO, n.d.). The ARISTO project includes 9 different PhD projects, that explore different

sides of pesticide microbial ecotoxicology.

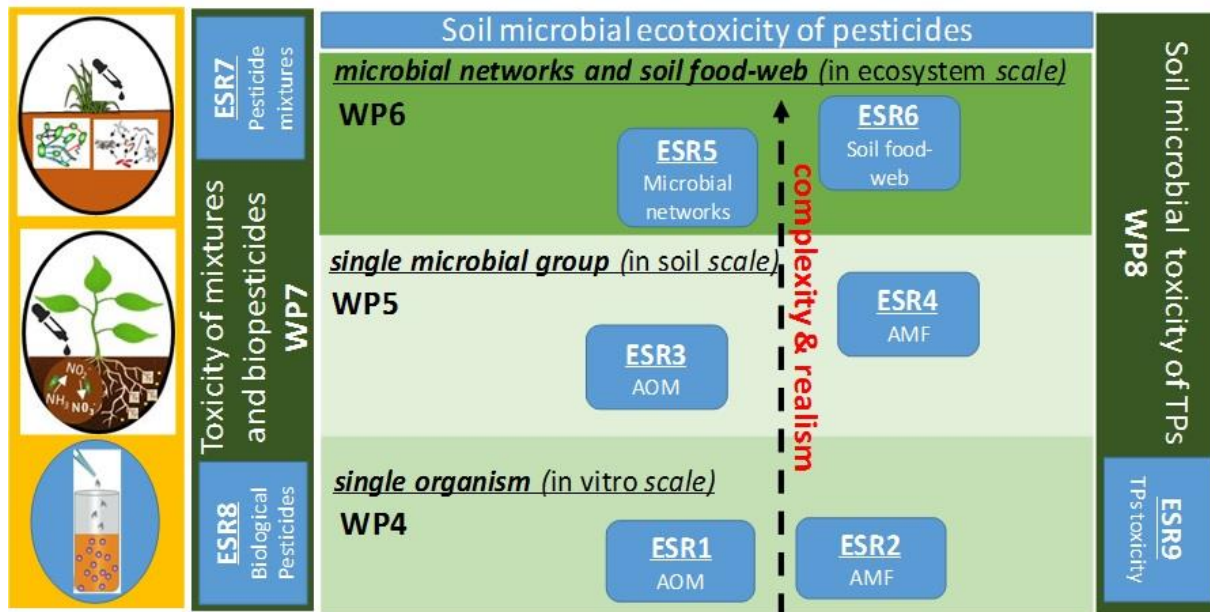


Figure 5. The ARISTO project structure.

In this context, my PhD aims at studying the effect of pesticide mixtures on soil microbes in interaction with free-living nematodes, to have a better understating of pesticide residue cocktail effect on different layers of the soil food-web (ESR7, Fig. 5). To achieve this goal, three experiments were designed and carried out.

The first experiment (Chapter 2) investigated the effect of pesticides applied to previously environmentally disturbed microbial communities. Extreme climatic events are becoming increasingly frequent, and few studied have investigated the compounded effect of environmental disturbances and active ingredients mixture on soil microbes. We have chosen to study heat stress and heavy rainfall as environmental disturbances, while the active ingredients were clopyralid, cypermethrin and pyraclostrobin. These active ingredients are commonly used in agriculture and are considered low-moderately toxic towards soil organisms. Nothing is known about the effect of these three molecules applied in combination. The main research questions were: is the effect of the active

ingredients on the soil microbial community significantly different when applied to a previously environmentally disturbed community? Is the mixture impacting the community significantly differently as compared to the single active ingredients?

In the second experiment (Chapter 3), we aimed to investigate the impact of the same a.i. as those used in the first experiment, but formulated as commercial PPPs, and applied sequentially under field conditions (“higher tier approach”) on soil microbes and on the abundance of free-living nematodes. The main research question was: is the effect of the sequential application of PPPs significantly impacting the microbial community and nematode abundance as compared to the single PPPs?

In the third experiment (Chapter 4) we focused on the characterization of the impact of a pesticide mixture more specifically the soil free-living nematode community and on the N cycle microbial guilds. Nematodes live in close relationships with the soil microbial community, and have yet not been explored for pesticide ecotoxicology. To this end, we investigated the individual and the combined effect of a conventional insecticide tested previously in the field experiment, and a bio-insecticide on the free-living nematode community structures, and on the abundance of a series of microbial groups, among them bacteria, fungi, ammonia oxidizers and comammox. Even though biopesticides are marketed as environmental-friendly compounds because of their low persistence and low toxicity towards non-target organisms as compared to conventional pesticides, nothing is known about their effect in combination with a conventional insecticide. The main research question was: does the mixture of a synthetic insecticide and a bio-insecticide affect the free-living nematodes and microbial communities significantly as compared to the single formulation application?

I then gathered all these results in a general discussion and proposed possible perspectives of research, valorization and transfer to better assess the toxicity of pesticides towards in-soil living organisms and help to prevent possible alteration of ecological functions and supported soil ecosystemic services.

Chapter 2

Evaluating the Effects of Environmental Disturbances and Pesticide Mixtures on Soil Microbial Endpoints

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ABSTRACT

Pesticides are widely used in conventional agriculture, either applied separately or in combination during the culture cycle. Due to their occurrence and persistence in soils, pesticide residues may have an impact on soil microbial communities and on supported ecosystem services. In this regard, the EFSA (European Food Safety Authority) recently published a scientific opinion inciting to change pesticide risk assessment to better protect soil microbe-mediated processes. Climate change is another major concern for all living organisms including soil microbial community stability. Extreme climatic events, such as heat waves or heavy rainfalls, are becoming more and more frequent and their impact on soil microbial diversity and functions have already been demonstrated.

The objectives of this study were to evaluate the effects of temperature and humidity disturbances and pesticide active ingredients exposure on soil microbial community structure and functions. To this end, 250 soil microcosms were exposed to either a heat disturbance, a high humidity to mimic heavy rain, or no environmental disturbance. After three days of recovery, soil microcosms were treated with different active ingredients: clopyralid (herbicide), cypermethrin (insecticide) and pyraclostrobin (fungicide). The treatments were applied alone or in combination at 1x or 10x of the agronomical dose. We then evaluated the effects of the disturbances and the active ingredients on various microbial endpoints related to the diversity and the structure of soil microbial communities, and with a specific focus on microbial guilds involved in nitrification.

Overall, we demonstrated that the impact of environmental disturbances applied to soil microcosms, especially heat, on microbial endpoints was stronger than that of the active ingredients applied alone or in combinations. We also did not observe synergistic effects of environmental disturbances and active ingredients application on measured microbial endpoints.

Keywords: ecotoxicology, global change disturbance, pesticide mixture, bacterial communities

INTRODUCTION

Soil is a non-renewable resource at human life span that carries out several ecosystem services supporting the life of many organisms: it sustains food and fibers production, stores CO₂ and water, and helps in disease suppression (Dominati et al., 2010; Aislabie and Deslippe, 2013; Moebius-Clune et al., 2016). Many of those services, like the nitrogen cycle (N-cycle) which regulates the availability of N in soil, are carried out by microbes (Dominati et al., 2010; Aislabie and Deslippe, 2013; Whitman et al., 1998; Singh, 2015). Indeed, the N₂ in the atmosphere is not available for direct use by plants, while symbiotic nitrogen-fixing bacteria can fix it into plants as ammonia that is then used to build amino acids and proteins (Moebius-Clune et al., 2016). The ammonia oxidizing bacteria and archaea take part to the nitrification converting the NH₄⁺ derived from organic nitrogen into NO₂⁻ (Lehtovirta-Morley, 2018), while the newly discovered comammox can transform ammonia directly into NO₃⁻ (Koch et al., 2019). The inverse process from NO₂⁻ to N₂ is performed by the denitrifiers or anammox bacteria (Kuypers et al., 2018). Overall, the N-cycle involves a wide diversity of microbial taxa that are important to ensure its functioning all along seasonal changes of soil temperature and humidity.

Climate change and extreme climatic events can induce shifts in microbial community composition and taxa abundance (Barnard et al., 2013; Jurburg et al., 2017a; Mooshammer et al., 2017; Calderón et al., 2018; Meisner et al., 2018), with effects on nutrient cycles (Philippot et al., 2013) and ecosystem resilience (Jurburg et al., 2017a). Frey et al. (2008) in their long-term experiment in mixed forest concluded that 12 years of soil heating reduced total microbial biomass, and induced a shift in community composition, with a negative impact on substrate use. The N-cycle is also deeply influenced by temperature (Calderón et al., 2018; Mooshammer et al., 2017; Sahrawat, 2008; Szukics et al., 2010), and the

perturbation of one of the steps, *e. g.* nitrification, poses challenges for nitrogen supply in the soil, leading to the disruption of nitrogen availability.

Pesticides are commonly applied in conventional agriculture and in integrated pest management to ensure an adequate production of food and fibres. According to the Food and Agriculture Organization (FAO, 2023), 354'082 tonnes of pesticides were sprayed for agricultural use in the EU in 2021, of which only a part of the applied compounds gets to the target. The rest (30-50%) can reach the soil environment (Rodríguez Eugenio et al., 2018) where it may impact non-target organisms and transfer to other environmental compartments, including water resources. Several studies have already documented that pesticide residues can impact soil microbial community leading to *i*) the emergence of pesticide-degrading strains able to use pesticide as carbon source for their growth, or *ii*) the alteration of microbial abundance and diversity and possibly affecting ecosystems stability and their resilience (Karas et al., 2018; Kumar et al., 2019; Puglisi, 2012; Storck et al., 2018; Zabaloy et al., 2010). During the entire crop cycle, often more than one pesticide is applied at the same field. Persistent residues of active ingredients as well as their transformation products accumulate to a cocktail of chemicals to which soil living organisms are exposed (Geissen et al., 2021; Tang and Maggi, 2021). The overall ecotoxicological impact resulting from the exposure of non-target organisms to chemical cocktails is the result of the combination of additive, antagonistic or synergistic effects, that until now have been rarely studied with regard to soil microorganisms. Cedergreen (2014) reviewed the available literature on chemical mixtures, concluding that additive interactions generally dominate whereas evidences for synergistic interactions were limited to cholinesterase inhibitors (insecticide) and cytochrome P450 inhibitors (fungicide). Toxicity endpoints of aquatic organisms dominated this review (Cedergreen 2014), while microbial endpoints were underrepresented.

Hence, there is a strong need for a better understanding of the impact of accumulated pesticide residues particularly on the soil microbial community.

Ecosystems are usually exposed to more than one disturbance, that act independently or influence each other, with different effects on the ecological recovery of the ecosystem functions. Very often, a legacy effect of disturbances (Paine et al., 1998) can be observed and might decrease the ability of species to resist and then recover to subsequent perturbations. Jurburg et al. (2017b) demonstrated that a microbial community needed at least 29 days to recover from a heat shock. In addition, the disturbance history (heat, and heat or cold shock) affects the microbial community response to the subsequent perturbations (Jurburg et al., 2017a). Extensive works have been done to assess the effect of factors mimicking extreme climate events (Bardgett and Caruso, 2020; Calderón et al., 2018; Meisner et al., 2018; Mooshammer et al., 2017; Philippot et al., 2021), but up to now, only a few studies have considered the potential combined effect of environmental disturbances and pesticides on soil microbial communities (Franco-Andreu et al., 2016; Pesaro et al., 2004).

Within this context, the objective of the present work is, therefore, to evaluate the compounded effect of global change-related environmental disturbances and impacts of pesticidal active ingredients on soil microbial community structure and functioning. We hypothesize that, as compared to undisturbed soil *i*) extreme climatic events, such as elevated temperature and heavy rainfall will change microbial community's structure and functioning; *ii*) subsequent exposure to different pesticidal active ingredients applied alone or in combination may lead to compounded effects on the structure and functioning of some key microbial community members.

MATERIAL AND METHODS

Soil sampling and microcosms set up

Soil from the surface layer (0-20 cm depth) was sampled from Sayens field site located in the vicinity of the INRAE experimental farm in Bretenière, France (47° 14' 03.1" N, 5° 06' 37.9" E) during August 2021. This soil is silty clay loam, characterized by 34.6% clay, 59.0% silt, and 6.3% sand, with an organic carbon content of 2.7%. The water holding capacity (WHC) was 64. The soil was homogenized and sieved to 4 mm and stored at 4°C until further use. The soil humidity after sieving was 26%. The fresh soil was divided into 250 microcosms, each containing 63 g fresh soil (corresponding to 50 g dry soil), and incubated at room temperature for 6 days prior to exposure, or not, to environmental disturbance.

Environmental disturbances and active ingredients application

We studied two different environmental disturbance factors: elevated temperature (heat) and heavy rainfall (high humidity). Respectively, 80 soil microcosms were subjected to the heat disturbance, 80 to high humidity and 80 received no environmental disturbance (control). Heat and high humidity disturbances were applied over a window of 7 days. The heat disturbance consisted in two periods of 30 h at 42°C, separated by a period of 40 h at room temperature. At the end of the disturbance window, the humidity was adjusted to 22%. The high humidity disturbance consisted in watering soil microcosms to reach 35% of humidity, followed by an evaporation period of 7 days. Three days after the end of the disturbance window, *i. e.* at day 19 of the experiment, five microcosms per each disturbance condition tested (heat, high humidity and control) were treated with one of the three pesticides (technical material of active ingredients, a.i.) or with the mixture of the three a.i. at 1x or 10x the agronomical dose. The pesticides were: 3,6-dichloropyridine-2-carboxylic acid (*i. e.* clopyralid, pesticide analytical standard, CAS 1702-17-6, supplier: Sigma-

Aldrich, purity $\geq 98.0\%$, agronomical dose: $4.5 \mu\text{g}/50 \text{ g dry soil}$), [cyano-(3-phenoxyphenyl)methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate (*i. e.* cypermethrin, pesticide analytical standard, CAS 52315-07-8, supplier: Sigma-Aldrich, purity $\geq 90.0\%$, agronomical dose: $3.4 \mu\text{g}/50 \text{ g dry soil}$), and methyl *N*-[2-[[1-(4-chlorophenyl)pyrazol-3-yl]oxymethyl]phenyl]-*N*-methoxycarbamate (*i. e.* pyraclostrobin, pesticide analytical standard, CAS 175013-18-0, supplier: Sigma-Aldrich, purity $\geq 98.0\%$, agronomical dose: $7.1 \mu\text{g}/50 \text{ g dry soil}$). Clopyralid was dissolved in water, and cypermethrin and pyraclostrobin were dissolved in acetone, and equal quantities of water (1 mL) and acetone (10 μL) a.i. solutions were applied to all microcosms with the respective treatment (single a.i or a.i. mixture). The solutions were applied onto the soil surface, without mixing the soil after application. In a similar manner, water and acetone were applied to 10 untreated control microcosms. Soil moisture was adjusted and kept to 26% in all microcosms. All the treated microcosms and the untreated controls were done in five independent replicates, for a total of 250 microcosms (Fig. S1).

From each treatment combination and from the untreated controls, five microcosms were destructively sampled at day 29 (T30: 10 days after a.i application), and the remaining five at day 61 (T60: 42 days after a.i application). The soil samples were processed fresh or stored at -20°C for further analyses.

Nitrogen pools quantification

The nitrogen species NO_3^- and NH_4^+ were extracted from 10 g (fresh weight) of the sampled soil using 50 ml of K_2SO_4 0.5 M. The soil suspension was shaken for one hour, allowed to settle for two-three hours at room temperature, and then decanted. The supernatant was filtered and kept frozen until quantification. The quantification was performed by colometry according to ISO 14256-2:2005. Two blanks per series were included. The results of the NO_3^- and NH_4^+ quantifications were expressed as mg of N/kg of dry soil.

DNA extraction

DNA was extracted from 250 mg of dry weight soil using the DNeasy PowerSoil DNA extraction Kit (Qiagen, FR) following the kit's instructions. The DNA was quantified with the Quant-iT™ dsDNA Assay Kit (Thermo Fischer Scientific, FR), and stored at -20°C until further use.

Assessment of microbial community composition and diversity

Bacterial community composition and diversity were monitored via sequencing the 16S rDNA V3-V4 hypervariable region. We use a two-step PCR amplification method (Berry et al., 2011). In the first step, 25 amplification cycles were performed using the fusion primers U341F (5'-CCTACGGGRRSGCAGCAG-3') and 805R (5'-GACTACCAGGGTATCTAAT-3') as described in Takahashi et al., (2014), with overhang adapters (forward: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3', adapter: 5'-GTCTCGTG GGCTCGGAGATGTGTATAAGAGACAG-3'). The thermal cycling condition of the first PCR were: 3 min at 94°C, 25*(30 sec at 94 °C, 30 sec at 55 °C, and 30 sec at 72 °C), and 10 min at 72°C. Duplicate of the first PCR were pooled and used in a second PCR, where the amplification linked a unique combination of multiplex primer pair to the overhang adapters for each sample. The thermal cycling condition of the second PCR were: 3 min at 98°C, 8*(15 sec at 98°C, 30 sec at 55°C, 30 sec at 72°C), and 10 min at 72°C.

The amplicons from the second PCR were pooled and visualized in a 2% agarose gel for size and amplification check. They were then pooled and purified with the SequelPrep Normalization Plate kit (Invitrogen, Frederick, MD, USA). Sequencing was performed by GenoScreen (Lille, FR) on MiSeq (Illumina 2*250 bp) using the MiSeq reagent kit v2 (500 cycles).

Quantification of microbial communities

The total abundances of bacteria (16S), fungi (ITS), and protists (18S), as well as the N-cycle microbial guilds were estimated through real-time quantitative PCR (qPCR). The 16S rDNA and ITS2 primers described by Muyzer et al. (1993) and White et al. (1990) were used to estimate the total 16S and ITS communities abundances, respectively. The 18S community abundance was estimated from the 18S rDNA using the primers EK-565F (5'-GCAGTTAAAAAGCTCGTAGT-3') and 18S-EUK-1134-R-*UnonMet* (5'-TTTAAGTTTCAGCCTTGCG-3') with cycling condition of 3 min at 95°C, 35*(10 sec at 95 °C, 30 sec at 58 °C, 30 sec at 72 °C and 20 sec at 80°C). The ammonia oxidizing archaea (AOA) and bacteria (AOB) were quantified through gene *amoA* (Bru et al., 2011), comammox A (ComaA) and B (ComaB) were targeted with the clade A and B *amoA* genes (Pjevac et al., 2017), respectively.

The quantifications were carried out in a ViiA7™ (Life Technologies, Carlsbad, CA, USA) with a reaction volume of 15 µL containing 7.5 µL of Takyon Master Mix (Eurogentec, Liège, BE), 1 µM of each primer, 250 ng of T4 gene 32 (MP Biomedicals, Santa Ana, CA, USA), 3 ng of DNA. Two independent replicates were used for each qPCR assay. Prior to qPCR analysis, an inhibition test was performed by mixing the soil DNA extracts with a control plasmid DNA (pGEM-T Easy Vector, Promega, Madison, WI, USA) or water. No inhibition was detected. The results from the quantification analysis were expressed as number of gene copies/ng of DNA.

Bioinformatics analysis

Sequencing data were analyzed using an in-house developed Jupyter Notebooks (Kluyver et al., 2016) piping together different bioinformatics tools. Briefly, for 16S, R1 and R2 sequences were assembled using PEAR (Zhang et al., 2014) with default settings. Further quality checks were conducted using the QIIME pipeline (Caporaso et al., 2010) and short sequences were removed (< 400 bp for 16S). Reference based and de novo chimera detection, as well as clustering

in OTUs were performed using VSEARCH (Rognes et al., 2016) and the SILVA v132 reference database. The identity thresholds were set at 94% for 16S rDNA data based on replicate sequencings of a bacterial mock community containing 40 bacterial species. Representative sequences for each OTU were aligned using infernal (Nawrocki and Eddy, 2013) and a 16S phylogenetic tree was constructed using FastTree (Price et al., 2009). Taxonomy was assigned using BLAST (Altschul et al., 1990) and the SILVA reference database v132 (Quast et al., 2013). Diversity metrics, that is, Faith's Phylogenetic Diversity (Faith, 1992), richness (observed species) and evenness (Simpson's reciprocal index), describing the structure of microbial communities were calculated based on rarefied OTU tables (8300 sequences per sample). Weighted UniFrac distance matrices (Lozupone and Knight, 2005) were also computed to detect global variations in the composition of microbial communities.

Statistical analysis of the microbial endpoints and of the nitrogen pools

The statistical analysis was carried out using RStudio statistical software version 4.2.1. The data were checked for normal distribution of residuals with Shapiro Wilk test ($p < 0.05$), and the homogeneity of variance with Levene's test ($p < 0.05$). Data were log-transformed when necessary.

The values from 16S, ITS, 18S, AOA, AOB, ComaA, ComaB abundances, NO_3^- and NH_4^+ concentration, and α -diversity indices which are observed species (OS), Faith's Phylogenetic Diversity (PD) and Simpson's reciprocal index (SR) were analyzed with linear ANOVAs ($n=5$ per treatment at each sampling date) to determine any significant variance in the measured variables that can be attributed to the time of sampling, environmental disturbance (heat, high humidity, control), pesticide treatment (three different a.i and their mixture) and dose (1x, 10x) combinations on microbial parameters. Post-hoc analyses were performed with Tukey's honestly significant difference test (p value < 0.05) using the *TukeyHSD()* function from the *stats* package in RStudio.

The data from β -diversity weighted UniFrac were analyzed with PerMANOVA ($n=5$) using the function `adonis` from the R package `vegan` to detect significant differences between time, environmental disturbance, pesticide and dose combinations on bacterial community composition. Post-hoc analyses with pairwise comparisons were performed when significant differences were observed (p value < 0.05).

To estimate which OTUs significantly differ between our experimental conditions, we used a generalized linear mixed model. This model accounts for the non-normal distribution of abundance data that typically follow a Poisson distribution, and contains both fixed effects (experimental conditions: time of sampling, environmental disturbances, pesticide treatments and doses) and random effects (sampling effects). Multiple pairwise comparisons were performed with the `emmeans()` function of the `emmeans` package with the Bonferroni correction for p-value adjustment.

RESULTS

We assessed here the impact of environmental disturbances (heat or high humidity) and a.i exposure (three different a.i and their mixture) on several endpoints: *i*) abundance of 16S, ITS and 18S communities, *ii*) structure and composition of the soil bacterial community (α - and β -diversity) and *iii*) N-cycle functional guilds, by targeting the nitrification step by different means (NH_4^+ and NO_3^- pools, AOA, AOB, ComaA and ComaB abundances).

Effect of environmental disturbances on microbial endpoints

We performed a Principal Component Analysis (PCA) to decipher the contribution of each measured endpoint to the observed response of soil microcosms to environmental disturbances as compared to the control at each time of sampling (T30 and T60, Fig.6 panel A and panel B, respectively). Most of the

variance was explained by the two first axes (respectively ~75 % and ~65 % at T30 and T60) and separated the heat-disturbed microcosms from the high humidity and the control ones (Fig. 6). At both timepoints, this was mostly explained by α -diversity indices and NO_3^- pool (Tab. S1).

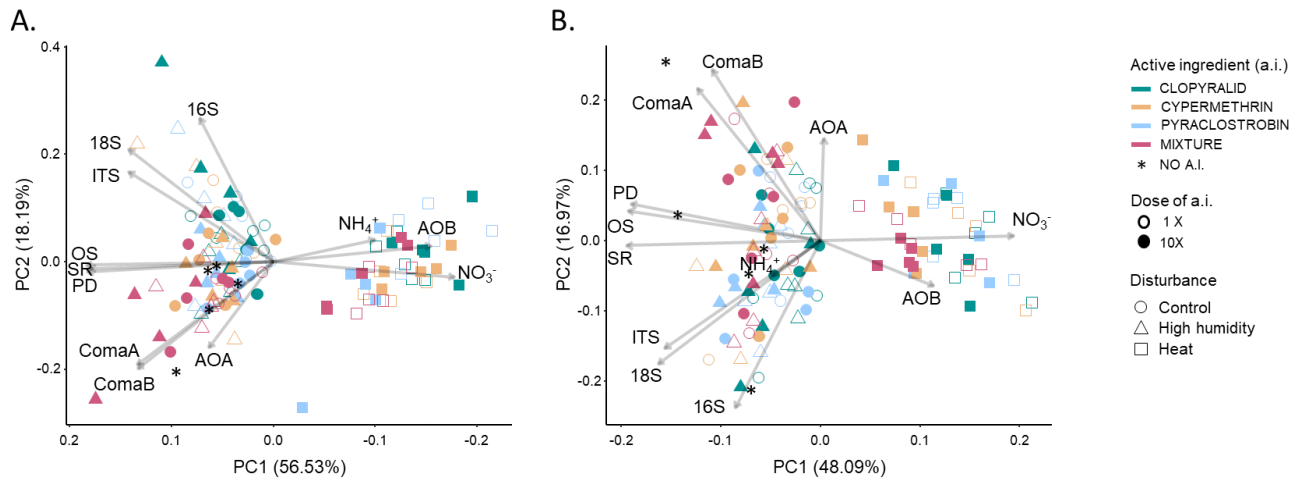


Figure 6. Principal Component Analysis (PCA) of the microbial endpoints measured at T30 (panel A) and T60 (panel B). PC1 and PC2 axes represent the two main principal components capturing the maximum variability in the dataset. Arrows indicate the direction and magnitude of each endpoint's contribution to the first and second principal components. Each soil microcosm is depicted by a symbol whose shape, color and fill respectively indicate the disturbance, the active ingredient (a.i) and the a.i. doses that were applied.

Focusing on disturbed and control microcosms irrespectively of the applied a.i, we observed significant differences between heat-disturbed microcosms and the others (Tab. 1). The abundances of 16S, ITS and 18S communities, as well as the diversity of the bacterial community (OS, PD and SR) were significantly lower in heat-disturbed microcosms (ANOVAs followed by Tukey HSD tests; $p < 0.05$) as compared to the control. When focusing on N-cycle, we also observed a significant decrease in the abundance of the ComaA and ComaB guilds and a significant increase in NH_4^+ (only at T30) and NO_3^- pools, as well as in the abundance of AOB in heat-disturbed microcosms. For most of the endpoints, the high humidity disturbance did not induced changes as compared to control

microcosms, except for the diversity level of the bacterial community at T30, and the abundance of the protist community (18S).

Table 1. Microbial endpoints measured at T30 and T60 on disturbed (heat, humidity) and control soil microcosms. For each endpoint, the mean \pm the standard error across all pesticide treated microcosms (n= 40 for each disturbance treatment and control) measured at each time point and for each soil disturbance is indicated. For a given time, an asterisk (*) indicates that the endpoint is significantly different from the one measured in the undisturbed soil (ANOVA followed by a Tukey HSD test; $p < 0.05$).

		T30			T60		
		CTRL	HIGH HUMIDITY	HEAT	CTRL	HIGH HUMIDITY	HEAT
Microbial abundance	ITS (x 10 ³ per ng)	10.9 \pm 0.5	11.9 \pm 0.7	5.2 \pm 0.3*	13.5 \pm 0.8	15.5 \pm 0.8	7.2 \pm 0.4*
	18S (x 10 ³ per ng)	52.6 \pm 1.9	56.7 \pm 3.2*	23.3 \pm 0.9*	65.8 \pm 3.5	76.6 \pm 3.5*	31.7 \pm 1.3*
	16S (x 10 ³ per ng)	128 \pm 4	140 \pm 7	110 \pm 4*	134 \pm 6	145 \pm 5	123 \pm 4*
Bacterial α -diversity	SR	240 \pm 3	247 \pm 3	140 \pm 2*	262 \pm 3	265 \pm 2	179 \pm 4*
	PD	139 \pm 1	142 \pm 1*	122 \pm 1*	143 \pm 1	142 \pm 0	128 \pm 1*
	OS	1799 \pm 8	1832 \pm 7*	1542 \pm 9*	1840 \pm 8	1836 \pm 6	1626 \pm 9*
N-cycle functional guild	ComaA (per 10 ⁴ 16S)	71.3 \pm 1.6	76.2 \pm 3.1	52.5 \pm 1.6*	80.0 \pm 1.7	83.3 \pm 2.6	67.0 \pm 1.6*
	ComaB (per 10 ⁴ 16S)	14.3 \pm 0.4	15.4 \pm 0.5	10.5 \pm 0.3*	21.5 \pm 0.5	22.5 \pm 0.8	18.6 \pm 0.5*
	AOA (per 10 ⁴ 16S)	276 \pm 12	301 \pm 12	270 \pm 12	325 \pm 10	326 \pm 12	358 \pm 12
	AOB (per 10 ⁴ 16S)	27 \pm 1.0	28 \pm 0.9	57 \pm 3.0*	30 \pm 1.0	33 \pm 1.3	45 \pm 2.8*
	NO ₃ ⁻	1.8 \pm 0.3	1.3 \pm 0.2	46.2 \pm 0.6*	1.6 \pm 0.2	1.6 \pm 0.2	46.8 \pm 1.1*
	NH ₄ ⁺	1.1 \pm 0.1	0.9 \pm 0.1	2.1 \pm 0.3*	1.4 \pm 0.1	1.7 \pm 0.2	1.2 \pm 0.1

We also performed a Principal Coordinates Analysis (PCoA) of the weighted UniFrac distance matrix to examine bacterial compositional changes in response to environmental disturbances applied to soil microcosms (Fig. 7). In line with results presented above, heat-disturbed microcosms were significantly different from the other two disturbance treatments (high humidity and control) at both timepoints (PermANOVAs; $p < 0.05$). In addition, the high-humidity-disturbed microcosms were significantly slightly different from control microcosms at T30. Overall, among the 336 dominant OTUs (defined as those having abundance $> 0.1\%$), $\sim 80\%$ showed at both T30 and T60, significant differences in their abundance between heat-disturbed and control microcosms, while less than 5% displayed significant differences between high-humidity-disturbed and control ones (Fig. S2).

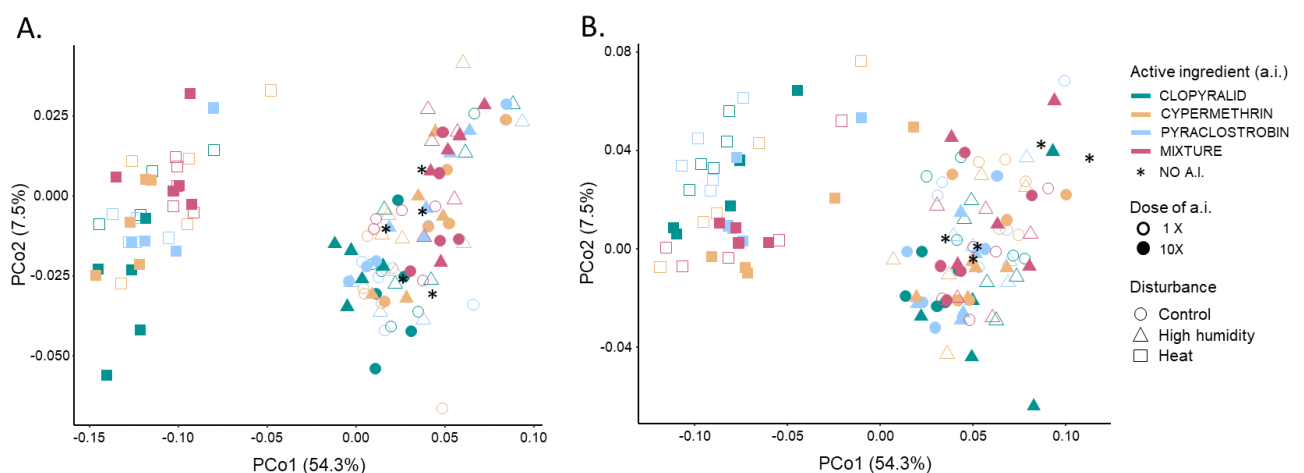


Figure 7. Principal Coordinate Analysis (PCoA) of the bacterial community structure in the different soil microcosms based on the weighted UniFrac distance metric. The two main principal coordinates PCo1 and PCo2 captured respectively 54.3 % and 7.5 % of the observed variability between bacterial communities. For T30 (panel A) and T60 (panel B), each sample is represented by a symbol whose shape, color and fill respectively indicate the disturbance, the active ingredient and the a.i. dose that were applied.

Evaluating the impact of active ingredients on microbial endpoints

We then focused on the results obtained for the disturbance control microcosms exposed to a.i applied alone (clopyralid, *i. e.* CLO; cypermethrin *i. e.*

CYP; pyraclostrobin, *i. e.* PYR) or as mixture (*i. e.* MIX) to evaluate their ecotoxicological impacts on measured endpoints. When considering all the endpoints together in a PCA (Fig. 8, panels A and B), we did not observe a strong clustering of the samples, neither according to the a.i or mixture of the three a.i, nor to the dose of applications (1x or 10x) at both timepoints (T30 and T60). However, when considering the individual endpoints, we detected only at T30 a significant effect of a.i treatments on NH_4^+ and NO_3^- pools, as well as on the abundance of ComaA and ComaB (ANOVAs, *Pesticide_Dose* and/or *Pesticide_Dose-by-Time* effects $p < 0.05$). The NO_3^- pool was the most sensitive endpoint, with significantly higher levels detected in the CLO1x, CLO10x, CYP1x, PYR1x and MIX1x treated soil microcosms as compared to the untreated controls (Tab. 2). The NH_4^+ pool was significantly higher only in the CLO1x and MIX1x treated microcosms as compared to the untreated control. At T60, the 16S abundance was the sole endpoint that was significantly decreased in response to exposure to CYP1x and PYR1x.

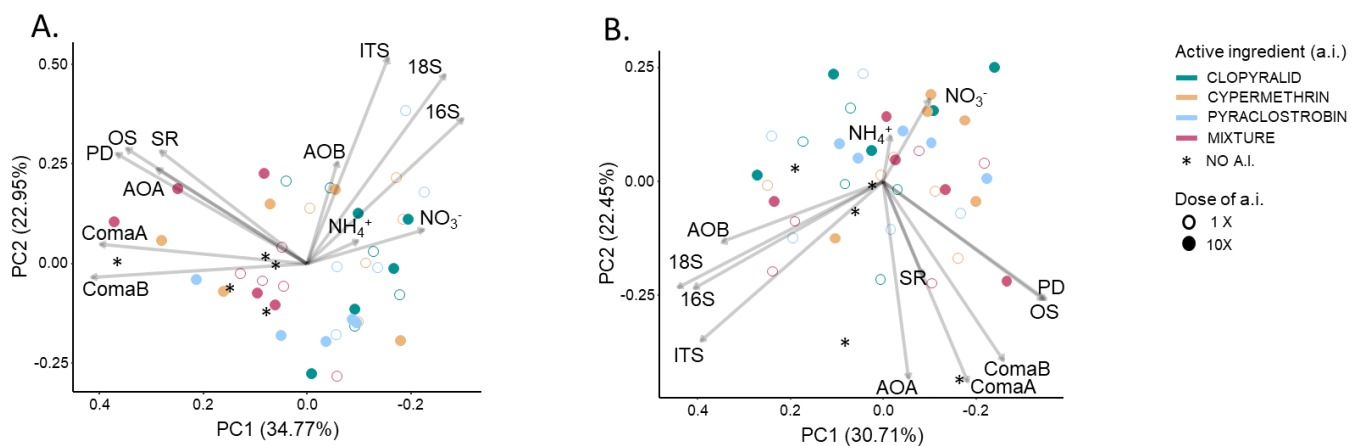


Figure 8. Principal Component Analysis (PCA) performed on the microbial endpoints measured in the undisturbed microcosms at T30 (panel A) and T60 (panel B). The PC1 and PC2 axes represent the two main principal components capturing the maximum variability in the dataset. Arrows indicate the direction and magnitude of each endpoint's contribution to the first and second principal components. Each soil microcosm is depicted by a circle whose color denotes the active ingredient that it received. Empty circles represent samples having received the a.i. at the agronomical dose while the full circle represent the one who received 10 times the agronomical dose.

Table 2. Microbial endpoints measured at T30 and T60 on the undisturbed treated and untreated control (u.c.) soil microcosms. For each endpoint, the mean \pm the standard error across all control treated microcosms and u.c. (n=5 for each active ingredient-dose and u.c.) measured at each time point and for each active ingredient is indicated. For a given time, an asterisk (*) indicates that the endpoint is significantly different from the one measured in the u.c. soil (ANOVA followed by a Tukey HSD test; $p < 0.05$).

Part 1		T30								
		U.C.	CLO1x	CLO10x	CYP1x	CYP10x	PYR1x	PYR10x	MIX1x	MIX10x
Microbial abundance	ITS (x 10 ³ per ng)	10.0 \pm 0.5	11.9 \pm 1.3	10.5 \pm 1.2	13.3 \pm 1.8	11.0 \pm 1.3	11.8 \pm 2.1	8.7 \pm 0.6	9.6 \pm 0.7	10.4 \pm 1.1
	18S (x 10 ³ per ng)	46.9 \pm 3.1	55.3 \pm 3.6	56.3 \pm 6.3	61.9 \pm 4.8	49.7 \pm 4.0	61.3 \pm 8.5	44.4 \pm 1.8	42.2 \pm 3.0	49.5 \pm 5.0
	16S (x 10 ³ per ng)	102 \pm 6	132 \pm 7	139 \pm 12	137 \pm 9	131 \pm 13	142 \pm 21	115 \pm 7	113 \pm 4	115 \pm 5
Bacterial α -diversity	SR	244 \pm 6	244 \pm 12	229 \pm 6	232 \pm 8	251 \pm 6	240 \pm 6	234 \pm 12	240 \pm 6	254 \pm 8
	PD	142 \pm 1	139 \pm 2	138 \pm 1	138 \pm 1	140 \pm 3	139 \pm 0	137 \pm 2	138 \pm 2	144 \pm 2
	OS	1827 \pm 12	1792 \pm 22	1777 \pm 7	1780 \pm 11	1790 \pm 35	1793 \pm 12	1771 \pm 23	1787 \pm 26	1872 \pm 20
N-cycle functional guild	ComaA (per 10 ⁴ 16S)	73.7 \pm 5.6	64.9 \pm 2.0	62.4 \pm 2.7	65.0 \pm 2.7	80.4 \pm 4.1	62.7 \pm 2.1	71.0 \pm 2.5	81.4 \pm 2.9	83.1 \pm 4.3
	ComaB (per 10 ⁴ 16S)	20.1 \pm 1.6	12.5 \pm 0.3	13.6 \pm 0.8	13.5 \pm 0.7	15.5 \pm 1.5	12.1 \pm 0.5	14.8 \pm 0.5	15.2 \pm 0.6	16.9 \pm 1.1
	AOA (per 10 ⁴ 16S)	265 \pm 21	233 \pm 26	235 \pm 7	274 \pm 41	287 \pm 31	249 \pm 34	284 \pm 26	313 \pm 37	330 \pm 49
	AOB (per 10 ⁴ 16S)	26.7 \pm 0.9	30.9 \pm 3.9	24.9 \pm 2	27.9 \pm 2.3	31.8 \pm 3.0	29.2 \pm 3.0	22.0 \pm 0.6	25.9 \pm 3.4	27.2 \pm 2.9
	NO ₃ ⁻	0.1 \pm 0.1	1.4 \pm 0.5*	3.1 \pm 0.7*	3.8 \pm 1.0*	0.1 \pm 0.0	4.4 \pm 1.0*	0.1 \pm 0.0	1.3 \pm 0.6*	0.6 \pm 0.4
	NH ₄ ⁺	0.8 \pm 0.1	1.7 \pm 0.3*	0.8 \pm 0.1	0.9 \pm 0.2	0.6 \pm 0.1	1.4 \pm 0.2	0.8 \pm 0.1	1.9 \pm 0.4*	1.1 \pm 0.1

Part 2		T60								
		U.C.	CLO1x	CLO10x	CYP1x	CYP10x	PYR1x	PYR10x	MIX1x	MIX10x
Microbial abundance	ITS (x 10 ³ per ng)	18.7 ± 2.1	11.4 ± 2.6	15.1 ± 1.2	11.4 ± 2.7	13.8 ± 1.9	10.9 ± 1.4	15.9 ± 2.4	13.1 ± 2.0	18.5 ± 2.5
	18S (x 10 ³ per ng)	79.4 ± 6.2	60.7 ± 12.3	70.2 ± 4.2	54.0 ± 12.0	72.2 ± 9.2	54.9 ± 7.1	83.4 ± 10.5	67.4 ± 10.8	80.0 ± 10.1
	16S (x 10 ³ per ng)	200 ± 16	133 ± 25	129 ± 7	107 ± 4*	128 ± 18	119 ± 10*	148 ± 13	136 ± 20	163 ± 20
Bacterial α-diversity	SR	259 ± 2	264 ± 9	264 ± 6	266 ± 7	265 ± 10	258 ± 8	257 ± 9	268 ± 8	251 ± 5
	PD	142 ± 1	142 ± 1	141 ± 2	144 ± 1	143 ± 2	142 ± 2	143 ± 2	144 ± 2	142 ± 2
	OS	1852 ± 7	1825 ± 20	1814 ± 24	1868 ± 16	1839 ± 21	1833 ± 21	1848 ± 30	1861 ± 25	1830 ± 23
N-cycle functional guild	ComaA (per 10 ⁴ 16S)	77.3 ± 3.3	69.5 ± 1.6	86.4 ± 4.8	79.0 ± 3.3	96.3 ± 3.5	80.7 ± 6.5	85.0 ± 12.3	73.8 ± 3.4	76.7 ± 2.3
	ComaB (per 10 ⁴ 16S)	20.5 ± 1	19.2 ± 1.5	22.7 ± 1.2	21.4 ± 1.0	24.5 ± 1.7	22.4 ± 1.5	26.8 ± 3.9	19.5 ± 1.6	21.6 ± 1.2
	AOA (per 10 ⁴ 16S)	357 ± 35	253 ± 15	329 ± 33	310 ± 29	395 ± 23	320 ± 23	344 ± 23	318 ± 18	315 ± 26
	AOB (per 10 ⁴ 16S)	33.7 ± 3.2	30.7 ± 4	29.7 ± 1.4	26.7 ± 2.1	31.1 ± 3.7	27.1 ± 1.4	26.5 ± 1.1	30.9 ± 3.9	27.7 ± 1.9
	NO ₃ ⁻	1.1 ± 0.1	1.8 ± 0.8	1.4 ± 0.2	3.4 ± 0.2	2.6 ± 0.8	0.8 ± 0.2	0.7 ± 0.1	1.4 ± 0.2	0.7 ± 0.1
	NH ₄ ⁺	0.8 ± 0.1	2.4 ± 0.5	0.8 ± 0.1	1.8 ± 0.1	1.3 ± 0.2	1.5 ± 0.2	1.4 ± 0.1	1.4 ± 0.2	1.5 ± 0.2

PCoA on the Weighted UniFrac distance matrix (Fig. 9) showed that in the absence of heat or humidity disturbance the bacterial community structure was not changed by a.i exposure as compared to the untreated control. We noticed that the structure of the bacterial communities of the microcosms exposed to CLO10x were significantly different from those exposed to CYP10x (Pairwise PermANOVAs, $p < 0.05$). When looking at the dominant OTUs, at both sampling times, we found only 12 OTUs that showed significant differences in relative abundance in a.i treated microcosms versus the untreated control ones. Among those, only four were consistently and significantly impacted at both timepoints: they were observed only in CLO-treated microcosms (three in CLO10x and one in CLO1x) and three of them are related to uncultured members of the Acidobacteria phylum.

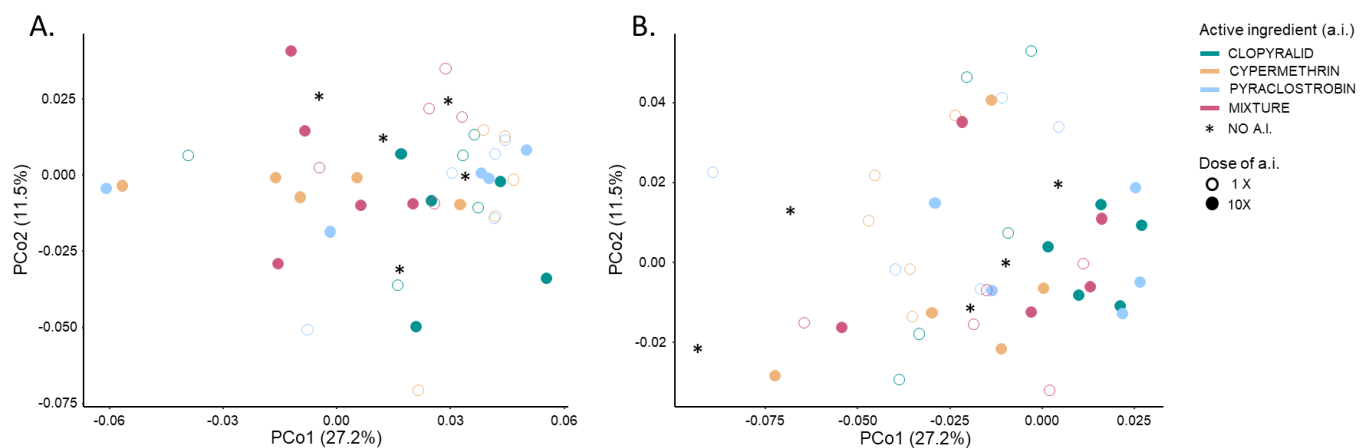


Figure 9. Principal Coordinate Analysis (PCoA) of the bacterial community structure in the undisturbed microcosms based on weighted UniFrac distance metric. The two main principal coordinates PCo1 and PCo2 captured respectively 27.2% and 11.5% of the variability is represented by a symbol whose color and fill respectively indicate the active ingredient and its dose applied in soil microcosms.

Estimating compounded effects of environmental disturbances and pesticides inputs on microbial endpoints

A compounded effect in our ANOVA model would manifest with a significant two-way ‘*Disturbance-by-Pesticide_Dose*’ or three-way

'Disturbance-by-Pesticide_Dose-by-TimeOfSampling' effect, indicating that the effect of a given *'Pesticide_Dose'* at a given *'TimeOfSampling'* is conditioned by the *'Disturbance'*. The AOB abundance and the OS of the bacterial community were the only two endpoints showing a significant three-way interaction (Tab. 3). However, none of the pairwise comparisons were significant for OS, while the only significant, but slight, difference observed for AOB was between T30-Heat-PYR10x (38 ± 8 copies per 10^4 copies 16S rDNA) and T30-Heat-CLO10x (75 ± 2 copies per 10^4 copies 16S rDNA) samples, indicating that the heat disturbance conditioned the relative effects of PYR and CLO at the 10x dose. The two-way interaction was only significant for the abundance of 16S, and NH_4^+ and NO_3^- pools. More specifically, CYP1x-treated microcosms showed significantly higher levels of NO_3^- compared to PYR1x, PYR10x and CYP10x-treated microcosms, but only in the treated controls, while PYR10x-treated microcosms displayed significantly higher levels of NH_4^+ than PYR1x, CYP1x, CLO1x and MIX10x-treated ones, but only for the heat-disturbed microcosms. Overall, compounded effects are relatively sparse, idiosyncratic and of small scale which is in line with the relatively small ecotoxicological impact detected for the chosen pesticides in undisturbed environmental conditions.

Table 3. Analysis of variance (ANOVA) results assessing the impact of time of sampling, the disturbance, and the applied pesticide (nature/dose) on various microbial endpoints. Only the endpoints for which a significant 2-way ‘Disturbance-by-Pesticide_Dose’ or 3-way ‘Disturbance-by-Pesticide_Dose-by-TimeOfSampling’ interactions are presented ($p < 0.05$).

	Df	16S			OS			AOB			NO3			NH4		
		Mean Sq	F value	Pr(>F)	Mean Sq $\times 10^3$	F value	Pr(>F)	Mean Sq	F value	Pr(>F)	Mean Sq	F value	Pr(>F)	Mean Sq	F value	Pr(>F)
<i>TimeOfSampling</i>	1	0.04	4.3	0.040	114	60	< 0.001	0.00	0	0.985	1.90	4.70	0.031	0.537	13.4	< 0.001
<i>Disturbance</i>	2	0.14	13.5	< 0.001	1569	821	< 0.001	1.31	114	< 0.001	1.35	3.32	0.038	0.130	3.2	0.041
<i>Pesticide_Dose</i>	7	0.02	2.2	0.037	10	5	< 0.001	0.01	1	0.554	0.55	1.36	0.224	0.151	3.8	0.001
<i>Disturbance-by-TimeOfSampling</i>	2	0.01	0.8	0.466	32	17	< 0.001	0.15	13	< 0.001	0.06	0.16	0.852	0.964	24.1	< 0.001
<i>Pesticide_Dose-by-TimeOfSampling</i>	7	0.01	1.1	0.373	3	2	0.089	0.02	2	0.053	0.81	1.99	0.059	0.145	3.6	0.001
<i>Disturbance-by-Pesticide_Dose</i>	14	0.02	1.9	0.030	3	1	0.192	0.01	1	0.263	1.37	3.40	< 0.001	0.133	3.3	< 0.001
<i>Disturbance-by-Pesticide_Dose-by-TimeOfSampling</i>	14	0.01	0.7	0.781	4	2	0.013	0.03	2	0.007	0.68	1.69	0.061	0.064	1.6	0.084

DISCUSSION

The present study aimed to evaluate the effects of elevated temperature (heat) and heavy rainfall (high humidity), two global change related environmental disturbances, and of three commonly used a.i., CLO (herbicide), CYP (insecticide), PYR (fungicide) and their mixture on soil microbial community abundance, structure, composition and functioning. We used qPCR techniques to detect any effect on the abundance of some important microbial guilds, and Illumina next generation sequencing of the 16S rDNA amplicons to study the effects on the bacterial community composition.

Most of the measured microbial endpoints were affected by the heat stress, with significant effects at T30 (short term). The α -diversity indices and important N-cycle-related microbial guilds were the endpoints mostly impacted by the heat disturbance. We found decreasing α -diversity indices and accumulation of NO_3^- , which is in line with former studies in which the disruption of the N-cycle was described as a consequence of a lower functional redundancy due to community shifts (Calderón et al., 2018). In another research, the heat-disturbed microbial communities never recovered, and were always different from the control, independently from the soil disturbance history (Jurburg et al., 2017a). This response is in line with our observations, where the effect of heat stress persisted all along the experiment. We also measured a higher AOB abundance in the heat disturbed samples compared to the control, which suggests a higher sensitivity to heat of the AOB community compared to other members of N microbial guilds. This finding is supported by other studies, in which AOB were more sensitive to soil warming than AOA (Szukics et al., 2010; Wang, 2021). We observed that ComaA and ComaB abundances were negatively impacted by the heat stress: not much is known about the effect of heat on these two microbial guilds, but a recent research suggests that commamox are favoured at low temperature in wastewater treatment plants (Gonzalez-Martinez et al., 2016). We can therefore conclude that

the N-cycle was impacted by heat stress at several levels, and exhibited a limited recovery, *i. e.* low resilience, at the time scale of the experiment.

The effect of pesticides on soil microbial communities has been studied for decades (Bollen, 1961). The microbial response to pesticide application is variable, and depends on many factors such as the pesticidal mode of action, the experimental set up and the studied endpoints (Jacobsen and Hjelmsø, 2014; Puglisi, 2012). Indeed, the few responses observed in our experiment are not consistent across the various endpoints analysed. Most are related to the N-cycle and exhibit a transient effect, *i. e.* recovery in the longer term. Some functional guilds, e.g. AOA and AOB, have been described to be sensitive to pesticide application (Karas et al., 2018). To our best knowledge, no studies have explored the influence of pesticides on comammox. Even though we could not detect a strong effect, our findings indicate that the application of technical a.i might affect these N-cycle microbial guilds. The exposure of soil to the a.i, and particularly to the herbicide CLO, led to a significant increase of the NO_3^- and NH_4^+ pools which is supported by previous observations reporting the disruption of the N-cycle in response to pesticide application (Brochado et al., 2023; Damin and Trivelin, 2011; Hernández et al., 2011). Interestingly, one could observe that exposure to a.i had significant effects on the abovementioned endpoints already at agronomical dose, contrary to what is reported in the existing literature where effects are mostly detected at higher doses (Crouzet et al., 2010; Puglisi, 2012; Romdhane et al., 2019a). The application of a.i alone or in a mixture had no strong impact on microbial community composition. This might be due to the large variability observed between biological replicates within given experimental conditions and to the limited effect of the pesticide. Among all the OTUs, just three affiliated to the Acidobacteria were responsive to one pesticide (CLO). This phylum is very abundant and ubiquitous in many ecosystems, and takes part to various metabolic pathways like carbon and nitrogen cycle (Kalam et al., 2020).

Previous studies described this phylum to be sensitive to various toxic metals deriving from pesticides (Kim et al., 2021).

The three a.i used in our study are considered from EFSA as showing low toxicity towards soil living organisms (EFSA et al., 2018a, 2018b, 2018c), but nothing is known about the effect of these three compounds applied in combination. Contrary to our expectation, the exposure of soil microcosms to the mixture of these three a.i at the agronomic application rate and at 10-fold this rate did not affect the measured endpoints differently than exposure to the respective single a.i. Studies on the effect of pesticide mixtures on the soil microbial community are scarce (Baćmaga et al., 2015; Joly et al., 2015, 2012; Schuster and Schröder, 1990), and the responses are variable. Based on knowledge for other endpoints (Cedergreen 2014), the probability of detecting synergistic effects is low. Hence, we can conclude that the present experiment confirms the findings of Cedergreen (2014) as no exceptionally stronger effect was found in the mixture treatments (pointing at a potential synergistic interaction) compared to the single a.i. treatments.

A compounded disturbance is the phenomenon in which ecosystems are subjected to multiple disturbances occurring over time. The effect of the initial disturbance may have legacy effect and consequently alter the ability of the microbial community to cope with the following ones. The responses to the following stresses are variable (Vinebrooke et al., 2004): *i. e.* the exposure to multiple perturbations of the same nature might lead to microbial selection and high community resilience to that specific stress (Calderón et al., 2018). However, if the subsequent perturbation is of a different nature, the disturbed community might either become more resistant to the following stress because of community priming (Rillig et al., 2015), or more sensitive (Calderón et al., 2018) because of negative species co-tolerance (Vinebrooke et al., 2004). In our study, the application of different a.i to a previously environmentally disturbed community

had no apparent further impact on the microbial communities. Responses to a.i application were few and transient, but it is worth mentioning that the heat stress seemed to condition the response to the different tested a.i. This was the case of AOB or NH_4^+ , especially to PYR treatment. According to the literature, abiotic disturbances, such as extreme temperatures show a great potential into shaping and modifying the microbial community (Bardgett and Caruso, 2020; Castro et al., 2010; Islam et al., 2020) as compared to pesticides where the effects are often variable and confined to certain endpoints.

Overall, the temperature disturbance had a major and persisting impact on microbial communities. On the opposite, the transient increase in soil humidity was without effects. Similarly, the effects of the tested a.i and their mixture on the control community were only slight and transient, and seem to impact mainly some N-cycle endpoints. We did not find any strong and persisting effect on microbial endpoints of the compounded disturbances. Consideration of denitrifiers and N_2O emitters in future studies would be of interest to give a more comprehensive understanding of the compounded effect of environmental disturbances and pesticides on N-cycle in soils.

SUPPLEMENTARY MATERIAL

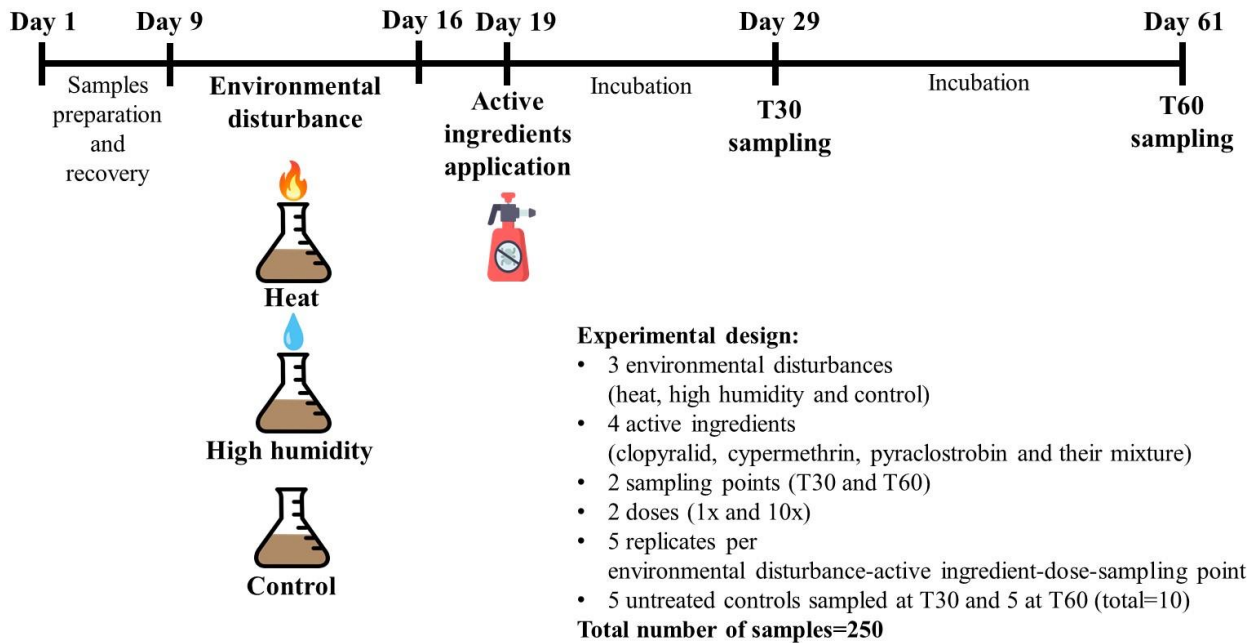


Figure S1. Graphic representation of the experimental timeline with a description of the experimental design.

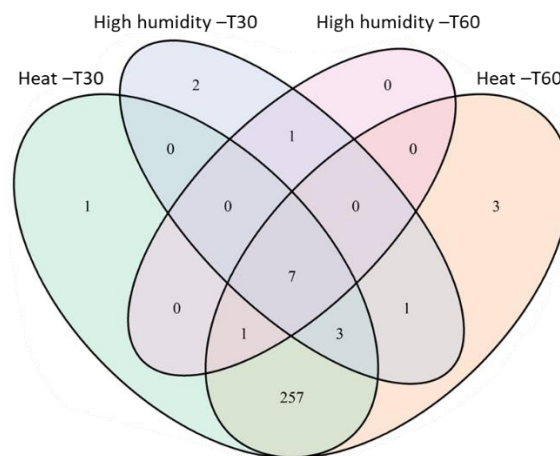


Figure S2. Venn Diagram illustrating the number of Operational Taxonomic Units (OTUs) impacted by heat or high humidity disturbances at T30 and T60 across all pesticide treated microcosms. OTUs whose relative abundance was significantly impacted by disturbances were identified by performing a GLM model on the most abundant 336 OTUs (abundance > 0,1% and present at least in 5/5 of the samples of one Time-A.I.-Dose-Disturbance condition). Each circle represents the set of OTUs impacted by one disturbance (high humidity or heat) at one time of sampling (T30 or T60).

Table S1. Contribution of the microbial endpoints to the principal components of the PCA at T30 and T60. For each axis, values represent the percentage of variance explained by each endpoint in the dataset.

	T30		T60	
	Axis 1	Axis 2	Axis 1	Axis 2
OS	13.5	0.0	15.0	0.7
PD	13.3	0.1	14.5	1.1
SR	13.0	0.1	15.2	0.0
16S	2.1	28.7	2.9	22.7
18S	8.0	17.6	10.6	12.4
ITS	8.1	11.2	9.8	9.5
AOA	1.6	10.3	0.0	8.5
AOB	9.6	0.3	5.2	1.7
ComaA	7.1	14.7	6.1	18.8
ComaB	7.1	15.9	4.7	23.7
NH ₄ ⁺	4.0	0.7	0.9	1.0
NO ₃ ⁻	12.6	0.3	15.0	0.0

PD: Faith's Phylogenetic Diversity; OS: observed species; SR: Simpson's reciprocal index.

Chapter 3

Evaluation of the Effects of a Sequential Application of Plant Protection Products on Soil Microbes and Free-Living Nematodes in a Field Experiment

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ABSTRACT

During the growth cycle of a crop, several different pesticides are usually applied, in combination or separately. Such sequential applications result in a mixture of residues in the soil that can disperse in the environment and reach non-target organisms in the soil, including soil microbial and nematodes communities, and may affect ecosystem services they are supporting. This mixture scenario, rather frequent, has rarely been addressed experimentally at field scale.

The objective of this study was therefore to evaluate in realistic conditions the effect of individual and sequential application of pesticides on soil microbial communities, and on the abundance of free-living nematode. To do so, triplicated field plots were treated either with an herbicidal, an insecticidal, a fungicidal plant protection product, or their sequential mixture at 1x or 10x the agronomical dose. Plots were sampled prior to the respective applications and after 7 and 28 days. Untreated control plots were also sampled at each timepoint. The ecotoxicological impact of pesticides application was evaluated on various microbial endpoints including bacterial and fungal communities' diversity and structure, and on free-living nematode abundance.

We showed that the fungal community was the most responsive to pesticide application, while bacterial community composition was mainly driven by soil spatial heterogeneity. Transient effects of pesticide applications were detected on nematode abundance. Higher tier ecotoxicological studies offer greater ecological relevance compared to the standard laboratory tests but are challenged by soil spatial heterogeneity and environmental variations that should be factored in when evaluating the ecotoxicity of pesticides on soil organisms.

Keywords: ecotoxicological impact; pesticide mixture; microbial endpoints; bacterial community, fungal community, nematode abundance

INTRODUCTION

Soil is a complex substrate that hosts a great biological diversity, which is vital to keep soil healthy and resilient. It has been estimated that one gram of soil may harbor up to a billion bacteria, around 200 meters of fungal hyphae (Wagg et al., 2014), but also nematodes, enchytraeids, mites, and other microorganisms. The interplay between communities of different species is essential as they provide mutual benefits to each other (Wall et al., 2012). Soil microbial communities are greatly shaped by intrinsic physicochemical properties and aboveground perturbations. Concerns derive from the intensive use of agricultural lands relying on the application of agrochemicals that depletes soil communities overtime (Murray et al., 2006), impacting their functioning and resilience (Bardgett and van der Putten, 2014; Bender et al., 2016) and threatening the production of food products. To tackle this critical issue, the European Commission adopted a Soil Monitoring Law to protect and restore soils and ensure that they are used sustainably. Within this framework, the EU has therefore set a target of achieving soil healthiness for 75% of its soils by 2050. To achieve this goal, identification and restoration measures and enhancement of sustainable soil management regulations will have to be launched within the EU (European Commission, Soil and Land, n.d), like the LUCAS project that allowed the identification of risk indicators for different pesticide mixtures found all over Europe (Franco et al., 2024).

Pesticides are among the most detected pollutants in arable soils (FAO and UNEP, 2021), as they have been and still are a crucial input in agriculture to ensure high yield of crops for an increasing world population (Sharma, 2019; Tudi et al., 2021). According to the Food and Agriculture Organization (FAO), in 2021 more than 3,500,000 tons of active ingredients were sprayed worldwide for agricultural use, with Europe accounting for 14.3% (FAOSTAT, 2023). Of this, 30 to 50% reach the environment or undergoes different process such as leaching

(Rodríguez Eugenio et al., 2018) and enter in contact with non-target organisms, with the selection of resistant organisms but also impact on ecosystem functioning (Mahmood et al., 2016; Carvalho, 2017; Tudi et al., 2021). Additionally, pesticides are frequently applied sequentially during the crop development which may impact the ability of the soil communities to recover from previous pesticide treatments applied to the crop, an aspect that has received limited attention from the scientific community (Schuster and Schröder, 1990b), and has not been adequately addressed within the legislative framework for the release on the market of pesticides. The current environmental risk assessment (ERA) of pesticides, requires *a priori* toxicity tests of active ingredients and their marketed products on defined model organisms, hence before the release of the pesticide on the market. Very little is known about the effect of unintended pesticide mixtures on natural field communities after the pesticides have been sprayed in the environment (Weisner et al., 2021).

Microbes are fundamental providers of key ecosystem services (Nannipieri et al., 2017; Schloter et al., 2018; Singh, 2015; Ward and Jensen, 2014). However, their community structure and functions can be impacted by pesticide application (Storck et al., 2018; Gallego et al., 2019; Romdhane et al., 2019a). Despite their importance and potential sensitivity to agrochemicals, the current ERA of pesticides lacks tests assessing impacts on microbial community composition. Such testing would offer a more comprehensive set of information compared to the existing microbial toxicity assessment which focuses solely on a functional parameter, via the Nitrogen transformation test, which is described as insufficiently sensitive (Karpouzias et al., 2022). Nematodes, the most abundant metazoan in soil (Sellami et al., 2011), occur worldwide and interact with a broad range of organisms like plant, fungi, bacteria, small animals (van den Hoogen et al., 2019; Neilson et al., 2020; van den Hoogen et al., 2020). Due to their ubiquity and suitability as indicators of soil health, they have been extensively utilized for

soil health monitoring purposes. Despite evidence pointing to the potential adverse effects of pesticides on free-living nematodes (Zhao et al., 2013; Waldo et al., 2019; Grabau et al., 2020; Höss et al., 2022), the current ERA of pesticides does not mandate tests on any nematode endpoint. Recently the EFSA (European Food and Safety Authority) acknowledged these weaknesses and published a scientific opinion (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017) in which the need of improving the ERA of pesticides on microbial endpoints was emphasized and proposing a range of possible endpoints (including microorganisms and nematodes) that can be considered to assess the ecotoxicity of active ingredient towards in-soil living microorganisms.

The objective of this experiment was to assess the ecotoxicity of a sequential application of individual formulated pesticides and their mixture on soil bacteria, fungi and nematodes in a field context. The different selected compounds were an herbicidal (clopyralid), an insecticidal (zeta-cypermethrin) and a fungicidal (pyraclostrobin) active substance, and they were applied sequentially according to their terms of use. We hypothesized that *i*) application of individual compounds will induce shift in the abundance, the diversity and the composition of microbial communities as well as on the abundance of soil free-living nematodes, especially at high concentration and that *ii*) application of mixture, especially at high concentration, will induce stronger shifts in measured microbial and nematode endpoints.

MATERIALS AND METHODS

Study site and pesticide application

The field study took place in an agricultural area in Hochheim am Main (50° 00' 58.1" N 8° 23' 41.0" E), Germany. The soil is a silty loam, characterized by 20.6% of clay, 24.8% of silt, and 54.6% of sand. Before the beginning of the experiment, the soil was harrowed to clear the surface from crop residues. The field was not irrigated during the experiment and the weeds were not removed as their growth was homogeneous over the plots.

The field site was partitioned into 27 plots, each measuring 2 m by 2 m with an interplot distance of 0.5 m. The arrangement followed a Latin square design. Each plot was treated with either a single active ingredient (a.i), or the sequential application of three a.i at 1x or 10x the agronomical dose. The studied a.i were applied in the form of three commercial formulations and were: clopyralid (CAS: 1702-17-6, herbicide, commercial formulation: Cliophar, a.i concentration: 100 g/L, agronomical dose: 1.5 L/ha, commercial supplier: Agriphar), zeta-cypermethrin (CAS: 1315501-18-8, insecticide, commercial formulation: Minuet® 10EW, a.i concentration: 100 g/L, agronomical dose: 0.75 L/ha, commercial supplier: Belchim Crop Protection France) and pyraclostrobin (CAS: 175013-18-0, fungicide, commercial formulation: Comet® 200, a.i concentration: 200 g/L, agronomical dose: 1.25 L/ha, commercial supplier: BASF). The treatment application was performed with a hydraulic sprayer (Agrotop GmbH, Obertraubling, DE) with a water volume of 350 L/ha. The sequential application of the three a.i was performed with one-week interval, first the clopyralid, then the zeta-cypermethrin and lastly the pyraclostrobin. Three plots served as control and did not receive any treatment.

Soil was sampled at the beginning of the experiment from all the plots, one day before the respective pesticide application only from the plot that received the treatment, and also 7 and 28 days after the respective applications. Control and

mixture plots were sampled all the sampling days over the duration of the experiment. Details of the experimental design and sampling schedule are shown in the Figs. S3 & S4. Five soil cores of 2 cm diameter and 5 cm depth were taken per plot. The cores were pooled together and mixed to create a composite sample. The soil was then sieved to 4 mm, divided and stored according to the endpoint until use. Soil subsamples were extracted from composite samples of each plot at the start of the experiment to analyze pH, organic material (OM), organic carbon (OC), cation exchange capacity (CEC), C/N ratio (RATIOCN), and total nitrogen (NTOT) content for soil property characterization. Soil subsamples for nematode was stored at 4°C up to 4 weeks and for DNA extraction at -20°C until their use.

Nematode extraction and identification

Nematodes were then extracted from 100 g fresh soil using a modification of Cobb's decanting and sieving method (1918). Briefly, 100 g of fresh soil were mixed with 800 mL of water and soaked for about 30 minutes to detach the nematodes from soil particles. Thereafter, the soil suspension was stirred and decanted for 30 seconds. The supernatant was then poured in a collection plastic bowl. This procedure was repeated 3 times. At the end the remaining sediment was discarded.

The supernatant with nematodes went through several filters with decreasing pore size: 1000, 350, 175, 100 and 45 μm (repeated 4 times). The collected impurities on the 1000 μm filter were rinsed and discarded. The nematodes on the 350, 175, 100 and 45 sieves were rinsed with tap water and transferred to a separate bowl. The nematode suspension was then transferred to an extraction sieve with 3 layers of milk filters. The nematodes suspension was incubated for 46 hours at room temperature to allow the migration of the nematodes through the filters to the collection tray. Nematode were counted from three 5 mL aliquots and expressed as nematodes number/100 g of soil dry weight.

DNA extraction

The DNA was extracted from 250 mg of dry weight soil using the DNeasy PowerSoil DNA extraction Kit (Qiagen) following the kit's instructions. The DNA was quantified with the Quant-iT™ dsDNA Assay Kit (Thermo Fischer Scientific, France), and stored at -20°C until use.

Assessment of microbial community composition

Bacterial and fungal communities' compositions and diversities were monitored via Illumina Miseq 2x250 bp by sequencing the 16S rRNA V3-V4 hypervariable region and the Internal Transcribed Spacer 2 (ITS2) region of the ribosomal RNA gene cluster, with a two-steps PCR amplification method (Berry et al., 2011). In the first step of bacterial community composition assessment, 25 amplification cycles were performed using the fusion primers 341F (5'-CCTACGGGRSGCAGCAG-3') and 805R (5'-GACTACCAGGGTATCTAAT-3'). The ITS gene was targeted with ITS3F (5'-GCATCGATGAAGAACGCAGC-3') and ITS4R (5'-TCCTCSSCTTATTGATATGC-3'). The two libraries were amplified with overhanging adapters (forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG, adapter: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG).

The thermal cycling condition of the first PCR for bacteria were: 3 min at 98°C, followed by 25 cycles *(15 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C), and 10 min at 72°C, and that for fungi were: 3 min at 95°C, followed by 30 cycles *(15 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C), and 7 min at 72°C. In the second PCR, duplicates of the first PCR were pooled and used as a template to link a unique combination of multiplex primer pair to the overhang adapters of each sample. The thermal cycling condition of the second PCR for both bacteria and fungi were: 3 min at 98°C, then 8 cycles *(15 sec at 98°C, 30 sec at 55°C, 30 sec at 72°C), and 10 min at 72°C.

The amplicons from the second PCR were pooled and visualized in a 2% agarose gel for size and amplification check. They were then pooled together and purified with the SequalPrep Normalization Plate kit (Invitrogen, Frederick, MD, USA). Sequencing was performed by GenoScreen (Lille, FR) on MiSeq (Illumina 2*250 bp) using the MiSeq reagent kit v2 (500 cycles).

Bioinformatics analysis

The sequence data were analyzed using an in house developed Jupyter Notebooks (Kluyver et al., 2016) piping together different bioinformatics tools. Briefly, for both 16S and ITS data, R1 and R2 sequences were assembled using PEAR (Zhang et al., 2014) with default settings. Further quality checks were conducted using the QIIME pipeline (Caporaso et al., 2010) and short sequences were removed (< 400 bp for 16S and for ITS). Reference-based and *de novo* chimera detection, as well as clustering in OTUs were performed using VSEARCH (Rognes et al., 2016) and the adequate reference databases (SILVA' representative set of sequences for 16S and SILVA's ITS2 reference dynamic dataset for ITS). The identity thresholds were set at 94% for 16S data based on replicate sequencing of a bacterial mock community containing 40 bacterial species, and 97% for ITS data for which we did not have a mock community). For 16S data, representative sequences for each OTU were aligned using MAFFT (Kato and Standley, 2013) and a 16S phylogenetic tree was constructed using FastTree (Price et al., 2009). Taxonomy was assigned using BLAST (Altschul et al., 1990) and the SILVA reference database v138 for 16S (Quast et al., 2013). For ITS, the taxonomy assignment was performed using BLAST (Altschul et al., 1990) and the UNITE reference database (Abarenkov et al., 2021). Diversity metrics, that is, Faith's Phylogenetic Diversity (PD) for 16S data only (Faith, 1992), richness (observed species-OS) and evenness (Simpson's reciprocal index-SI), describing the structure of microbial communities were calculated based on rarefied OTU tables (22000 and 15000 sequences per sample for 16S and ITS

respectively). Bray-Curtis and UniFrac distance matrices (Lozupone and Knight, 2005) were also computed to detect global variations in the composition of microbial communities for ITS and 16S, respectively.

Quantification of microbial communities

The total abundances of bacteria (16S), fungi (ITS), ammonia oxidizing archaea (AOA) and bacteria (AOB), and comammox A (COMAA) and B (COMAB) were quantified through real-time quantitative PCR (qPCR) using the ViiA7™ thermocycler (Life Technologies, Carlsbad, CA, USA). The reaction volume was 15 µL per sample, and contained 7.5 µL of Takyon Low Rox SYBR MasterMix dTTP blue (Eurogentec, Seraing, Belgium), 1 µM of each primer, 250 ng of T4 gene 32 protein (MP Biomedicals, Illkirch, France), and 1.5 ng of DNA. Total bacterial and fungal community were quantified according to Muyzer et al. (1993) and White et al. (1990), respectively. The abundance of AOA and AOB were estimated targeting the *amoA* gene (Bru et al., 2011). COMAA and COMAB were quantified targeting the clade A and B *amoA* (Pjevac et al., 2017). Non-template controls (NTC) were analyzed too and no quantification could be detected. Standard curves were created with serial dilutions of linearized plasmids containing cloned genes from bacterial strains or environmental clones. Two independent runs were performed per each gene quantification. The qPCRs efficacy ranged from 78% to 107%. The final abundances were expressed in number of numbers of copies/g of soil dry weight. Prior to analysis, an inhibition test was performed mixing the soil DNA with a control plasmid (pGEM-T Easy Vector, Promega, Madison, WI, USA) or water to detect the presence of inhibitors of the qPCR assays. No inhibition could be detected.

Statistical analysis

Statistical analyses were performed using R statistical software version 2023.12.1+402. We first assessed the spatial variability of soil properties within the experimental field (*i. e.* pH, OM, OC, NTOT, CEC and RATIOCN),

employing ANOVAS with the plot positions (X and Y coordinates) as explaining factors.

To assess the impact of single and mixture treatments on soil microbial communities and nematode abundance, we used multiple generalized linear mixed models (GLMMs) using the `glmmTMB` function from the `glmmTMB` package (version 1.1.8), which allow to account for both fixed effects and random effects. Since we observed a spatial variability for certain soil properties, we incorporated them as fixed effects across all models. This approach allows to distinguish the treatment effect from those of the soil properties spatial variability. To reduce collinearity among variables, only soil properties with low correlation were included in the models (*i. e.* OM, pH, RATIOCN and CEC) (Fig. S5). To account for the temporal variability, we included the sampling day into the models as random effects. Three models were then constructed to evaluate the effects of *i)* pesticides mixture across all sampling days, *ii)* single treatments (*i. e.* for herbicide, insecticide and fungicide altogether), and *iii)* single and mixture treatments (*i. e.* for herbicide, insecticide and fungicide, individually). For models *ii)* and *iii)*, we only considered the day preceding the application of the respective pesticide (day -1), 7 and 28 days following the pesticide application (day 7, day 28, respectively). Normality and homogeneity of the residual distribution were verified, and log-transformations were performed when necessary for gene copy abundances. We then implemented multiple pairwise comparisons for each significant treatment-dose effect using the `emmeans` function from the `emmeans` package (version 1.10.0). The p-values were then adjusted using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995).

We then conducted a Partial Redundancy Analysis (P-RDA) model to explore the impact of the Treatment-by-Dose and soil properties (explanatory variables) on the composition of the bacterial and fungal communities (response variables), while considering the variation due to the sampling day as a covariate).

To perform this analysis, low-abundance OTUs were filtered out, retaining only those representing $> 0.01\%$ of the sequences across all samples which resulted in 1280 OTUs for 16S and 587 OTUs for ITS. The significance of the P-RDA model and its variables was assessed using *the anova.cca* function (with 1000 permutations) in the *vegan* package.

RESULTS

Effect of spatial variability on plot properties

All soil properties, except for the pH, were influenced by the plot coordinates ($p \leq 0.05$), meaning that OC, OM, NTOT, RATIOCN and CEC were heterogeneous within the studied field according to the X and/or Y coordinates (Tab. 4 and Fig. S5). Specifically, OC, OM, and NTOT were significantly influenced by both X and Y coordinates, while RATIOCN and CEC varied only along the Y and X axis, respectively. Notably, the ninth row on the Y coordinate demonstrates the most pronounced differences, with higher values of OC, OM, NTOT, and RATIOCN. On the other hand, these variables exhibit less variability along the X axis, except for CEC, which shows a clear increasing gradient from the first to the third row (Fig. S6). To overcome spatial variability of soil properties, a Latin square design was used to set up the experimental fields site.

Table 4. Result of the ANOVA analysis of the spatial variability of soil physicochemical properties observed in the field (pH, soil pH; OC, organic carbon; OM, organic matter).

PLOT COORDINATE	pH			OC			OM		
	Df	F	<i>p</i>	Df	F	<i>p</i>	Df	F	<i>p</i>
X	2	0.936	0.413	2	4.095	<0.05	2	4.374	<0.05
Y	8	1.021	0.459	8	6.435	<0.05	8	6.484	<0.05

PLOT COORDINATE	NTOT			RATIOCN			CEC		
	Df	F	<i>p</i>	Df	F	<i>p</i>	Df	F	<i>p</i>
X	2	5.316	<0.05	2	0.830	0.454	2	11.239	<0.05
Y	8	2.960	<0.05	8	4.673	<0.05	8	1.396	0.271

X: coordinate X, Y: coordinate Y, Df: degrees of freedom, F: F value, *p*: *p*-value

Effect of the treatments on bacterial and fungal community structures

Results from the five GLMM models used to evaluate the effects of single or mixture of pesticide on bacterial α -diversity suggest that none of the pesticide treatment had an impact on either PD, OB or SI (Tab. S2, Figs. S7 & S8 & S9). Regarding the fungal α -diversity (Tab. S2), the SI was the most impacted endpoint by the pesticide applications (Fig. 10). Even though fluctuating overtime, pairwise comparisons showed overall significantly higher values in the Mixture-1x treatment compared to the Control plots (Mixture-1x: 27.7 ± 2.2 , Control: 20.7 ± 1.8). Pairwise comparisons of the single application model showed significantly lower SI in Control samples compared to the Herbicide-10x (Control: 20.1 ± 1.7 , Herbicide-10x: 30.1 ± 2.7). When comparing the herbicide single application versus the mixture application, fungal SI showed consistently lower values in Control compared to Herbicide-1x and Mixture-10x (Control: 19.6 ± 2.2 , Herbicide-1x: 29.8 ± 2.3 , Mixture-10x: 30.8 ± 2.4). When comparing the insecticide single application, as well as the fungicide one, versus the mixture application, no statistically significant differences could be detected for SI. For OB, the treatment-by-dose effect was detected as significant however, none of the pairwise comparisons yielded statistically significant differences (Tab. S2, Fig. S10).

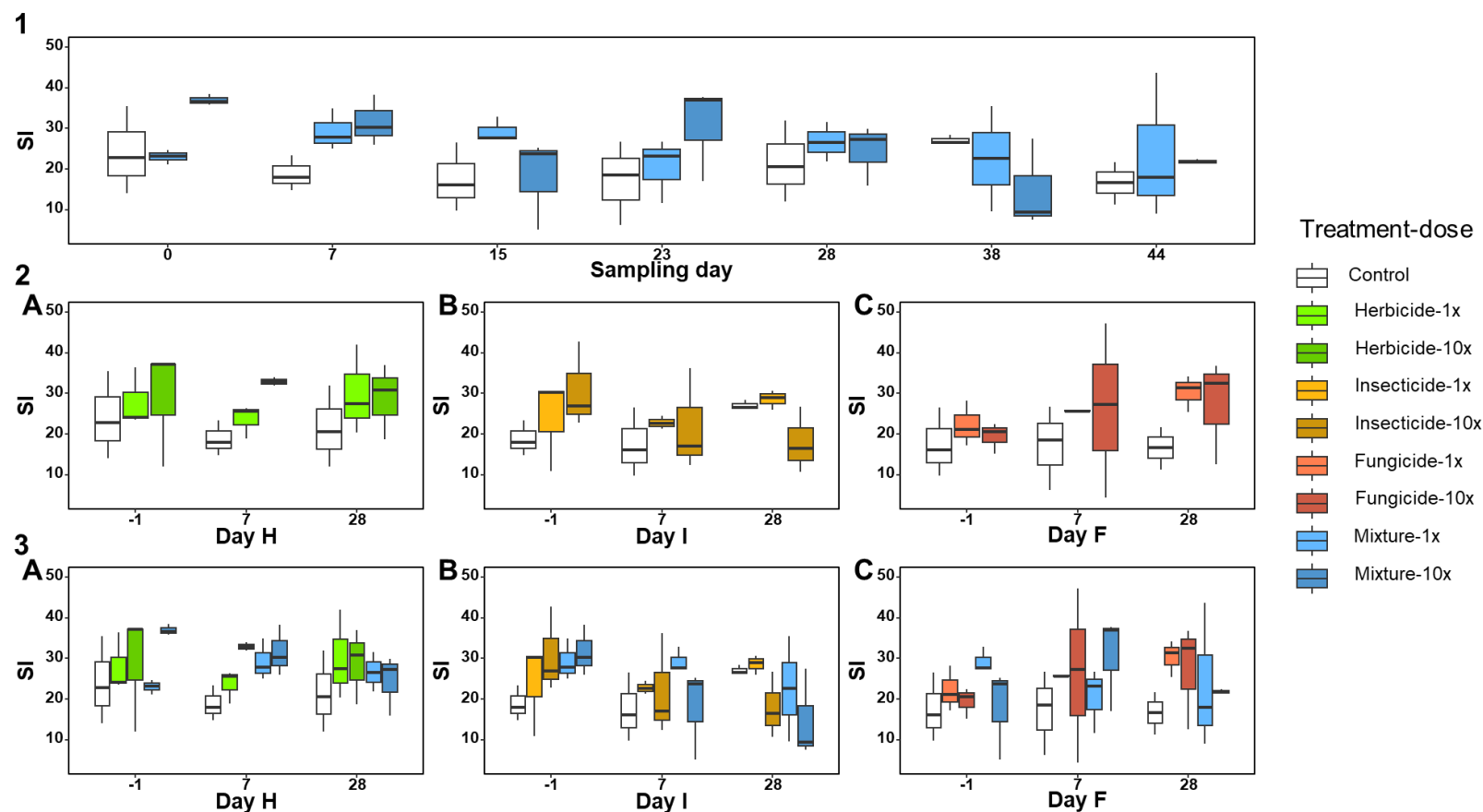


Figure 10. Effect of the sequential mixture of pesticides (panel 1), single pesticides (panel 2, A-B-C) and comparison between sequential mixture and single pesticide applications (panel 3, A-B-C) on Fungal Simpson index (SI) on the different sampling dates. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

We conducted a partial-RDA analysis to determine which explanatory variables were contributing to the observed differences in bacterial and fungal community compositions. We evaluated the contribution of various physico-chemical properties and of the pesticide treatments, and detected that 21% and 18% of the observed constrained variance in the bacterial and fungal community compositions respectively, was attributable to those variables (Tab. 5). We then evaluated the significance of constraints (*i. e.* soil physico-chemical properties and pesticide treatments) using the *anova.cca* function and showed that, of the constrained variances, respectively 4% and 20% was inferred to the pesticide treatments for bacterial and fungal community composition, respectively (Tab. 6). This is visually exemplified by the partial-RDA plot where the Control samples tend to cluster on the opposite of most of the treated ones when looking at the fungal community (Fig. 11-panel B). On the other hand, the bacterial communities' composition seems to be mainly structured by the soil properties (Fig. 11-panel A).

Table 5. Results of the conditioned, constrained and unconstrained variances explained by the P-RDA model for bacterial and fungal communities' composition analyses assessed by 16S rDNA and ITS metabarcoding, respectively.

Variance	Bacteria		Fungi	
	Inertia	Proportion	Inertia	Proportion
Total	0.05	1.00	0.21	1.00
Conditioned	0.01	0.08	0.01	0.05
Constrained	0.01	0.21	0.04	0.18
Unconstrained	0.04	0.70	0.16	0.77

Table 6. Results of the anova.cca analysis on the constrained variance for the soil physicochemical parameters (OM, organic matter; pH, soil pH ; RATIOCN, ratio C/N; CEC, cationic exchange capacity) bacterial and fungal communities' composition analyses assessed by 16S rDNA and ITS metabarcoding, respectively.

Soil property	Bacteria				Fungi			
	df	Variance	F	Pr(>F)	df	Variance	F	Pr(>F)
Treatment	8	0.004	1.5774	0.001	8	0.020	1.6981	0.001
OM	1	0.001	4.0470	0.001	1	0.005	3.0842	0.001
pH	1	0.003	8.1166	0.001	1	0.005	3.2340	0.001
RATIOCN	1	0.001	2.2207	0.004	1	0.002	1.6558	0.042
CEC	1	0.002	6.6938	0.001	1	0.006	4.3393	0.001
Residual	110	0.038			110	0.160		

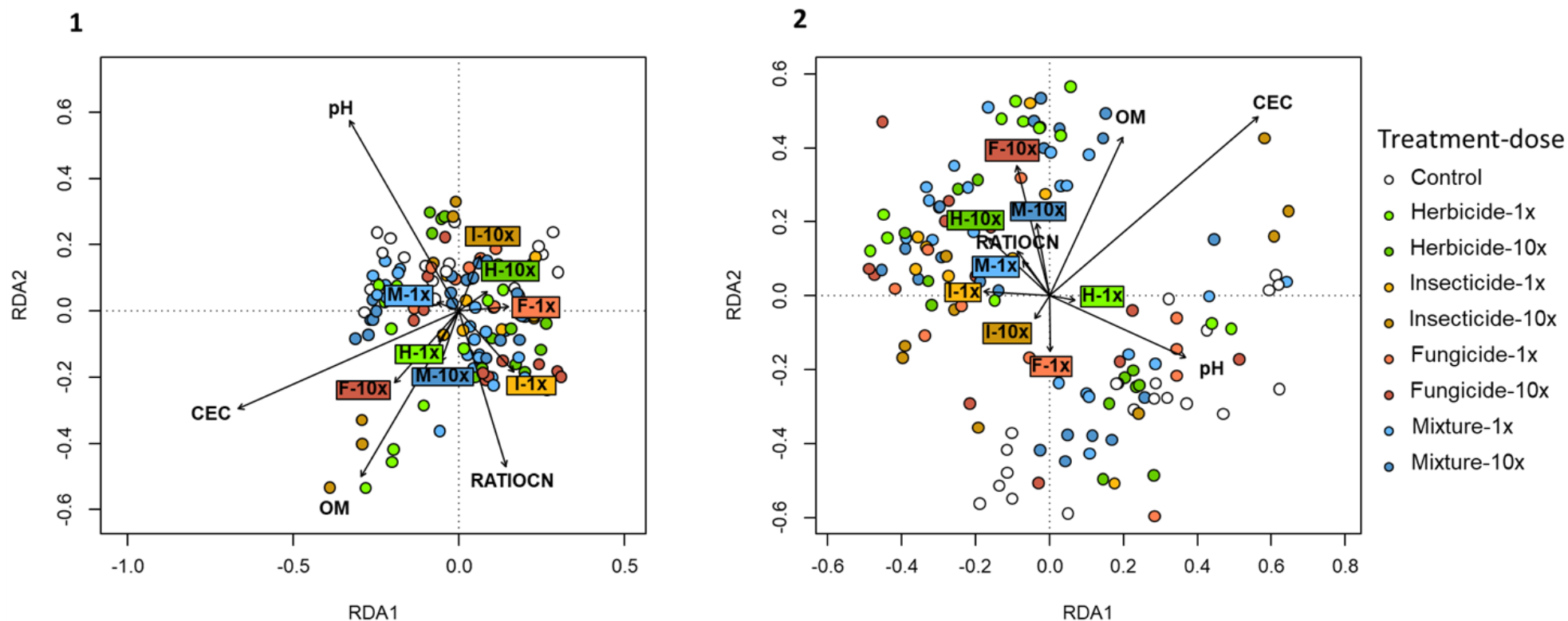


Figure 11. Partial Redundancy Analysis (P-RDA) of the impacts of pesticide treatment-dose (control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose) and soil properties on the bacterial (panel 1, 16SrDNA amplicon) and fungal (panel 2, ITS amplicon) communities' composition. The different points colors represent the different treatment-doses. The length and direction of the arrows illustrate the contribution of the factors to the variation observed between the communities impacted by the treatments.

Effect of the treatments on the abundance of bacteria, fungi and nitrifiers

The 16S and ITS abundances displayed overall a relatively small range of variations (from 1.4×10^8 copies number/g dry soil to 1.2×10^9 copies number/g dry soil for 16S, and from 9.0×10^7 copies number/g dry soil to 7.4×10^8 copies number/g dry soil for ITS, Tab. S4, Figs. S11 & S12). Significant differences were detected when comparing the fungicide *vs* mixture treatments, in which Mixture-1x displayed lower 16S abundance compared to the Control and Fungicide-10x (Mixture-1x: $4.3 \times 10^5 \pm 2.2 \times 10^3$ copies number/g dry soil, Control: $4.4 \times 10^5 \pm 2.2 \times 10^3$ copies number/g dry soil, Fungicide-10x: $4.4 \times 10^5 \pm 2.2 \times 10^3$ copies number/g dry soil). Significantly lower ITS abundance was detected in the insecticide *vs* mixture analysis, where the Mixture-10x had a significantly higher ITS abundance compared to Control, Insecticide-1x and Insecticide-10x (Mixture-10x: $4.3 \times 10^5 \pm 2.2 \times 10^3$ copies number/g dry soil, Control: $4.3 \times 10^5 \pm 2.2 \times 10^3$ copies number/g dry soil, Insecticide-1x: $4.2 \times 10^5 \pm 2.2 \times 10^3$ copies number/g dry soil, Insecticide-10x: $4.2 \times 10^5 \pm 2.2 \times 10^3$ copies number/g dry soil).

The AOA/AOB*100 remained stable in the Control treatment over the duration of the experiment, while it sharply increased at day 15 and 44 for the Mixture-1x treatment, resulting in an overall significant higher AOA abundance compared to Mixture-10x (Mixture-1x: 458.0 ± 31.6 , Mixture-10x: 292.0 ± 32.9) (Tab. S4, Fig. S13-panel 1). The AOA/AOB*100 quantified in the Mixture-1x was also significantly higher compared to Fungicide-10x and Mixture-10x according to the fungicide *vs* mixture analysis (Mixture-1x: 535.0 ± 45.3 , Fungicide-10x: 269.0 ± 48.2 , Mixture-10x: 294.0 ± 42.2).

After 28 and 38 days the AOB/16S*100 measured in Mixture-10x treatment were similar to that observed at day 0, thus indicating its recovery at the end of the experiment (Tab. S4, Fig. 12-panel 1). However, the AOB/16S*100 measured in Mixture-1x treatment was statistically lower than that of Mixture-

10x treatment all over the duration of the experiment (Mixture-1x: 0.6 ± 0.1 , Mixture-10x: 1.1 ± 0.1), and also significantly lower compared to Fungicide-10x in the fungicide *vs* mixture comparison (Mixture-1x: 0.6 ± 0.1 , Fungicide-10x: 1.2 ± 0.1).

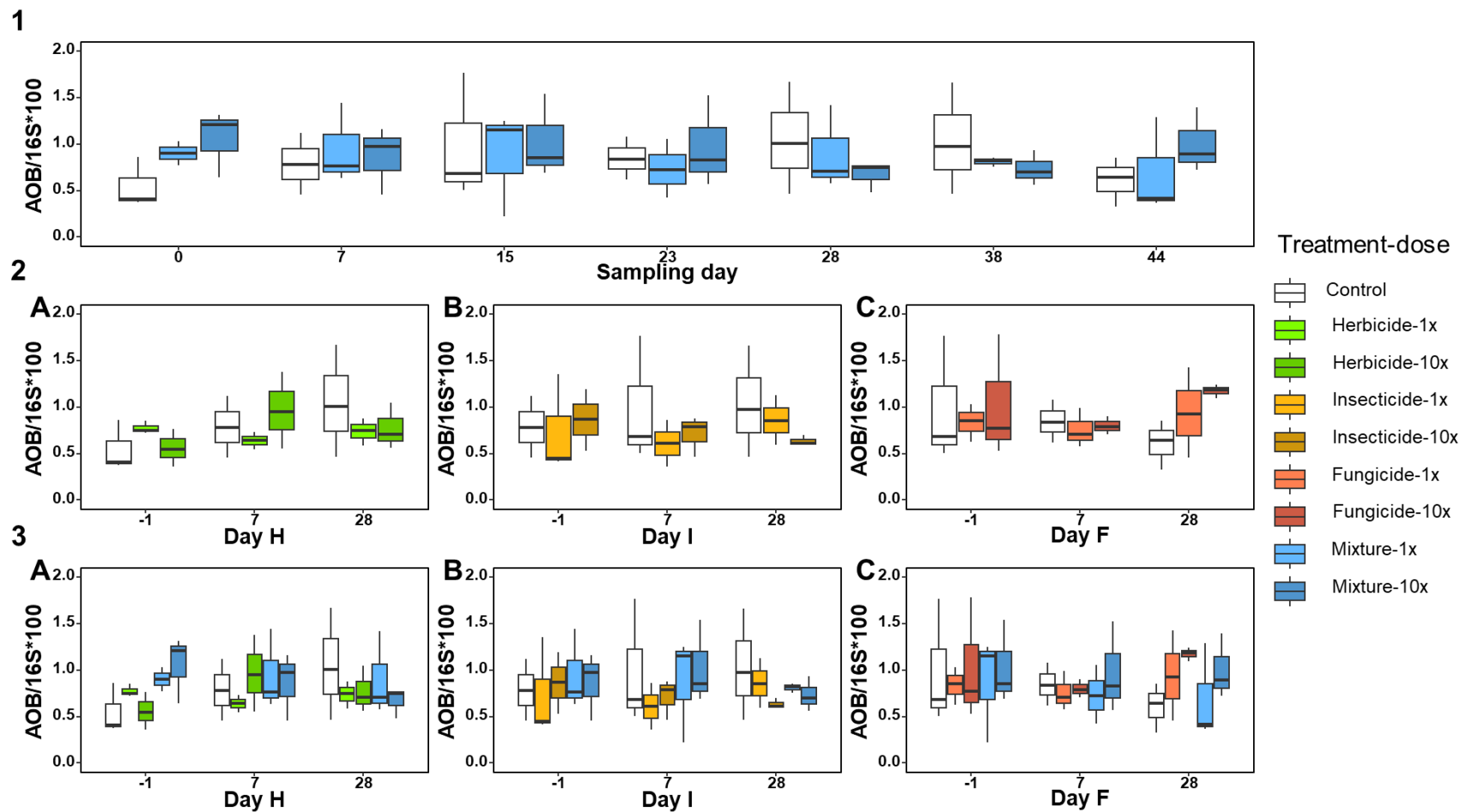


Figure 12. Effect of the sequential mixture of pesticides (panel 1), single pesticides (panel 2, A-B-C) and comparison between sequential mixture and single pesticide applications (panel 3, A-B-C) on the relative abundance of AOB (AOB/16S*100) on the different sampling dates. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

The COMAA/16S*100 (Tab. S4, Fig. S14-panel 1) and COMAB/16S*100 (Tab. S4, Fig. S15-panel 1) exhibited similar patterns characterized by a sharp increase in Mixture-1x at day 15. This increase led to a significantly higher COMAB abundance in Mixture-1x compared to the Control (Control: 1.0 ± 0.1 , Mixture-1x: 1.3 ± 0.1). Both COMAA/16S*100 and COMAB/16S*100 were significantly increased by the Herbicide-1x treatment as compared to the Control (COMAA: Control: 1.6 ± 0.1 , Herbicide-1x: 2.2 ± 0.2 , COMAB: Control: 1.0 ± 0.1 , Herbicide-1x: 1.4 ± 0.1). Interestingly, in the herbicide *vs* mixture comparison, a different Herbicide-1x effect was observed on the two comammox communities. For COMAA it led to a significantly lower COMAA compared to the Control, Herbicide-10x, and Mixture 1x (Herbicide-1x: 2.4 ± 0.1 , Control: 1.4 ± 0.1 , Herbicide-10x: 1.7 ± 0.1 , Mixture-1x: 1.6 ± 0.1). In contrast, for COMAB Herbicide-1x caused a significant enhancement compared to the Control (Control: 0.9 ± 0.1 , Herbicide-1x: 1.5 ± 0.1).

Overall, results from the quantification of the abundance bacteria, fungi and nitrifiers indicated limited temporal variations over the duration of the experiment, with idiosyncratic impacts of pesticide applications of relatively low effect sizes that might probably not be biologically relevant.

Effect of the treatments on nematode abundance

The nematode abundance remained fairly stable in the control until day 23, when it increased and stabilized over the duration of the experiment (Tab. S5, Fig. 13-panel 1). The abundance of nematodes in the mixture treatments was fairly constant over the duration of the experiments. Results of the GLMM model of the mixture analysis show significantly higher nematode abundance in the Control and Mixture-10x compared to Mixture-1x (Control: 1132.0 ± 48.3 , Mixture-10x: 1179.0 ± 57.2 , Mixture-1x: 910.0 ± 53.7). No significant differences could be detected between the Control and the single treatment applications. Significant differences could be detected when comparing the single treatments and the

mixture. In the insecticide *vs* mixture comparison, the Mixture-10x had a significant higher nematode abundance compared to Insecticide-1x, Insecticide-10x and Mixture-1x and (Mixture-10x: 1309.0 ± 85.3 , Insecticide-1x: 974.0 ± 81.8 , Insecticide-10x: 855.0 ± 82.7 , Mixture-1x: 869.0 ± 78.5). In the fungicide *vs* mixture, the Fungicide-10x at day 7 has a sharp increase in nematode abundance, which decrease at day 28, and resulted in significantly higher nematode abundance compared to Fungicide-1x and Mixture-1x (Fungicide-1x: 943.0 ± 85.7 , Mixture-1x: 872.0 ± 95.3 , Fungicide-10x: 1403.0 ± 101.5).

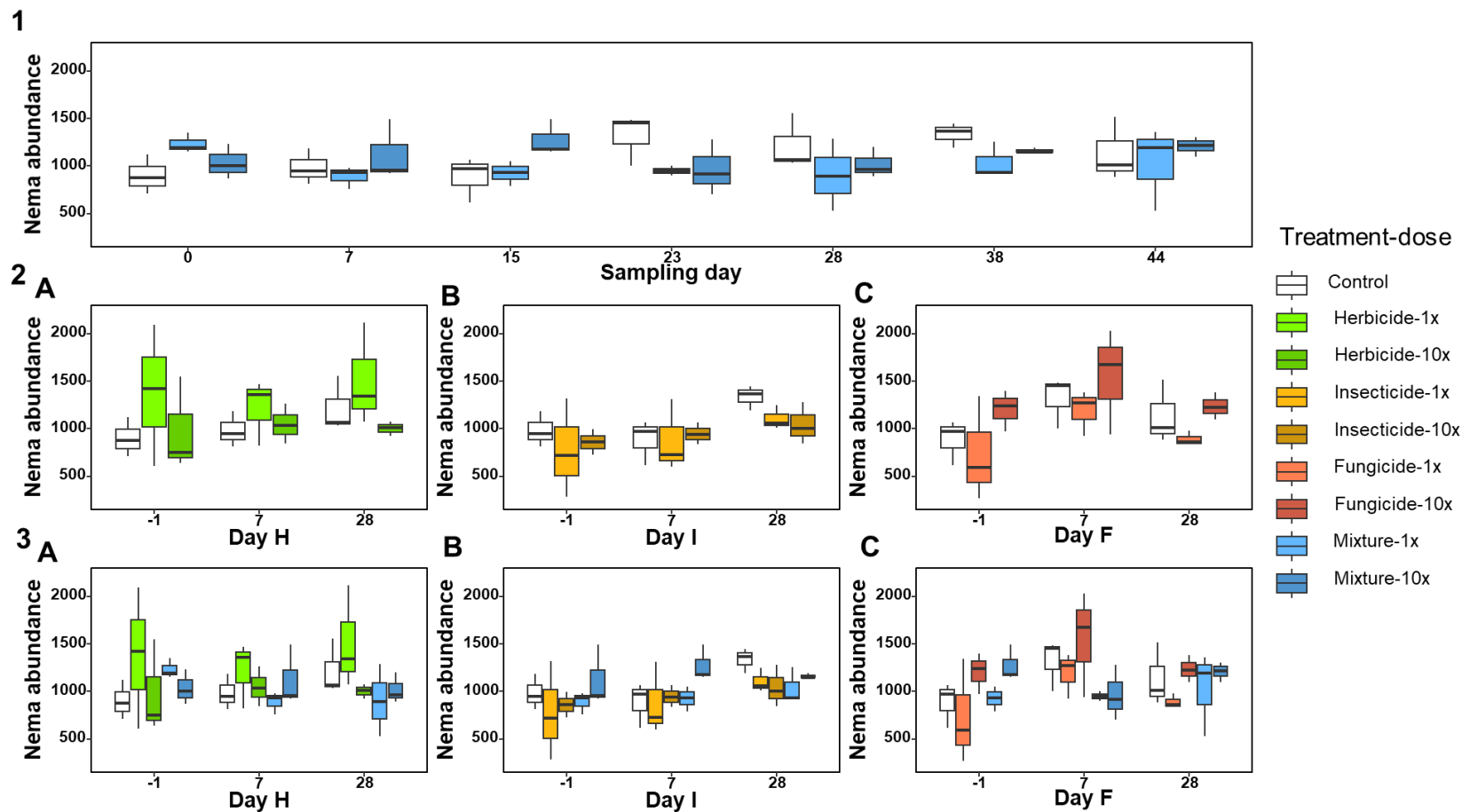


Figure 13. Effect of the sequential mixture of pesticides (panel 1), single pesticides (panel 2, A-B-C) and comparison between sequential mixture and single pesticide applications (panel 3, A-B-C) on the nematode abundance (Nema abundance) on the different sampling dates. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

DISCUSSION

The aim of this study was to investigate in a field experiment the effect of the sequential application on bare agricultural field of three commercial formulations of plant protection products on the structure and diversity of the bacterial and fungal soil communities, as well as on the total abundances of bacteria, fungi and nematodes, with also a specific focus on microbial guilds involved in nitrification. We employed a combination of molecular tools for bacterial and fungal community analyses, and classical counting method under the microscope for measuring the nematode abundances. We also assessed the spatial heterogeneity at the field scale of several soil physicochemical properties.

Overall, we observed a relatively large spatial heterogeneity of certain soil physico-chemical properties between the field subplots that may have caused the relatively high variability between biological triplicates for many measured endpoints. We therefore included those subplot properties into the statistical analyses as exemplified in Romdhane et al. (2019b), to properly evaluate the effects of the pesticide treatments on the endpoints measured. Belowground microbial communities are indeed deeply influenced and shaped by soil physico-chemical characteristics, which can be variable in a field site even at relatively small scale (Goovaerts, 1998). For example, Klironomos et al. (1999) demonstrated that biotic and abiotic factors can vary within 1 meter, and are not randomly distributed in the field. In a given agricultural field, one can find different degrees of soil compaction due to tillage operations (Alaoui and Diserens, 2018), but also different weed species and organic material residues unevenly distributed (Berg and Smalla, 2009), and field slope creating nutrient gradients that offer then multiple microhabitats hosting different microbial communities (Kuramae et al., 2011), resulting in spatial variability of soil microbial communities (Cavigelli et al., 2005). Among the most variable parameters in the studied site was the organic matter content, whose

decomposition is directly mediated by microbes (Liang et al., 2019). The distribution of organic matter across the field can then influence the abundance of specific microbial groups in soil (Inagaki et al., 2023). In an agricultural field where the organic residues might be unevenly dispersed on the soil, *i. e.* after crop harvest, this can result in “hotspots” with high enzymatic potential and peaks of microbial abundance (Heitkötter and Marschner, 2018; Inagaki et al., 2023). The field where this experiment took part was arrowed before the experiment started, but some areas were characterized by higher organic matter residues compared to others which might have impacted the abundance and the structure of the microbial communities (Yao et al., 2024). Implementing the Latin square design in this experiment enabled us to evenly distribute the field spatial variability among the different treatments, decreasing the bias due to correlation of the measured biological parameters with the soil properties.

Accordingly, results of the partial-RDA analysis regarding the variability of the bacterial community structure showed that most of the constrained variance was explained by variation in the soil physicochemical properties, rather than by the pesticide applications. Many studies attributed to soil pH as being the main driver of bacterial community composition (Lauber et al., 2009, 2008; Philippot et al., 2023; Shen et al., 2013), as it influences the conditions that enable the microbes to survive (Jin and Kirk, 2018). In our study, soil pH was fairly homogenous within the field.

On the other hand, the fungal community diversity and composition appeared to be the most sensitive endpoint to pesticide application in this study, as shown by the abundance and partial-RDA analysis. Significant differences consistently indicate that the sequential mixture and herbicide treatments led to the increase in the fungal abundance and to the modification of the fungal diversity compared to the control. Fungal responses to pesticides are less studied compared to bacteria, and a large part of the research focuses on arbuscular

mycorrhiza fungi obligate symbiont of most of higher plants, which exhibit non univocal responses to pesticide application (Hage-Ahmed et al., 2019). Generally, fungi have been also described to be resistant and resilient to pesticide application (Kalia and Gosal, 2011), even though adverse pesticide effects were reported in previous studies on the fungal community diversity and composition (Streletskii et al., 2023, 2022). The variable responses to pesticide application reported in the literature could be influenced by the community's historical exposure to pesticides, potentially indicating existing adaptation to certain active ingredients entering in the composition of pesticides. Unfortunately, no information was available to us on the field pesticide treatment history, but it is plausible that this field was previously exposed to the tested active ingredients as in this region corn is often cropped.

The N cycle guilds, particularly the ammonia oxidizers, have been described to be sensitive to pesticide application (Karas et al., 2018; Karpouzas et al., 2022; Jowenna X. F. Sim et al., 2022). In our study, the abundance of comammox increased in response to pesticide application, especially in the case of COMAB which exhibit particular sensitivity to the herbicide already at low dose. It is plausible that the herbicidal formulation containing clopyralid had a stimulatory effect on this microbial guild. This finding is in contrast with other studies that reported adverse effect of herbicides on the N cycle (Damin and Trivelin, 2011).

Soil nematodes live in the water layer of the soil pores having diameter of 25-100 μm (Neher, 2010). Like for soil microbes, their distribution in the field is deeply influenced by soil properties, among which organic carbon and pH seem to be the most impacting, together with the soil porosity (Liu et al., 2019). The substantial variation in nematode abundance in our study may stem from the pronounced variability observed in soil characteristics. Moreover, the spatial distribution of soil nematodes could be linked to the distribution of their food

sources, such as microbes (Liu et al., 2019). Given the high variability in bacterial abundance, it is plausible that bacterivore nematodes, the most abundant feeding group in the nematode community (van den Hoogen et al., 2020), were distributed in accordance with their food sources. From our observation one can conclude that the observed variance of the nematode abundance was mainly explained by the soil physicochemical characteristics and not influenced by the pesticide application. Even though field studies on the effects of pesticides on the nematode community are rare (Waldo et al., 2019), most of the pesticide effects are visible on the community composition rather than on the abundance. Therefore, a deeper exploration of nematode community composition, which would enable the characterization of functional aspects, would be advantageous for a comprehensive assessment of pesticide toxicity on nematodes.

Another possible explanation for the variable effects of pesticides among treatment replications could be linked to pesticide's properties, such as the adsorption potential and persistence. Indeed, clopyralid has the lowest absorption/desorption and low-moderate persistence in soil (K_{foc}: 0.26–4.1 mL/g, DT₅₀: 0.16 –23.7 days). This implies a high mobility in soil, and so bioavailability. On the other hand, zeta-cypermethrin and pyraclostrobin are moderately persistent in soil, where they tend to highly adsorb (K_{foc}: 72405-285562 mL/g and 6000-16000 mL/g, respectively; DT₅₀: 6-24 days and 33 days, respectively). The higher organic matter concentration can be correlated with a higher cation exchange capacity (CEC), as the organic matter contributes to up to 60% of the CEC (Hayashi et al., 2023). CEC is the total exchangeable cations that soil can hold at a specific pH (Seybold et al., 2005), and it influences pesticide availability in soil, *i. e.* herbicides are generally more effective in soil with low organic material, and so with low CEC as molecules are less likely to be absorbed and therefore remain available for their intended purpose (Kerr et al., 2004). Additionally, the absorption of a fungicide and an insecticide have been reported

to be positively correlated to the organic matter content and CEC (Han et al., 2019). Generally, the literature reports many cases of absorption of pesticide molecules on the organic matter (Boivin et al., 2005; Rodriguez-Cruz et al., 2007; Yu and Zhou, 2005). The uneven distribution of organic matter in our experimental field, together with the different pesticide properties, might have influenced their bioavailability and subsequently exerting different effects on the microbial and nematode communities. Nevertheless, the active ingredients in the tested formulations are considered to be low toxic towards non-target organisms, another reason for why we could not observe a strong pesticide effect (EFSA, 2008; EFSA et al., 2018c).

To conclude, in-field studies to estimate the effects of pesticide mixtures on soil microbes and free-living nematodes are still rare. Here, we report that the fungal community was the most sensitive to pesticide application, which appears to be positively impacted by the pesticides. The structure and diversity of the total bacterial community were insensitive and mainly driven by the soil physicochemical properties. The abundance of the nematode community was mildly affected by the pesticide applications and effects on the dynamics of the nematode community seemed overall transient. While higher tier ecotoxicological studies offer greater ecological relevance compared to the standard laboratory tests, challenges arise from the interpretation of pesticide effects due to the inherent and strong dependence of bacterial, fungal and nematode communities on soil characteristics and their typically high spatial heterogeneity at the field scale. Soil spatial heterogeneity should be considered to derive proper conclusions when assessing the ecotoxicological effects of pesticides on soil microorganisms.

SUPPLEMENTARY MATERIAL CHAPTER 3

Table S2. Results of the GLMM models and pairwise comparisons on the bacteria alpha diversity. Significant difference is indicated in bold.

1	PD_16S				OB_16S				SI_16S			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Mixture-1x	0.12	3.48	0.03	0.97	4.72	27.14	0.17	0.86	-16.68	14.55	-1.15	0.25
Mixture-10x	0.02	3.86	0.01	1.00	-24.99	30.11	-0.83	0.41	-6.18	16.14	-0.38	0.70
OM	0.98	0.96	1.03	0.31	10.42	7.48	1.39	0.16	1.29	4.01	0.32	0.75
pH	6.85	20.84	0.33	0.74	53.39	162.52	0.33	0.74	84.76	87.14	0.97	0.33
RATIOCN	0.57	6.20	0.09	0.93	33.41	48.35	0.69	0.49	4.15	25.93	0.16	0.87
CEC	0.05	0.67	0.08	0.94	2.85	5.23	0.54	0.59	5.13	2.80	1.83	0.07

2	PD_16S				OS_16S				SI_16S			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Herbicide-1x	2.47	4.61	0.54	0.59	-1.23	34.26	-0.04	0.97	13.94	15.82	0.88	0.38
Herbicide-10x	2.82	4.43	0.64	0.52	15.15	32.96	0.46	0.65	3.95	15.21	0.26	0.80
Insecticide-1x	-1.44	4.65	-0.31	0.76	-15.15	34.49	-0.44	0.66	28.07	15.99	-1.76	0.08
Insecticide-10x	5.49	4.43	1.24	0.22	51.51	32.88	1.57	0.12	1.66	15.24	0.11	0.91
Fungicide-1x	-1.49	4.66	-0.32	0.75	-5.74	34.72	-0.17	0.87	-15.34	16.01	-0.96	0.34
Fungicide-10x	-3.23	5.10	-0.63	0.53	-15.22	37.97	-0.40	0.69	-24.79	17.54	-1.41	0.16
OM	-1.10	0.71	-1.56	0.12	-3.56	5.22	-0.68	0.50	-1.95	2.43	-0.80	0.42
pH	-10.14	12.74	-0.80	0.43	-91.25	94.25	-0.97	0.33	-82.34	43.82	-1.88	0.06
RATIOCN	0.74	5.29	0.14	0.89	-8.04	39.17	-0.21	0.84	7.97	18.21	0.44	0.66
CEC	-0.81	0.52	-1.55	0.12	-4.62	3.88	-1.19	0.23	1.42	1.80	0.79	0.43

3	PD_16S				OB_16S				SI_16S			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Herbicide-1x	1.62	5.04	0.32	0.75	6.31	42.31	0.15	0.88	28.98	22.06	1.31	0.19
Herbicide-10x	2.05	4.55	0.45	0.65	16.28	38.15	0.43	0.67	4.26	19.90	0.21	0.83
Mixture-1x	-4.87	4.87	-1.00	0.32	-12.83	40.87	-0.31	0.75	-10.73	21.31	-0.50	0.61
Mixture-10x	6.29	5.06	1.24	0.21	35.46	42.43	0.84	0.40	20.89	22.13	0.94	0.35
OM	-0.90	0.86	-1.05	0.29	-1.05	7.19	-0.15	0.88	-0.87	3.75	-0.23	0.82
pH	40.0	22.37	1.79	0.07	204.45	187.68	1.09	0.28	-18.94	97.87	-0.19	0.85
RATIOCN	-2.78	7.09	-0.39	0.69	-46.42	59.47	-0.78	0.44	-59.83	31.01	-1.93	0.05
CEC	-0.44	0.75	-0.59	0.55	-4.66	6.32	-0.74	0.46	3.95	3.29	1.20	0.23

4	PD_16S				OB_16S				SI_16S			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Insecticide-1x	-2.24	5.52	-0.41	0.68	-39.59	40.98	-0.97	0.33	-35.64	20.94	-1.70	0.09
Insecticide-10x	6.23	5.39	1.16	0.25	51.96	40.02	1.30	0.19	15.82	20.45	0.77	0.44
Mixture-1x	7.15	5.20	1.38	0.17	47.61	38.63	1.23	0.22	5.07	19.74	0.26	0.80
Mixture-10x	-1.35	5.70	-0.24	0.81	-64.36	42.37	-1.52	0.13	-32.03	21.65	-1.48	0.14
OM	-0.07	1.27	-0.06	0.95	0.50	9.45	0.05	0.96	5.47	4.83	1.13	0.26
pH	-12.74	21.70	-0.59	0.56	-139.67	161.20	-0.87	0.39	-215.70	82.38	-2.62	0.01
RATIOCN	5.46	6.81	0.80	0.42	76.95	50.61	1.52	0.13	-14.09	25.87	-0.55	0.59
CEC	-1.06	0.75	-1.41	0.16	-6.43	5.56	-1.16	0.25	2.35	2.84	0.83	0.41

5	PD_16S				OB_16S				SI_16S			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Fungicide-1x	2.76	4.75	0.58	0.56	23.11	30.85	0.75	0.45	-1.73	13.57	-0.13	0.90
Fungicide-10x	-2.51	5.55	-0.45	0.65	-10.58	36.09	-0.29	0.77	-1.96	15.87	-0.12	0.90
Mixture-1x	3.86	4.62	0.84	0.40	26.03	30.04	0.87	0.39	-16.78	13.21	-1.27	0.20
Mixture-10x	3.17	4.94	0.64	0.52	18.42	32.09	0.57	0.57	-1.77	14.11	-0.13	0.90
OM	0.71	1.12	0.63	0.53	9.22	7.31	1.26	0.21	-0.32	3.21	-0.10	0.92
pH	-8.34	13.13	-0.64	0.53	-49.65	85.30	-0.58	0.56	53.70	37.51	1.43	0.15
RATIOCN	-1.98	4.74	-0.42	0.68	-5.17	30.82	-0.17	0.87	2.86	13.55	0.21	0.83
CEC	-0.49	0.65	-0.76	0.45	-3.56	4.23	-0.84	0.40	3.79	1.86	2.04	0.04

Table S3. Results of the GLMM models and pairwise comparisons on the fungi alpha diversity. Significant difference is indicated in bold.

1	OS_ITS				SI_ITS			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Mixture-1x	47.28	24.40	1.94	0.05	6.97	2.71	2.58	0.01
Mixture-10x	-7.20	26.95	-0.27	0.79	1.94	2.99	0.65	0.52
OM	21.72	6.77	3.21	<0.01	1.85	0.75	2.47	0.01
pH	-137.16	145.25	-0.94	0.34	-6.86	16.10	-0.43	0.67
RATIOCN	-37.12	44.22	-0.84	0.40	0.68	4.90	0.14	0.89
CEC	2.58	4.76	0.54	0.59	-0.90	0.53	-1.70	0.09

Pairwise comparison-1	SI_ITS			
	Emmean	SE	df	group
Control	20.70	1.80	52	B
Mixture-1x	27.70	2.05	52	A
Mixture-10x	22.70	2.18	52	AB

2	OB_ITS				SI_ITS			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Herbicide-1x	14.11	29.34	0.48	0.63	8.24	3.18	2.59	0.01
Herbicide-10x	7.86	29.27	0.27	0.79	9.97	3.17	3.15	<0.01
Insecticide-1x	-13.69	30.13	-0.45	0.65	4.74	3.26	1.45	0.15
Insecticide-10x	28.71	28.56	1.01	0.31	4.08	3.09	1.32	0.19
Fungicide-1x	62.62	30.72	2.04	0.04	6.32	3.33	1.90	0.06
Fungicide-10x	20.51	33.05	0.62	0.53	2.49	3.58	0.70	0.49
OM	10.09	4.80	2.10	0.04	0.49	0.52	0.95	0.34
pH	-20.45	87.16	-0.24	0.81	-10.34	9.44	-1.10	0.27
RATIOCN	2.66	37.08	0.07	0.94	1.13	4.02	0.28	0.78
CEC	-5.24	3.62	-1.45	0.15	-1.32	0.39	-3.36	<0.01

Pairwise comparison-2	OB_ITS				SI_ITS			
	Emmean	SE	df	group	Emmean	SE	df	group
Control	813	15.80	60	A	20.1	1.71	60	B
Herbicide-1x	827	24.90	60	A	28.4	2.70	60	AB
Herbicide-10x	821	25.30	60	A	30.1	2.74	60	A
Insecticide-1x	799	24.50	60	A	24.9	2.66	60	AB
Insecticide-10x	841	25.20	60	A	24.2	2.73	60	AB
Fungicide-1x	875	25.50	60	A	26.5	2.76	60	AB
Fungicide-10x	833	27.30	60	A	22.6	2.96	60	AB

3	OB_ITS				SI_ITS			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Herbicide-1x	53.45	31.91	1.68	0.09	10.24	3.31	3.10	< 0.01
Herbicide-10x	12.32	29.62	0.42	0.68	8.42	3.07	2.74	0.01
Mixture-1x	88.32	30.87	2.86	< 0.01	7.82	3.20	2.44	0.01
Mixture-10x	89.36	31.94	2.80	0.01	11.22	3.31	3.39	< 0.01
OM	8.69	5.45	1.60	0.11	0.68	0.56	1.20	0.23
pH	-112.38	145.80	-0.77	0.44	-22.22	15.11	-1.47	0.14
RATIOCN	-108.07	45.57	-2.37	0.02	-10.48	4.72	-2.22	0.03
CEC	-2.99	4.78	-0.63	0.53	-1.48	0.50	-2.99	< 0.01

Pairwise comparison-3	OB_ITS				SI_ITS			
	Emmean	SE	df	group	Emmean	SE	df	group
Control	791	21.00	33	A	19.6	2.18	33	B
Herbicide-1x	844	22.00	33	A	29.8	2.28	33	A
Herbicide-10x	803	22.60	33	A	28.0	2.34	33	AB
Mixture-1x	879	22.20	33	A	27.4	2.30	33	AB
Mixture-10x	880	23.50	33	A	30.8	2.43	33	A

4	OB_ITS				SI_ITS			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Insecticide-1x	-22.39	39.92	-0.56	0.57	4.63	4.12	1.13	0.26
Insecticide-10x	44.60	39.13	1.14	0.25	3.08	4.04	0.76	0.44
Mixture-1x	44.86	38.83	1.16	0.25	7.98	4.00	1.99	0.05
Mixture-10x	-18.27	41.29	-0.44	0.66	-1.10	4.26	-0.26	0.80
OM	20.01	9.50	2.11	0.04	0.60	0.98	0.61	0.54
pH	-183.87	158.27	-1.16	0.25	-3.59	16.32	-0.22	0.83
RATIOCN	-56.76	49.99	-1.14	0.26	0.63	5.15	0.12	0.90
CEC	0.03	5.56	0.01	1.00	-1.02	0.57	-1.78	0.07

Pairwise comparison-4	SI_ITS			
	Emmeans	SE	df	group
Control	20.9	2.70	33	A
Insecticide-1x	25.5	3.01	33	A
Insecticide-10x	23.9	3.07	33	A
Mixture-1x	28.8	3.00	33	A
Mixture-10x	19.8	3.20	33	A

5	OB_ITS				SI_ITS			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Fungicide-1x	72.32	29.74	2.43	0.02	10.28	4.07	2.53	0.01
Fungicide-10x	0.96	33.96	0.03	0.98	2.52	4.64	0.54	0.59
Mixture-1x	35.38	28.27	1.25	0.21	10.75	3.87	2.78	0.01
Mixture-10x	-2.14	30.39	-0.07	0.94	2.86	4.16	0.69	0.49
OM	23.13	6.86	3.37	<0.01	2.29	0.94	2.44	0.01
pH	-33.54	80.29	-0.42	0.68	-11.47	10.98	-1.05	0.30
RATIOCN	-0.71	30.09	-0.02	0.98	0.85	4.11	0.21	0.84
CEC	-4.52	4.02	-1.12	0.26	-1.41	0.55	-2.56	0.01

Pairwise comparison-5	OB_ITS				SI_ITS			
	Emmean	SE	df	group	Emmean	SE	df	group
Control	816	20.50	32	A	17.8	2.80	32	A
Fungicide-1x	889	21.30	32	A	28.1	2.91	32	A
Fungicide-10x	817	24.00	32	A	20.3	3.28	32	A
Mixture-1x	852	22.30	32	A	28.6	3.04	32	A
Mixture-10x	814	21.60	32	A	20.7	2.96	32	A

Table S4. Results of the GLMM models and pairwise comparisons on the microbial abundance parameters. Significant difference is indicated in bold. + indicates log transformation of the data.

1a	16S+				ITS+				AOA/AOB*100			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Mixture-1x	-0.15	0.13	-1.21	0.22	0.10	0.13	0.82	0.41	86.67	41.36	2.10	0.04
Mixture-10x	-0.01	0.14	-0.09	0.93	0.34	0.14	2.41	0.02	-79.44	45.68	-1.74	0.08
OM	0.01	0.03	0.03	0.98	-0.01	0.04	-0.22	0.83	17.70	11.47	1.54	0.12
pH	-0.02	0.75	-0.03	0.98	-0.03	0.75	-0.04	0.97	-472.00	245.99	-1.92	0.06
RATIOCN	-0.08	0.22	-0.34	0.73	-0.12	0.22	-0.52	0.60	-18.90	73.17	-0.26	0.80
CEC	0.01	0.02	0.34	0.73	0.02	0.02	0.92	0.36	-29.57	8.07	-3.66	<0.01

1b	AOB/16S*100				COMAA/16S*100				COMAB/16S*100			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Mixture-1x	-0.23	0.10	-2.40	0.02	0.44	0.19	2.32	0.02	0.29	0.11	2.54	0.01
Mixture-10x	0.20	0.11	1.88	0.06	0.31	0.21	1.45	0.15	0.09	0.12	0.69	0.49
OM	-0.06	0.03	-2.23	0.03	0.04	0.05	0.75	0.45	0.05	0.03	1.69	0.09
pH	1.92	0.58	3.33	<0.01	2.05	1.14	-1.79	0.07	-0.98	0.67	-1.46	0.14
RATIOCN	0.30	0.17	1.77	0.08	-0.37	0.34	-1.10	0.27	-0.21	0.20	-1.05	0.30
CEC	0.09	0.02	4.55	<0.01	0.02	0.04	0.63	0.53	-0.01	0.02	-0.20	0.84

Pairwise comparison-1a	ITS				AOA/AOB*100				AOB/16S*100			
	Emmean	SE	df	group	Emmean	SE	df	group	Emmean	SE	df	group
Control	19.1	0.08	53	A	371	27.70	53	AB	0.86	0.07	53	AB
Mixture-1x	19.2	0.10	53	A	458	31.60	53	A	0.63	0.07	53	B
Mixture-10x	19.5	0.10	53	A	292	32.90	53	B	1.06	0.08	53	A

Pairwise comparison-1b	COMAA/16S*100				COMAB/16S*100			
	Emmean	SE	df	group	Emmean	SE	df	group
Control	1.55	0.13	53	A	1.00	0.08	53	A
Mixture-1x	2.00	0.15	53	A	1.29	0.09	53	B
Mixture-10x	1.86	0.15	53	A	1.09	0.09	53	AB

2a	16S+				ITS+				AOA/AOB*100			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Herbicide-1x	-0.28	0.13	-2.07	0.04	0.03	0.15	0.20	0.84	-0.07	43.96	-0.07	0.95
Herbicide-10x	-0.19	0.13	-1.50	0.13	0.04	0.14	0.29	0.78	2.25	42.03	2.25	0.02
Insecticide-1x	-0.10	0.14	-0.77	0.44	0.05	0.15	0.31	0.75	0.63	45.11	0.63	0.53
Insecticide-10x	-0.15	0.13	-1.13	0.26	0.11	0.15	0.73	0.47	0.45	42.78	0.45	0.65
Fungicide-1x	-0.27	0.13	-1.98	0.05	-0.10	0.15	-0.68	0.50	0.85	44.59	0.85	0.40
Fungicide-10x	0.05	0.15	0.34	0.73	0.25	0.17	1.50	0.13	-1.49	49.34	-1.49	0.14
OM	0.05	0.02	2.48	0.01	0.05	0.02	1.96	0.05	1.71	7.17	1.71	0.09
pH	-0.17	0.39	-0.45	0.66	-0.63	0.44	-1.43	0.15	0.02	129.50	0.02	0.99
RATIOCN	-0.13	0.16	-0.82	0.41	-0.05	0.18	-0.25	0.80	0.14	53.81	0.14	0.89
CEC	0.02	0.02	1.42	0.15	0.04	0.02	2.19	0.03	-3.69	5.33	-3.69	<0.01

2b	AOB/16S*100				COMAA/16S*100				COMAB/16S*100			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Herbicide-1x	-0.09	0.13	-0.70	0.49	0.69	0.19	3.62	<0.00	0.42	0.14	3.08	<0.01
Herbicide-10x	-0.11	0.12	-0.93	0.35	0.24	0.18	1.32	0.19	0.27	0.13	2.04	0.04
Insecticide-1x	-0.13	0.13	-1.04	0.30	0.07	0.20	0.37	0.71	0.09	0.14	0.65	0.52
Insecticide-10x	-0.16	0.12	-1.31	0.19	0.37	0.19	1.99	0.05	0.29	0.13	2.21	0.03
Fungicide-1x	-0.01	0.13	-0.06	0.95	0.62	0.20	3.17	<0.01	0.39	0.14	2.82	<0.01
Fungicide-10x	0.25	0.14	1.80	0.07	0.24	0.22	1.10	0.27	0.06	0.15	0.38	0.70
OM	-0.05	0.02	-2.35	0.02	-0.04	0.03	-1.21	0.23	-0.02	0.02	-0.80	0.43
pH	0.23	0.37	0.64	0.53	0.17	0.55	0.31	0.76	0.23	0.39	0.60	0.55
RATIOCN	-0.08	0.15	-0.55	0.59	0.03	0.23	0.13	0.89	0.01	0.16	0.06	0.95
CEC	0.05	0.02	3.19	<0.01	0.01	0.02	0.11	0.91	-0.01	0.02	-0.68	0.50

Pairwise comparison-2	16S				AOA/AOB*100				COMAA/16S*100				COMAB/16S			
	Emmean	SE	df	group	Emmean	SE	df	group	Emmean	SE	df	group	Emmean	SE	df	group
Control	19.9	0.07	62	A	364	23.80	62	A	1.55	0.10	62	B	0.99	0.08	62	B
Herbicide-1x	19.7	0.11	62	A	361	37.40	62	A	2.24	0.17	62	A	1.41	0.12	62	A
Herbicide-10x	19.7	0.11	62	A	459	35.70	62	A	1.79	0.16	62	AB	1.25	0.12	62	AB
Insecticide-1x	19.8	0.11	62	A	393	36.70	62	A	1.62	0.16	62	AB	1.08	0.12	62	AB
Insecticide-10x	19.8	0.12	62	A	384	37.80	62	A	1.92	0.17	62	AB	1.28	0.12	62	AB
Fungicide-1x	19.7	0.11	62	A	402	36.20	62	A	2.17	0.16	62	AB	1.37	0.12	62	AB
Fungicide-10x	20.0	0.12	62	A	291	40.60	62	A	1.78	0.18	62	AB	1.04	0.13	62	AB

3a	16S+				ITS+				AOA/AOB*100			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Herbicide-1x	-0.20	0.15	-1.32	0.19	0.19	0.19	1.00	0.32	-19.83	53.14	-0.37	0.71
Herbicide-10x	-0.14	0.13	-1.05	0.29	0.07	0.17	0.44	0.66	96.26	46.48	2.07	0.04
Mixture-1x	0.11	0.14	0.77	0.44	0.40	0.18	2.19	0.03	-38.66	50.62	-0.76	0.45
Mixture-10x	-0.07	0.15	-0.44	0.66	0.27	0.19	1.44	0.15	-56.62	51.77	-1.09	0.27
OM	0.06	0.03	2.32	0.02	0.06	0.03	1.83	0.07	2.77	8.78	0.32	0.75
pH	-1.41	0.66	-2.13	0.03	-2.29	0.83	-2.76	0.01	289.21	232.48	1.24	0.21
RATIOCN	-0.24	0.21	-1.14	0.26	-0.49	0.26	-1.86	0.06	138.14	72.60	1.90	0.06
CEC	0.02	0.02	0.84	0.40	0.04	0.03	1.37	0.17	-18.32	7.80	-2.35	0.02

3b	AOB/16S*100				COMAA/16S*100				COMAB/16S*100			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Herbicide-1x	-0.04	0.13	-0.31	0.75	0.97	0.21	4.72	<0.00	0.57	0.16	3.54	<0.01
Herbicide-10x	-0.07	0.11	-0.61	0.54	0.29	0.19	1.55	0.12	0.32	0.14	2.22	0.03
Mixture-1x	0.017	0.12	0.14	0.89	0.21	0.20	1.04	0.30	0.24	0.16	1.55	0.12
Mixture-10x	0.23	0.13	1.81	0.07	0.53	0.21	2.55	0.01	0.27	0.16	1.69	0.09
OM	-0.03	0.02	-1.42	0.16	-0.04	0.04	-1.05	0.29	-0.03	0.03	-1.10	0.27
pH	-0.20	0.57	-0.36	0.72	-0.98	0.92	-1.07	0.29	-0.40	0.71	-0.56	0.58
RATIOCN	-0.30	0.18	-1.68	0.09	-0.49	0.29	-1.71	0.09	-0.14	0.22	-0.61	0.54
CEC	0.06	0.02	3.19	<0.01	-0.03	0.03	-0.91	0.36	-0.02	0.02	-0.68	0.50

Pairwise comparison-3	ITS				AOA/AOB*100				COMAA/16S*100				COMAB/16S*100			
	Emmean	SE	df	group	Emmean	SE	df	group	Emmean	SE	df	group	Emmean	SE	df	group
Control	19.1	0.12	33	A	377	33.70	32	A	1.38	0.14	33	A	0.88	0.10	33	B
Herbicide-1x	19.3	0.13	33	A	357	38.10	32	A	2.36	0.14	33	B	1.45	0.11	33	A
Herbicide-10x	19.2	0.13	33	A	474	34.80	32	A	1.67	0.14	33	A	1.20	0.11	33	AB
Mixture-1x	19.5	0.14	33	A	339	37.70	32	A	1.59	0.15	33	A	1.12	0.12	33	AB
Mixture-10x	19.4	0.14	33	A	321	37.60	32	A	1.91	0.15	33	AB	1.15	0.12	33	AB

4a	16S+				ITS+				AOA/AOB*100			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Insecticide-1x	-0.12	0.16	-0.78	0.44	0.12	0.15	0.83	0.40	12.50	59.60	0.21	0.83
Insecticide-10x	-0.17	0.16	-1.10	0.27	0.05	0.15	0.34	0.73	32.76	58.21	0.56	0.57
Mixture-1x	0.09	0.15	0.60	0.55	0.37	0.14	2.64	0.01	44.35	56.19	0.79	0.43
Mixture-10x	0.12	0.16	0.75	0.45	0.64	0.15	4.11	<0.01	-78.59	61.62	-1.28	0.20
OM	0.01	0.04	0.25	0.80	-0.01	0.03	-0.31	0.75	14.18	13.75	1.03	0.30
pH	-0.42	0.62	-0.68	0.50	-0.39	0.59	-0.67	0.51	-69.88	234.45	-0.30	0.77
RATIOCN	-0.21	0.20	-1.02	0.31	-0.29	0.18	-1.55	0.12	111.96	73.62	1.52	0.13
CEC	0.01	0.02	0.33	0.74	0.02	0.02	0.88	0.38	23.93	8.09	-2.96	<0.01

4b	AOB/16S*100				COMAA/16S*100				COMAB/16S*100			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Insecticide-1x	-0.19	0.14	-1.32	0.19	0.03	0.25	0.12	0.90	0.02	0.16	0.14	0.89
Insecticide-10x	-0.29	0.14	-2.13	0.03	0.57	0.25	2.30	0.02	0.30	0.16	1.90	0.06
Mixture-1x	-0.20	0.13	-1.52	0.13	0.57	0.24	2.41	0.02	0.23	0.15	1.48	0.14
Mixture-10x	0.14	0.15	0.96	0.34	0.32	0.26	1.23	0.22	-0.09	0.17	-0.51	0.61
OM	-0.05	0.03	-1.62	0.11	0.01	0.06	0.11	0.91	0.01	0.04	0.28	0.78
pH	0.72	0.56	1.30	0.19	-1.29	0.99	-1.29	0.20	-0.09	0.64	-0.15	0.88
RATIOCN	-0.11	0.17	-0.65	0.52	-0.32	0.31	-1.04	0.30	0.01	0.20	0.00	1.00
CEC	0.06	0.02	2.90	<0.01	0.05	0.03	1.48	0.14	0.01	0.02	0.13	0.90

Pairwise comparison-4	ITS				AOB/16S*100				COMAA/16S*100			
	Emmean	SE	df	group	Emmean	SE	df	group	Emmean	SE	df	group
Control	19.1	0.10	34	B	0.94	0.09	34	A	1.49	0.17	34	A
Insecticide-1x	19.2	0.11	34	B	0.75	0.10	34	A	1.52	0.19	34	A
Insecticide-10x	19.1	0.11	34	B	0.64	0.11	34	A	2.05	0.19	34	A
Mixture-1x	19.5	0.10	34	AB	0.74	0.10	34	A	2.06	0.18	34	A
Mixture-10x	19.7	0.12	34	A	1.08	0.11	34	A	1.81	0.20	34	A

5a	16S+				ITS+				AOA/AOB*100			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Fungicide-1x	0.31	0.15	-2.05	0.04	-0.10	0.17	-0.59	0.56	6.29	58.66	0.11	0.91
Fungicide-10x	0.09	0.18	0.49	0.62	0.35	0.20	1.75	0.08	-112.85	68.62	-1.65	0.10
Mixture-1x	-0.57	0.15	-3.81	<0.01	-0.17	0.17	-1.00	0.32	153.14	57.13	2.68	0.01
Mixture-10x	-0.20	0.16	-1.26	0.21	0.03	0.18	0.20	0.84	-87.33	61.01	-1.43	0.15
OM	0.03	0.04	0.78	0.43	0.02	0.04	0.63	0.53	3.20	13.89	0.23	0.82
pH	0.41	0.42	0.98	0.33	0.15	0.47	0.33	0.74	-279.75	162.22	-1.73	0.08
RATIOCN	0.03	0.15	0.23	0.82	0.17	0.17	0.98	0.33	13.53	58.60	0.23	0.82
CEC	0.03	0.02	1.53	0.13	0.05	0.02	2.03	0.04	-22.27	8.04	-2.77	0.01

5b	AOB/16S*100				COMAA/16S*100				COMAB/16S*100			
	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)	Estimate	SE	Z value	Pr(> z)
Fungicide-1x	0.13	0.16	0.81	0.42	0.43	0.27	1.59	0.11	0.32	0.16	1.98	0.05
Fungicide-10x	0.42	0.19	2.28	0.02	-0.05	0.32	-0.16	0.87	-0.04	0.19	-0.21	0.84
Mixture-1x	-0.16	0.16	-1.04	0.30	0.56	0.26	2.11	0.03	0.31	0.16	1.96	0.05
Mixture-10x	0.35	0.17	2.09	0.04	0.29	0.28	1.02	0.31	0.20	0.17	1.18	0.24
OM	-0.02	0.04	-0.45	0.65	-0.04	0.06	-0.62	0.54	-0.02	0.04	-0.45	0.65
pH	1.14	0.44	2.58	0.01	-0.53	0.75	-0.71	0.48	-0.05	0.45	-0.11	0.92
RATIOCN	-0.04	0.16	-0.28	0.78	0.13	0.27	0.50	0.62	-0.01	0.16	-0.08	0.94
CEC	0.02	0.02	1.02	0.31	0.04	0.04	0.97	0.33	0.01	0.02	0.20	0.85

Pairwise comparison-5a	16S				AOA/AOB*100				AOB/16S*100			
	Emmean	SE	df	group	Emmean	SE	df	group	Emmean	SE	df	group
Control	20.0	0.11	34	A	382	41.60	34	AB	0.74	0.11	34	AB
Fungicide-1x	19.7	0.11	34	AB	388	40.70	34	AB	0.86	0.11	34	AB
Fungicide-10x	20.1	0.13	34	A	269	48.20	34	B	1.16	0.13	34	A
Mixture-1x	19.4	0.12	34	B	535	45.30	34	A	0.57	0.12	34	B
Mixture-10x	19.8	0.11	34	AB	294	42.20	34	B	1.08	0.12	34	AB

Pairwise comparison-5b	COMAA/16S*100				COMAB/16S*100			
	Emmean	SE	df	group	Emmean	SE	df	group
Control	1.70	0.20	34	A	1.03	0.12	34	A
Fungicide-1x	2.13	0.20	34	A	1.35	0.12	34	A
Fungicide-10x	1.65	0.23	34	A	0.99	0.14	34	A
Mixture-1x	2.26	0.22	34	A	1.34	0.13	34	A
Mixture-10x	1.99	0.20	34	A	1.23	0.12	34	A

Table S5. Results of the GLMM models and pairwise comparisons on the nematode abundance. Significant difference is indicated in bold.

1	Nematode abundance			
	Estimate	SE	Z value	Pr(> z)
Mixture-1x	-222.46	71.43	-3.11	<0.01
Mixture-10x	46.64	79.24	0.59	0.56
OM	-26.31	19.68	-1.34	0.18
pH	783.08	427.73	1.83	0.07
RATIOCN	149.74	127.26	1.18	0.24
CEC	41.62	13.76	3.03	<0.01

Pairwise comparison-1	Nematode abundance			
	Emmean	SE	df	group
Control	1132	48.30	54	A
Mixture-1x	910	53.70	54	B
Mixture-10x	1179	57.20	54	A

2	Nematode abundance			
	Estimate	SE	Z value	Pr(> z)
Herbicide-1x	189.21	113.84	1.66	0.10
Herbicide-10x	-56.80	109.44	-0.52	0.60
Insecticide-1x	-125.90	114.37	-1.10	0.27
Insecticide-10x	-138.46	108.93	-1.27	0.20
Fungicide-1x	-150.04	114.12	-1.32	0.19
Fungicide-10x	235.08	125.20	1.88	0.06
OM	14.54	17.47	0.83	0.41
pH	507.05	315.37	1.61	0.11
RATIOCN	-62.82	131.05	-0.48	0.63
CEC	47.16	12.99	3.63	<0.01

3	Nematode abundance			
	Estimate	SE	Z value	Pr(> z)
Herbicide-1x	187.71	128.62	1.46	0.14
Herbicide-10x	-8.81	115.98	-0.08	0.94
Mixture-1x	-121.03	124.23	-0.97	0.33
Mixture-10x	53.55	128.99	0.42	0.68
OM	28.79	21.85	1.32	0.19
pH	459.87	570.54	0.81	0.42
RATIOCN	-50.78	180.78	-0.28	0.78
CEC	51.59	19.21	2.69	0.01

4	Nematode abundance			
	Estimate	SE	Z value	Pr(> z)
Insecticide-1x	-99.67	94.72	-1.05	0.29
Insecticide-10x	-218.19	92.50	-2.36	0.02
Mixture-1x	-204.08	89.29	-2.29	0.02
Mixture-10x	235.20	97.93	2.40	0.02
OM	-46.28	21.85	-2.12	0.03
pH	966.29	372.59	2.59	0.01
RATIOCN	95.99	116.99	0.82	0.41
CEC	10.30	12.86	0.80	0.42

Pairwise comparison-4	Nematode abundance			
	Emmean	SE	df	group
Control	1073	75.70	34	AB
Insecticide-1x	974	81.80	34	B
Insecticide-10x	855	82.70	34	B
Mixture-1x	869	78.50	34	B
Mixture-10x	1309	85.30	34	A

5	Nematode abundance			
	Estimate	SE	Z value	Pr(> z)
Fungicide-1x	-176.62	123.43	-1.43	0.15
Fungicide-10x	283.79	144.38	1.97	0.05
Mixture-1x	-247.05	120.20	-2.06	0.04
Mixture-10x	45.40	128.38	0.35	0.72
OM	-33.56	29.23	-1.15	0.25
pH	610.19	341.31	1.79	0.07
RATIOCN	212.23	123.30	1.72	0.09
CEC	48.59	16.91	2.87	<0.01

Pairwise comparison-5	Nematode abundance			
	Emmean	SE	df	group
Control	1119	87.50	34	AB
Fungicide-1x	943	85.70	34	B
Fungicide-10x	1403	101.50	34	A
Mixture-1x	872	95.30	34	B
Mixture-10x	1165	88.70	34	AB

	X1	X2	X3
Y1	Control	Insecticide 10x	Insecticide 1x
Y2	Herbicide 1x	Herbicide 10x	Fungicide 1x
Y3	Insecticide 1x	Mixture 1x	Herbicide 10x
Y4	Fungicide 1x	Mixture 10x	Insecticide 10x
Y5	Herbicide 10x	Control	Fungicide 10x
Y6	Insecticide 10x	Herbicide 1x	Mixture 1x
Y7	Fungicide 10x	Insecticide 1x	Mixture 10x
Y8	Mixture 1x	Fungicide 1x	Control
Y9	Mixture 10x	Fungicide 10x	Herbicide 1x

Figure S3. Schematic representation of the field plot design, with the corresponding X and Y plot coordinates.

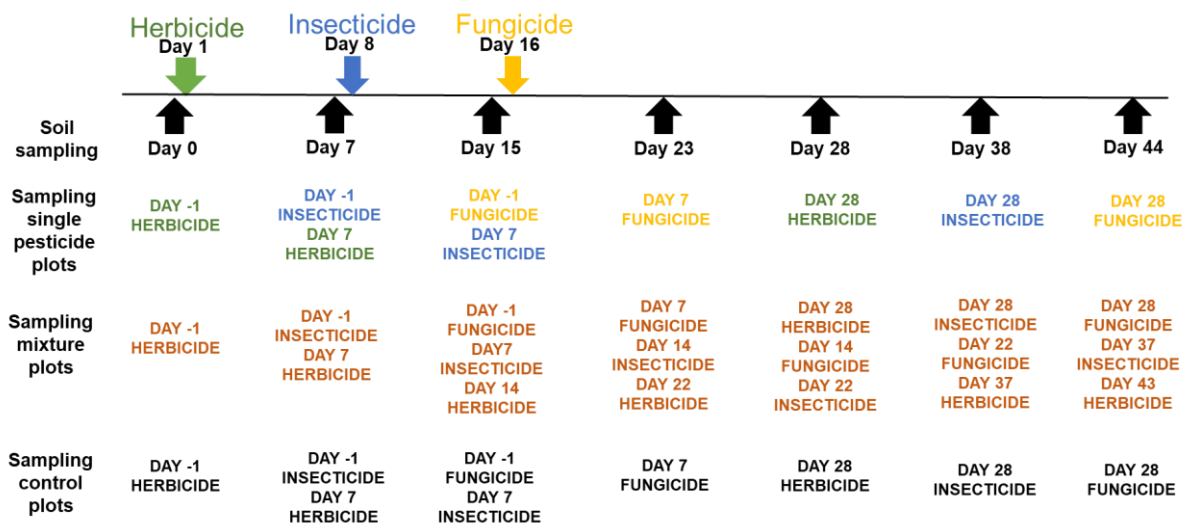


Figure S4. Schematic representation of the treatment and sampling schedule. Single pesticide treatment plots sampled one day before the respective pesticide applications and day 7 and 28 after the respective applications. Control and mixture plots sampled at each timepoint over the 44 days.

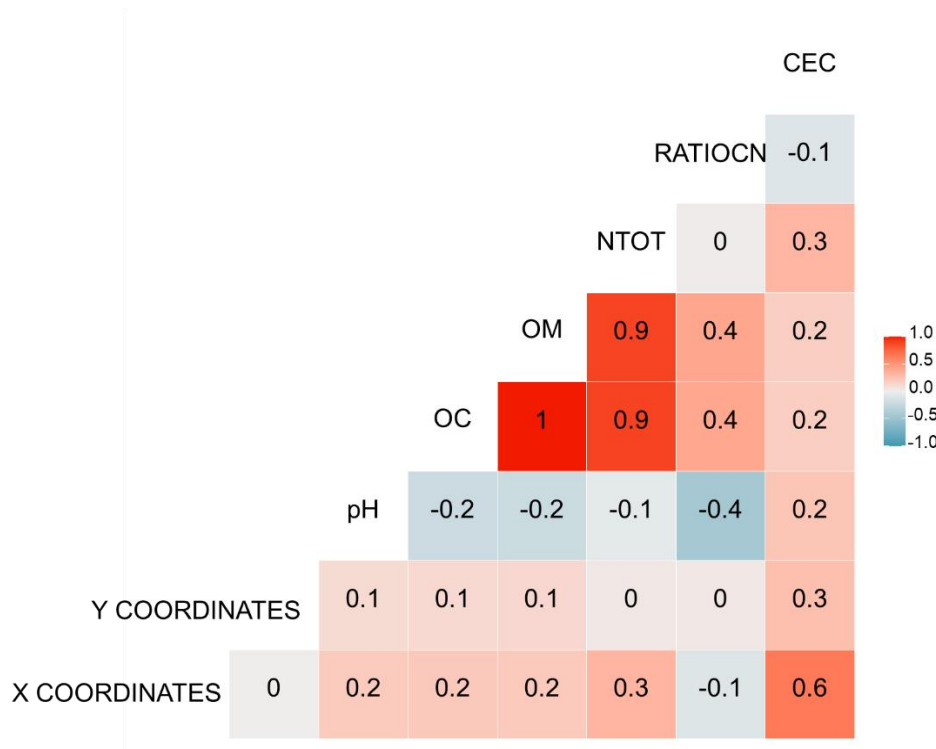


Figure S5. Pearson correlation analysis between the plot coordinates and the soil properties. CEC: cation exchange capacity, RATIOCN: ratio C/N, NTOT: total nitrogen, OM: organic material, OC: organic carbon.

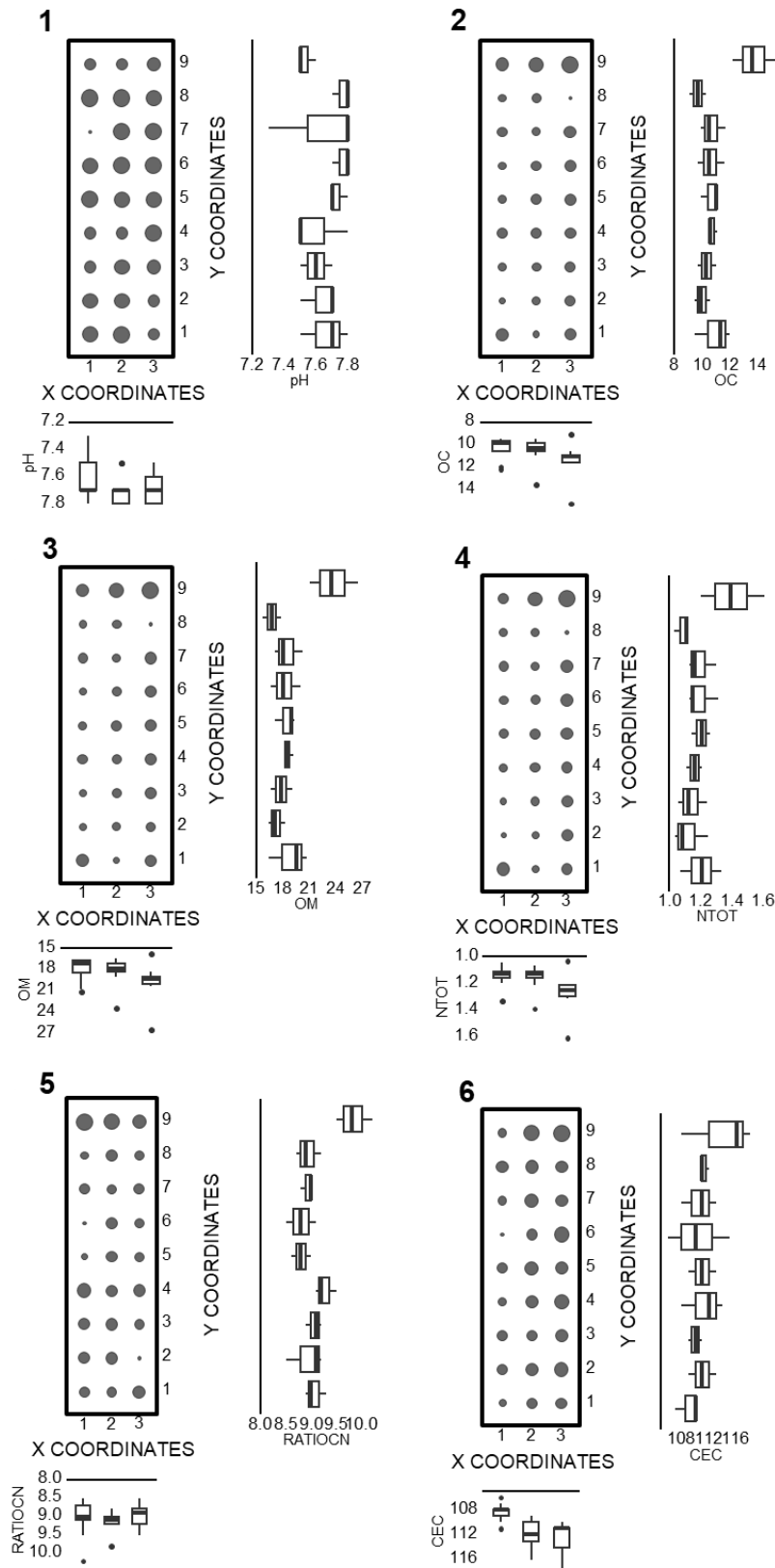


Figure S6. Changes in pH, OC, OM, NTOT, CNRATIO and CEC according to the X and Y axis in the field site.

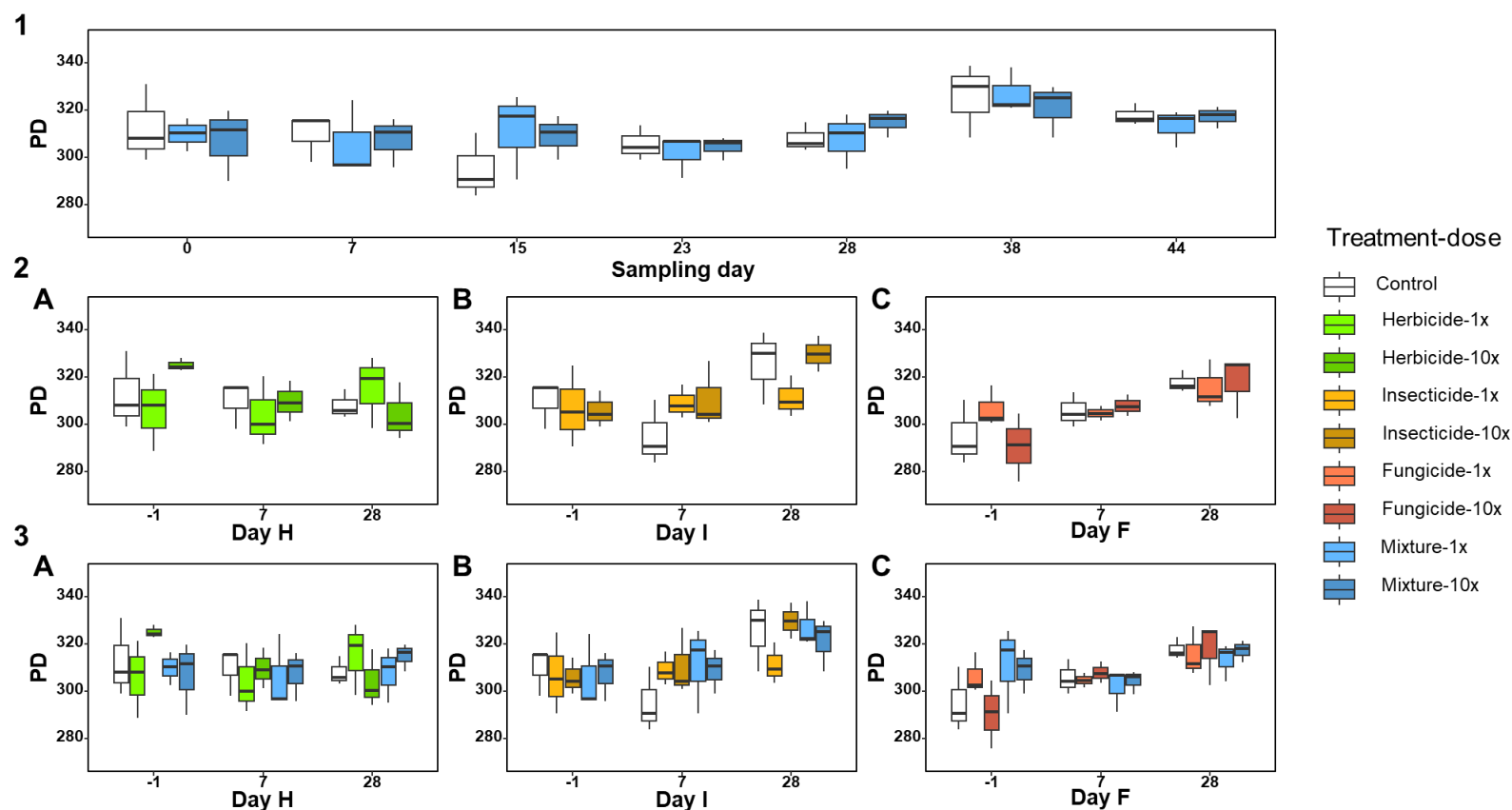


Figure S7. Effect of the mixture (panel 1), single pesticides (panel 2, A-B-C) and mixture and single treatments (panel 3, A-B-C) on the 16S phylogenetic diversity (PD) on the different sampling days. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

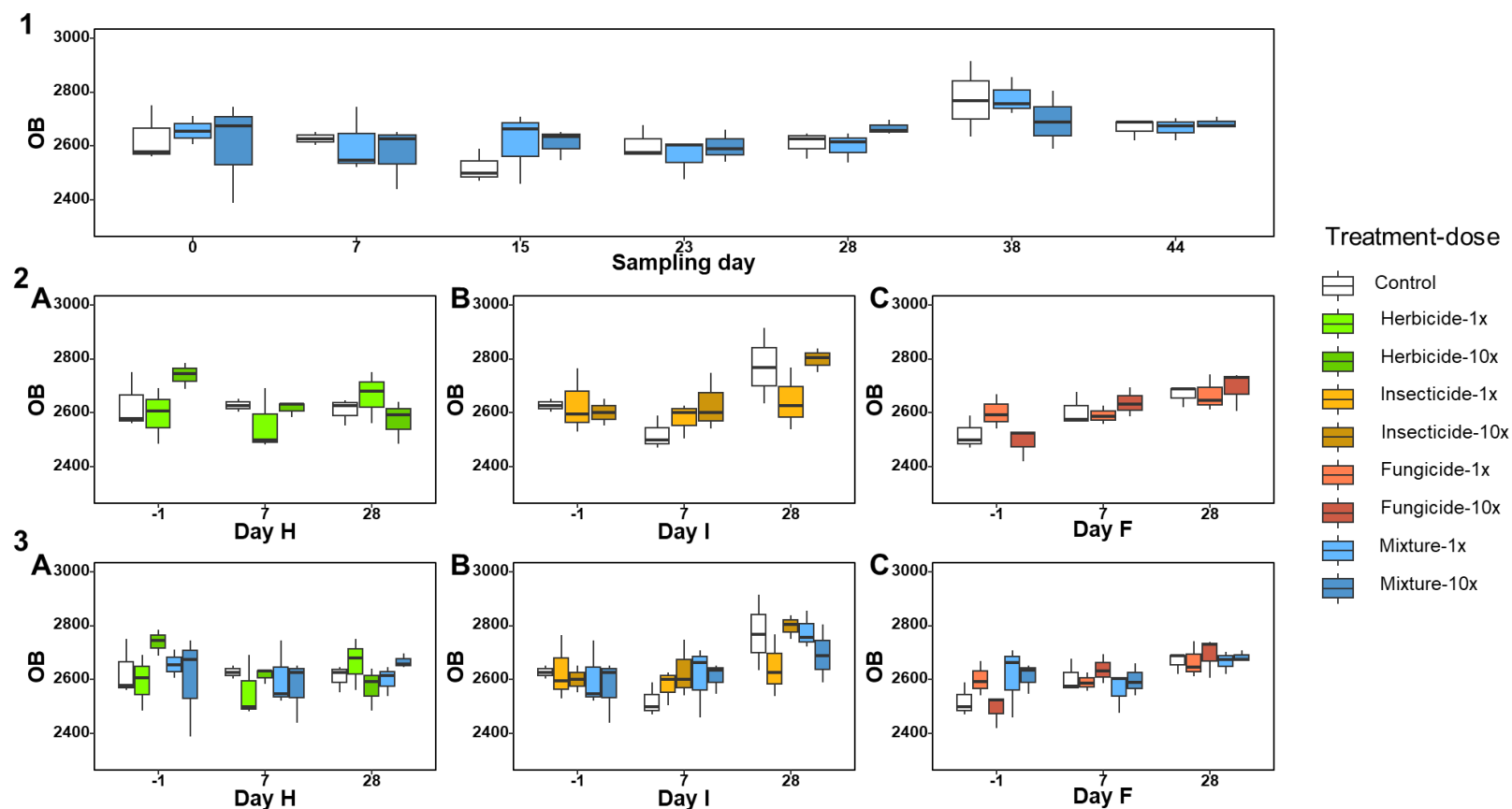


Figure S8. Effect of the mixture (panel 1), single pesticides (panel 2, A-B-C) and mixture and single treatments (panel 3, A-B-C) on the 16S observed species (OB) on the different sampling days. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

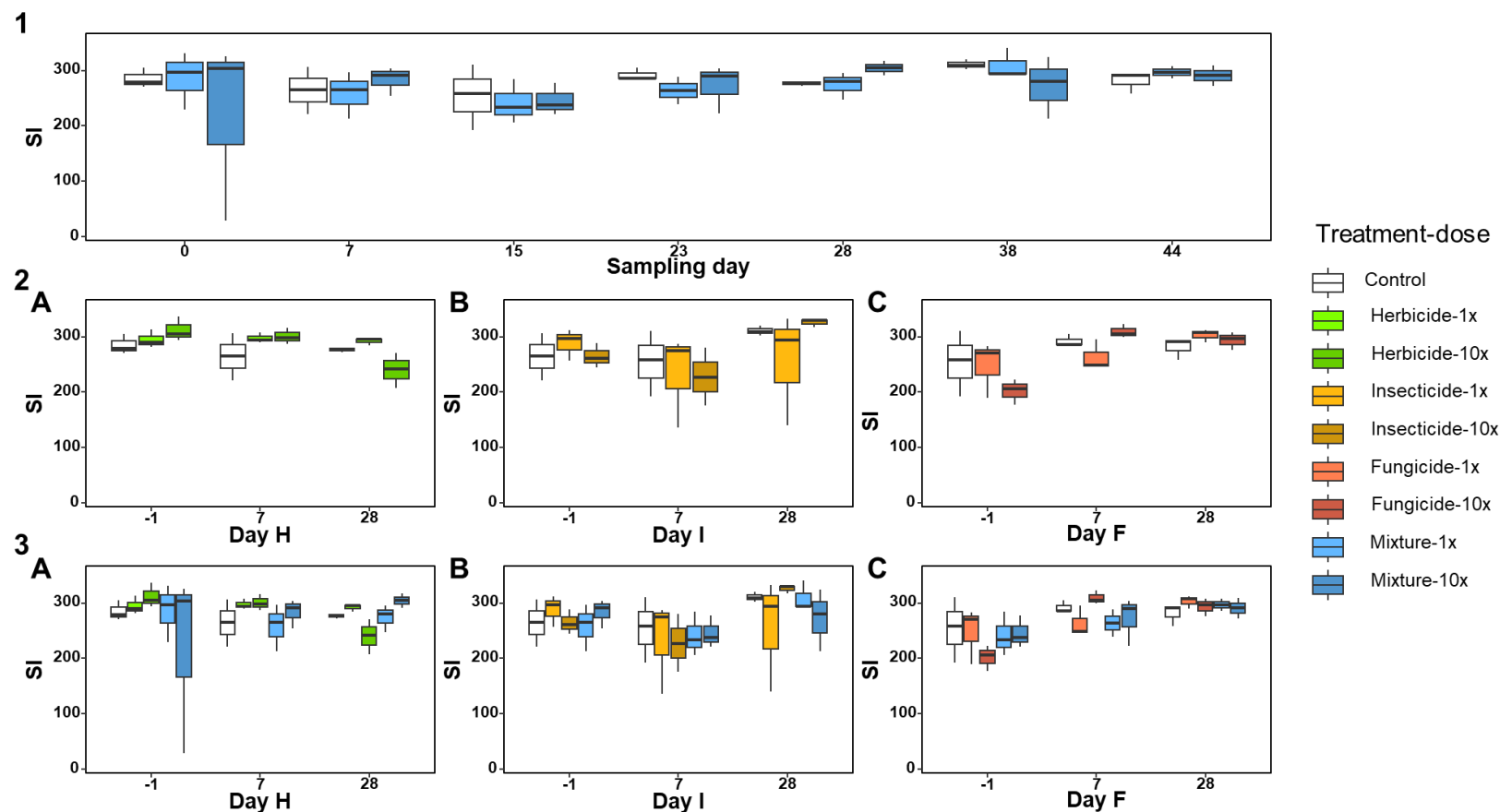


Figure S9. Effect of the mixture (panel 1), single pesticides (panel 2, A-B-C) and mixture and single treatments (panel 3, A-B-C) on the 16S Simpson index (SI) on the different sampling days. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

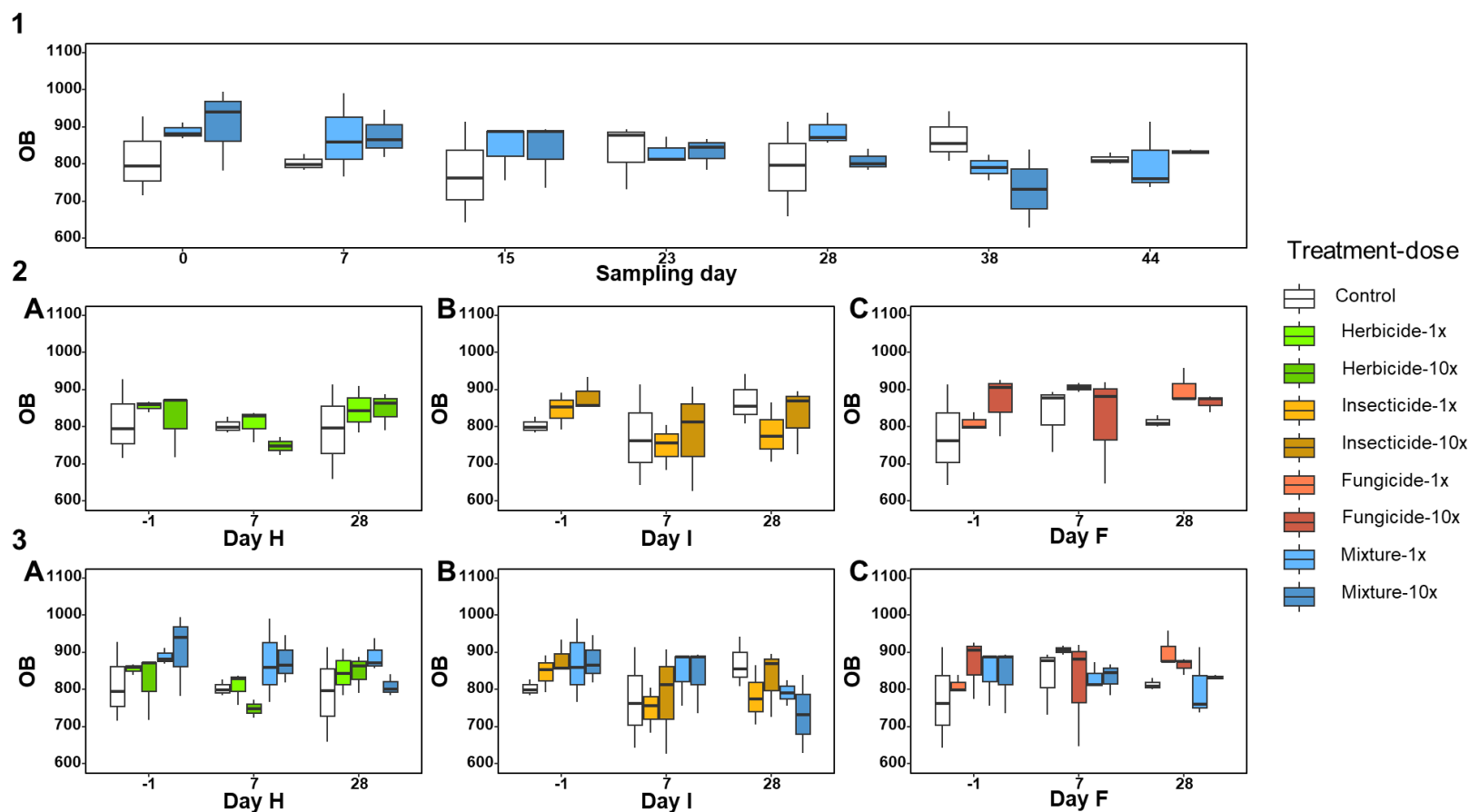


Figure S10. Effect of the mixture (panel 1), single pesticides (panel 2, A-B-C) and mixture and single treatments (panel 3, A-B-C) on the ITS Observed species (OB) on the different sampling days. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

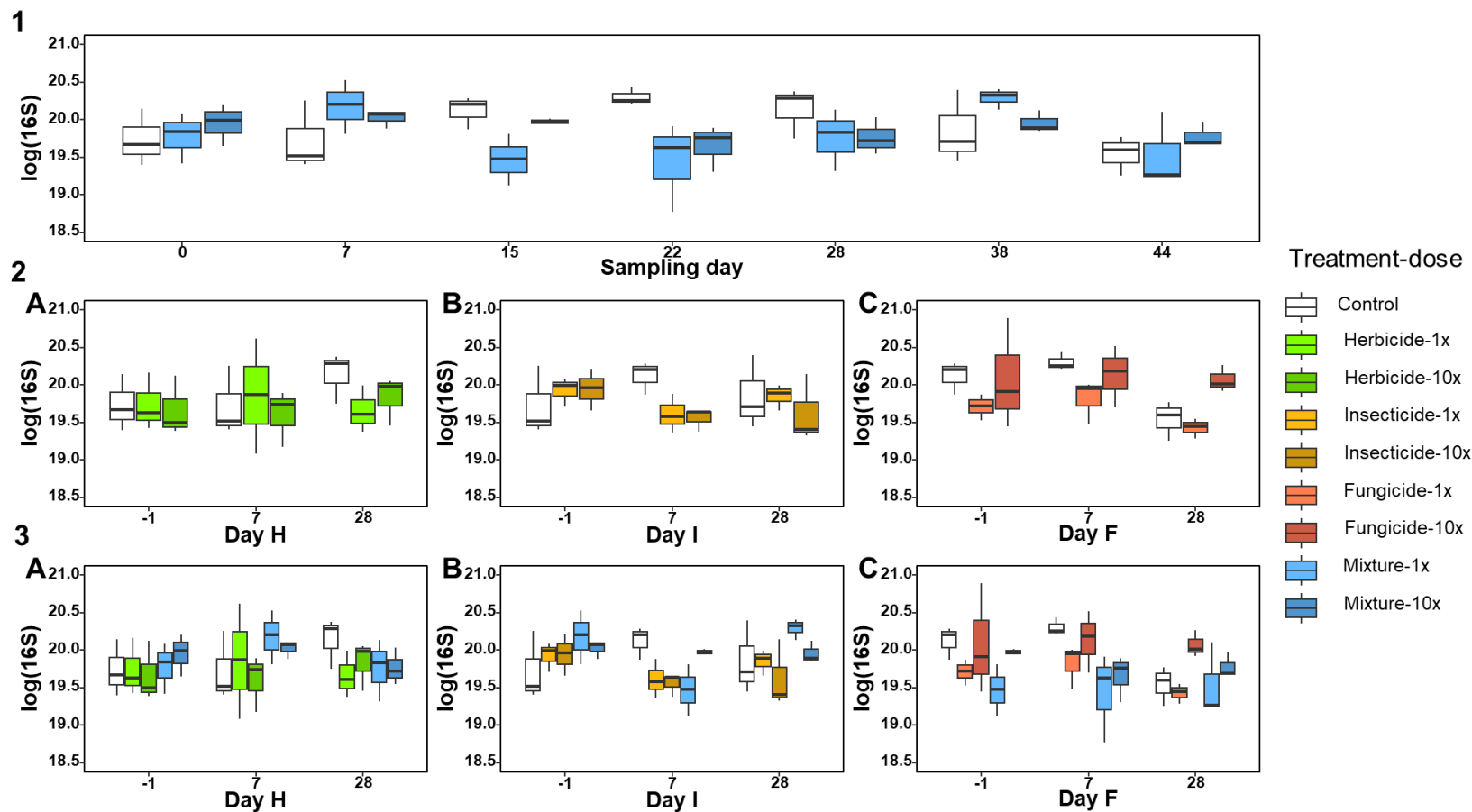


Figure S11. Effect of the mixture (panel 1), single pesticides (panel 2, A-B-C) and mixture and single treatments (panel 3, A-B-C) on the 16S abundance expressed as copy number/g of dry soil on the different sampling days. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

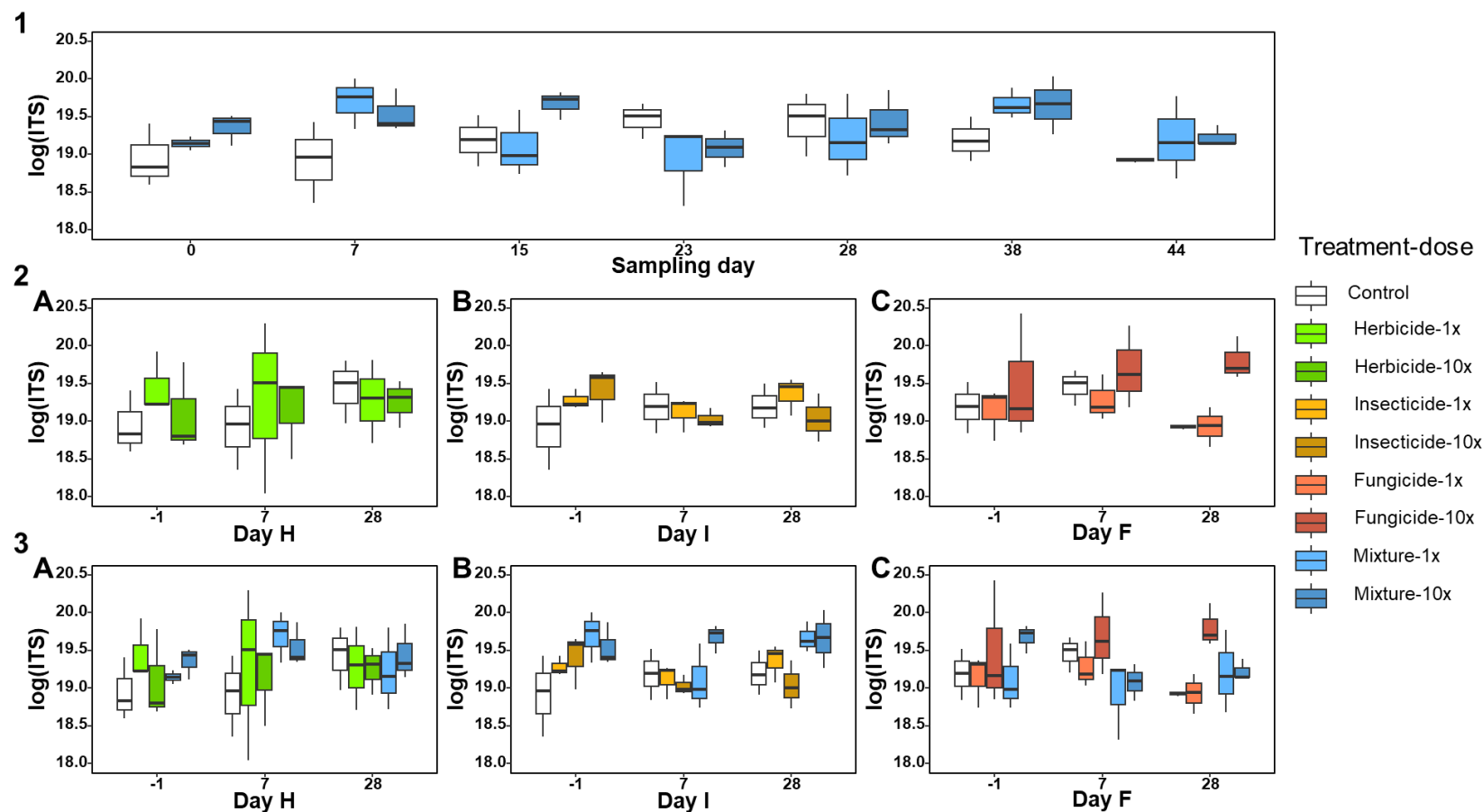


Figure S12. Effect of the mixture (panel 1), single pesticides (panel 2, A-B-C) and mixture and single treatments (panel 3, A-B-C) on the ITS abundance expressed as copy number/g of dry soil on the different sampling days. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

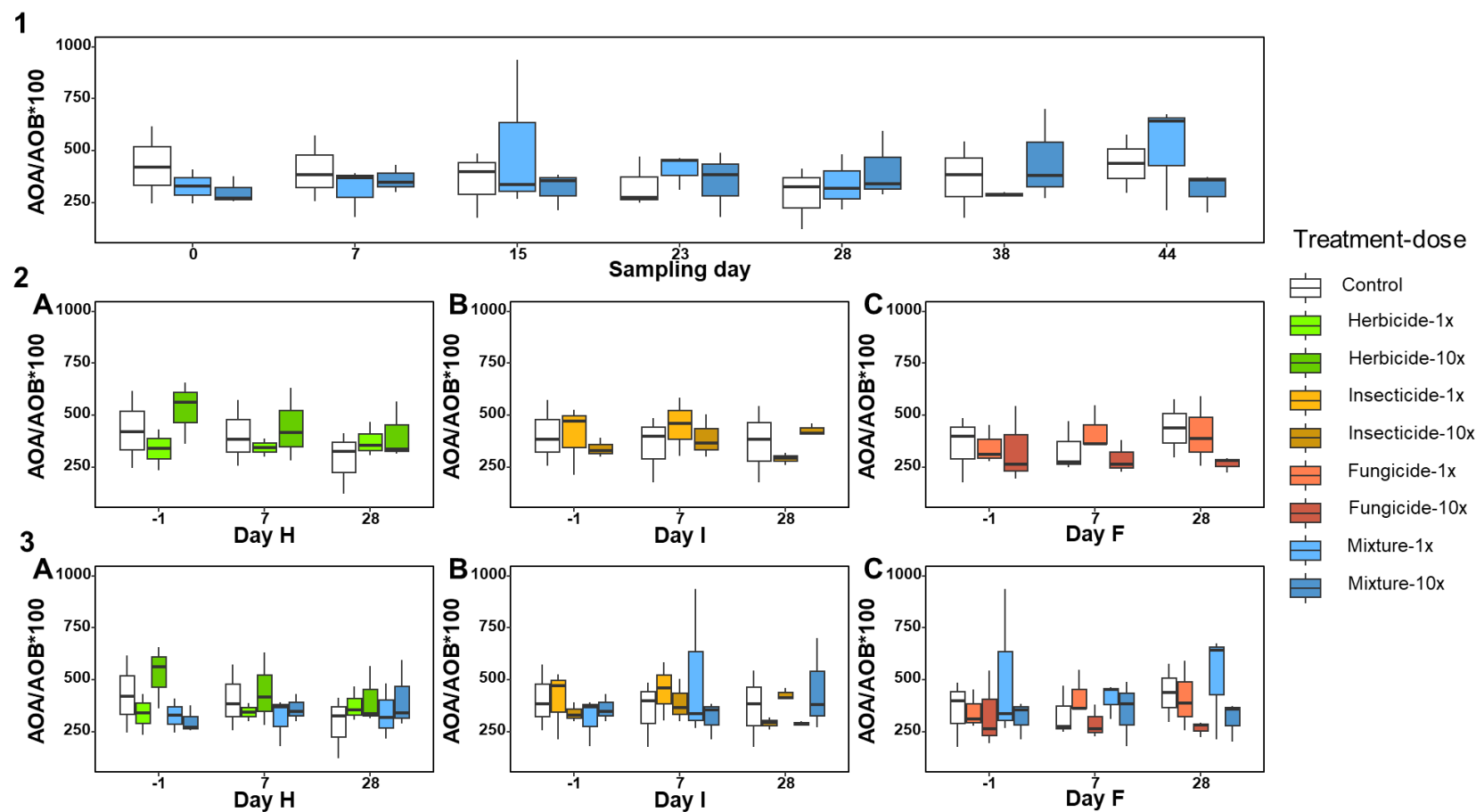


Figure S13. Effect of the mixture (panel 1), single pesticides (panel 2, A-B-C) and mixture and single treatments (panel 3, A-B-C) on the relative abundance of AOA (AOA/AOB*100) on the different sampling days. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

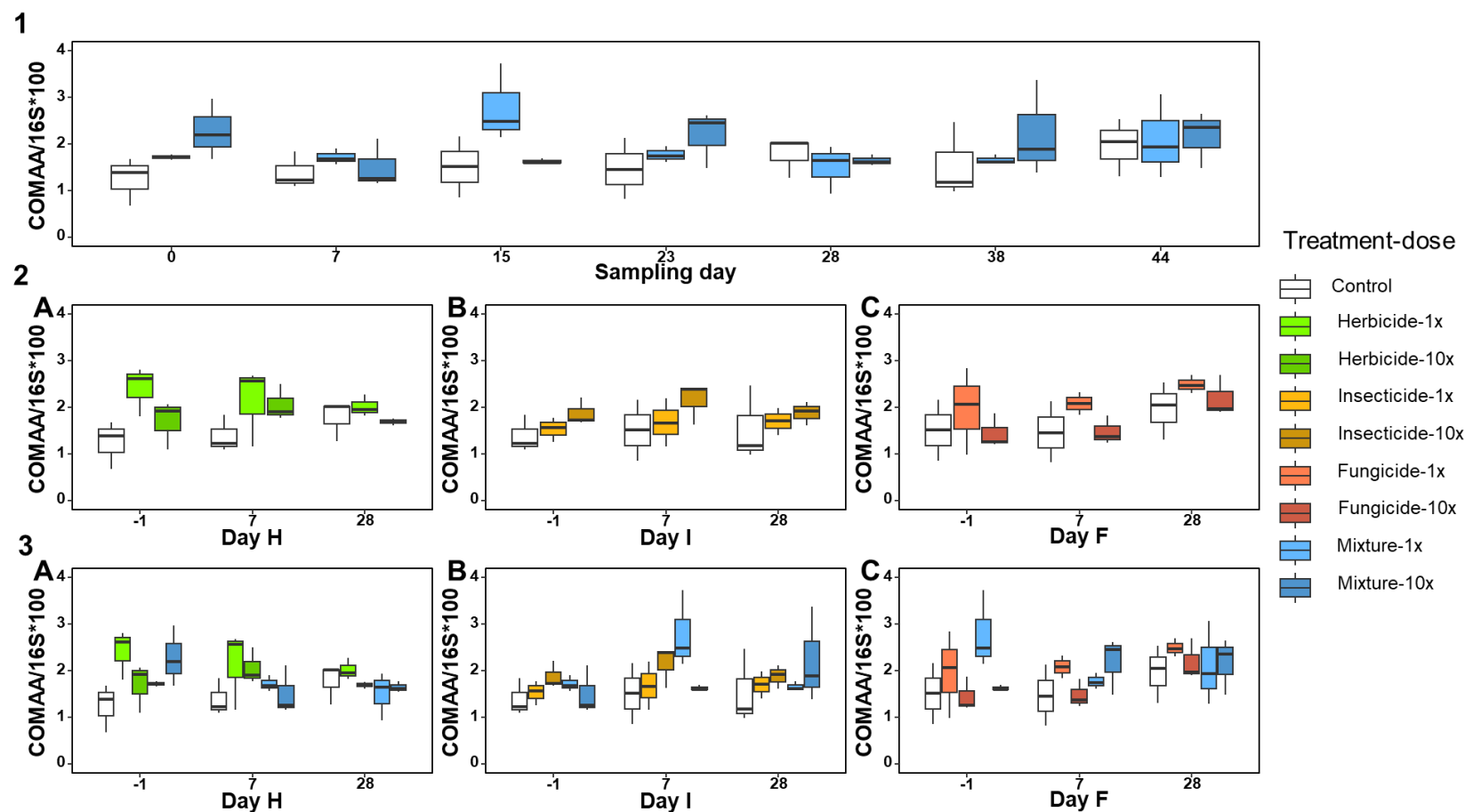


Figure S14. Effect of the mixture (panel 1), single pesticides (panel 2, A-B-C) and mixture and single treatments (panel 3, A-B-C) on the relative abundance of comamox-A (COMAA/16S*100) on the different sampling days. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

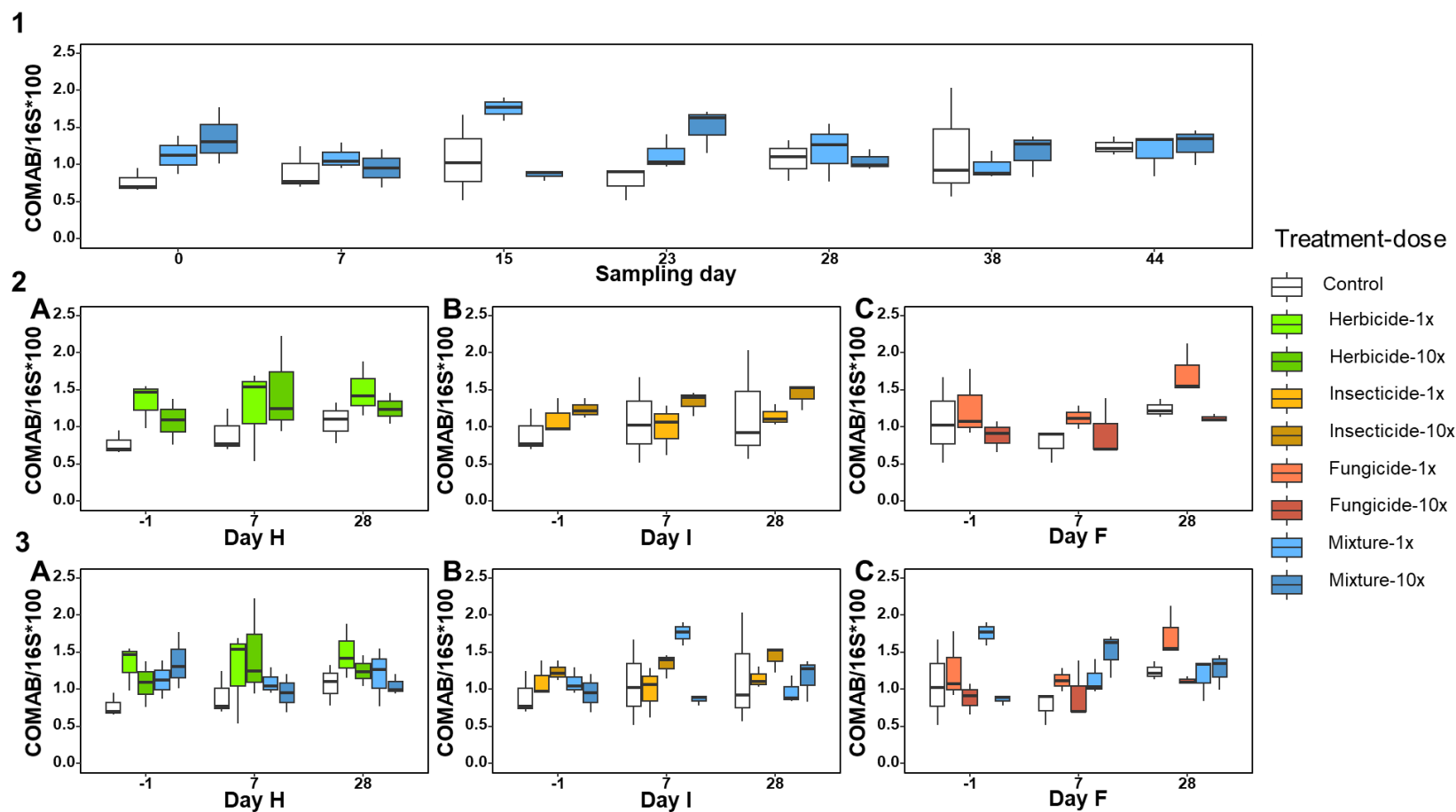


Figure S15. Effect of the mixture (panel 1), single pesticides (panel 2, A-B-C) and mixture and single treatments (panel 3, A-B-C) on the relative abundance of comamox-B (COMAB/16S*100) on the different sampling days. Day H, Day I and Day F represent the sampling day relative to the individual pesticides (-1: before relative pesticide application, 7: 7 days after relative pesticide application, 28: 28 days after relative pesticide application). The treatments are control, herbicide (clopyralid), insecticide (zeta-cypermethrin), fungicide (pyraclostrobin) and sequential mixture, all tested at 1x or 10x the agronomical dose.

Chapter 4

Development of Soil Microbial and Free-Living Nematode Communities in Laboratory Microcosms Upon Exposure to Two Insecticides and Their Mixture

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ABSTRACT

Free-living nematode and microbial communities are powerful soil health indicators, and potentially sensitive to pesticide application. While the effect of agrochemicals has been broadly investigated on the structure and function of the soil microbial communities, studies on the effect of pesticides on soil nematodes remain rare. To date there has been no research simultaneously examining these two interconnected communities after pesticide application, thus missing opportunities to explore the pesticide effect on multiple layers of the soil food web. Despite their ecological importance, nematode testing is not mandatory in current environmental risk assessments (ERA) of pesticides required by European Food Safety Authority. The N-transformation test is the only microbial endpoint evaluated and has been deemed insufficiently sensitive.

The objective of this study was to evaluate at the laboratory scale whether the application of a synthetic organic (conventional) and a biosimilar insecticide applied solely or as a mixture has an impact on natural communities of microbes and free-living nematodes in soil. Soil microcosms were either untreated (control) or treated at 1x, 10x or 100x the agronomical rate with a synthetic organic insecticide (a.i. zeta-cypermethrin), a biosimilar insecticidal product (containing alpha-terpinene, +D-limonene, and para-cymene) or both insecticidal products.

The ecotoxicological impact of these three pesticide treatments has been evaluated by comparison to untreated control on the abundance of bacteria, fungi and on key nitrogen cycle microbial guilds such as complete ammonia oxidizers, ammonia oxidizing bacteria and archaea using molecular tools (q-PCR), and on the abundance and community composition of nematodes.

Our results show that the biosimilar and the mixture applied at high doses prevent the natural development towards a more structured nematode community. Microbes responded ambiguously to pesticide application. In general, the test design and the investigated soil community proved suitable for evaluating the effect of pesticides on the development of the nematode community at the laboratory scale, making it promising candidate for inclusion in the ERA.

Keywords: terpenoids, zeta-cypermethrin, biosimilar, pesticide mixture, free-living nematodes, bacteria, fungi, ammonia oxidizers, commamox.

INTRODUCTION

Prompted by the recently introduced Farm to Fork strategy, part of the European Green Deal, established by the European Commission, which aims to accelerate the transition towards a more sustainable agriculture in Europe, the demand and market of more sustainable pest control strategies, including biopesticides, is expected to be increasing (Acheuk et al., 2022; Villaverde et al., 2016). According to the US Environmental Protection Agency (EPA), biopesticides are of natural origin and include nature-identical active substances, so-called ‘biosimilars’ (Czaja et al., 2015; Marrone, 2019). Because of their natural origin, it is argued that biopesticides are eco-friendly substances, being rapidly degraded in soil and presenting low risk for non-target organisms (Acheuk et al., 2022; Czaja et al., 2015; Damalas and Koutroubas, 2018; Marrone, 2019). Despite this push towards more sustainable agricultural practices, conventional synthetic organic pesticides are still used in agriculture, either alone or in combination with others, which may result in a mixture of pesticide residues in the environment that can exhibit so-called ‘cocktail effects’ towards non-targeted organisms (Panizzi et al., 2017). While multiple pesticide residues are commonly found in agricultural soils (Froger et al., 2023; Geissen et al., 2021; Panico et al., 2022), their ecotoxicological assessment is not done as it is still conducted *a priori* on pure active substance entering in the composition of pesticide.

Soil is a key provider of ecosystem services (Moebius-Clune et al., 2016; Singh and Gupta, 2018; Wall et al., 2015), which relies on the highly complex network of interactions and interdependencies among soil communities for its delivery (Bardgett and van der Putten, 2014; Creamer et al., 2022; Lazarova et al., 2021; Wall, 2004). Microorganisms are at the bottom of the soil food web and have been found to be affected by pesticide application (Gallego et al., 2019; Kalia and Gosal, 2011; Puglisi, 2012; Romdhane et al., 2022; Storck et al., 2018), with consequences on ecological functions due to changes in microbial abundance and

diversity (Karas et al., 2018; Kumar et al., 2019; Puglisi, 2012; Storck et al., 2018; Zabaloy et al., 2010). The nitrogen cycle, and especially the nitrification (Karpouzas et al., 2022), has been described to be sensitive to various pesticides, which may cause major problems to nitrogen supply to plant.

Nematodes are among the most abundant macro-organisms in soil (Moser and Frankenbach, 2009). Due to their functional and taxonomic diversity and their worldwide occurrence, nematodes community structure is a powerful indicator for soil health and is successfully used in ecological studies (Lazarova et al., 2021). Indeed, nematodes are at the center of the soil food web as they interact with various organisms, either feeding on them or being predated by others, and thereby contributing actively to the carbon (C) and nitrogen (N) cycles in soil (Ferris, 2010). The group of bacterivorous nematodes, the most abundant feeding type in the soil nematode community (van den Hoogen et al., 2020) exhibits top-down control on the microorganism community by grazing on bacteria, and their complex interactions has been studied extensively (Bååth et al., 1981; Ingham et al., 1985; Jiang et al., 2017; Neilson et al., 2020). Additionally, nematodes rapidly respond to environmental changes due to their limited mobility and short generation times compared to other soil macro-organisms such as earthworms (Bongers and Ferris, 1999). The structure of a nematode community is often described, and related to soil health and disturbances, by indices and compositional abundance of the different feeding types (first introduced by Bongers (1990) and extended e. g. by Korthals et al. (1996) and Ferris et al. (2001)).

Despite their vital importance in ecological functions provision and their potential sensitivity to agrochemicals, information on effects on nematode and microbial community structures is not a standard data requirement in the environmental risk assessment (ERA) procedure of active substance. The assessment for the microbial community relies on a functional test measuring N-

transformation, which has been criticized for lacking suitable sensitivity to predict pesticide toxicity on soil microbes (Karpouzas et al., 2022). Notably, there is no requirement for a nematode test in the ERA of pesticides.

In this context, the present study aims to investigate the effects of a biosimilar and a conventional insecticide as well as their mixture on the nematode and microbial soil communities. Thereby, we explored an experimental approach to study potential impacts of pesticides on multiple trophic levels in a naturally complex soil food web by using replicated soil microcosms under laboratory conditions.

MATERIALS AND METHODS

Assay design

This study consisted in a multi-factorial laboratory experiment, entailing soil microcosms set-up with field-sampled soil exposed, or not exposed (control), to either a synthetic organic insecticide, a biosimilar insecticide or their mixture. The two pesticides and their mixture were tested at 1x, 10x or 100x of their respective agronomical application rates, resulting in a total of nine treatments plus the untreated control. To assess short-term impact on the structure of the nematode and the microbial communities as well as their recovery and effects persisting on a longer timescale, the microcosms were destructively sampled 7 days and 42 days after pesticide application.

Soil sampling

The field soil used for the experiment was sampled from a bare agricultural field in Hochheim am Main, Germany (50°00'58.1" N, 8°23'41.0" E) in December 2022. The soil was characterized by 20.6% of clay, 24.8% of silt, and 54.6% of sand, with a carbon content of 1.1%. The soil was air-dried at room temperature for 15 days, sieved to 1 cm, homogenized and kept at 4°C until use. The maximum

water holding capacity (mWHC) determined shortly before test start was 41.7% of the soil dry weight, the pH 7.1 (measured in 0.01 M CaCl₂ according to DIN EN 15933:2012), and the humidity based on dry soil weight was 40.5% of the mWHC.

Investigated pesticides

Two insecticides were selected for the present study. Both were used as their commercial formulations and in relation to their agronomical application rate. One was a preparation of zeta-cypermethrin (CAS number: 1315501-18-8, commercial formulation: Minuet 10 EW, 100 g a.s./L). This pyrethroid insecticide is a mixture of stereoisomers and acts on insects as non-systemic neurotoxin via interaction with sodium channels. The approval for agricultural use of zeta-cypermethrin in the European Union expired in 2020, but cypermethrin is currently approved until 2029. According to the label information, the agricultural rate for the selected reference crop Brassicaceae of the zeta-cypermethrin formulation is 0.2 L/ha per year. Zeta-cypermethrin represents in the present study conventional synthetic organic insecticides (named in the following the ‘conventional’ insecticide).

Terpenoid blend QRD 460 (commercial formulation: Requiem Prime ®, consisting of alpha-Terpinene 91 g/L, +D-limonene 27.3 g/L, +Para-Cymene 34 g/L) was selected as the biosimilar insecticide. The components of the active substance terpenoid blend QRD 460 are identical to molecules produced by the plant *Chenopodium ambrosioides*. The product information states as mode of action impairment of insect mobility and respiration due to degradation of the soft cuticles of the target insects. The maximum application rate per treatment on the selected reference crop Brassicaceae is 5 L/ha, which can be repeatedly applied on greenhouse crops for a maximum of 12 times per crop growth cycle. However, due to concerns about potential issues such as the viscosity of the pesticide solution during treatment application, we considered a maximum of 2 applications

per year, resulting in an application rate of 10 L/ha. The terpenoid blend QRD 460 represents in the present study the increasingly important group of pesticides with natural origin as its active ingredients are identical to naturally occurring substances; it is called in the following the ‘biosimilar’ insecticide.

Experimental set-up

The homogenized field soil was divided in aliquots (corresponding to 2.4 kg of dry soil), which were treated with one of the pesticide formulations or their mixture, each dissolved in 110 mL of demineralized water. The control received 110 mL of demineralized water. With these applications, the final humidity was adjusted to 49.3% of the mWHC. The insecticides were applied at 0.2 L/ha for the conventional and 10 L/ha for the biosimilar. At 1x, the application resulted in 0.27 $\mu\text{L/kg}$ dry soil (26.66 $\mu\text{g a.s./kg soil d.w.}$) for the conventional insecticide and in 13.33 $\mu\text{L/kg}$ dry soil (2030.7 $\mu\text{g a.s./kg soil d.w.}$) for the biosimilar. This was calculated considering a soil depth of 5 cm and a soil density of 1.5 g/cm^3 . Each batch of soil was well mixed after application and 360.7 g of fresh soil (corresponding to 299.2 g of dry soil) was distributed to each of $n=8$ replicate 580 mL glass vessels per treatment. Vessels were closed with a loose glass lid and incubated at $20\pm 3^\circ\text{C}$ at a 16/8-h light/dark photoperiod for up to 42 days. During this incubation, soil humidity was adjusted weekly by adding demineralized water without mixing the soil. From each treatment, four replicates were destructively sampled on day 7, and the remaining four replicates on day 42. The untreated soil was sampled additionally in 4 replicates at the beginning of the experiment before pesticide application to characterize the initial communities. Destructive sampling consisted in first mixing all soil within each sampled replicate and taking a subsample for analysis of microbial endpoints, which was stored at -20°C until DNA extraction. The remainder of the soil was stored at 4°C up to 15 days before being processed for analysis of nematode endpoints.

Microbial endpoints analysis

The DNA was extracted from 250 mg of soil dry weight using the DNeasy 96 PowerSoil Pro kit (Qiagen, France) according to the manufacture's indication. DNA purity and concentration were measured with the Quant-iT™ dsDNA Assay Kit (Thermo Fischer Scientific, France). The DNA was diluted to 0.5 µg/µl. An inhibition test was performed prior to DNA usage to detect any extract inhibitors of the qPCR assay. No inhibitors could be detected.

The abundance of bacteria (16S), fungi (ITS), ammonia oxidizing archaea (AOA) and bacteria (AOB), and that of comammox clade A (COMAA) and B (COMAB) were analyzed with real-time qPCR using the ViiA7™ thermocycler (Life Technologies, Carlsbad, CA, USA). The reaction volume was 15 µL per sample, and contained 7.5 µL of Takyon Low Rox SYBR MasterMix dTTP blue (Eurogentec, Seraing, Belgium), 1.5 µM of each primer, 250 ng of T4 gene 32 protein (MP Biomedicals, Illkirch, France), and 1.5 ng of DNA. Total 16S and ITS community were quantified according to Muyzer et al. (1993) and White et al. (1990), respectively. The abundances of some nitrogen cycle microbial guilds were estimated targeting the *amoA* gene for the quantification of AOA and AOB, COMAA and COMAB according to Bru et al. (2011) and Pjevac et al. (2017), respectively. Non-template controls (NTC) were analyzed too and no quantification could be detected. Standard curves were obtained with serial dilutions of linearized plasmids containing cloned genes from bacterial strains or environmental clones. Two independent runs were performed per each gene quantification. The qPCRs efficacy ranged from 78.2% to 101.4% depending on the target considered. The final abundances were expressed in number of gene sequence/g of dry soil.

Nematode endpoints analysis

The free-living nematodes were extracted from the soil using a modification of Cobb's decanting and sieving method (Cobb, 1918; Coors et al., 2016) Briefly, 100 g of fresh soil were soaked in 800 mL of tap water for 30

minutes, stirred, let settled for 15 seconds and then decanted. This procedure was repeated three times, the decanted solutions containing the nematodes were pooled and further processed by several filtration steps with decreasing pore sizes (1 mm to remove plant residues, followed by 350, 175, 100 and 45 (repeated four times) μm sieves) to collect and rinse nematodes. The resulting nematode suspensions were then transferred to an extraction sieve with two layers of heavy-duty paper towels, and incubated for 46 hours at room temperature. The nematode that migrated into the tray were then collected.

The total abundance of nematodes per 100 g dry soil was calculated from the mean abundance of nematode counted from 3 aliquots of 5 mL of the nematode suspension. If the nematode number in one of the aliquots was more than 100% different from another one, a fourth additional aliquot was counted and a mean of four replicates calculated. The collected nematodes were then transferred to a 15 mL falcon tube and preserved in 4% formaldehyde at 4°C until taxonomic identification. At least 100 randomly selected individuals per sample were identified to family level according to Bongers (1994). The nematode community characterization and calculation of indices was done as described in Coors et al. (2016). The calculated indexes were: enrichment index (EI), structure index (SI) and channel index (CI), maturity index (MI), maturity index 2.5 (MI2.5), and fungivore-bacterivore ratio (FB ratio). Other additional calculated indexes were the Shannon Weaver index (H'), family richness (FR) and plant-parasite index (PPI). Proportional abundance was summarized for nematode feeding types (bacterivores BAC%, fungivores FUN%, omnivores OMN%, and herbivores HER%).

Statistical analysis

The microbial endpoints and the nematode indexes and feeding groups percentages were statistically analyzed with R studio (version 2023.12.1+402) through multiple ANOVAs ($n=4$). Fisher's LSD post hoc tests were performed to

detect significant differences among treatments at each of the two sampling time points. Normal distribution of residuals was checked with Levene's Test ($p=0.01$) and homogeneity of variances with Shapiro Wilk Test ($p=0.01$). The data were arcsine transformed, and outliers removed from the microbial endpoints' datasets, where necessary. If the data were still not normally distributed after the transformation, the Kruskal Wallis test was performed.

Pearson's correlation was used to check for any correlation between the absolute number of bacteria and that of bacterivores per each treatment, independently from the dose and time of sampling.

RESULTS

Effects on the microbial community

The ITS and nitrifiers (AOA, AOB, comaA, comaB) abundances in the control samples remained stable all over the duration of the experiment. That of 16S slightly increased from the beginning of the experiment to 7 days after pesticide application, and remained stable until the end of the experiment.

Similar to the control, the abundance of 16S increased in most of pesticide treatments within the first 7 days of the experiment, *i. e.* on the short-term scale (Fig.14-panel 1B). Only in two treatments, the lowest concentrations of the conventional insecticide (I) and the mixture (M), bacterial abundance was significantly lower than in the control and most other treatments (Tab. S6). This effect was transient as bacterial abundances observed for these treatments were similar to that of control after 42 days of incubation. In the longer term, *i. e.* after 42 days, the bacterial abundance of soil exposed to 10x and the 100x biosimilar (B) treatments, as well as to the mixture 10x treatment, remained significantly lower than that of the control.

With regard to the abundance of fungal community (ITS), there were no significant differences at day 7 no matter the treatments considered as compared to the control (Fig. 14-panel 2B). At day 42, I-10x was the only treatment in which fungal abundance was significantly reduced as compared to the control. Overall, the abundances of the various microbial groups (bacteria, fungi) never significantly exceeded in any of the treatments the abundance observed in the respective control. For the insecticidal treatments for which a significant reduction was observed, the size of the reduction ranged between 15-20% compared to the control. Details are provided in Tabs. S6 & S7.

Any of the three insecticidal treatments has a significant impact on the relative abundance of the nitrifiers (AOA, AOB, COMAA and COMAB) as compared to the control (Tab. S7).

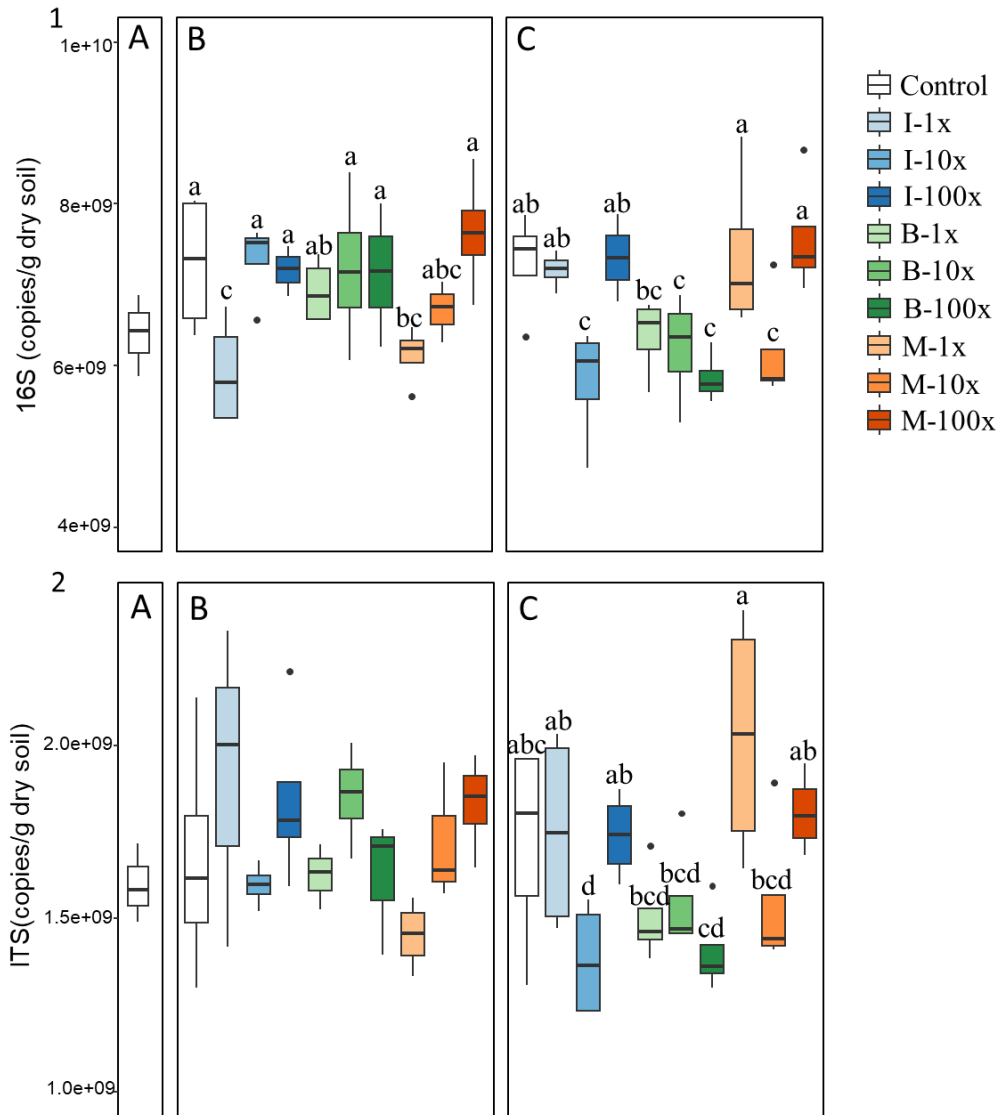


Figure 14. The 1) bacterial and 2) fungal abundances at experiment start (A), 7 days after experiment start (B) and 42 days after experiment start (C) after the application of a synthetic insecticide (I), a biosimilar insecticide (B) or their mixture (M) at 1x, 10x or 100x the agronomical dose. Letters denote statistically significant different groups according to the Fisher LSD post hoc test. Outliers are represented by points outside of the boxes.

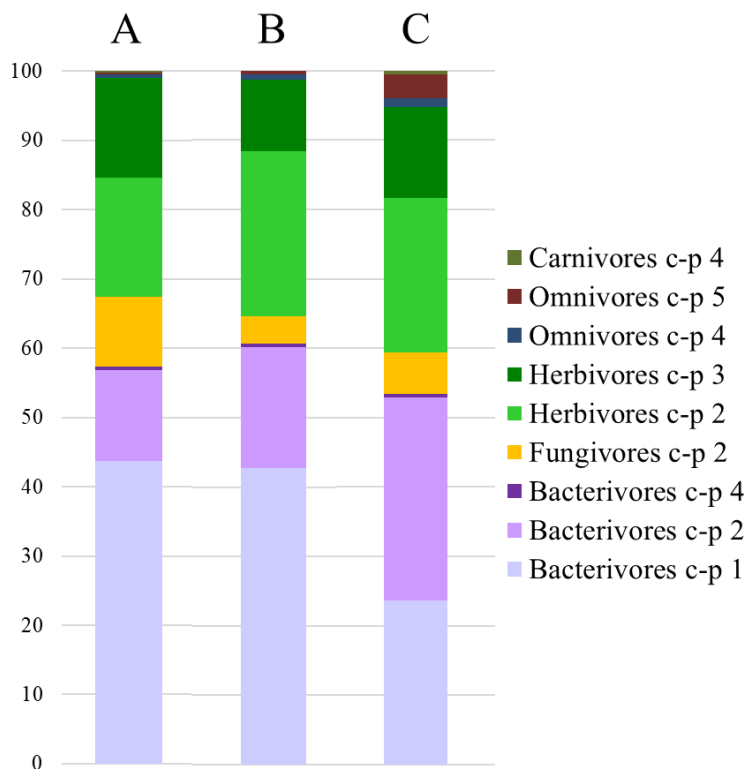
Effects on the nematode community

Figure 15. The nematode community composition of the control at experiment start (A), 7 days after experiment start (B) and 42 days after experiment start (C). The colors correspond to the different feeding groups according to their c-p values, and are expressed as a % of the whole community.

The natural nematode community in the sampled field soil at test start (Fig. 15-panel A, Tab S9) was mainly composed by bacterivores (57.4%), among which Rhabditidae dominated followed by Panagrolaimidae. These families are classified as enrichment opportunists (c-p value 1). Herbivores, mainly family Tylenchidae (c-p value 2), were the second most abundant feeding type

(31.5%). Fungivores accounted for the 10% of the initial field community, with the general opportunistic family Aphelenchoididae (c-p value 2) dominating. Omnivorous and carnivorous nematode families accounted for the lowest proportion with 0.8% and 0.3%, respectively. The high abundance of opportunistic families in the initial field community is reflected in the high EI (87.9 ± 6.7), and low MI (1.4 ± 0.2) and SI (20.4 ± 7.3). Details of the indices are showed in Table 7. Altogether, this indicates that this arable soil is hosting a highly nutrient-enriched, disturbed and poorly structured nematode community (quadrat A described in Ferris et al. (2001)). Over the course of the experiment, the nematode abundance and family richness in the control remained stable, while

there were changes in community structure. After 7 days of exposure, the proportion of the feeding groups hardly changed no matter the treatment considered. The family Cephalobidae (c-p value 2) became more representative among the bacterivores, accounting for the 17.3% of the whole nematode community. Among herbivores, the percentage of the family Tylenchidae increased to 21.1%. The proportion of fungivores, and so of the general opportunistic, decreased to 4%, and that of omnivores just slightly increase to 1.2%, resulting in higher SI (27.4 ± 3.0). The major changes were evident in the longer term: the bacterivores decreased to 53%, and were mainly represented by Cephalobidae and Rhabditidae, indicating decreasing disturbance and enrichment conditions compared to the beginning of the experiment and to after 7 days of exposure. The omnivores, now dominated by Aporcelaimidae, increased to almost 5%. There was a clear development into a less nutrient-enriched, more structured ($EI=72.7 \pm 10.7$; $SI=45.1 \pm 14.8$) and less disturbed ($MI=1.9 \pm 0.1$) nematode community in the control microcosms over the full duration of the experiment (Fig. 15-panels A&C).

Table 7. Mean and standard deviation of the nematode endpoints at experiment start (T0), 7 days (T7) and 42 days (T42) after experiment start for the control, biosimilar insecticide (B), conventional insecticide (I), and their mixture (M) applied at 1x, 10x or 100x their agronomical dose. Letters denote statistically significant different groups according to the Fisher LSD post hoc test.

Time	Treat-dose	Abundance	FR	H'	BAC%	FUN%	HERB%	OMN%	CAR%	MI	MI 2.5	PPI	EI	CI	SI
T0		981±159	16	2.1±0.2	57.3±11.4	10.1±4.5	31.5±6.1	0.8±1.0	0.2±0.5	1.4±0.2	2.1±0.0	2.4±0.1	87.9±6.7	6.4±5.0	20.4±7.3
T7	control	975±108	13	1.9±0.1	60.6±8.5	3.9±3.1	34.2±6.8	1.2±0.5	0.0	1.4±0.1	2.2±0.0	2.3±0.2	88.7±3.6	2.5±2.5	27.4±3.0
	B-1x	744±202	14	1.9±0.1	61.5±3.8	5.9±1.5	31.6±4.0	1.0±1.1	0.0	1.5±0.1	2.1±0.1	2.3±0.2	84.2±3.9	3.7±0.8	12.5±15.2
	B-10x	695±104	15	2.0±0.1	56.1±4.7	7.4±3.0	34.5±5.5	2.0±1.8	0.0	1.6±0.1	2.2±0.2	2.4±0.1	80.9±4.8	5.5±2.2	23.5±22.2
	B-100x	732±209	14	1.9±0.1	58.1±4.0	6.2±4.2	33.7±5.5	1.2±0.5	0.7±0.9	1.5±0.1	2.2±0.1	2.4±0.1	86.8±3.1	3.8±2.8	29.0±7.2
	I-1x	853±150	17	2.0±0.0	59.4±5.7	8.9±4.4	30.0±7.0	1.5±1.3	0.2±0.5	1.5±0.1	2.2±0.1	2.4±0.1	85.9±1.9	5.1±1.9	25.2±18.7
	I-10x	902±142	16	2.0±0.2	63.9±3.6	5.2±3.9	27.1±2.1	3.5±3.5	0.2±0.5	1.5±0.2	2.4±0.2	2.4±0.2	90.6±4.1	2.8±2.0	43.3±20.4
	I-100x	968±164	18	1.9±0.1	61.9±4.6	4.5±0.6	32.6±4.3	0.5±0.6	0.5±1.0	1.4±0.1	2.1±0.1	2.3±0.1	88.8±1.6	2.5±0.3	22.3±14.6
	M-1x	1003±160	14	2.0±0.1	57.8±6.6	7.5±2.4	33.2±5.8	0.8±1.0	0.7±0.5	1.5±0.1	2.1±0.1	2.4±0.1	85.2±6.1	4.9±2.2	23.3±16.2
	M-10x	1085±263	16	2.0±0.1	58.3±5.0	6.2±2.2	34.0±4.9	1.5±1.7	0.0	1.5±0.1	2.1±0.1	2.3±0.2	83.1±4.2	4.3±1.8	18.2±15.5
M-100x	1031±363	16	1.7±0.2	70.7±7.9	6.0±3.3	22.9±6.5	0.2±0.5	0.2±0.5	1.4±0.1	2.1±0.1	2.4±0.1	89.3±5.9	3.1±2.4	16.3±12.8	
T42	control	902±128	17	2.1±0.1 ^a	53.4±3.0 ^{bcd}	6.0±2.1	35.3±6.0 ^{abc}	4.8±2.5 ^{ab}	0.5±0.6	1.9±0.1 ^a	2.4±0.2	2.4±0.1	72.7±10.7	6.9±4.6	45.1±14.8
	B-1x	712±99	15	2.0±0.1 ^a	46.8±4.8 ^d	7.0±2.4	39.0±2.8 ^a	7.0±2.7 ^a	0.2±0.5	1.9±0.2 ^a	2.5±0.2	2.4±0.1	74.9±10.5	7.1±1.7	51.9±17.9
	B-10x	586±231	16	1.9±0.1 ^{ab}	58.6±8.8 ^{abc}	4.7±2.2	32.2±4.8 ^{bcd}	4.2±3.3 ^{abc}	0.2±0.5	1.7±0.1 ^{ab}	2.4±0.2	2.3±0.1	80.6±5.5	3.9±2.2	44.7±21.4
	B-100x	816±97	14	1.7±0.0 ^c	63.2±4.0 ^{ab}	6.5±1.2	27.3±4.5 ^{de}	2.8±0.5 ^{bc}	0.3±0.5	1.6±0.1 ^b	2.2±0.1	2.2±0.1	81.0±5.2	4.4±1.0	31.8±8.1
	I-1x	866±212	17	2.0±0.1 ^a	52.4±8.7 ^{cd}	4.7±1.7	36.9±5.4 ^{ab}	5.7±2.7 ^{ab}	0.2±0.5	1.9±0.2 ^a	2.4±0.2	2.3±0.1	75.6±6.4	5.1±3.6	48.4±11.7
	I-10x	931±226	16	2.0±0.2 ^{ab}	59.5±7.0 ^{abc}	6.0±2.2	29.2±5.0 ^{cde}	4.7±1.9 ^{ab}	0.5±1.0	1.8±0.2 ^{ab}	2.4±0.2	2.3±0.1	80.8±5.0	4.7±2.2	47.3±17.4
	I-100x	1039±180	17	2.0±0.2 ^a	55.5±6.6 ^{bcd}	7.0±4.4	31.7±3.3 ^{bcd}	5.2±2.2 ^{ab}	0.5±0.6	1.9±0.1 ^a	2.3±0.1	2.4±0.1	71.5±3.9	6.8±3.7	41.6±13.2
	M-1x	861±269	15	2.0±0.1 ^a	51.7±8.1 ^{cd}	8.0±4.2	37.1±3.8 ^{ab}	3.2±0.9 ^{bc}	0.0	1.8±0.1 ^{ab}	2.3±0.1	2.3±0.1	75.0±6.1	8.2±5.7	36.4±10.4
	M-10x	913±171	15	2.0±0.0 ^a	54.5±9.3 ^{bcd}	8.5±3.0	34.2±6.9 ^{abc}	2.7±2.1 ^{bc}	0.0	1.7±0.1 ^{ab}	2.2±0.1	2.3±0.1	74.4±4.4	7.7±2.3	30.6±11.2
M-100x	960±150	15	1.8±0.2 ^{bc}	68.5±4.9 ^a	6.9±3.4	23.1±1.7 ^e	1.5±1.3 ^c	0.0	1.6±0.2 ^b	2.1±0.0	2.4±0.1	79.4±9.6	5.1±3.5	18.5±4.4	

After 7 days of exposure, the nematode community of the treated soils no matter the concentration applied was similar to that of the control (Fig. 16, Tab S8). Accordingly, since the composition of the nematode community 7 days after treatment application did not drastically change, the MI remained overall stable, as well as the EI (Tab. 7).

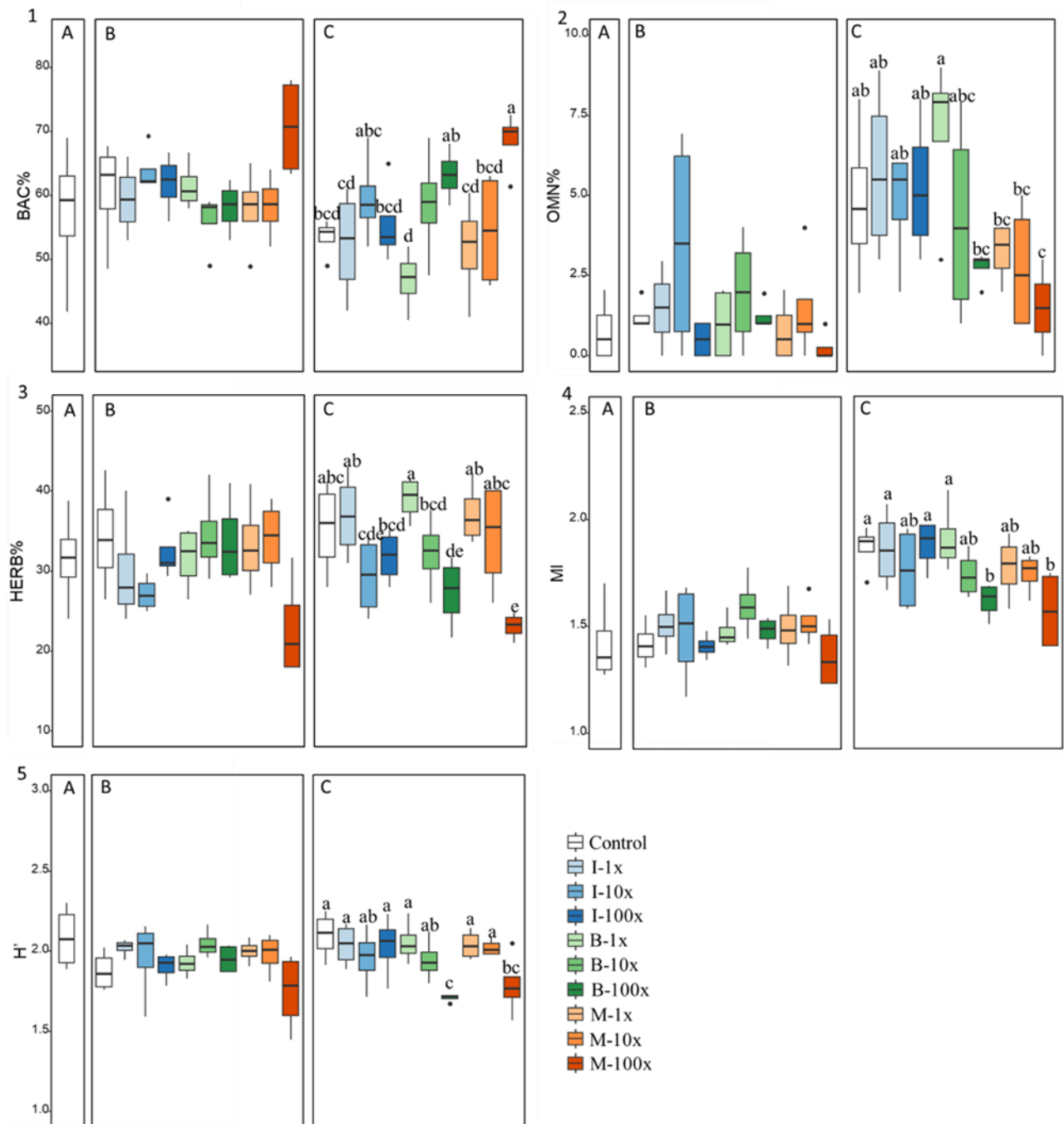


Figure 16. The 1) BAC%, 2) OMN%, 3) HERB%, 4) MI and 5) H' at experiment start (A), 7 days after experiment start (B) and 42 days after experiment start (C) after the application of a synthetic insecticide (I), a biosimilar insecticide (B) or their mixture (M) at 1x, 10x or 100x the agronomical dose. Letters denote statistically significant different groups according to the Fisher LSD post hoc test. Outliers are represented by points outside the box.

After 42 days of exposure, several parameters of the nematode community observed in treated soils showed statistically significant differences as compared

to the control (Tab. S9). The variation in the proportion of bacterivores (BAC%) increased compared to day 7 (Fig. 16-panel 1), and the M-100x was the only treatment with a significantly higher BAC%, still mainly represented by Rhabditidae (33.2%), compared to the control. This treatment had also a significantly lower OMN% compared to the control. This is reflected in the MI (Fig. 16-panel 4), where M-100x and B-100x have significantly lower values compared to the control. Similarly, the H' was significantly lower in B-100x and M-100x than in the control. The changes in these nematode community parameters in the biosimilar and mixture treatments appeared to be concentration-dependent in all cases, while the difference to the control was only statistically significant for the highest concentration of each pesticide treatment applied to soil microcosms.

After 42 days of exposure for HERB% values of M-100x and B-100x samples were significantly lower than that of the control. In addition, the proportion of herbivore families such as Dolichodoridae and Hoplolaimidae decreased of the 71%, and that of Paratylenchidae disappeared in the B-100x. Similarly, in M-100x the abundances of Tylenchidae and Paratylenchidae were lower as compared to those observed in the control.

Soils treated with the biosimilar insecticide had the lowest abundance of nematodes across all timepoints (T0, T7 and T42) and treatments. However, absolute nematode abundances indicated no significant differences between pesticide treated soils and the control, neither after 7 days nor after 42 days of exposure.

No significant correlations were detected between the absolute number of bacteria and that of bacterivorous nematodes for any treatment, except for the mixture (Fig. S16), based on the Pearson's correlation.

We conducted a Principal Component Analysis (PCA) to explore how the nematode and microbial endpoints contribute to the response following pesticide

7 and 42 days exposure to the three pesticide treatments and the control (Fig. S17). The PC1 axis in both graphs accounted for a significant portion of the sample variance, approximately 32% at each time point. While after 7 days of exposure no clear clustering of the various treatments was observed, after 42 days B-100x and M-100x treatments were separated from the control along PC1 axis. Notably, nematode endpoints appear to have the strongest association with PC1 at both time points, so explaining most of the variance observed between the treatments. Interestingly, microbial endpoints show no correlation with nematode endpoints, except for SI and OMN% after 7 days of exposure. Additionally, EI and BAC% display negative correlations with MI and H', aligning with their respective ecological interpretations and validating our analysis.

DISCUSSION

Nematodes and microbes represent functionally diverse and important groups in the soil food web (Mikola and Setälä, 1998). Until now they have been used separately as indicators for soil health (Yeates, 1970). Despite their importance in soil ecology, microbial and nematode abundance, structure and diversity are currently not considered as endpoints in the process of a priori ERA of pesticidal active substances. To date only a few numbers of studies explored the use of natural free-living nematode communities in pesticide ecotoxicology studies (Waldo et al., 2019; Grabau et al., 2020; Höss et al., 2022), and none of them investigated the effect of pesticide on the nematodes in interaction with the soil microbial community.

In the present study, we worked on soil collected from bare agricultural field located in Germany which was found to host a highly disturbed and poorly structured nematodes community, characterized mainly by families with c-p value of 1 (quadrat A described in Ferris et al. (2001)). This observation is coherent with the type and use of soil and the season of sampling (winter): indeed, the number

of bacterivores, especially Rhabditidae (Bongers, 1990), is generally high agricultural soil in winter (Wasilewska, 1971). In the control microcosms, nematode abundance showed no change all over the duration of the experiment, but evolved towards a more structured community, with greater abundance of high c-p value families. The progressively higher abundance of c-p 2 families like Cephalobidae has previously been described by Korthals et al., (1996) as a response after a period of nutrient enrichment (type I response), where enrichment of opportunistic nematodes decreases in abundance due to increasingly limiting food availability. However, the bacterial abundance remained stable overtime in the control samples therefor not accounting for the hypothesis of food limitation of bacterivore nematodes. The type I response is indeed typical under fertilization regimes, liming, eutrophication or physical stress (Korthals et al., 1996), which do not apply to the control samples. We might then conclude that the evolution of the control community towards less enriched condition is not a consequence of a stress, but rather a natural development towards a structured and undisturbed community under favorable laboratory-controlled conditions.

All the statistically significant impacts of pesticide treatments (I, B and M) tested in this experiment on the nematode community parameters were detected only after 42 days of exposure as compared to control. Indeed, the control and the pesticide treated communities were similar after 7 days of exposure. This suggests that the pesticide treatments had no detectable immediate effects on nematodes, but caused rather a delayed impact. This might be attributed to the enriched condition of the initial community, composed by few nematodes with high cp-values. Therefore, the insecticides applications may not have contributed much to shift towards even more enriched conditions in the short term, and to the selection of resistant taxa, an effect that might be observed after pesticide application (Höss et al., 2022). Indeed, even the MI2.5 index that is normally used as a pollution indicator could not capture significant differences among the treatments in the

various sampling points, due to the lack of high c-p values families. The used experimental microcosm setup and the analyzed community appear therefore to be highly appropriate for studying the impact of chemical stressors on the natural development of nematode (and microbial) communities under laboratory condition. Impacts induced by chemical stressors are unlikely to be hidden by a simultaneous deterioration of the control community due to stress imposed by the laboratory environment, but would be detectable in comparison to the control when the community becomes more structured. On the other hand, the initial nematode community is not suited to study immediate impacts of chemical stressors, as it already represented a rather deteriorated state typical for bare agricultural soil sampled in winter.

The strongest impact on nematodes was observed for the highest application rate of the biosimilar insecticide and the mixture of the two insecticides. For these two treatments, the nematode community was not mature as indicated by the still high EI and unchanged SI, domination by bacterivorous Rhabditidae, low proportion of omnivores, and low structural diversity (H').

Among the herbivores, the order Tylenchida has been described to be resistant to pollution, in particular to copper, due to their impermeable cuticula (Korthals et al., 1996). On the other hand, terpenoids are known for their effect on some herbivorous nematodes (Abdel-Rahman et al., 2013; Chitwood, 2002; Echeverrigaray et al., 2010; Wuyts et al., 2006), which partially aligns with our findings, as effects can be only observed at high doses in the mixture. This underscores the need for substantial pesticide doses to elicit an effect. Indeed, no significant differences could be detected in the plant parasites index (PPI), which might not be suitable to capture changes in the nematode community sampled from a bare agricultural field in winter, due to the absence of vegetation.

According to EFSA, terpenoid blend as the biosimilar insecticide used in this experiment is considered of low toxicity for micro- and macro-organisms, with

almost no effect on the nitrogen transformation test (EFSA, 2014). Zeta-cypermethrin is also considered to show low toxicity to microorganisms and earthworms (EFSA, 2008). Previous research has concluded that cypermethrin can stimulate some bacterial groups (Borowik et al., 2023; Zhang et al., 2009), while terpenoids can exhibit an antimicrobial activity (Guimarães et al., 2019). In the present study, the abundance of bacteria and fungi has been ambiguously impacted by pesticides application, with no clear dose-dependent response. When statically significant changes induced by the pesticide treatments on microbial abundance, their observed size effect was small, suggesting a minor impact of the tested pesticides on these two microbial communities.

The effects observed on the nematode community in response to mixture treatment resembled to those observed with the biosimilar insecticide. On the other hand, the exposure to conventional insecticide at concentration has no effects on nematode community. Hence, at highest dose applied the mixture effect on the nematode community was dominated by the biosimilar rather than the conventional insecticide. The microbial community exhibited a slightly different pattern with effects from both the conventional and the biosimilar insecticide on various endpoints. Furthermore, these statistically observed effects were variable over time and did not show a concentration-dependence patterns in contrast to the nematode endpoints. The nematode endpoints stand out as primary factors describing the variability among treatments, serving as unequivocal indicators of exposure to these pesticide treatments, a conclusion supported by the PCA analysis. Increasing the number of repetitions could improve the statistical analysis of microbial endpoints. Though interpreting effects on microbial endpoints' abundance is challenging, the planned bacteria, fungi, and protists community compositions analysis might offer clearer insights into the microbial community's response to pesticide application.

CONCLUSIONS

The aim of this study was to assess the impact of two insecticides and their mixture on soil microbes and free-living nematodes sampled from a bare agricultural field. Our results underscore the importance a well-described control community under laboratory conditions as a prerequisite for detecting and interpreting effects of chemical stressors. While a highly-structured initial nematode community rich in assumed pollution-sensitive taxa allows the detection of short-term adverse impacts, as demonstrated by the study of Höss et al. (2022), it is difficult to disentangle pollution-induced stress from that induced by the laboratory condition in such a setting. Starting with a poorly structured nematode community as in the present study, which closely resembles that of a bare farm soil, limits the possibility to detect short-term adverse effects. Yet, this approach enables to detect impacts on the natural development towards a structured and mature community which occurs when a soil is left undisturbed, even under laboratory conditions. Our findings suggest that the application of the biosimilar insecticide and the mixture at high dose prevent the nematode community from developing towards a more complex structure in the long term. Introducing an additional sampling point for extended-term evaluation may be beneficial for monitoring any further evolution of the community. Most of the microbial endpoints were not responsive, not even at high dose, and observed responses lacked of dose-dependent pattern. Hence, the analysis of the nematode community emerges as a distinct indicator of soil pollution.

In conclusion, studies on the nematode and microbial communities can be easily carried out at the laboratory scale, using morphology-based taxonomic identification of nematodes and rRNA-based molecular tools for microbial endpoints. This makes such an experimental setup a potential tool to complement standard endpoints for environmental risk assessments. However, the selection of the soil used in such a study is of importance as it defines the initial composition

of both nematode and microbial communities which account for a large degree the effects that are possible to detect in response to pesticide exposure.

SUPPLEMENTARY MATERIAL CHAPTER 4

Table S6. Results of the ANOVA analysis of the effect of the treatment-doses on the microbial endpoints 7 day after experiment start/short term (T7) and 42 days after experiment start/longer term (T42). The * indicates statistically significant difference.

Endpoint	T7			T42		
	Df	F	<i>p</i>	Df	F	<i>p</i>
16S	9	2.7496	0.0194*	9	4.3745	0.0011*
ITS	9	1.5365	0.1875	9	3.2683	0.0069*
AOA/16S	9	1.8320	0.1048	9	1.9430	0.0881
AOB/16S	9	1.3918	0.2404	9	0.7335	0.6752
COMAA/16S	9	1.3234	0.2679	9	0.8115	0.6097
COMAB/16S	9	1.1268	0.3761	9	1.7877	0.1173

Table S7. Mean and standard deviation of the microbial endpoints at experiment start (T0), 7 days/short term (T7) and 42 days/longer term (T42) after the application of a synthetic insecticide (I), a biosimilar insecticide (B) or their mixture (M) at 1x, 10x or 100x the agronomical dose. Letters denote statistically significant different groups according to the Fisher LSD post hoc test. If the ANOVA test did not yield significance, no letters indicating statistical differences between groups are present in the graph. 16S and ITS are expressed as gene copies/g of dry soil.

Time	Treat-dose	16S	ITS	AOA/16S	AOB/16S	COMAA/16S	COMAB/16S
T0		$6.4 \times 10^9 \pm 5.0 \times 10^8$	$1.6 \times 10^9 \pm 1.1 \times 10^8$	$6.1 \times 10^{-2} \pm 1.4 \times 10^{-3}$	$1.2 \times 10^{-2} \pm 8.9 \times 10^{-4}$	$5.8 \times 10^{-3} \pm 6.9 \times 10^{-4}$	$3.4 \times 10^{-3} \pm 2.5 \times 10^{-4}$
T7	control	$7.3 \times 10^9 \pm 8.7 \times 10^8$ a	$1.7 \times 10^9 \pm 3.5 \times 10^8$	$6.4 \times 10^{-2} \pm 7.6 \times 10^{-3}$	$1.3 \times 10^{-2} \pm 9.5 \times 10^{-4}$	$6.4 \times 10^{-3} \pm 6.4 \times 10^{-4}$	$6.4 \times 10^{-3} \pm 6.4 \times 10^{-4}$
	B-1x	$6.9 \times 10^9 \pm 4.1 \times 10^8$ ab	$1.6 \times 10^9 \pm 8.2 \times 10^8$	$5.9 \times 10^{-2} \pm 6.4 \times 10^{-3}$	$1.2 \times 10^{-2} \pm 1.2 \times 10^{-3}$	$7.0 \times 10^{-3} \pm 5.5 \times 10^{-4}$	$3.6 \times 10^{-3} \pm 3.7 \times 10^{-4}$
	B-10x	$7.2 \times 10^9 \pm 9.7 \times 10^8$ a	$1.9 \times 10^9 \pm 1.4 \times 10^8$	$6.4 \times 10^{-2} \pm 4.8 \times 10^{-3}$	$1.3 \times 10^{-2} \pm 6.5 \times 10^{-3}$	$7.0 \times 10^{-3} \pm 1.3 \times 10^{-3}$	$3.2 \times 10^{-3} \pm 3.6 \times 10^{-4}$
	B-100x	$7.1 \times 10^9 \pm 7.6 \times 10^8$ a	$1.6 \times 10^9 \pm 8.3 \times 10^8$	$5.5 \times 10^{-2} \pm 4.9 \times 10^{-3}$	$1.2 \times 10^{-2} \pm 1.0 \times 10^{-3}$	$6.1 \times 10^{-3} \pm 1.8 \times 10^{-4}$	$3.2 \times 10^{-3} \pm 4.3 \times 10^{-4}$
	I-1x	$5.9 \times 10^9 \pm 6.8 \times 10^8$ c	$1.9 \times 10^9 \pm 1.0 \times 10^9$	$6.6 \times 10^{-2} \pm 8.6 \times 10^{-3}$	$1.4 \times 10^{-2} \pm 1.1 \times 10^{-3}$	$6.5 \times 10^{-3} \pm 7.7 \times 10^{-4}$	$3.4 \times 10^{-3} \pm 2.4 \times 10^{-4}$
	I-10x	$7.3 \times 10^9 \pm 5.0 \times 10^8$ a	$1.6 \times 10^9 \pm 6.1 \times 10^7$	$6.2 \times 10^{-2} \pm 5.5 \times 10^{-3}$	$1.2 \times 10^{-2} \pm 1.1 \times 10^{-3}$	$6.4 \times 10^{-3} \pm 1.2 \times 10^{-3}$	$3.5 \times 10^{-3} \pm 5.0 \times 10^{-4}$
	I-100x	$7.3 \times 10^9 \pm 3.6 \times 10^8$ a	$1.8 \times 10^9 \pm 2.6 \times 10^8$	$6.5 \times 10^{-2} \pm 9.7 \times 10^{-3}$	$1.3 \times 10^{-2} \pm 2.3 \times 10^{-3}$	$6.6 \times 10^{-3} \pm 1.1 \times 10^{-3}$	$3.2 \times 10^{-3} \pm 6.0 \times 10^{-4}$
	M-1x	$6.1 \times 10^9 \pm 3.6 \times 10^8$ bc	$1.5 \times 10^9 \pm 9.8 \times 10^7$	$5.6 \times 10^{-2} \pm 4.4 \times 10^{-3}$	$1.4 \times 10^{-2} \pm 2.1 \times 10^{-3}$	$6.6 \times 10^{-3} \pm 4.1 \times 10^{-4}$	$3.5 \times 10^{-3} \pm 1.3 \times 10^{-4}$
	M-10x	$6.7 \times 10^9 \pm 3.8 \times 10^8$ abc	$1.7 \times 10^9 \pm 8.8 \times 10^8$	$6.6 \times 10^{-2} \pm 3.4 \times 10^{-2}$	$1.4 \times 10^{-2} \pm 7.1 \times 10^{-3}$	$7.3 \times 10^{-3} \pm 3.7 \times 10^{-3}$	$3.8 \times 10^{-3} \pm 2.0 \times 10^{-3}$
	M-100x	$7.6 \times 10^9 \pm 7.4 \times 10^8$ a	$1.8 \times 10^9 \pm 1.4 \times 10^8$	$5.5 \times 10^{-2} \pm 5.9 \times 10^{-3}$	$1.2 \times 10^{-2} \pm 4.0 \times 10^{-4}$	$5.6 \times 10^{-3} \pm 3.0 \times 10^{-4}$	$3.1 \times 10^{-3} \pm 4.4 \times 10^{-4}$
T42	control	$7.3 \times 10^9 \pm 6.4 \times 10^8$ ab	$1.7 \times 10^9 \pm 3.1 \times 10^8$ abc	$6.7 \times 10^{-2} \pm 3.4 \times 10^{-2}$	$1.4 \times 10^{-2} \pm 1.4 \times 10^{-3}$	$6.7 \times 10^{-3} \pm 7.4 \times 10^{-4}$	$3.7 \times 10^{-3} \pm 2.7 \times 10^{-4}$
	B-1x	$6.4 \times 10^9 \pm 4.9 \times 10^8$ bc	$1.5 \times 10^9 \pm 1.4 \times 10^8$ bcd	$6.1 \times 10^{-2} \pm 5.9 \times 10^{-3}$	$1.4 \times 10^{-2} \pm 2.3 \times 10^{-3}$	$6.3 \times 10^{-3} \pm 5.4 \times 10^{-4}$	$3.4 \times 10^{-3} \pm 2.2 \times 10^{-4}$
	B-10x	$6.2 \times 10^9 \pm 6.8 \times 10^8$ c	$1.6 \times 10^9 \pm 1.7 \times 10^8$ bcd	$6.3 \times 10^{-2} \pm 7.4 \times 10^{-3}$	$1.3 \times 10^{-2} \pm 6.6 \times 10^{-4}$	$7.5 \times 10^{-3} \pm 1.2 \times 10^{-3}$	$3.3 \times 10^{-3} \pm 2.3 \times 10^{-4}$
	B-100x	$5.9 \times 10^9 \pm 3.1 \times 10^8$ c	$1.4 \times 10^9 \pm 1.3 \times 10^8$ cd	$6.0 \times 10^{-2} \pm 3.7 \times 10^{-3}$	$1.4 \times 10^{-2} \pm 7.9 \times 10^{-4}$	$6.9 \times 10^{-3} \pm 9.7 \times 10^{-4}$	$3.7 \times 10^{-3} \pm 3.1 \times 10^{-4}$
	I-1x	$7.2 \times 10^9 \pm 2.2 \times 10^8$ ab	$1.7 \times 10^9 \pm 3.0 \times 10^8$ ab	$6.4 \times 10^{-2} \pm 5.9 \times 10^{-3}$	$1.4 \times 10^{-2} \pm 8.9 \times 10^{-4}$	$6.8 \times 10^{-3} \pm 8.0 \times 10^{-4}$	$3.9 \times 10^{-3} \pm 5.8 \times 10^{-4}$
	I-10x	$5.8 \times 10^9 \pm 7.4 \times 10^8$ c	$1.4 \times 10^9 \pm 1.7 \times 10^8$ d	$6.6 \times 10^{-2} \pm 3.5 \times 10^{-3}$	$1.4 \times 10^{-2} \pm 1.3 \times 10^{-3}$	$6.9 \times 10^{-3} \pm 1.2 \times 10^{-3}$	$4.2 \times 10^{-3} \pm 2.5 \times 10^{-4}$
	I-100x	$7.3 \times 10^9 \pm 5.4 \times 10^8$ ab	$1.7 \times 10^9 \pm 1.2 \times 10^8$ ab	$5.9 \times 10^{-2} \pm 3.0 \times 10^{-2}$	$1.2 \times 10^{-2} \pm 6.2 \times 10^{-3}$	$7.3 \times 10^{-3} \pm 7.1 \times 10^{-4}$	$3.7 \times 10^{-3} \pm 5.2 \times 10^{-4}$
	M-1x	$7.4 \times 10^9 \pm 1.0 \times 10^9$ a	$2.0 \times 10^9 \pm 3.6 \times 10^8$ a	$6.7 \times 10^{-2} \pm 4.4 \times 10^{-3}$	$1.4 \times 10^{-2} \pm 9.9 \times 10^{-4}$	$7.2 \times 10^{-3} \pm 5.3 \times 10^{-4}$	$3.9 \times 10^{-3} \pm 6.2 \times 10^{-4}$
	M-10x	$6.2 \times 10^9 \pm 7.2 \times 10^8$ c	$1.5 \times 10^9 \pm 2.3 \times 10^8$ bcd	$6.4 \times 10^{-2} \pm 1.1 \times 10^{-3}$	$1.4 \times 10^{-2} \pm 9.8 \times 10^{-4}$	$7.2 \times 10^{-3} \pm 9.3 \times 10^{-4}$	$3.8 \times 10^{-3} \pm 3.9 \times 10^{-4}$
	M-100x	$7.6 \times 10^9 \pm 7.5 \times 10^8$ a	$1.8 \times 10^9 \pm 1.2 \times 10^8$ ab	$5.4 \times 10^{-2} \pm 3.7 \times 10^{-3}$	$1.3 \times 10^{-2} \pm 2.5 \times 10^{-3}$	$6.3 \times 10^{-3} \pm 8.6 \times 10^{-4}$	$3.4 \times 10^{-3} \pm 4.6 \times 10^{-4}$

Table S8. Abundance of the nematode families at experiment start (T0), 7 days/short term (T7) and 42 days/longer term (T42) after the application of a synthetic insecticide (I), a biosimilar insecticide (B) or their mixture (M) at 1x, 10x or 100x the agronomical dose.

Family% (c-p value)	T0	Control		B-1x		B-10x		B-100x		I-1x		I-10x		I-100x		M-1x		M-10x		M-100x	
		T7	T42	T7	T42	T7	T42	T7	T42	T7	T42	T7	T42	T7	T42	T7	T42	T7	T42	T7	T42
Bacterivores%	57.4	60.6	53.4	61.5	46.8	56.1	58.6	58.1	63.2	59.4	52.4	63.9	59.5	61.9	55.5	57.8	51.7	58.3	54.5	70.7	68.5
<i>Alaimidae</i> (4)	0.5	0.5	0.5	-	-	-	1.2	0.3	-	0.5	-	0.5	1.3	0.5	0.3	0.5	0.7	0.3	0.8	0.2	0.5
<i>Cephalobidae</i> (2)	11.6	17.3	27.8	22.9	22.9	22.9	25.2	18.3	25.0	18.1	25.4	14.7	25.5	17.6	31.3	18.2	25.4	22.6	26.0	17.4	29.3
<i>Diplogastridae</i> (1)	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Monhysteridae</i> (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-
<i>Neodiplogastridae</i> (1)	-	-	1.8	-	-	-	-	-	-	-	1.0	-	-	0.3	0.5	-	-	0.3	-	0.3	-
<i>Panagrolaimidae</i> (1)	15.9	14.3	4.3	17.0	3.2	12.9	5.5	12.4	2.2	14.2	5.2	17.4	3.8	18.3	3.0	14.5	6.0	12.9	2.5	10.4	4.2
<i>Plectidae</i> (2)	1.5	-	1.5	0.2	1.0	1.0	0.8	-	2.3	0.5	2.0	-	0.3	0.3	1.0	0.5	1.0	0.3	2.8	-	1.2
<i>Rhabditidae</i> (1)	27.9	28.5	17.6	21.1	19.6	19.3	25.9	27.2	33.7	25.9	18.7	31.1	28.8	24.7	19.5	24.1	18.7	22.1	22.5	41.6	33.2
<i>Teratocephalidae</i> (3)	-	-	-	-	-	-	-	-	-	0.2	-	0.3	-	0.3	-	-	-	-	-	0.5	-
Fungivores%	10.1	4.0	6.0	5.9	7.0	7.5	4.7	6.2	6.5	8.9	4.7	5.2	6.0	4.5	7.0	7.5	8.0	6.2	8.5	6.0	7.0
<i>Anguinidae</i> (2)	1.3	-	0.8	-	0.5	0.5	0.8	-	0.2	0.5	0.3	0.3	0.5	0.5	1.5	-	0.8	0.3	1.3	-	0.7
<i>Aphelenchidae</i> (2)	2.8	2.0	3.2	2.2	2.0	2.5	1.2	2.7	1.3	5.9	1.5	2.5	2.0	1.7	2.0	4.0	2.7	3.0	4.3	3.5	3.0
<i>Aphelenchoididae</i> (2)	6.1	2.0	2.0	3.7	4.5	4.5	2.7	3.5	5.0	2.5	3.0	2.5	3.5	2.2	3.5	3.5	4.5	3.0	3.0	2.5	3.2
Herbivores%	31.5	34.2	35.3	31.6	39.1	34.5	32.2	33.7	27.3	30.0	36.9	27.1	29.3	32.6	31.8	33.2	37.1	34.0	34.3	22.9	23.1
<i>Dolichodoridae</i> (3)	2.8	4.2	3.8	2.3	3.2	4.0	3.2	2.5	0.7	2.5	5.5	3.7	2.0	3.0	2.5	3.7	5.0	2.5	3.3	0.7	1.7
<i>Hoplolaimidae</i> (3)	5.5	3.2	6.3	2.7	7.7	6.4	3.8	6.2	1.8	4.2	4.0	5.5	3.8	4.7	4.3	5.7	3.0	3.8	3.0	2.7	3.0
<i>Paratylenchidae</i> (2)	2.3	2.7	1.5	3.4	1.7	1.0	0.5	1.7	-	1.7	0.8	1.5	3.0	1.7	1.8	2.0	3.0	4.4	2.3	2.2	0.2
<i>Psilenchidae</i> (2)	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Pratylenchidae</i> (3)	6.1	3.0	3.0	5.2	5.0	3.7	3.5	3.2	3.0	4.4	3.5	2.7	3.0	2.5	4.8	2.5	3.7	5.2	4.0	4.7	4.2
<i>Tylenchidae</i> (2)	14.9	21.1	20.8	18.0	21.4	19.1	21.2	20.1	21.7	17.1	23.2	13.7	17.5	20.7	18.5	19.3	22.4	18.1	21.8	12.4	13.9
Omnivores%	0.8	1.2	4.8	1.0	7.0	2.0	4.2	1.2	2.8	1.5	5.7	3.5	4.8	0.5	5.3	0.8	3.2	1.5	2.8	0.2	1.5
<i>Aporcelaimidae</i> (5)	-	0.5	3.5	0.3	2.7	1.5	2.0	0.5	1.8	0.7	3.2	1.5	2.5	0.3	2.8	-	2.2	0.3	1.5	0.2	0.5
<i>Qudsianematidae</i> (4)	0.5	0.7	1.3	0.7	4.2	0.5	2.2	0.7	1.0	0.7	2.2	2.0	2.3	0.3	2.5	0.8	1.0	1.2	1.3	-	1.0
<i>Thornenematidae</i> (5)	0.3	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-
Carnivores%	0.3	-	0.5	-	0.2	-	0.3	0.7	0.3	0.2	0.3	0.3	0.5	0.5	0.5	0.8	-	-	-	0.3	-
<i>Mononchidae</i> (4)	0.3	-	0.5	-	0.2	-	0.3	0.7	0.3	0.2	0.3	0.3	0.5	0.5	0.5	0.8	-	-	-	0.3	-

Table S9. Results of the ANOVA analysis of the effect of the treatment-doses on the nematode endpoints 7 days after experiment start/short term (T7) and 42 days after experiment start/longer term (T42). The * indicates statistically significant difference.

Endpoint	T7			T42		
	Df	F	<i>p</i>	Df	F	<i>p</i>
Abundance	9	1.8700	0.0968	9	1.9602	0.0808
H'	9	1.5001	0.1931	9	3.1432	0.0087*
BAC%	9	2.1380	0.0575	9	3.3161	0.0064*
FUN%	9	0.9049	0.5336	9	0.7334	0.6755
HERB%	9	1.9096	0.0890	9	4.5373	0.0008*
OMN%	9	1.0517	0.4246	9	2.2737	0.0443*
CAR%	NA	NA	NA	NA	NA	NA
MI	9	1.0299	0.4398	9	2.3362	0.0393*
MI 2.5	9	1.4899	0.1968	9	2.1956	0.0515
PPI	9	0.4113	0.9190	9	1.1534	0.3586
EI	9	2.1661	0.0545	9	0.9835	0.4733
CI	9	1.1957	0.3336	9	0.8270	0.5968
SI	9	1.1654	0.3514	9 ⁺	16.3780 ⁺	0.0594 ⁺

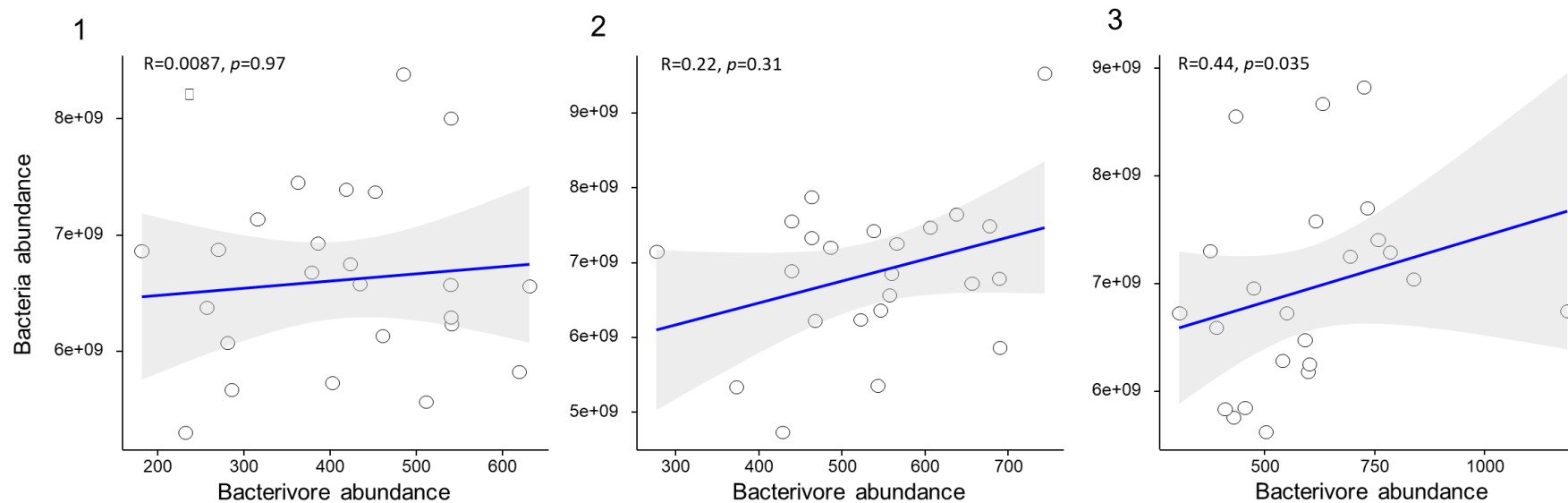


Figure S16. Pearson correlations between the absolute bacterivore abundance (total number of bacterivores/100 g of dry soil), and the absolute number of bacteria (gene copies/g of dry soil) for the soil treated with the biosimilar insecticide (1), conventional insecticide (2) and their mixture (3), independently from the dose and time of sampling. R is the correlation coefficient, and p is the p-value.

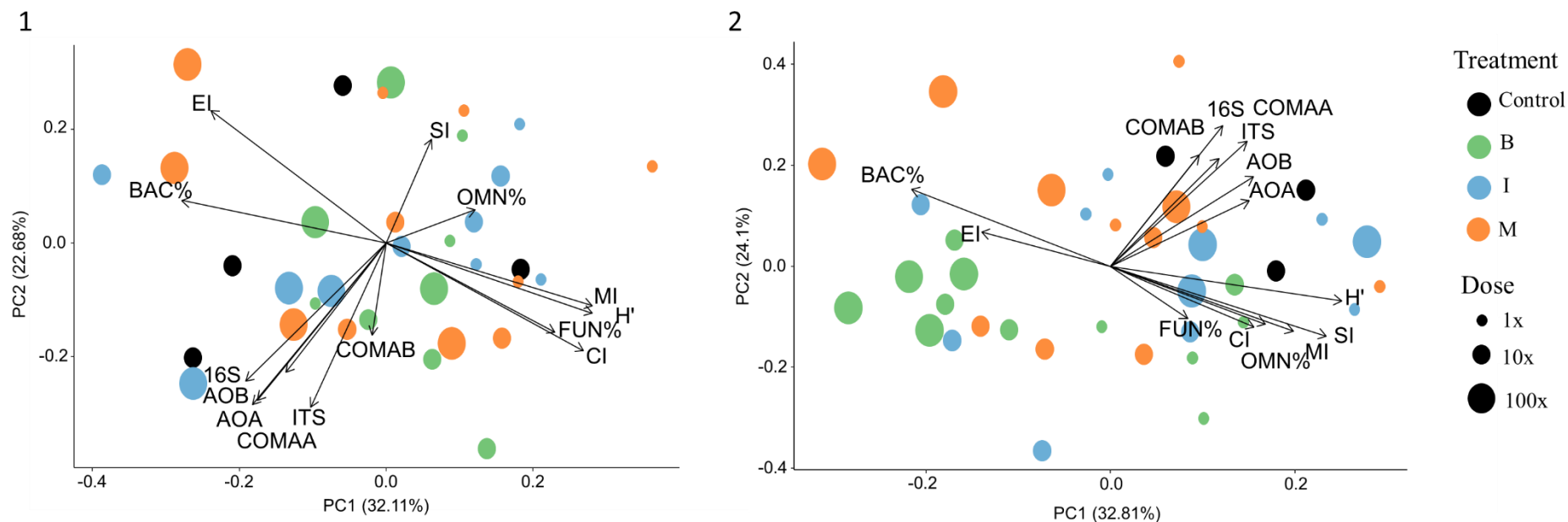


Figure S17. PCAs of the nematode and microbial endpoints and indices measured 7 days/short term (1) and 42 days/longer term (2) after the application of a synthetic insecticide (I), a biosimilar insecticide (B) or their mixture (M) at 1x, 10x or 100x the agronomical dose. The PC1 and PC2 axes represent the two main principal components capturing the maximum variability of the data. Arrows are the direction and magnitude of each endpoint's contribution to the PC1 and PC2. Each point corresponds to a sample, the color indicates the treatment and the point dimension the dose.

Chapter 5

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Worldwide conventional agriculture remains the dominant production system, which is highly dependent on the use of agrochemicals to grow (chemical fertilizers) and protect (synthetic pesticides) the crops. The intensive crops production allows the provision of food and fibers for a growing world population, but has numerous side effects on environment and human's health. Therefore, there is an urgent need to decrease the agrochemicals dependency by developing and adopting new cropping systems, such as agroecology, which implement crop diversification and bio-based solutions, and would enable to reach a more sustainable agriculture in the long term. Nonetheless, cropping systems with any agrochemicals input (*i. e.* zero pesticides) appear today difficult to reach due to numerous agronomical, ecological, economical and societal factors. One could imagine future cropping systems to be more diversified, mobilizing new technologies like robotics and bio-based solutions (biocontrol and biostimulation products), but also chemical based solutions applied as ultimate solutions to protect the crops as recommended by integrated pest management principles (IPM).

There is a need to better understand agrochemicals side effects. To do so, new tools must be developed to fulfill the precaution principle and be conservative enough to preserve the One Health. Until now, the pesticides' side effects are assessed *a priori* (before their release to the market), to evaluate their ecotoxicity on a range of non-target organisms living in different environmental compartments. When studying the effect of pesticides on soil microorganisms, only effects on C and N mineralization are/were measured up to now. This is far too few considering the vast knowledge on microorganism ecological functions accumulated during the past two decades. Other important key players in the soil food web, such as free-living nematodes, are not even considered in the current ERA of pesticides. The *a priori* ERA evaluates the active ingredients which are registered at the European level. The formulation, which comprise other chemical

ingredients, are also subjected of ERA, but regulated at the member state level as established by the Reg. (EC) No 1107/2009. Contrary to water and air compartments which are protected by the EU water and air framework directives, a *posteriori* risk assessment of pesticides on in-soil living organisms is not done at all. This is mainly due to the absence of regulatory requirements, although projects like “LUCAS” (Orgiazzi et al., 2022) highlighted the presence of multiple pesticide residues in most of the European soils. Furthermore, a *posteriori* ERA of pesticide residue mixtures is seldomly assessed in academic research. Understanding the fate and the impact of multiple pesticide residues on the different ecosystem compartments is a major challenge to preserve the One Health and to maintain sustainable cropping production system in the global change context. There is an urgent need to better assess the ecotoxicological effect of pesticide mixture on in soil living organisms and their supported ecological functions in interaction with abiotic stresses related to climate change. With this general aim in mind, I conducted my PhD work to assess the impact of pesticide mixtures on in soil living organisms (microbes and nematodes endpoints) under a lab-to-field experimental design following the tier scenario of exposure recommended by EFSA to assess ERA of pesticides.

Pesticide mixture risk assessment

As mentioned before, mixtures of pesticides residues are often found in soils. This has been reported in several studies conducted worldwide (Froger et al., 2023; Silva et al., 2019). These complex mixtures originate from the sequential application of plant protection products applied during the cropping cycles, and their long-term persistence. Therefore, trying to assess the impact of pesticide mixtures is not a trivial task and needs to draw original experimental design to tackle this challenge. We were able to investigate this topic under different aspects and with increasing complexity by adopting a lab-to-field approach, *i. e.* first evaluating the effect of the mixture of three different active

ingredients (one herbicide, one fungicide and one insecticide plausibly applied to protect corn crop) in combination with abiotic stresses related to climate change (heat and drought stresses) on the abundance, structure, diversity and activity of microbial community (Chapter 2). We studied then the effect of a sequential application of formulated pesticides on the abundance, structure, diversity of indigenous soil microbial community and on nematode abundance (Chapter 3). Lastly, we focused more specifically on the nematode community, to assess the ecotoxicological effect of a mixture of a conventional and a biosimilar insecticide (Chapter 4). The result show that none of the studied active ingredients/pesticide mixtures had a stronger impact on the microbial and nematode communities as compared to the single active ingredients/pesticide tested. In Chapter 2, we demonstrated that abiotic stresses, such as heat, have a major impact on the microbial community, compared to the active ingredients and their mixture which transitionally affect the microbial community. In Chapter 3, where we tested formulations containing similar active ingredients to that tested in Chapter 2, we observed that the bacterial community was mostly impacted by the field soil properties, while the fungal community responded more sensitively to pesticide application. Limited effects were shown on the nematode abundance. In Chapter 4, the mixture effect on the nematode community can be inferred to the biosimilar insecticide, with significant effects only at higher dosages.

Over the past years, models such as the concentration addition (CA) and independent action (IA) have been proposed to predict pesticide mixture toxicity. Both models assume that each pesticide entering in the composition of a given mixture acts additively. Consequently, these models can only correctly predict additive effects. The CA model was developed for mixture of molecules having the same mode of action, wherein each constituent's impact is determined by its concentration. The IA model was designed for mixture of molecules with dissimilar mode of actions and the components below their NOEC do not

contribute in the mixture toxicity (Taenzler et al., 2023). Studies on the application of these models to aquatic organisms dominate the literature. In 2013, Rodney et al. reviewed the available literature on the application of these models on aquatic tests. The CA model was able to successfully predict the toxicity of most of the reported studies (Belden et al., 2007; Coors and Frische, 2011; Deneer, 2000), and the authors concluded that the CA can be used to predict pesticide mixture at environmentally relevant concentration effect in aquatic systems. On the other hand, the literature lacks studies proving the effectiveness of these models on terrestrial organisms, for which the responses are not always well predicted (Gomez-Eyles et al., 2009; Guimarães et al., 2018; Martin et al., 2009). While our experimental were not suited for the application of the CA and IA models, as no dose-response curve could be withdrawn for lack of tested doses, our statistical results suggest that no stronger effect than additive are likely to have occurred within our experiments. With our lab-to-field approach we were not able to quantify experimentally the synergism and additive ecotoxicological effects on the endpoints measured targeting microbes and nematodes, neither under controlled conditions using pure active ingredients, nor in real conditions with formulated PPPs. We showed that the effect of abiotic stresses related to climate change was stronger than that of active ingredient. Only transient effects of either active ingredient or formulated PPPs were recorded. This is somehow in agreement with biogeographical studies carried out in the framework of the French soil monitoring survey showing that the factors affecting the abundance, structure and diversity of soil microorganisms are highly influenced by soil physicochemical properties and land use (Christel et al. 2024). In addition, the pesticides tested in the experiments were not enough persistent to test their synergisms.

Despite the efforts to bring new clues to tackle the pesticide mixture toxicity effect on soil living organisms, I must recognize that I was not able to

quantify experimentally any synergism, rather just conclude that no stronger effect than additivity could be observed. This conclusion derives from three experiments in which we studied only few active ingredients (over the 400 available on the market) on two different endpoints. One must recognize the significant amount of work hidden under the presented results, which suggest the impracticability to impose so for all active ingredients authorized in EU, applied on different crops under different pedoclimatic conditions.

Many step forward on the mixture problem were made with the publication of the EFSA *Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals* (EFSA Scientific Committee et al., 2019). The Guidance's concepts for addressing mixture toxicity were applied by Franco et al., (2024), which combined the residue data of the LUCAS project with the residue's NOEC from toxicity databases to retrieve hazard metrics of 81 out of the 118 pesticide residues detected in each sampling. The residues' mixture toxicity (RI) was calculated by summing the risk quotient of each residue using the formulas:

$$RI_{\text{soil,NOEC}_{\text{min}}} = \sum_{i=1}^n \frac{C_{\text{soil},i}}{\text{NOEC}_{\text{soil}_{\text{min}},i}} \times 5,$$

$$RI_{\text{soil,NOEC}_{50}} = \sum_{i=1}^n \frac{C_{\text{soil},i}}{\text{NOEC}_{\text{soil}_{50},i}},$$

Where $C_{\text{soil},i}$ is the concentration of the substance i in the mixture, $\text{NOEC}_{\text{soil}_{\text{min}},i}$ and $\text{NOEC}_{\text{soil}_{50},i}$ are the minimum and median soil NOECs of the active ingredient i in the mixture and 5 is the assessment factor. This study helped not only withdrawing ecological risk metrics of pesticide residues mixtures, but also understating that their risk is mostly driven only by one residue, while few others insignificantly contributed to the RI. Given the impracticality of assessing every potential unintentional pesticide combination ending up in the environment,

studies like that of Franco et al. (2024), together with recurrent sampling project like LUCAS serve as virtuous example for detecting the most relevant pollutants and deriving of risk metrics using indications from EFSA Scientific Committee et al. (2019).

Eventually, employing sustainable pest control methods such as IPM and biopesticides, which are designed to rapidly degrade in the environment, is possibly the only way to practically reduce the persistence and occurrence of active ingredients in the environment. One potential drawback is that biopesticides may require more frequent applications compared to conventional pesticides. This could potentially lead to increased environmental impact if not managed properly. It is important to carefully consider the overall sustainability of these practices and to monitor their impact on the environment over time.

Improving the current environmental risk assessment for soil microbes and nematodes

The primary objective of the ARISTO project to which my PhD is taking part is to investigate new tools and bioassays that could *in fine* be implemented to improve the current ERA of pesticides framework, to be more protective for in-soil living organisms, including various microbial functional guilds including ammonia oxidizers and mycorrhizal fungi. This process follows a tiered-approach, progressively advancing from basic *in vitro* assessments to comprehensive investigations at lab and field scales to assess ecotoxicological impact of active ingredient on the abundance, structure, diversity and activity of microbial communities. Contemporary molecular tools based on nucleic acid extraction and purification from soils and further analyses based on polymerase chain reaction (PCR) to amplify and quantify, or sequence amplicons to assess the abundance or the structure and diversity of a microbial guild, can be used to investigate the ecotoxicological effect of active ingredient entering in the composition pesticide on soil communities. These molecular tools offer a robust

means of analysis which enables detecting shifts of community and diversity loss, aspects that can undermine soil microbial functioning and eventually soil health. However, until now they are suffering from a lack of normal operating range values (NOR), which would delineate the extent to which the community can be affected before experiencing a significant functional effect, allowing the interpretation of the results obtained in terms of significance of effect. In addition, despite the DNA extraction and quantification of microbial groups by qPCR have been standardized at International Standard Organization (ISO 11063 and ISO 17601, respectively), amplicon sequencing and further bioinformatic analyses have not yet been standardized by ISO. Only recommendations have been delivered by the Earth Microbiome Project. Therefore, the results obtained by the different labs cannot be easily compared. Additionally, the protocols and materials applied diverged, which make even more difficult the deduction of general conclusions. These technical issues combined with the lack of a clearly defined safe NOR for each microbial endpoint make it difficult to implement it right now in the ERA of pesticides. Therefore, further work is needed to standardize high throughput sequencing methods, build database and model to establish normal operating range and referential system to interpret the effects observed following pesticide exposure.

Nonetheless, despite the current limitations mentioned above, quantification (qPCR) and sequencing (Hi-Seq or Mi-Seq) data are of particular interest for the registration of microbial pesticides within the European regulatory framework as they can be used to monitor their persistence in the soil environment. Indeed, like conventional pesticides, microbial pesticides are governed by Regulation 1107/2009, which is coupled with Regulation 2022/1439, 1440 and 1441 for specific data requirements for microbial pesticides. When considering the environmental impact of microbial pesticides, it is crucial to assess their fate and related ecotoxicological impact in the soil as they may

compete with the indigenous soil microbiota and, if successful, colonize habitats in soil and displace indigenous populations. Therefore, the *a priori* ERA of microbial pesticide must ensure that they do not pose any risk to the natural microbial community. The required data can be sourced from literature reviews, or experimental studies. However, until now there is no specific guidance provided on which experimental tests to be utilized. In this case, qPCR approaches can be viewed as a way to monitor the persistence of microbial pesticides and amplicon sequencing as a tool to monitor the effect of microbial pesticides on soil microbial community as compared to untreated soil or to soil treated with the equivalent chemical active ingredient. This would provide information on its persistence in the soil and on its effect on soil microbial community overtime.

Nitrifier guild

Functional microbial guilds involved in N-cycle such as ammonia oxidizing organism have been identified as suitable endpoints by EFSA (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017) to be included in the ERA of pesticides (Karpouzas et al., 2022). The reasons include their relevant ecological function, and their proven sensitivity to various pesticides (Fang et al., 2018; Karas et al., 2018; Singh et al., 2015). In Chapter 2 we showed that the application of different active ingredients can induce changes in the nitrogen cycle to different levels. The changes were mainly related to the N mineral pools, but also to some N cycle guilds, and recovered in the long term. The differences were evident already at low dose of pesticides and prove the connection between N guilds abundance and their functionality in the N cycle.

Nematode guild

In Chapter 4 we demonstrated that the application of a mixture of a biosimilar insecticide and a conventional insecticide, as well as of the biosimilar alone lead to a poor development of the nematode community in the long term. The communities in the mixture and biosimilar at high dose were mainly

composed by bacterivores with low c-p value, which resulted into a highly disturbed nematode community. The Shannon index indicated a poorly balanced community, where few bacterivores families were very abundant. These observations indicate that nematodes are responsive to pesticide exposure and that generally they recover from the stress caused by pesticide exposure.

As mentioned earlier in this thesis, ecotoxicological studies on the nematode community are scarce, especially for what concerns pesticide application. In addition, no nematode endpoint is analyzed in the current ERA of pesticides. Consequently, the impacts of the agrochemicals on nematodes are not well understood and most likely underestimated, making it difficult to develop effective risk assessment strategies and protection goals. From our results it is not possible to identify a specific family sensitive to pesticides, as the whole bacterivore community was strongly boosted by it and its composition changed overtime. Therefore, using a single nematode taxon, as the reference nematode *Caenorhabditis elegans* (fam. Rhabditidae) in the risk assessment would not be suitable. On the other hand, the few researches available on the effect of pesticides on nematode always detect community shifts to many levels after pesticide application (Höss et al., 2022; Waldo et al., 2019). Different responses are due to the initial community compositions, just like the expected response. One would expect a strong short-term impact on a well structure community, with the perishing of slow growing nematodes. Incorporating a comprehensive assessment of the entire nematode community into the ERA of pesticides would be the ideal approach for evaluating the risks posed to the soil nematode community. Moreover, nematode community studies offer the advantage of indirectly characterizing all interacting compartments by simply profiling the entire community, which would indirectly provide many information on different layers of the soil food web dynamics. Given the possibility of observing a decline in the number of nematodes "persisters" in microcosm studies (Höss et al., 2022), it

might be more appropriate to use a less mature community for this type of research such as the one analyzed in Chapter 4, which demonstrated resilience to laboratory conditions. On the other hand, no conclusions can be drawn regarding the responses of nematodes classified as sensitive to perturbations, such as carnivores and omnivores in the short term. One of the limitations of taking on board nematodes is the fact that their observation is time consuming and not automatized yet. Despite the availability of molecular markers to determine the taxonomy of nematode, it is difficult to apply it to soil because of the problem of sampling and of sample representativeness. Indeed, actual direct soil DNA extraction start from a few hundred of mg to a few g of soils which is far not enough for having a sampling effort representative of nematodes community in soil. eDNA (extracellular) extraction from soil samples can overcome this limitation as larger amount of soil samples can be washed with different solutions (TE Buffer or other) and from which eDNA can be recovered (Bairoliya et al. 2022). Recently, eDNA metabarcoding was used to characterize nematode communities of 48 glacier forelands from five continents (Guerrieri et al. 2024) starting from 75g sample per site, close to the values proposed by Wiesel et al. (2015) to study nematode communities, suggesting that in a near future it might be implemented to assess the impact of pesticide exposure on nematode communities.

CONCLUSION AND FUTURE PERSPECTIVES

Soil microbes and nematodes contribute to the delivery of major soil ecosystem services. Their activity and function can be perturbed by agricultural practices such as pesticide application. The results presented in my PhD manuscript showed the pattern of responses of microbial and nematode to the exposure to different pesticides applied in a mixture in lab experiment or sequentially one after the other during a cropping cycle in a field experiment. Our main findings are that:

- Environmental stressors, such as heat stress, have a major impact on the microbial community, especially on its diversity and on the nitrogen cycle, with little recovery overtime. The pesticide active ingredients and their mixture have few and transient effects on the microbial community, affecting mainly the nitrogen cycle. These observations, suggest that ERA of pesticides should be carried out in systems submitted to other abiotic and biotic stresses related to global change and that the use of endpoints related to functional guilds is of interest for pesticide toxicity assessment.
- The soil spatial heterogeneity in field studies has a great impact on the microbial and nematode communities, and modulate the pesticide effect because of local properties impacting the fate of active ingredients.
- The application of a biosimilar insecticide at high dose contributes to major shifts of the nematode community, which is prevented from developing towards a more diverse and equilibrate community.
- The few mixture effects detected in this thesis could be regarded as additive, because no sign of synergism or antagonism could be statistically detected.

Up to now, the ERA of pesticides relies only on the N mineralization test as standard soil microbial endpoint, while no nematode endpoint is considered. From this thesis it can be inferred that molecular analyses are offering powerful tools that provide a comprehensive amount of information, which would otherwise remain concealed by solely relying on nitrogen transformation tests. For what concerns nematodes, we have proven that a small-scale microcosm experiment is suitable to assess pesticide toxicity towards nematodes. Consequently, it would be of interest to include them as endpoint in the ERA of pesticide. At present, identifying nematode communities necessitates trained specialists. However, with the continuous advancement of standardized protocols, this task could be accomplished in a near future with molecular based approaches

using eDNA metabarcoding. Higher tiers test using microbes and nematodes as endpoints should be planned carefully as the effects investigated could be biased and masked by the field spatial heterogeneity. Implementing sufficient treatment replicates and incorporating plot properties into the statistical analysis could aid in mitigating spatial variability. Concerning the assessment of pesticide mixtures on soil organisms, more research is needed to gain a better understanding of the combined effect of these mixtures on soil organisms, as well as the frequency of synergistic interactions. Following this, predictive models like those used in Franco et al. (2024), can continued to be used to forecast mixture toxicity also on soil organisms.

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