Metadata of the chapter that will be visualized online

Chapter Title	Assessing the Effects of Pesticides on the Soil Microbial Community: Advances, Standardization of Methods and the Need for a New Regulatory Framework	
Copyright Year	2021	
Copyright Holder	The Author(s), under exclusive license to Springer Nature Switzerland AG	
Corresponding Author	Family Name	**Karpouzas**
	Particle	
	Given Name	**Dimitrios G.**
	Suffix	
	Division	Laboratory of Plant and Environmental Biotechnology, Department of Biochemistry and Biotechnology
	Organization	University of Thessaly
	Address	Larissa, Greece
	Email	dkarpouzas@uth.gr
Abstract	Upon their application pesticides end up in soil where they interact with the soil microbial community. Considering the pivotal role of soil microorganisms in ecosystem homeostasis and the growing evidence about their potential toxicity response to pesticide exposure, there is an urgent need to revisit the relevant regulatory framework. This is necessary in light of the enormous methodological and standardization advances in soil microbial ecology in the last 20 years and the outdated assessment scheme currently in place. In this chapter we highlight the key elements of a new risk assessment scheme including (a) the definition of microbial indicator groups like ammonia-oxidizing microorganisms and arbuscular mycorrhizal fungi (b) the parallel determination of the level and the duration of the exposure including transformation products (c) the need for implementation in environmental risk analysis of advanced and standardized tools. Based on all these a new tiered-risk assessment scheme is proposed. Emerging issues in soil microbial ecotoxicology are discussed including (a) the assessment of pesticide soil microbial toxicity at ecosystem level and (b) the assessment of the soil microbial toxicity of biopesticides, pesticide mixtures and pesticide transformation products on soil microorganisms. We conclude by highlighting emerging scientific questions that are expected to puzzle the soil microbial ecotoxicologists working with pesticides in the next decade.	
Keywords (separated by '-')	Ammonia-oxidizing microorganisms - Arbuscular mycorrhizal fungi - Microbial ecotoxicity - Pesticides - Risk assessment - Soil microbial community	

Assessing the Effects of Pesticides on the Soil Microbial Community: Advances, Standardization of Methods and the Need for a New Regulatory Framework

Dimitrios G. Karpouzas

Contents

Abstract Upon their application pesticides end up in soil where they interact with the soil microbial community. Considering the pivotal role of soil microorganisms in ecosystem homeostasis and the growing evidence about their potential toxicity response to pesticide exposure, there is an urgent need to revisit the relevant regulatory framework. This is necessary in light of the enormous methodological and standardization advances in soil microbial ecology in the last 20 years and the outdated assessment scheme currently in place. In this chapter we highlight the key elements of a new risk assessment scheme including (a) the definition of microbial

D. G. Karpouzas (✉)
Laboratory of Plant and Environmental Biotechnology, Department of Biochemistry and Biotechnology, University of Thessaly, Larissa, Greece
e-mail: dkarpouzas@uth.gr

M. Sonia Rodríguez-Cruz and M. Jesús Sánchez-Martín (eds.),
Pesticides in Soils: Occurrence, Fate, Control and Remediation,
Hdb Env Chem, DOI 10.1007/698_2021_797,

indicator groups like ammonia-oxidizing microorganisms and arbuscular mycorrhizal fungi (b) the parallel determination of the level and the duration of the exposure including transformation products (c) the need for implementation in environmental risk analysis of advanced and standardized tools. Based on all these a new tiered-risk assessment scheme is proposed. Emerging issues in soil microbial ecotoxicology are discussed including (a) the assessment of pesticide soil microbial toxicity at ecosystem level and (b) the assessment of the soil microbial toxicity of biopesticides, pesticide mixtures and pesticide transformation products on soil microorganisms. We conclude by highlighting emerging scientific questions that are expected to puzzle the soil microbial ecotoxicologists working with pesticides in the next decade.

Keywords Ammonia-oxidizing microorganisms, Arbuscular mycorrhizal fungi, Microbial ecotoxicity, Pesticides, Risk assessment, Soil microbial community

1 Introduction

Pesticides are still the cornerstone of pest control in modern agriculture. However the focus of the pesticide market has shifted in the last 20 years from high dose–low potency chemicals, like triazines and organophosphates, to low dose–high potency active ingredients like neonicotinoids and sulfonylureas. This change coincided, at EU level and beyond, with the implementation of a stringent regulatory framework for pesticide registration reflecting the growing concern of the general public about the frequent detection of pesticide residual levels in fresh produce [1], natural water resources [2, 3] and soil [4]. This regulatory framework was built around the hallmark EC Directive 91/414 (available at https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX% 3A31991L0414), which describes the tests and procedures required for placing in the market a plant protection product (PPP). This was supplemented by (1) the water framework directive (2000/60/EC, available at https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX: 32000L0060), which identified several pesticides as priority water pollutants and (2) the EC Directive 128/2009 (available at https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32009L0128), which put into force procedures for the sustainable use of pesticides. Eventually, in 2009 91/414/EC was replaced by the EC Regulation 1107/2009 (available at https://eur-lex.europa.eu/ legal-content/EN/ TXT/?uri=celex%3A32009R1107), which now dictates how a PPP is granted authorization for use in the EU market.

Environmental risk assessment is based on a direct comparison between environmental exposure, determined by mathematical models fed with experimentally obtained environmental fate data, and ecotoxicological outputs derived from relevant studies. This assessment is performed along tiers of increasing complexity and

reduced conservatism, which determine the potential risk associated with the use of the given pesticide for the suggested use [5].

Aquatic and terrestrial ecotoxicology were pioneers in establishing a robust set of standardized methods to assess the toxicity of pesticides on macroorganisms. In contrast the potential toxicity of pesticides on soil microorganisms has been overlooked. Soil microorganisms are known as the growth engines of terrestrial ecosystems by (1) controlling several key reactions in nutrient cycling which modulate soil fertility and the production of greenhouse gases (GHG) [6], (2) interacting with crops with the outcome being often beneficial [7] (3) supporting soil structure [8] and (4) providing a wealth of functional biodiversity which could be exploited by biotechnology. In support of all these, the European Food Safety Authority (EFSA) highlighted the presence of regulatory gaps in the assessment of the toxicity of pesticides on soil microorganisms and identified soil microbes as a specific protection goal [9]. Still the assessment of the toxicity of pesticides on soil microorganisms is based on an outdated, crude and insensitive N mineralization test (OECD 216), which offers a lumped measurement of ammonification and nitrification rate in soil.

Hence there is an urgent need for revisiting the current framework regarding pesticides toxicity on soil microorganisms, especially in light of the major methodological advances that have occurred in soil microbiology in the last 15 years. In accordance with this, EFSA issued a scientific opinion [10] acknowledging the need for revising the relevant regulatory framework and suggested that (1) the N mineralization test is still inclusive and should be maintained for pesticide testing (2) new molecular and biochemical tools are available but not yet standardized, hence ready to be implemented in environmental risk assessment (3) functional microbial endpoints are more easily quantified compared to microbial diversity and (4) arbuscular mycorrhizal fungi (AMF) are good potential bioindicators for assessing the soil microbial toxicity of pesticides. In this review we will (1) define the key elements of a comprehensive assessment of the toxicity of pesticides on soil microorganisms (2) provide an update on the current knowledge regarding toxicity of pesticides on soil microorganisms, the methodological toolbox that is available and the level of its standardization and (3) highlight emerging research questions that should be the focus of future research in soil microbial ecotoxicology. Finally we conclude with our suggestion for a tiered risk assessment approach that could act as a core in the foreseen revision of the current regulatory framework regarding the toxicity of pesticides on soil microorganisms.

2 Key Elements of an Accurate Assessment of the Toxicity of Pesticides on Soil Microorganisms

The assessment of the toxicity of pesticides on the soil microbial community will require a list of necessary elements as follows:

- A thorough and detailed determination of the level and the duration of the exposure of the soil microbial community to the studied pesticides
- The utilization of advanced, high resolution methods which will be adequately standardized to determine with high accuracy the toxicity of pesticides on soil microorganisms
- The selection of appropriate endpoints and soil microbial groups that could act as bioindicators of the toxicity of pesticides to soil microorganisms
- A relevant tiered risk assessment scheme supported by guidelines for experimental setup at each tier

We will further present an update on each of these key elements and define which pieces of the puzzle are still missing.

2.1 Pesticide Exposure Measurement

Monitoring of pesticide dissipation and transformation in soil studies should constitute an integral part of any experiment aiming to assess the toxicity of pesticides on soil organisms, including soil microbiota. Such measurements enable us to define the level and the duration of exposure of the soil microbial community to the pesticide in question. Furthermore, determination of the transformation products (TPs) formed in soil during pesticide dissipation could clarify the role of TPs on effects observed. Correlation testing between the measured soil concentrations of pesticides and their TPs with temporal microbial measurements would point to the causal agent of the potential toxicity on the soil microbial community (parent vs TPs). Using such an approach Karas et al. [11] identified two demethylated products of the herbicide isoproturon, MD-IPU and DD-IPU, as key drivers of the reduced activity of acid and alkaline phosphatases in soils treated with various dose rates of isoproturon. Using a similar approach other research groups managed to distinguish the toxicity of (1) iprodione and 3,5-dichloroaniline [12] (2) tebuconazole and its TPs [13] (3) chlorpyrifos and trichloropyridinol [11].

2.2 Use of Advanced, High Resolution and Standardized Methods

Methodological advances in soil microbiology have revolutionized our view of soil microorganisms and their role in ecosystem functioning. The new molecular tools that became available from 1995 onwards unravelled an enormous microbial diversity in soil ecosystems, which was previously unattained due to our limited knowledge of their special nutritional needs [14]. The molecular and biochemical methods that are currently available could be categorized into two broad groups: (a) *functional tools* that measure either the activity of key microbial processes or

the dynamics of functional microbial groups and (b) *structural tools* that measure the diversity of the overall soil microbial community and its phylogenetically or functionally distinct components.

Standardization of methods constitutes a prerequisite for their implementation in pesticide environmental risk analysis. As EFSA suggested [10], standardization of functional methods in soil microbial ecology is more advanced compared to molecular tools for measuring microbial diversity. Biochemical tools measuring the rates of microbially-mediated reactions (e.g. nitrification, denitrification), the overall microbial activity (e.g. respiration) or the activity of microbial enzymes involved in biogeochemical cycling (phosphatases, arylsulfatases, aminopeptidases, chitinases, etc.) have been extensively used for assessing the toxicity of pesticides on soil microbial functioning [11, 12, 15–17]. These methods are characterized by high standardization level with several relevant ISO standards being available (Table 1). In addition, several of these methods, like the determination of activity of soil microbial exoenzymes [18] and soil microbial respiration through MicroResp® [19], have been modified for high-throughput use facilitating their implementation in rapid toxicity screening assays. Despite their high level of standardization these methods are still not used in pesticide environmental risk assessment. A possible reason for this is the general lack of consistency in their response to pesticide exposure. This has been demonstrated in a range of soil studies where pesticides applied at increasing dose rates did not impose a clear dose-dependent response [11, 20].

Molecular methods have been used in soil microbial ecology to determine the abundance (q-PCR), activity (RT-q-PCR) and diversity (PCR-based techniques) of soil microorganisms. The implementation of these methods in pesticide environmental risk analysis was until recently blocked by the lack of standardization [21]. This has changed in the last 10 years where ISO standards for soil DNA extraction (ISO11063) and determination of the soil microbial biomass via q-PCR (ISO 17601) were introduced, challenging the recent scientific opinion of EFSA regarding the low level of standardization of molecular methods [10]. Indeed several soil studies have used q-PCR methods to determine the effects of pesticides on the abundance of phylogenetical distinct microbial groups like bacteria, fungi or archaea or most importantly the abundance of functional microbial groups like ammonia-oxidizing microorganisms (AOM) [22–24], sulphur-oxidizing bacteria [11] and degraders of biogenic aromatic compounds [13].

Several tools are currently available for the determination of microbial diversity in soil, which vary in their level of standardization and phylogenetic resolution. Phospholipid Fatty Acid analysis (PLFAs) is a well-standardized method (ISO/TS29843-1 and -2), which provides information about the composition of the soil microbial community at low phylogenetic resolution. On the other hand, PCR-based molecular methods suffer from limited standardization, but they provide a deeper phylogenetic characterization of the composition of the soil microbial community. The depth of analysis of the soil microbial community offered by these methods has increased from the lower resolution of earlier fingerprinting methods like Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal

t1.1 **Table 1** A list of the ISO standardized methods that are currently available in soil microbiology

t1.2 Year	ISO code	Full title of standardized method
1997 t1.3	ISO14240:1	Determination of soil microbial biomass – part 1 substrate induced respiration method
1997 t1.4	ISO14240:2	Determination of soil microbial biomass – part 2 fumigation-extraction method
t1.5 2002	ISO16072	Laboratory methods for determination of microbial soil respiration
t1.6 2009	ISO10832	Effects of pollutants on mycorrhizal fungi- germination test
2010 t1.7	ISO/TS29843-1	Determination of soil microbial diversity – part 1: Method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis
2011 t1.8	ISO/TS29843-2	Determination of soil microbial diversity – part 2: Method by phospholipid fatty acid analysis (PLFA) using the simple PLFA extraction method
2012 t1.9	ISO15685	Determination of potential nitrification and inhibition of nitrification – rapid test by ammonium oxidation
2012 t1.10	ISO14238	Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes
2012 t1.11	ISO17155	Determination of abundance and activity of soil microflora using respiration curves
2016 t1.12	ISO17601	Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil
2016 t1.13	ISO18187	Contact test for solid samples using the dehydrogenase activity of *Arthrobacter globiformis*
2018 t1.14	ISO/TS20131-1	Easy laboratory assessments of soil denitrification, a process source of N_2O emissions – part 1: Soil denitrifying enzymes activities
2018 t1.15	ISO/TS20131-2	Easy laboratory assessments of soil denitrification, a process source of N_2O emissions – part 2: Assessment of the capacity of soils to reduce N_2O
2018 t1.16	ISO20130	Measurement of enzyme activity patterns in soil samples using colorimetric substrates in micro-well plates
2019 t1.17	ISO23753:1 ISO23753:1/AMD1:2020	Determination of dehydrogenase activity in soils – part 1: method using triphenyltetrazolium chloride (TTC) Determination of dehydrogenases activity in soils – part 1: Method using triphenyltetrazolium chloride (TTC) – Amendment 1
2019 t1.18	ISO23753:2 ISO23753:2/AMD1:2020	Determination of dehydrogenase activity in soils – part 2: method using iodotetrazolium chloride ~~(INT)~~ Determination of dehydrogenase activity in soils – part 2: method using iodotetrazolium chloride ~~(INT)~~ – amendment 2
2019 t1.19	ISO/TS22939	Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates
t1.20 2020	ISO11063	Method to directly extract DNA from soil samples

AU1

188 Restriction Fragment Length Polymorphism (TRFLP) [25, 26], to the higher reso-
189 lution of recent next generation amplicon sequencing approaches or the so-called
190 metataxonomics [27]. DGGE and TRFP, either as stand-alone approaches or in
191 combination with clone libraries, have been heavily used in the period of
192 2000–2015 to determine effects of pesticides on the diversity of phylogenetically

and functionally distinct microbial groups [28–31]. However these methods fail to provide accurate quantitative information on pesticide effects, especially on less abundant members of the soil microbiota. Since 2015 several studies have used metataxonomic approaches to identify effects of pesticides on the diversity of bacteria, fungi [32, 33] and distinct functional microbial groups like ammonia-oxidizing microorganisms (AOM) [12]. Benchmarking protocols for the preparation and setup of metataxonomic analysis of the soil bacterial (https://earthmicrobiome.org/protocols-and-standards/16s/) and fungal diversity (https://earthmicrobiome.org/protocols-and-standards/its) were developed by the Earth Microbiome Project (ECM) [34] and have been largely adopted by most recent pesticide soil ecotoxicity studies. Despite this major standardization step, we are still missing standardization at the bioinformatic handling of the sequencing data. A standardized pipeline for the bioinformatic analysis of amplicon sequencing data will make possible the full implementation of these powerful tools in the pesticide regulatory framework.

2.3 Microbial Endpoints and Bioindicators

Assessment of the toxicity of pesticides on aquatic organisms but also on terrestrial macrobiota relies on tests performed with single species from different trophic levels identified as bioindicators. Such examples are *Daphnia magna* for aquatic invertebrates, *Oncorhynchus mykiss* for fishes and *Eisenia fetida* for earthworms [10, 35]. All these bioindicator species were selected based on (1) their key ecological role (2) their higher sensitivity, compared to other species in the same group of organisms (3) their meaningful ecotoxicological response to toxicants (4) our good knowledge of their life cycle and (5) the existence of assays, protocols and methods to determine their response to pesticides. In accordance with all these, we need to identify microbial groups which fulfil all or most of the above criteria as candidate bioindicators for assessing the toxicity of pesticides on soil microorganisms.

Several groups of soil microorganisms have been proposed or used such as microbial indicators including AOM [36], AMF [10, 23], N-fixing bacteria [37], protists [38] and microalgae [39]. Most studies have focused on the first two microbial groups, in line with their key functional role in terrestrial ecosystems, although protists have also attracted attention in recent years due to their important role as mediators of bacterial and fungal populations in soils [40].

AOM control the rate-limiting step of nitrification, the energy-gain oxidation of ammonia to hydroxylamine which is further transformed to nitric oxide and eventually to nitrite [41]. Nitrite is subsequently oxidized to nitrate by nitrite-oxidizing bacteria (NOB) [42] (Fig. 1). Nitrification constitutes one of the most important inputs of N in soil contributing 330 Tg of N per year [43], hence perturbations in its operation are expected to adversely affect N balance in soil. Beyond nitrification, AOM have been found to contribute to N_2O emissions, a major GHG, through a process called nitrifiers denitrification [44]. Ammonia oxidation constitutes a specialized process controlled by (1) ammonia-oxidizing bacteria (AOB), mostly

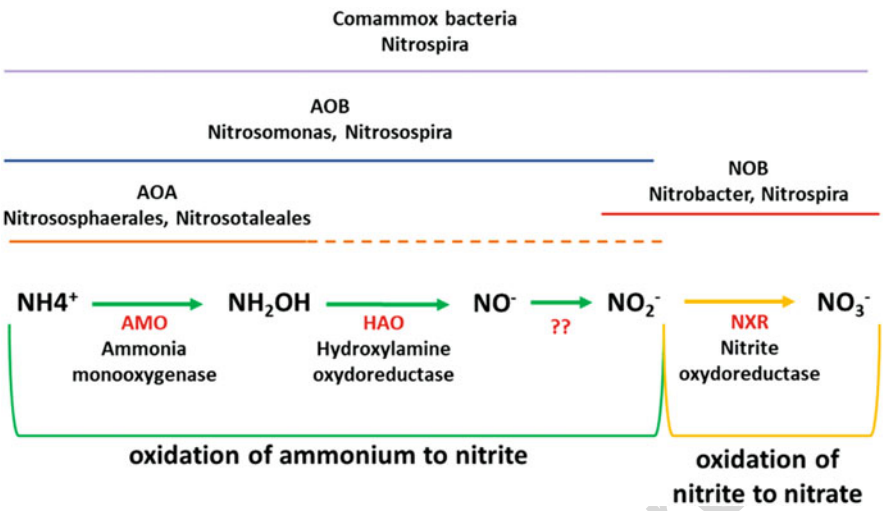

Fig. 1 A schematic representation of the microbially mediated transformation of nitrogen during nitrification along with the groups involved in each step of the process. *AOA* Ammonia oxidizing archaea, *AOB* Ammonia oxidizing bacteria, *NOB* Nitrite oxidizing bacteria, *Comammox* Complete ammonia oxidation bacteria

belonging to β-proteobacteria and specifically to *Nitrosomonas* and *Nitrosospira* [45] (2) ammonia-oxidizing archaea (AOA) belonging to the phylum Thaumarchaeota with lineages Nitrososphaerales and Nitrosotaleales dominating in soil ecosystems [46] and (3) comammox bacteria which perform the full nitrification process in one cell [47] and was recently shown to actively participate in ammonia oxidation in soil [48]. The contribution of the different groups of AOM in ammonia oxidation in soil is largely determined by soil physicochemical attributes like pH [49] and ammonia concentrations pointing to an AOM niche specialization [50, 51]. All AOM share an enzyme called ammonia monooxygenase, a periplasmic enzyme which is responsible for the oxidation of ammonia to hydroxylamine [43]. The gene encoding the alpha subunit of ammonia monooxygenase (*amoA*) provides a thorough phylogenetic classification of AOM [45, 46]. This gene constitutes the key marker gene for the design of group-specific primers for AOA, AOB and comammox [52–54] that have been used in q-PCR, RT-q-PCR and amplicon sequencing to determine the abundance, activity and diversity of AOM in soil ecosystems [12, 20].

The responsiveness of nitrifiers to pesticides was first reported by Domsch [55] who observed a high sensitivity of *Nitrosomonas* and *Nitrobacter* to pesticides. Since then, further studies reinforced the sensitivity of AOM to abiotic stressors and suggested their use as microbial bioindicators [36]. Indeed, several studies have explored the response of AOM to pesticide exposure by measuring their abundance via q-PCR [24, 56–58], their activity via measurements of potential ammonia oxidation (or potential nitrification) [20], and their diversity via DGGE, TRFLP or

amplicon sequencing of the *amoA* gene [12, 31, 59]. Recent studies by Hund-Rinke et al. [60] tested the toxicity of silver nanoparticles, as potential pesticides, on soil microorganisms using a range of standardized methods including potential nitrification (or so-called potential ammonia-oxidation method ISO 15685), MicroResp®, and exoenzymes activity. The former was the most sensitive endpoint providing a consistent dose-effect response allowing the calculation of EC_{50} values. Similar studies have also reported the sensitivity of potential nitrification to pesticide exposure [20, 61]. Potential nitrification is a measure of the rate of the microbial transformation of ammonia to nitrite, in contrast to the N-mineralization test which is a lump measurement of ammonification and nitrification compromising its sensitivity to abiotic stressors. All the above evidence along with its ISO standardization (ISO 15685) reinforce its potential for implementation in the battery of tests that could be used to assess the toxicity of pesticides on soil microorganisms.

Most studies to date have tested the effect of different pesticides to AOM abundance via q-PCR of the *amoA* gene, but only a few have worked at transcription level. In one of these few studies, Papadopoulou et al. [20] observed a clear temporal inhibition of the activity of AOM in soil by ethoxyquin only when *amoA* transcripts were measured. Considering the high turnover rates of RNA in soil, RNA-based approaches are expected to provide a more accurate view of the effects of pesticides on the activity of functional microbial groups like AOM. However, the labile nature of RNA has precluded its wider use in pesticide microbial ecotox studies and maybe its use could be considered when refinement of the ecotox part of the risk assessment is requested. Overall AOM constitute good potential bioindicators for the assessment of the toxicity of pesticides on soil microorganisms since (1) they control a very significant function in soil N cycling (2) they are particularly sensitive to pesticide exposure providing a consistent and relevant ecotoxicological response (3) we have a good knowledge of their life cycle, ecology, biochemistry and physiology and (4) we have well-standardized methods to measure their activity and abundance in an accurate way.

Apart from AOM other N-cycling microbial groups have been considered as valuable toxicity endpoints including denitrifying bacteria [62] and N-fixing bacteria [37]. Pesticide effects on denitrification has been measured through q-PCR analysis of bacterial genes involved in the different steps of denitrification, although results were contrasting and no clear ecotoxicological response was seen [11, 62, 63]. Pesticide effects on N-fixing bacteria have been also investigated, mostly via *nifH*-based q-PCR, with the results varying from no inhibition by trifluralin (especially compared to AOM) [64], to temporal inhibition by chlorothalonil [65] and to strong inhibition by 1,3-dichloropropene [66].

AMF were identified as potential bioindicators for assessing the toxicity of pesticides on soil microorganisms [10, 16]. AMF are the most ubiquitous plant symbiotic microbes on earth with up to 80% of plants colonized by obligate biotrophic fungi of the phylum Glomeromycota [67]. They colonize roots and derive plant photosynthates in exchange for nutrients (up to 80% of plant P is of AMF origin), offering plant tolerance to biotic and abiotic stress [68]. Besides improving plant fitness, AMF also contribute to the formation and stabilization of soil

aggregation [69] and improve soil carbon stocks [70]. At the ecosystem level AMF could affect the composition and productivity of plant communities with reciprocal effects on nutrient cycling [71].

AMF sensitivity to pesticides has been extensively studied at various experimental scales [16, 72, 73]. However, their obligatory symbiotic nature and their biological cycle which involves intraradical and extraradical life stages require the use of complementary experimental approaches to identify the nature of the inhibitory effects observed: direct on AMF or indirect stemming from phytotoxicity effects on the plant host. Direct effects on AMF are expected to be imposed mostly by fungicides, unlike herbicides whose effects on AMF are expected to be indirect by exerting their toxicity to the plant host. In addition, the different life stages of AMF are not expected to be equally exposed to pesticides, while the consequences of pesticide exposure for AMF survival are expected to vary in the different life stages. Extraradical AMF life stages are more prone to pesticide exposure, unlike intraradical stages which are less exposed. The standard ISO-10832 «Effects of pollutants on mycorrhizal fungi- germination test» could be used to assess effects of pesticides on extraradical stages of AMF, using spore germination of *Funneliformis mosseae* as a relevant toxicity endpoint. Giovanneti et al. [74] tested the effect of 14 pesticides on spore germination and pre-symbiotic mycelial growth and observed that fungicides were more toxic than the other pesticide groups tested. Mallman et al. [75] proposed an optimization of the ISO test with the use of *Gigaspora albida* and *Rhizophagus clarus*, to cover a wider diversity of AMF, and boric acid as negative control instead of cadmium nitrate.

Pot and field studies have been also employed to assess the toxicity of pesticides on natural assemblages of AMF. In those studies, plant roots mycorrhizal colonization, P content and other plant physiological attributes (root and shoot biomass) are often used as ecotoxicological endpoints to identify potential adverse effects on AMF [72, 76]. These plant-soil studies introduce realism and complexity in ecotoxicological assessment, but at the same their outcome is affected by several confounding pesticide- and AMF-related factors. A classic example is provided by the study of Karpouzas et al. [16] which showed that nicosulfuron when applied repeatedly in soil at rates higher than x10 the recommended dose rate led to a dramatic decrease in maize roots colonization by AMF, although it was not possible to distinguish if effects were direct on AMF or indirect driven by plant host phytotoxicity. The use of in vitro tests with AMF could complement pot studies and provide a conservative estimate of the potential toxicity of pesticides on the different life stages of AMF, and hence clarify the nature of the effects observed.

In vitro cultivation of AMF is possible on self-propagating mycorrhized Ri tDNA-transformed roots of *Daucus carota* or *Medicago truncatula* growing in sterilized minimal medium [77, 78]. These mono-compartmental axenic culture systems have been used to assess the toxicity of pesticides at the symbiotic phase and allow the calculation of IC_{50} values. Wan et al. [77] calculated the IC_{50} values for a range of pesticides using reduction in extraradical mycelium sporulation as the most conservative endpoint. Benomyl, chlorothalonil and glyphosate were the most toxic pesticides with IC_{50} values <1 mg/L compared to AMPA ($IC_{50} = 4.2$ mg/L),

the major transformation product of glyphosate, and the biopesticide azadirachtin (IC_{50} = 230 mg/L). Subsequently, Zocco et al. [79] used a modified bi-compartmental system composed of a root compartment (RC) and hyphae compartment (HC) to test the toxicity of fenpropimorph and fenhexamid on the symbiotic phase, the hyphae and the spores at the post-symbiotic phase and also on the root biomass. A three-compartment AM-P system composed of a shoot compartment (SC) where a plantlet shoot grows, and the RC and SC described above were proposed by Dupré de Boulois et al. [80]. AM-P systems have been used to test the toxicity of pesticides like fenpropimorph and fenhexamid on the capacity of extraradical hyphae and spores to colonize roots, while it offers the opportunity to determine effects on P uptake using ^{33}P ~~Zocco et al.~~ [78]. These AM-P systems could be further advanced including a second SC associated with the HC to systematically test the effect of pesticides on the capacity of extraradical hyphae to sporulate and colonize plant crops where the pesticide tested is destined for use ~~Buysens et al.~~ [73]. Besides just determining toxicity endpoint values for AMF, these ~~AMF~~ in vitro cultivation systems could be used for the determination of the toxicity mechanism, the nature of the effect observed, and also of potential effects on the physiology of symbiosis. Campagnac et al. [81] used the single compartment axenic culture system to determine the effects of fenhexamid and fenpropimorph on the sterol biosynthesis in mycorrhized plantlets. Zocco et al. [78] used the AM-P system to define the toxicity mechanism of the same two fungicides focusing on plant P uptake machinery.

The introduction of molecular tools in the study of AMF unravelled an enormous diversity which revolutionized the taxonomy of Glomeromycota ~~Oehl et al.~~ [82]. Still only a few studies have looked into the effects of pesticides on the diversity of AMF. Karpouzas et al. [16] using a DGGE – cloning approach showed that nicosulfuron, when applied at rates multiple times higher than the recommended, could result in a dramatic decrease in the diversity of AMF in maize roots. Rivera-Becerril et al. [83] studied the effect of a mixture of fenhexamid, folpet and deltamethrin applied at x1, x10 and x20 dose rates on the soil diversity of AMF, via clone library with taxon-specific primers and observed a reduction of AMF soil diversity with increasing dose rates. Jin et al. [76] constitutes the only study to date that used amplicon sequencing to determine the effects of a range of pesticides on AMF intraradical diversity. They observed pesticide-specific effects on AMF community with *Gigaspora hoi* and *Acalauspora uera* showing increasing sensitivity to fludioxonil in pea and chickpea, respectively.

Overall, AMF appear to be also good candidate bioindicators to assess the toxicity of pesticides on soil microorganisms since (1) they control a series of key functions in soil ecosystems (2) we have standardized tools to define effects of pesticides on their growth (3) we have a good knowledge of their life cycle and biology and (4) they are generally sensitive to pesticides. However, we should note that due to their symbiotic nature a combination of in vitro and soil-plant studies are often required in order to define the true extent of pesticides toxicity on AMF.

2.4 Tiered-Risk Assessment Scheme and Standardization of Experimental Planning

To date environmental risk assessment of pesticides relies on a tiered system starting from simple and highly conservative Tier I assays and moving gradually to less conservative and more realistic Tiers II and III. To date no such risk assessment scheme is available for soil microorganisms. Pioneering studies by Jonhen and Drew [84] and Atlas et al. [15] proposed for the first time a set of experimental procedures and rules on how to determine the toxicity of pesticides on soil microorganisms. Both studies agreed that if significant inhibitory effects on soil microbial functioning are observed at lab scale, the toxicity of pesticides on soil microorganisms should be further examined at field tests. Following the same philosophy, Karpouzas et al. [23] established a two-tiered risk assessment procedure where lab soil microcosms are employed at Tier I to determine the toxicity of pesticides on soil microorganisms. If effects are observed a Tier II assessment at field scale should be undertaken (Fig. 2a). Subsequently Karpouzas et al. [85] provided a more conclusive tiered system composed of three tiers of increasing experimental complexity based on the ecotoxicological response of key soil functional groups like AOM and AMF (Fig. 2b). This scheme is composed of (1) a Tier I highly conservative in vitro screening of pesticides against a set of soil derived AOM and AMF strains that cover the different ecophysiological and phylogenetic variants of these microbial groups (2) a Tier II toxicity assessment in lab soil microcosms (or pot studies when AMF are considered) against natural assemblages of AOM and AMF and (3) a Tier III toxicity assessment at field scale against natural assemblages of AOM and AMF. In case where an unacceptable risk for soil microorganisms is still evident refinement of exposure could be an option to minimize risk. Fast track in vitro tests for AOM and AMF are available and have been used in the past to assess pesticides toxicity [12, 73, 78]. However certain aspects of these tests should be standardized (e.g. selection of the most ecotoxicological relevant strains for testing) before considered for inclusion in the regulatory framework.

AU2

Several studies have assessed the toxicity of pesticides on soil microbial diversity or functioning. However inconsistencies in experimental planning have prevented the systematic characterization of the potential risk associated with the use of pesticides for soil microorganisms. Below we will identify the most common problems in experimental planning and we will propose certain solutions for a more systematic and thorough determination of the soil microbial toxicity of pesticides.

- In most ecotox studies pesticides are applied at increasing dose rates and the effects on selected endpoints are followed. Several studies have used particularly high pesticides levels, up to x100 and x1000 the recommended dose rates or application schemes that are not relevant to the registered application scheme of the tested pesticide [23, 28, 86]. Effects observed under these experimental conditions are not ecotoxicologically relevant and do not substantiate a potential

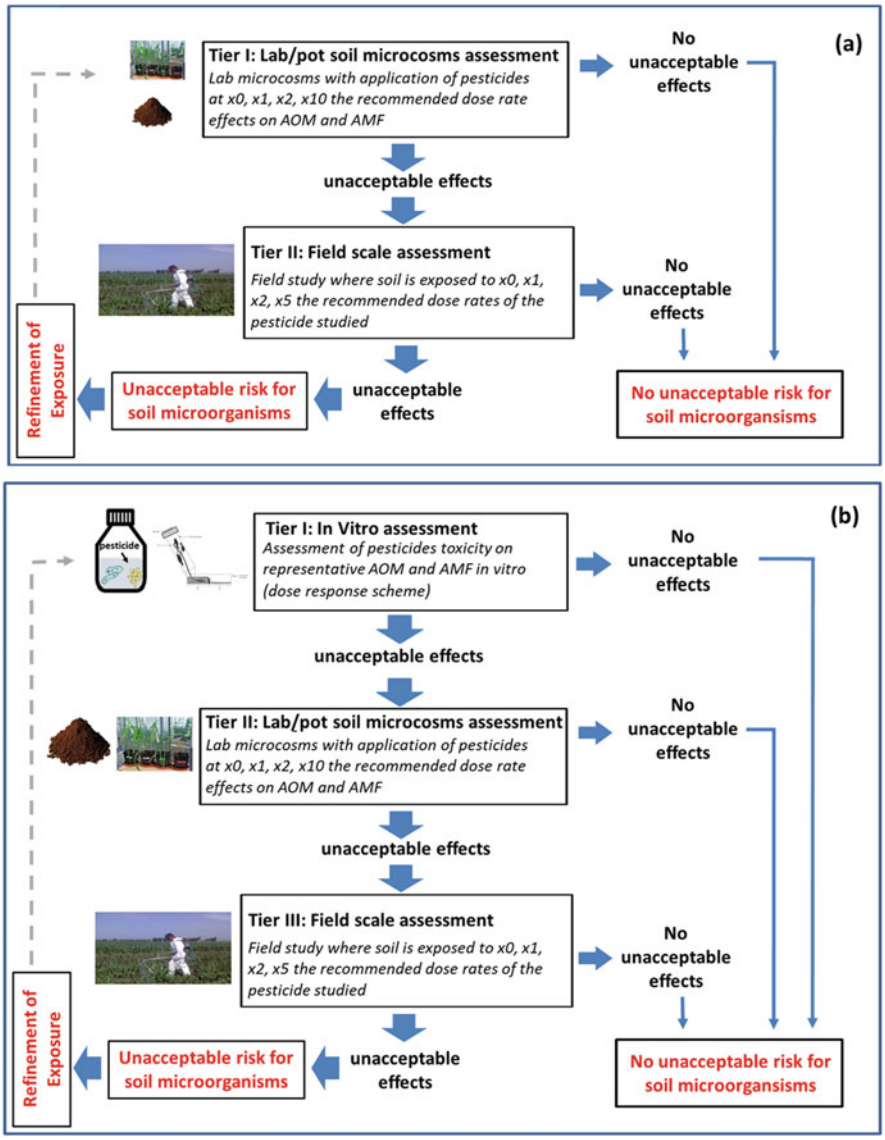

Fig. 2 Two-tier risk assessment (**a**) and three-tier risk assessment schemes (**b**) proposed for assessing the risk associated with the use of pesticides for soil microorganisms. *AOM* Ammonia-oxidizing microorganisms, *AMF* Arbuscular mycorrhizal fungi

risk for soil microorganisms. We propose (1) in lab tests pesticides will be tested at x1, x2 and x10 the recommended dose rate and (2) in field tests pesticides will be tested at x1, x2 and x5 the recommended dose rate (3) at both experimental scales a non-treated control treatment should be included.

- Several soil studies investigating the effects of pesticides on soil microorganisms are often of limited duration (e.g. 30 days). This does not allow a potential recovery of the soil microbial community from temporal effects that might be evident at the first 30 days of exposure. We propose that (1) soil lab tests will be extended to at least 70 days depending on the persistence of the pesticide tested, and temporal measurements of microbial toxicity endpoints will be performed along the experimental duration (2) field tests should be extended for the whole growing season of the crop used. Such a monitoring setup will allow potential recovery to be observed.
- Several of the currently available studies have explored the effects of pesticides on selected functional and diversity microbial endpoints in a single soil limiting the applicability of the results. We propose that all lab tests to be performed at three soils with varying pH and organic carbon content, two parameters that are known to affect pesticide behaviour [87] but also microbial activity and diversity [6].
- When summarizing studies looking at the effects of pesticides on AOM we noted that only a few of them have considered amending soil with ammonium prior to pesticide exposure [12, 22, 59]. This practice is essential to trigger the proliferation and activity of AOM in soil. However due care should be taken regarding the source (inorganic or organic) and the level of ammonium added in the soil. Both of these parameters are known to strongly affect the activity of the different microbial players in ammonia oxidation in soil [50].
- A final tip that we believe it is essential for studies focusing on the effects of pesticides on AMF is the inclusion of plant species which are relevant for the agronomic use of the tested pesticides.

3 Emerging Issues in the Assessment of the Soil Microbial Toxicity of Pesticides

A new risk assessment scheme for defining in a robust way the potential risk for soil microorganisms due to pesticide application is urgently required. The use of the tiered risk assessment scheme proposed here, combined with the use of AOM and AMF as bioindicators of the toxicity of pesticides on soil microorganisms and the implementation of advanced and standardized tools would fill the gap in pesticide regulatory process. The initial focus on functional microbial endpoints could further expand to diversity endpoints when the on-going standardization of modern metataxonomic tools will be finalized. Still there are emerging issues regarding the study of the toxicity of pesticides on soil microorganisms that should be the focus of studies in the next few years. Some of these emerging issues are described in the following paragraphs.

3.1 Toxicity of Pesticides at Soil Ecosystem Level

Most studies to date have assessed the toxicity of pesticides on individual taxa or functional groups separately or in the context of a specific biochemical pathway ignoring the ecological dimension of the effects observed. However, in soil microorganisms are assembled in subpopulations which are intertwined by metabolic links or other types of interactions and can be important for ecosystem homeostasis [88, 89]. Resilience and robustness of microbial consortia to external perturbations has been attributed either to microbial diversity, which enables tolerant members to fill functional voids left by intolerant species [90], or to functional complementarity of network members resulting in better exploitation of resources and elevated resistance to stress [91]. Little is known about the impact of pesticides on soil microbial networks and the consequences for soil functioning. A functional microbial network that could be utilized in such an approach could be AOM, NOB and denitrifying bacteria. Previous studies have looked into the effect of pesticides in the above functional microbial network via gene abundance measurements which provided conflicting results [11, 65]. Measurements at activity level (RT-q-PCR) and determination of the concentrations of N intermediates supporting a metabolic flux analysis would provide a robust assessment of the potential toxicity of pesticides on soil microbial networks.

Microorganisms also interact with other organisms within the soil-food web. Predator–prey relations are particularly important for soil ecosystem functioning with protists-bacteria being the best-studied model system [92]. Predation by protists influences bacterial and fungal diversity and productivity with consequences on the flux organic nutrients into biomass at higher trophic levels [93, 94]. Recent studies showed that the diversity of both protists and bacteria interactively determines the performance of the predator [95]. External perturbations like the application of pesticides could affect diversity at both trophic levels with possible effects on ecosystem functioning [96, 97]. To date research on the impact of pesticides has overlooked potential effects on multiple levels in soil food webs and microbial interactions. The protists–bacteria relationship could be used as a model predator–prey system in a soil-food web centric assessment of the toxicity of pesticides on soil microbiota. This could be determined in simple synthetic microbial communities (quasi in vitro systems) and further to complex natural soil assemblages enabled by the major advancements in microbial diversity analysis at both trophic levels [98].

3.2 Toxicity of Pesticide Mixtures and Co-formulants

PPP contain, apart from the active ingredient, several co-formulants that ensure that maximum pesticide amount will reach the target. The identity of these co-formulants is rarely known but they seem to contribute partially or heavily to the toxicity of pesticides on soil microorganisms. The potential effects of co-formulants on the soil

microbiota could be experimentally addressed via a comparative assessment of effects triggered by the pure active ingredient and the corresponding commercial formulation. This is a practice that is not largely followed when assessing the toxicity of pesticides on soil microorganisms through the OECD 216 test. In such a comparative study, Crouzet et al. [99] showed that the commercial formulation of the herbicide mesotrione when applied at x10 or x100 the recommended dose induced stronger effects on the structure of soil cyanobacteria compared to the pure active ingredient. Similarly, Rousidou et al. [31] demonstrated that glucose and skimmed milk powder, contained as additives in a commercial formulation of the nematode parasitic fungus *Paecilomyces lilacinus,* were responsible for a temporal inhibition of AOM upon soil application of the commercial formulation BIOACT®.

Besides additives, PPP commonly contain more than one active substance (ca. 25% in Germany) [100], hence releasing mixtures of pesticides in the environment that may exhibit effects deviating from those seen when applied individually. Studies have explored the aquatic toxicity of such mixtures [101] and suggested that for mixtures composed of pesticides with the same or different mode of action the concentration addition (CA) or the independent action (IA) models, respectively, could predict toxicity [102]. In contrast, little is known about the toxicity of pesticide mixtures on soil ecosystems. Evaluating the applicability of CA and IA models for assessing the soil microbial toxicity of pesticides or devising new models, more relevant for soil ecosystems, could be a new frontier in pesticide soil microbial ecotoxicology.

3.3 Toxicity of Biopesticides

The growing public concern about the effects of synthetic pesticides on environmental quality and soil health has shifted attention to biopesticides which have gained ground in the pesticide market. Biopesticides is a broad group of pesticides of biological origin which could be broadly categorized to (a) microbials where the active agent is a microorganism that protect crops from fungal and insect infestations (b) natural products or biochemicals or botanicals that are biogenic compounds, products of the secondary metabolism of plants and microorganisms with strong biocidal activity. Due to their biological origin, biopesticides are a priori considered as low risk. However this remains to be verified by a number of specific studies. The most studied natural product regarding its off-target toxicity to soil microorganisms is azadirachtin, with the results obtained being not in support of a low-risk profile. In vitro tests suggested low risk of azadirachtin for AMF [77]. However soil studies showed that azadirachtin even at the recommended dose had a consistent inhibitory effect on the abundance and the transcriptional activity of AOM, N-fixing bacteria and denitrifying bacteria [56, 103] and negatively affected the diversity of bacteria, fungi and AMF [30, 57]. In fact the effects observed were equal or even higher than those induced by comparatively studied synthetic pesticides. An interesting study by Romdhane et al. [104] compared the effects of the natural triketone herbicide

leptospermone and its synthetic derivative sulcotrione on the soil microbial community. In line with the results of azadirachtin they noted that leptospermone induced a stronger perturbation on the soil bacterial community compared with its synthetic counterpart. These studies certainly challenge the general perception that natural products are characterized by lower off-target toxicity compared to synthetic pesticides.

Even less studies are available for microbial pesticides. Potential toxicity effects of microbials on the soil microbial community largely depend on the mode of action of the microbial pesticide itself. Hence microbial pesticides based on microorganisms which do not act through the production of biocidal compounds are not generally expected to affect soil microorganisms. This was clearly the case for the nematode parasitic fungi *P. lilacinus* strain PL251 which did not have a direct inhibitory effect on AOM [31]. In contrast Yu et al. [105] using a different *P. lilacinus* strain PL1210 showed strong inhibitory effects on nitrification and AOM abundance which were attributed to antimicrobial metabolites that the tested strain produces. Other relevant studies also suggested that microbial pesticides based on microorganisms acting through parasitism or antagonism (i.e. *Metarhizium brunneum, Fusarium oxysporum f.sp. stringae, Bacillus amyloliquefaciens*) did not appear to induce strong and persistent effects on the soil microbial community [106–108].

According to the registration framework in Europe biopesticides are collectively treated as low-risk compounds, still undergoing the same registration process as synthetic pesticides. Although this could be relevant for natural pesticides, we argue that the risk assessment procedure for microbial pesticides should be adjusted to account for the particularities of these products. This should certainly include parallel ecotoxicity tests between formulations and active microbial agents along with the implementation of tools for monitoring their fate in soil.

3.4 Toxicity of Transformation Products

In addition to parent compounds, pesticide environmental risk assessment extends to their TPs that are formed at levels >10% of the parent compound. Minor TPs (<10% of the parent compound) could evade risk assessment, although they might exhibit similar or even higher toxicity than the parent pesticide if they carry toxicophore moieties in their structure [109]. Several studies have showed that TPs could be more toxic than the parent compound. For example, Papadopoulou et al. [20] showed that quinone imine, a TP of ethoxyquin used as preservative in fruit packaging plants, was responsible for the inhibition ~~of abundance and diversity~~ of AOM ~~observed~~ in soils treated with ethoxyquin. Further studies by Vasileiadis et al. [12] suggested that 3,5-dichloroaniline, a major TP of iprodione, was responsible for the strong inhibition in the abundance and activity of AOM in soils treated with iprodione. Similar soil studies with chlorothalonil showed that the formation of 4-hydroxy-chlorothalonil resulted in strong inhibitory effects on microbial activity

[110]. Advances in analytical chemistry have enabled the detection of previously unknown TPs formed even in low concentrations [111]. Recently, enviPath, a database and prediction tool for the biotransformation of organic contaminants [112], has been updated with all freely accessible EU regulatory data on pesticide degradation in lab soil studies with the aim to develop more accurate prediction for pesticide biotransformation pathways [113]. Complementary tools (i.e., QSAR) enabling the prediction of the soil microbial toxicity of TPs could allow for a targeted investigation of the TPs toxicity.

4 Conclusions and Future Perspectives

The assessment of the soil microbial toxicity of pesticides constitutes a major gap in the current pesticide regulatory framework and corrective actions are urgently required. These should encompass the dramatic methodological advances in soil microbiology and their increasing level of standardization. In this quest we believe that key functional microbial groups like AOM and AMF should have a key role as bioindicators of the toxicity of pesticides on soil microorganisms. This short list of microbial indicators should be gradually enriched with other potential candidates like protists or other microbial groups that could be identified through the use of advanced ecotoxicological tools (i.e. Species Sensitivity Distributions) in a meta-analysis of high-throughput amplicon sequencing data. This will be facilitated by the development of a database of amplicon sequencing data derived from studies investigating the toxicity of pesticides on the soil microbial diversity. A first example of such an effort is the microbiome stress project presented by Roca et al. [114].

Function-based toxicity endpoints are more mature and standardized for immediate implementation in the regulatory process unlike diversity endpoints whose standardization is still on-going. Furthermore, we still lack a clear scientific evidence-based answer to the question “How much soil microbial diversity loss we could accept without compromising soil ecosystem functioning”. Studies providing evidence for decision making at this level will open the route for the implementation of microbial diversity endpoints in pesticide ecotoxicity assessment.

We are currently at the era of amplicon sequencing approaches which provide a high-resolution overview of the phylogenetic composition of the soil microbial community and of the response of its individual members to pesticide exposure. However, this approach could not provide any information about the functional role of the affected microbes which requires metaomic approaches. The introduction of metagenomic and most importantly metatranscriptomic analysis in studies looking at the effects of pesticides on soil microorganisms would provide a holistic view of the functional response of the soil microbial community to pesticide exposure identifying key responders and toxicity mechanisms.

Up to date literature evidence suggests that pesticides when used at the recommended dose rates are not expected to impose adverse effects on the soil microbial community. Still a concerted action is required to be able to identify

exceptions to this statement and this could be achieved through the establishment of a robust scheme of toxicity and risk assessment analysis.

Acknowledgements This work was supported by the EU's H2020 research and innovation program under the Marie Sklodowska-Curie grant agreement No. 956496 MSCA-ITN-H2020 project ARISTO.

References

1. EFSA (2019) Scientific report on the 2017 European Union report on pesticide residues in food. EFSA J 17(6):5743., 152 pp. https://doi.org/10.2903/j.efsa.2019.5743
2. Loos R, Gawlik BM, Locoro G, Rimaviciute E, Contine S, Bidoglio G (2009) EU-wide survey of polar organic persistent pollutants in European river waters. Environ Pollut 157(2):561–568
3. Herrero-Hernandez E, Simon-Egea AB, Sanchez-Martin MJ, Rodriguez-Cruz MS, Andrades MS (2020) Monitoring and environmental risk assessment of pesticide residues and some of their degradation products in natural waters of the Spanish vineyard region included in the denomination of origin Jumilla. Environ Pollut 264:114666
4. Silva V, Mol HGJ, Zomer P, Tienstra M, Ritsema CJ, Geissen V (2019) Pesticide residues in European agricultural soils – a hidden reality unfolded. Sci Total Environ 653:1532–1545
5. Schafer RB, Liess M, Altenburger R, Filser J, Hollert H, Rob-Nickoll M, Schaffer A, Scheringer M (2019) Future pesticide risk assessment: narrowing the gap between intention and reality. Environ Sci Eur 31:21. https://doi.org/10.1186/s12302-019-0203-3
6. Fierer N (2017) Embracing the unknown: disentangling the complexities of the soil microbiome. Nat Rev Microbiol 15:579–590
7. Trivedi P, Leach JE, Tringe SG, Sa T, Singh BK (2020) Plant–microbiome interactions: from community assembly to plant health. Nat Rev Microbiol 18:607–621
8. Rillig MC, Mummey DL (2006) Mycorrhizas and soil structure. New Phytol 171:41–53
9. EFSA (2010) Scientific opinion on the development of specific protection goal options for environmental risk assessment of pesticides, inparticular in relation to the revision of the guidance documents on aquatic and terrestrial ecotoxicology (SANCO/3268/2001&SANCO/10329/2002). EFSA panel on plant protection products and their residues. EFSA J 8:1821–1876
10. EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues) (2017) Scientific opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms. EFSA J. https://doi.org/10.2903/j.efsa.2017.4690
11. Karas PA, Baguelin C, Pertile G, Papadopoulou ES, Nikolaki S, Storck V, Ferrari F, Trevisan M, Ferrarini A, Fornasier F, Vasileiadis S, Tsiamis G, Martin-Laurent F, Karpouzas DG (2018) Assessment of the impact of three pesticides on microbial dynamics and functions in a lab-to-field experimental approach. Sci Total Environ 637–638:636–646
12. Vasileiadis S, Puglisi E, Papadopoulou ES, Pertile G, Suciu N, Papolla A, Tourna M, Karas PA, Papadimitriou F, Kasiotakis A, Ipsilanti N, Ferrarini A, Sulowic S, Fornasier F, Nicol GW, Trevisan M, Karpouzas DG (2018) Blame it on the metabolite: 3,5-dichloraniline rather than the parent compound iprodione induces strong effects on the diversity and function of soil microorganisms. Appl Environ Microbiol 84(22):e01536–e01518
13. El Azhari N, Dermou E, Barnard RL, Storck V, Tourna M et al (2019) The dissipation and microbial ecotoxicity of tebuconazole and its transformation products in soil under standard laboratory and simulated winter conditions. Sci Total Environ 637–638:892–906
14. Lewis WH, Tahon G, Geesink P, Sousa DZ, Ettema TJG (2021) Innovations to culturing the uncultured microbial majority. Nat Rev Microbiol 19:225–240

15. Atlas RM, Pramer D, Bartha R (1977) Assessment of pesticide effects on non-target soil microorganisms. Soil Biol Biochem 10:231–239
16. Karpouzas DG, Kandeler E, Bru D, Friedel I, Auer Y, Kramer S, Vasileiadis S, Petric I, Udikovic-Kolic N, Djuric S, Martin-Laurent F (2014) A tiered assessment approach based on standardized methods to estimate the impact of nicosulfuron on the abundance and function of the soil microbial community. Soil Biol Biochem 75:282–291
17. Zhang C, Zhou T, Zhu L, Du Z, Li B et al (2019) Using enzyme activities and soil microbial diversity to understand the effects of fluoxastrobin on microorganisms in fluvo-aquic soil. Sci Total Environ 66:89–93
18. Bell CW, Fricks BE, Rocca JD, Steinweg JM, McMahon SK, Wallenstein MD (2013) High-throughput fluorometric measurement of potential soil extracellular enzyme activities. J Vis Exp:e50961. https://doi.org/10.3791/50961
19. Wakelin S, Lombi E, Donner E, Macdonald L, Black A, O'Callaghan M (2013) Application of MicroResp™ for soil ecotoxicology. Environ Pollut 179:177–184
20. Papadopoulou ES, Tsachidou P, Sulowic S, Menkissoglu-Spiroudi U, Karpouzas DG (2016) Land spreading of wastewaters from the fruit packaging industry and potential effects on soil microbes: effects of the antioxidant ethoxyquin and its metabolites on ammonia oxidizers. Appl Environ Microbiol 82:747–755
21. Martin-Laurent F, Kandeler E, Pertic I, Djuric S, Karpouzas DG (2013) ECOFUN-MICROBIODIV: an FP7 European project for developing and evaluating innovative tools for assessing the impact of pesticides on soil functional microbial diversity-towards new pesticide registration regulation? Environ Sci Pollut Res 20:1203–1205
22. Feld L, Hjelmsø MH, Nielsen MS, Jacobsen AD, Rønn R, Ekelund F, Krogh PH, Strobel BW, Jacobsen CS (2015) Pesticide side effects in an agricultural soil ecosystem as measured by amoA expression quantification and bacterial diversity changes. PLoS One 10:e0126080. https://doi.org/10.1371/journal.pone.0126080
23. Karpouzas DG, Papadopoulou ES, Ipsilantis I, Friedel I, Petric I, Udikovic-Kolic N, Djuric S, Kandeler E, Menkissoglu-Spiroudi U, Martin-Laurent F (2014) Effects of nicosulfuron on the abundance and diversity of arbuscular mycorrhizal fungi used as indicators of pesticide soil microbial toxicity. Ecol Indic 39:44–53
24. Fang W, Yan D, Wang X, Huang B, Wang X, Liu J, Liu X, Li Y, Ouyang C, Wang Q, Cao A (2018) Responses of nitrogen-cycling microorganisms to Dazomet fumigation. Front Microbiol 9:2529. https://doi.org/10.3389/fmicb.2018.02529
25. Clement BG, Kehl LE, DeBord KL, Kitts CL (1998) Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. J Microbiol Methods 31:135–142
26. Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73:127–141
27. Harris JK, Sahl JW, Castoe TA, Wagner BD, Pollock DD, Spear JR (2010) Comparison of normalization methods for construction of large, multiplex amplicon pools for next-generation sequencing. Appl Environ Microbiol 76(12):3863–3868
28. Omirou M, Rousidou C, Bekris F, Papadopoulou KK, Ehaliotis C, Menkissoglu-Spiroudi U, Karpouzas DG (2011) The impact of biofumigation and chemical fumigation methods on the structure and function of the soil microbial community. Microb Ecol 61:201–213
29. Li X, Zhang H, Wu M, Su Z, Zhang C (2008) Impact of acetochlor on ammonia-oxidizing bacteria in microcosm soils. J Environ Sci 20:1126–1131
30. Ipsilantis I, Samourelis C, Karpouzas DG (2012) The impact of biological pesticides on arbuscular mycorrhizal fungi. Soil Biol Biochem 45:147–155
31. Rousidou C, Papadopoulou ES, Kortsinidou M, Giannakou IO, Singh BK, Menkissoglu-Spiroudi U, Karpouzas DG (2013) Bio-pesticides: harmful or harmless to ammonia oxidizing microorganisms? The case of a Paecilomyces lilacinus-based nematicide. Soil Biol Biochem 67:98–105

32. Storck V, Nikolaki S, Perruchon C, Chabanis C, Sacchi A et al (2018) Lab to field assessment of the ecotoxicology impact of chlorpyrifos, isoproturon or tebuconazole on the diversity and composition of the soil bacterial community. Front Microbiol 9:1412. https://doi.org/10.3389/fmicb.2018.01412
33. Katsoula A, Vasileiadis S, Sapountzi M, Karpouzas DG (2020) The response of soil and phyllosphere microbial communities to repeated application of the fungicide iprodione: accelerated biodegradation or toxicity? FEMS Microbiol Ecol 96(6):fiaa056
34. Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G et al (2016) Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. mSystems 1(1):e00009–e00015. https://doi.org/10.1128/mSystems.00009-15
35. EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues) (2013) Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. EFSA J 11(7):3290. https://doi.org/10.2903/j.efsa.2013.329
36. Wessén E, Hallin S (2011) Abundance of archaeal and bacterial ammonia oxidizers – possible bioindicator for soil monitoring. Ecol Indic 11:1696–1698
37. Lu C, Yang Z, Liu J, Liao Q, Ling W, Waigi MG, Odinga ES (2020) Chlorpyrifos inhibits nitrogen fixation in rice-vegetated soil containing *Pseudomonas stutzeri* A1501. Chemosphere 256:127098
38. Foissner W (1999) Soil protozoa as bioindicators: pros and cons, methods, diversity, representative examples. Agric Ecosyst Environ 74:95–112
39. Dupraz V, Stachowski-Haberkorn S, Wicquart J, Tapie N, Butzinski H, Ackha F (2019) Demonstrating the need for chemical exposure characterisation in a microplate test system: toxicity screening of sixteen pesticides on two marine microalgae. Chemosphere 221:278–291
40. Amacker N, Mitchell EAD, Ferrari BJD, Chevre N (2018) Development of a new ecotoxicological assay using the testate amoeba *Euglypha rotunda* (Rhizaria; Euglyphida) and assessment of the impact of the herbicide S-metolachlor. Chemosphere 201:351–360
41. Caranto JD, Lancaster KM (2017) Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. Proc Natl Acad Sci U S A 114:8217–8222
42. Pester M, Maixner F, Berry D, Rattei T, Koch H, Lucker S et al (2014) NxrB encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing Nitrospira. Environ Microbiol 16(10):3055–3071
43. Kuypers MMM, Marchant HK, Kartal B (2018) The microbial nitrogen cycling network. Nat Rev Microbiol 16:263–276
44. Wrage-Monnig N, Horn MA, Well R, Muller C, Velthof G, Oenema O (2018) The role of nitrifier denitrification in the production of nitrous oxide revisited. Soil Biol Biochem 123:A3–A16
45. Abell GCJ, Robert SS, Frampton DMF, Volkman JK, Rizwi F et al (2012) High-throughput analysis of ammonia oxidiser community composition via a novel, amoA-based functional gene array. PLoS One 7(12):e51542
46. Alves RJE, Minh BQ, Urich T, von Haeseler A, Schleper C (2018) Unifying the global phylogeny and environmental distribution of ammonia oxidising archaea based on amoA genes. Nat Commun 9:1517
47. Daims H, Lebedeva EV, Pjevac P, Han P, Herbold G et al (2015) Complete nitrification by Nitrospira bacteria. Nature 528:504–509
48. Wang Z, Cao Y, Zhu-Barker X, Nicol GW, Wright AL, Jia Z, Jiang X (2019) Comammox *Nitrospira* clade B contributes to nitrification in soil. Soil Biol Biochem 135:392–395
49. Nicol GW, Leininger S, Schleper C, Prosser JI (2008) The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environ Microbiol 10:2966–2978
50. Hatzenpichler R (2012) Diversity, physiology and niche differentiation of ammonia-oxidizing archaea. Appl Environ Microbiol 78(21):7501–7510

51. Kits KD, Sedlacek CJ, Lebedeva EV, Han P, Bulaev A et al (2017) Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. Nature 549(7671):269–272
52. Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63:4704–4712
53. Tourna M, Freitag TE, Nicol GW, Prosser JI (2008) Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environ Microbiol 10:1357–1364
54. Pjevac P, Schauberger C, Poghosyan L, Herbold CW, van Kessel MAHJ et al (2017) AmoA targeted polymerase chain reaction primers for the specific detection and quantification of comammox Nitrospira in the environment. Front Microbiol 8:1508
55. Domsch KH (1972) Interactions of soil microbes and pesticides. Symp Biol Hungary 11:337–347
56. Singh S, Gupta R, Kumari M, Sharma S (2015) Nontarget effects of chemical pesticides and biological pesticide on rhizospheric microbial community structure and function in *Vigna radiata*. Environ Sci Pollut Res 22:11290–11300
57. Singh S, Gupta R, Sharma S (2015) Effects of chemical and biological pesticides on plant growth parameters and rhizospheric bacterial community structure in *Vigna radiata*. J Hazard Mater 291:102–110
58. Fang W, Wang X, Huang B, Zhang D, Liu J et al (2020) Comparative analysis of the effects of five soil fumigants on the abundance of denitrifying microbes and changes in bacterial community composition. Ecotoxicol Environ Saf 187:109850
59. Hernández M, Jia Z, Conrad R, Seeger M (2011) Simazine application inhibits nitrification and changes the ammonia-oxidizing bacterial communities in a fertilized agricultural soil. FEMS Microbiol Ecol 78:511–519
60. Hund-Rinke K, Hummler A, Schlinkert R, Wege F, Broll G (2019) Evaluation of microbial shifts caused by a silver nanomaterial: comparison of four test systems. Environ Sci Eur 31:86. https://doi.org/10.1186/s12302-019-0268-z
61. Crouzet O, Besse-Hogan P, Batisson I, Bonnemoy F, Bohatier J, Mallet C (2009) Responses of soil microorganisms to the herbicide mesotrione in microcosm experiments: from whole microbial communities to functional groups. In: Proceedings of the symposium on pesticide behaviour in soils, water and air, York, UK, pp 12–13, 14–16 Sept 2009
62. Hu X, Wang Y, Su X, Chen Y (2018) Acute response of soil denitrification and N_2O emissions to chlorothalonil: a comprehensive molecular mechanism. Sci Total Environ 636:1408–1415
63. Su X, Wang Y, Peng G, He Q (2020) Long-term effects of chlorothalonil on microbial denitrification and N_2O emission in a tea field soil. Environ Sci Pollut Res 27:17370–17381
64. Du P, Wu X, Xu J, Dong F, Liu X, Zheng Y (2018) Effects of trifluralin on the soil microbial community and functional groups involved in nitrogen cycling. J Hazard Mater 353:204–213
65. Zhang M, Xu Z, Teng Y, Christie P, Wang J, Ren W, Luo Y, Li Z (2016) Non-target effects of repeated chlorothalonil application on soil nitrogen cycling: the key functional gene study. Sci Total Environ 543:636–643
66. Fang W, Yan D, Wang Q, Huang B, Ren Z et al (2019) Changes in the abundance and community composition of different nitrogen cycling groups in response to fumigation with 1,3-dichloropropene. Sci Total Environ 650:44–55
67. Kiers TE, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E et al (2011) Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. Science 333:880–882
68. Veresoglou SD, Rillig MC (2012) Suppression of fungal and nematode plant pathogens through arbuscular mycorrhizal fungi. Biol Lett 8:214–217
69. Rillig MC, Aguilar-Trigueros CA, Bergmann J, Verbruggen E, Veresoglou SD, Lehmann A (2015) Plant root and mycorrhizal fungal traits for understanding soil aggregation. New Phytol 205:1385–1388

70. Fernandez CW, Kennedy PG (2015) Moving beyond the black-box: fungal traits, community structure, and carbon sequestration in forest soils. New Phytol 205:1378–1380. https://doi.org/10.1111/nph.13289
71. van der Heijden MG, Bardgett RD, van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol Lett 11:296–310
72. Druille M, Cabello MN, Omacini M, Golluscio RA (2013) Glyphosate reduces spore viability and root colonization of arbuscular mycorrhizal fungi. Appl Soil Ecol 64:99–103
73. Buysens C, de Boulois HD, Declerck S (2015) Do fungicides used to control *Rhizoctonia solani* impact the non-target arbuscular mycorrhizal fungus *Rhizophagus irregularis*? Mycorrhiza 25:277–288
74. Giovannetti M, Turrini A, Strani P, Sbrana C, Avio L, Pietrangeli B (2006) Mycorrhizal fungi in ecotoxicological studies: soil impact of fungicides, insecticides and herbicides. Preven Today 2:47–62
75. Mallmann GC, Paulo SJ, Sundh I, Pieper S, Arena M, da Cruz SP, Klauberg-Filho O (2018) Placing arbuscular mycorrhizal fungi on the risk assessment test battery of plant protection products (PPPs). Ecotoxicology 27:809–818
76. Jin H, Germida JJ, Walley FL (2013) Suppressive effects of seed-applied fungicides on arbuscular mycorrhizal fungi (AMF) differ with fungicide mode of action and AMF species. Appl Soil Ecol 72:22–30
77. Wan MT, Rahe JE, Watts RG (1998) A new technique for determining the sublethal toxicity of pesticides to the vesicular-arbuscular mycorrhizal fungus *Glomus intraradices*. Environ Toxicol Chem 17:1421–1428
78. Zocco D, Van Aarle IM, Oger E, Lanfranco L, Declerck S (2011) Fenpropimorph and fenhexamid impact phosphorus translocation by arbuscular mycorrhizal fungi. Mycorrhiza 21:363–374
79. Zocco D, Fontaine J, Lozanova E, Renard L, Bivort C, Durand R, Grandmougin-Ferjani A, Declerck S (2008) Effects of two sterol biosynthesis fungicides (fenpropimorph and fenhexamid) on the development of an arbuscular mycorrhizal fungus. Mycol Res 112:592–601
80. Dupré de Boulois H, Voets L, Delvaux B, Jackobsen I, Declerck S (2006) Transport of radiocesium by arbuscular mycorrhizal fungi to *Medicago truncatula* under *in vitro* conditions. Environ Microbiol 8:1926–1934
81. Campagnac E, Fontaine J, Sahraoui ALH, Laruelle F, Durand R, Grandmougin-Ferjani A (2008) Differential effects of fenpropimorph and fenhexamid, two sterol biosynthesis inhibitor fungicides, on arbuscular mycorrhizal development and sterol metabolism in carrot roots. Phytochemistry 69:2912–2919
82. Oehl Z, Sieverding E, Palenzuela J, Ineichen K, Alves da Silva G (2011) Advances in Glomeromycota taxonomy and classification. IMA Fungus 2:191–199
83. Rivera-Becerril F, van Tuinen D, Chatagnier O, Rouard N, Beguet J, Kuszala C, Soulas G, Gianinazzi-Pearson V, Martin-Laurent F (2017) Impact of a pesticide cocktail (fenhexamid, folpel, deltamethrin) on the abundance of Glomeromycota in two agricultural soils. Sci Total Environ 577:84–93
84. Johnen BG, Drew EA (1977) Ecological effects of pesticides on soil microorganisms. Soil Sci 123(5):319–324
85. Karpouzas DG, Tsiamis G, Trevisan M, Ferrari F, Malandain C, Sibourg O, Martin-Laurent F (2016) 'LOVE TO HATE'-pesticides: felicity or curse for the soil microbial community? An FP7 IAPP Marie Curie project aiming to establish tools for the assessment of the mechanisms controlling the interactions of pesticides with soil microorganisms. Environ Sci Pollut Res 23:18947–18951
86. Chang Y-J, Hussain AKMA, Stephen JR, Mullen MD, White DC, Peacock A (2001) Impact of herbicides on the abundance and structure of indigenous β-subgroup ammonia-oxidizer communities in soil microcosms. Environ Toxicol Chem 20(11):2462–2468

87. Pantelelis I, Karpouzas DG, Menkissoglu-Spiroudi U, Tsiropoulos NG (2006) Influence of soil physicochemical and biological properties on the degradation and adsorption of the nematicide fosthiazate. J Agric Food Chem 54:6783–6789
88. Hug LA, Beiko RG, Rowe AR, Richardson RE, Edwards EA (2012) Comparative metagenomics of three Dehalococcoides-containing enrichment cultures: the role of the non-dechlorinating community. BMC Genomics 13:327
89. Romine MF, Rodionov DA, Maezato Y, Osterman AL, Nelson WC (2017) Underlying mechanisms for syntrophic metabolism of essential enzyme cofactors in microbial communities. ISME J 11:1434–1446
90. Loreau M, Naeem S, Inchausti P, Bengtsson J, Grime JP et al (2001) Biodiversity and ecosystem functioning: current knowledge and future challenges. Science 294:804–808
91. Savage VM, Webb CT, Norberg J (2007) A general multi-trait-based framework for studying the effects of biodiversity on ecosystem functioning. J Theor Ecol 247:213–229
92. Simek K, Vrba J, Pernthaler J, Posch T, Hartman P, Nedoma J, Psenner R (1997) Morphological and compositional shifts in an experimental bacterial community influenced by protists with contrasting feeding modes. Appl Environ Microbiol 63(2):587–595
93. Glücksman E, Bell T, Griffiths RI, Bass D (2010) Closely related protist strains have different grazing impacts on natural bacterial communities. Environ Microbiol 12:3105–3113
94. Geisen S, Mitchell EAD, Adl S, Bonkowski M, Dunthorn M, Ekelund F et al (2018) Soil protists: a fertile frontier in soil biology research. FEMS Microbiol Rev 42:293–323
95. Saleem M, Fetzer I, Harms H, Chatzinotas A (2013) Diversity of protists and bacteria determines predation performance and stability. ISME J 7:1912–1921
96. Karimi B, Maron PA, Boure NCP, Bernard N, Gilbert D, Ranjard L (2017) Microbial diversity and ecological networks as indicators of environmental quality. Environ Chem Lett 15:265–281
97. Zhao Z-B, He J-Z, Quan Z, Wu C-F, Sheng R, Zhang L-M, Geisen S (2020) Fertilization changes soil microbiome functioning, especially phagotrophic protists. Soil Biol Biochem 148:107863
98. Oliverio AM, Geisen S, Delgado-Baquerizo M, Maestre FT, Turner BL, Fierer N (2020) The global-scale distributions of soil protists and their contributions to belowground systems. Sci Adv 6:eaax8787
99. Crouzet O, Wizniowski J, Donnadieu F, Bonnemoy F, Bohatier J, Mallet C (2013) Dose-dependent effects of the herbicide mesotrione on soil cyanobacterial communities. Arch Environ Contam Toxicol 64:23–31
100. Coors A, Frische T (2011) Predicting the aquatic toxicity of commercial pesticide mixtures. Environ Sci Eur 23:22. https://doi.org/10.1186/2190-4715-23-22
101. Nowell NH, Norman JE, Moran PW, Martin JD, Stone WW (2014) Pesticide toxicity index – a tool for assessing potential toxicity of pesticide mixtures to freshwater aquatic organisms. Sci Total Environ 476–477:144–157
102. Belden JB, Gilliom RJ, Lydy MJ (2007) How well can we predict the toxicity of pesticide mixtures to aquatic life? Integr Environ Assess Manag 3:364–372
103. Gopal M, Gupta A, Arunachalam V, Magu SP (2007) Impact of azadirachtin, an insecticidal allelochemical from neem on soil microflora, enzyme and respiratory activities. Bioresour Technol 98:3154–3158
104. Romdhane S, Devers-Lamrani M, Beguet J, Bertrand C, Calvayrac C, Salvia M-V, Jrad AB, Dayan FE, Spor A, Barthelemebs L, Martin-Laurent F (2019) Assessment of the ecotoxicological impact of natural and synthetic β-triketone herbicides on the diversity and activity of the soil bacterial community using omic approaches. Sci Total Environ 651:241–249
105. Yu Z, Zhang Y, Luo W, Wang Y (2015) Root colonization and effect of biocontrol fungus *Paecilomyces lilacinus* on composition of ammonia-oxidizing bacteria, ammonia-oxidizing archaea and fungal populations of tomato rhizosphere. Biol Fertil Soils 51:343–351
106. Krober M, Wibberg D, Grosch R, Eikmeyer F, Verwaaijen B, Chowdhury SP, Hartmann A, Puhler A, Schluter A (2014) Effect of the strain *Bacillus amyloliquefaciens* FZB42 on the

microbial community in the rhizosphere of lettuce under field conditions analyzed by whole metagenome sequencing. Front Microbiol 5:252. https://doi.org/10.3389/fmicb.2014.00252
107. Mayerhofer J, Eckard S, Hartmann M, Grabenweger G, Widmer F, Leuchtmann A, Enkerli J (2017) Assessing effects of the entomopathogenic fungus *Metarhizium brunneum* on soil microbial communities in *Agriotes* spp. biological pest control. FEMS Microbiol Ecol 93: fix117
108. Zimmermann J, Musyoki MK, Cadisch G, Rasche F (2016) Biocontrol agent *Fusarium oxysporum* f.sp. strigae has no adverse effect on indigenous total fungal communities and specific AMF taxa in contrasting maize rhizospheres. Fungal Ecol 23:1–10
109. Storck V, Lucini L, Mamy L, Ferrari F, Papadopoulou ES, Nikolaki S, Karas PA, Servien R, Karpouzas DG, Trevisan M, Benoit P, Martin-Laurent F (2016) Identification and characterization of tebuconazole transformation products in soil by combining suspect screening and molecular typology. Environ Pollut 208:537–545
110. Wu X, Yin Y, Wang S, Yu Y (2014) Accumulation of chlorothalonil and its metabolite, 4-hydroxychlorothalonil, in soil after repeated applications and its effects on soil microbial activities under greenhouse conditions. Environ Sci Pollut Res 21:3452–3459
111. Storck V, Karpouzas DG, Martin-Laurent F (2017) Towards a new pesticide registration policy. Sci Total Environ 575:1027–1033
112. Wicker J, Lorsbach T, Gutlein M, Schmid E, Latino D, Kramer S, Fenner K (2016) enviPath – the environmental contaminant biotransformation pathway resource. Nucleic Acids Res 44: D502–D508
113. Latino DARS, Wicker J, Gutlein M, Schmid E, Kramer S, Fenner K (2017) Eawag-soil in enviPath: a new resource for exploring regulatory pesticide soil biodegradation pathways and half-life data. Environ Sci: Processes Impacts 19:449–464
114. Rocca JD, Simonin M, Blaszczak JR, Ernakovich JG, Gibbons SM, Midani FS, Washburne AD (2019) The microbiome stress project: toward a global meta-analysis of environmental stressors and their effects on microbial communities. Front Microbiol 9:3272. https://doi.org/10.3389/fmicb.2018.032

Author Queries

Chapter No.:

Query Refs.	Details Required	Author's response
AU1	Please check if the expansion of the abbreviation "INT" is fine as given in Table 1.	
AU2	Part of the Fig. 2 is blurred. Please provide revised figure with better quality.	