Pesticide soil microbial toxicity: setting the scene for a new pesticide risk assessment for soil microorganisms (IUPAC Technical Report)

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IUPAC Technical Report

Dimitrios G. Karpouzas*, Zisis Vryzas and Fabrice Martin-Laurent

Pesticide soil microbial toxicity: setting the scene for a new pesticide risk assessment for soil microorganisms (IUPAC Technical Report)

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Abstract: Pesticides constitute an integral part of modern agriculture. However, there are still concerns about their effects on non-target organisms. To address this the European Commission has imposed a stringent regulatory scheme for new pesticide compounds. Assessment of the aquatic toxicity of pesticides is based on a range of advanced tests. This does not apply to terrestrial ecosystems, where the toxicity of pesticides on soil microorganisms, is based on an outdated and crude test (N mineralization). This regulatory gap is reinforced by the recent methodological and standardization advances in soil microbial ecology. The inclusion of such standardized tools in a revised risk assessment scheme will enable the accurate estimation of the toxicity of pesticides on soil microorganisms and on associated ecosystem services. In this review we (i) summarize recent work in the assessment of the soil microbial toxicity of pesticides and point to ammonia-oxidizing microorganisms (AOM) and arbuscular mycorrhizal fungi (AMF) as most relevant bioindicator groups (ii) identify limitations in the experimental approaches used and propose mitigation solutions, (iii) identify scientific gaps and (iv) propose a new risk assessment procedure to assess the effects of pesticides on soil microorganisms.

Keywords: ecotoxicity; pesticide regulatory framework; pesticides; risk assessment; soil microorganism.

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1 Introduction

The use of pesticides is still considered a cornerstone of modern agriculture, despite major concerns about their undesirable effects on environment and human health. Soil is the main sink for pesticides used in agriculture. Upon their application, pesticides interact with soil microorganisms with the outcome of this interaction being pesticide toxicity to the soil microbiota or growth-linked microbial degradation of the pesticide, depending on a range of biotic and abiotic factors (Fig. 1). Potential toxicity effects on soil microorganisms often appears as a result of the exposure of soil microbiota to pesticide levels that they cannot effectively dissipate. This leads to a significant change in the rate of different microbial processes associated with ecosystem services. Conversely, the growth-linked microbial degradation of pesticides is driven by "specialist microbes" that often constitute a small fraction of the soil microbiota [1, 2] and carry specific enzymes for the degradation of these compounds. Classic examples of pesticide classes which are prone to growth-linked microbial degradation, often rendering them inefficient for pest control, are organophosphates [3, 4], carbamates [5], triazines [6], dicarboxamides [7] and substituted phenylureas [8].

Globally, the placement in the market of pesticide compounds is under the control of a regulatory framework, which should be regularly updated to meet the growing public concern about the frequent detection of residual pesticide levels in fresh produce [9], water resources [10] and soil [11] and to incorporate methodological and knowledge advances. At the European Union (EU) level, the placement of pesticides (plant protection products) in the market is dictated by the Regulation 1107/2009. All pesticides should go through a tedious procedure of tests, described in detail in Regulation 1107/2009, which aim to determine (a) the level of environmental exposure stemming from the agricultural use of plant protection products containing these pesticide compounds and (b) the toxicity or ecotoxicity of pesticides to organisms from different trophic levels. The direct comparison between environmental exposure and ecotoxicity constitutes the backbone of pesticide environmental risk assessment [12].

Today, a set of standardized ecotoxicity tests supporting the risk assessment are in place to assess the toxicity of pesticides on aquatic organisms and soil fauna, unlike soil microorganisms, whose ecotoxicological response to pesticides has not been set as a priority in the current risk assessment framework. This is surprising

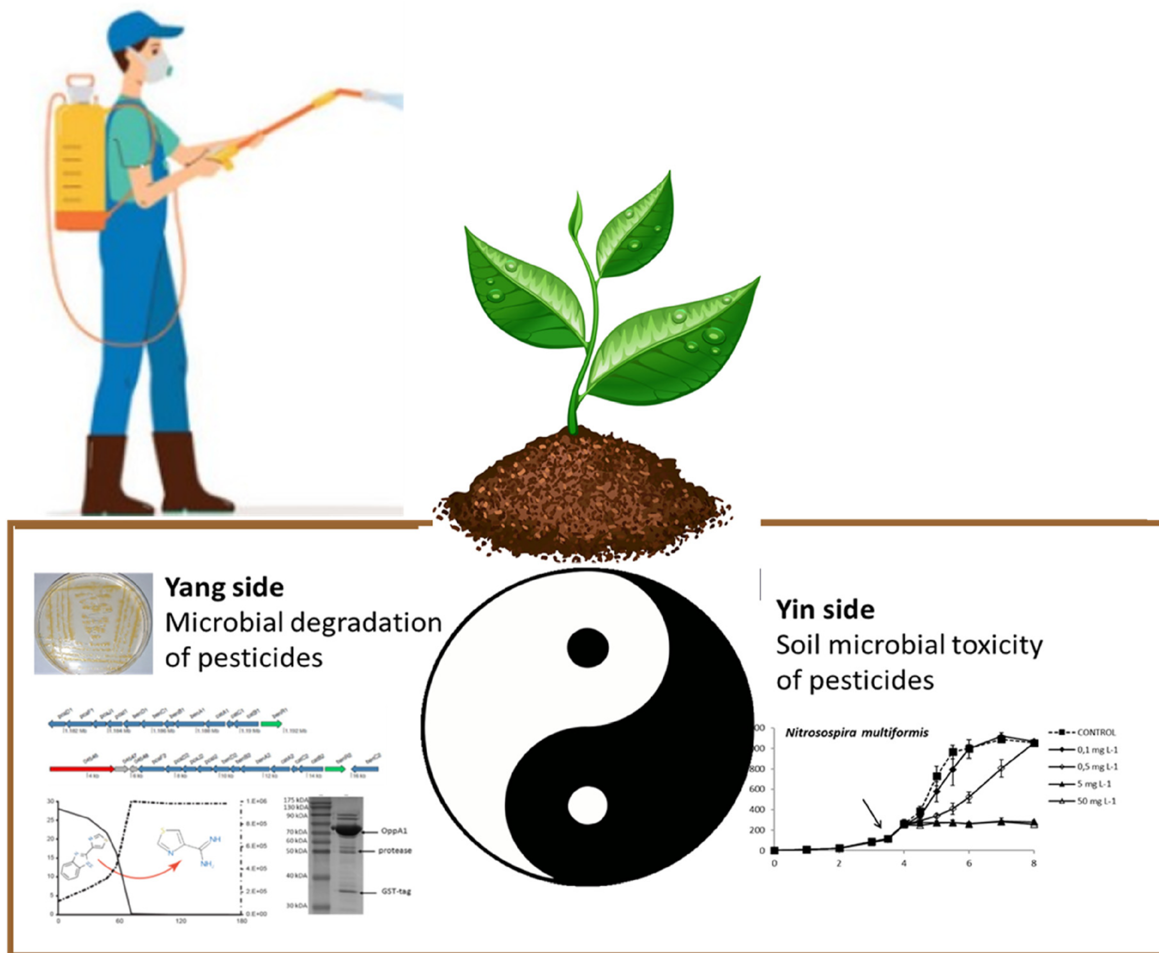

Fig. 1: A schematic representation of the type of interactions occurring between pesticides and soil microorganisms upon their agricultural application. Upon their application pesticides end up in soil with the outcome of the interactions being either (a) positive (yang side), where soil microorganisms have the capacity to actively degrade the pesticide, using it as an energy source, and (b) negative (yin side), where pesticides induce toxicity responses to the soil microorganisms appearing as inhibition of their growth with reciprocal effects on key soil microbial functions.

if we consider the number of key ecosystem services provided by microorganisms in soil. A non-exhaustive list of such services includes their role in: (i) nutrient cycling, modulating soil fertility [13]; (ii) increasing plant tolerance to biotic (pests and diseases) and abiotic (drought, salinity) stressors [14]; (iii) the uptake of nutrients by plants, hence promoting plant growth and agricultural productivity [15]; (iv) the maintenance of soil structure [16]. In response to this, the European Food Safety Authority (EFSA) verified the presence of regulatory gaps in the assessment of the toxicity of pesticides on soil microorganisms and identified soil microbes as a possible specific protection goal to avoid undesirable effects on ecosystem services [17]. Despite that, the assessment of the toxicity of pesticides on soil microorganisms at a regulatory level is still performed with the Organization for Economic Co-operation and Development (OECD) Test No. 216 (Soil Microorganisms: Nitrogen Transformation Test) [18], which offers a combined measurement of ammonification and nitrification rate in soil.

All the above highlight the need for a scheme to assess the toxicity of pesticides on soil microorganisms. This should consider the recent impressive methodological advances in soil microbial ecology coinciding with the upsurge of methods standardization. Still there seems to be a major lag (of almost 10 years for each step) between methods development, standardization and implementation in the regulatory process. In response

to all these advances, EFSA [19] issued a scientific opinion regarding the state of the science on risk assessment of plant protection products for in-soil organisms, where several suggestions regarding the toxicity of pesticides on the soil microbiota were put forward. Some of the proposed suggestions were controversial for the reasons described below: (i) the N transformation test is inclusive and should be retained as part of pesticide toxicity testing, despite being a rather crude and outdated approach. It was further suggested that the specific test should be upgraded to a dose response assay as it is applied for non-agrochemicals [19]; (ii) the new molecular methods provide a high resolution analysis of the soil microbiota, but they are still not considered standardized for use in environmental risk assessment, although the list of standards from the International Organization of Standardization (ISO) in soil microbial ecology has tripled in the last 15 years including molecular tools like direct soil DNA extraction (ISO 11063), q-PCR analysis of the abundance microbial groups (ISO 17601) etc. (Table 1); (iii) functional microbial endpoints are more relevant for environmental risk assessment compared to microbial diversity endpoints; and (iv) arbuscular mycorrhizal fungi (AMF) are good potential bioindicators for assessing the soil microbial toxicity of pesticides, but other key functional microbial groups like ammonia-oxidizing microbes (AOM)

Table 1: A list of the ISO standardized methods that are currently available in soil microbiology (details could be found in the website www.iso.org).

Year	ISO Code	Full title of standardized method
1997	ISO14240:1	Determination of soil microbial biomass – Part 1 Substrate induced respiration method
1997	ISO14240:2	Determination of soil microbial biomass – Part 2 Fumigation-extraction method
2002	ISO16072	Laboratory methods for determination of microbial soil respiration
2009	ISO10832	Effects of pollutants on mycorrhizal fungi- germination test
2010	ISO/TS29843-1	Determination of soil microbial diversity- Part 1: Method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis
2011	ISO/TS29843-2	Determination of soil microbial diversity- Part 2: Method by phospholipid fatty acid analysis (PLFA) using the simple PLFA extraction method
2012	ISO15685	Determination of potential nitrification and inhibition of nitrification – Rapid test by ammonium oxidation
2012	ISO14238	Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes
2012	ISO17155	Determination of abundance and activity of soil microflora using respiration curves
2016	ISO17601	Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil
2016	ISO18187	Contact test for solid samples using the dehydrogenase activity of *Arthrobacter globiformis*
2018	ISO/TS20131-1	Easy laboratory assessments of soil denitrification, a process source of N_2O emissions—Part 1: Soil denitrifying enzymes activities
2018	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	ISO20130	Measurement of enzyme activity patterns in soil samples using colorimetric substrates in micro-well plates
2019	ISO23753:1	Determination of dehydrogenase activity in soils – Part 1: Method using triphenyltetrazolium chloride (TTC)
	ISO23753:1/AMD1:2020	Determination of dehydrogenases activity in soils – Part 1: Method using triphenyltetrazolium chloride (TTC)—Amendment 1
2019	ISO23753:2	Determination of dehydrogenase activity in soils – Part 2: Method using iodotetrazolium chloride (INT)
	ISO23753:2/AMD1:2020	Determination of dehydrogenase activity in soils – Part 2: Method using iodotetrazolium chloride (INT) – Amendment 2
2019	ISO/TS22939	Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates
2020	ISO11063	Method to directly extract DNA from soil samples

might be equally good or even more appropriate toxicity bioindicators for reasons explained further in the paper (see Section 3.3).

In this review we will (i) define the main factors that drive the potential ecotoxicity of pesticides on the soil microbiota, (ii) describe the key elements of a comprehensive assessment of the ecotoxicity of pesticides on soil microorganisms, highlight microbial groups that could be utilized as microbial indicators in environmental risk assessment and propose a new relevant tiered risk assessment approach with appropriate testing procedures, (iii) highlight open questions in soil microbial ecotoxicology both at research and regulatory level (iv) identify new areas that should be the research focus in soil microbial ecotoxicology in the forthcoming years.

2 Factors controlling pesticides toxicity on the soil microbiota

Several factors determine the potential of a pesticide compound to be toxic on the soil microbiota including: (a) compound chemical structure, (b) pesticide mode of action and the target organism, (c) dose rates applied, and (d) mode of application.

Chemical structure has a major influence on the ecotoxicological profile of pesticides, either directly through the association of certain chemical moieties with the toxicity of pesticides on soil microorganisms or indirectly through its effects on the physicochemical properties of the given compound (water solubility, lipophilicity, etc.) that determine its **bioavailability and persistence**. Direct structure – toxicity relationships have been shown for pesticides and aquatic or terrestrial macro-organisms [20, 21]. These have acted as the basis for the development of Quantitative Structure-Activity Relationship (QSAR) models used in pesticide regulatory practice mostly to estimate the potential toxicity of pesticide transformation products (TPs) [22]. In this regard, Traore *et al.* [23] developed an *in-silico* tool called TyPoL which enables the classification of pesticide compounds into different ecotoxicological groups based on a series of molecular descriptors, environmental and toxicological parameters. The EFSA guidance document on the use of the Weight of Evidence approach in scientific assessments provides a framework for integrating *in silico* models and read-across extrapolation methods for predicting toxicity of pesticides [24]. Such an approach has been used in the assessment of pesticide risk to aquatic organisms; on the contrary little is known about potential associations of chemical structure with the toxicity of pesticides on soil microorganisms. This is very much reflected in the lack of QSAR *in silico* tools facilitating the estimation of the soil microbial toxicity of pesticides according to molecular descriptors.

The **pesticide mode of action** strongly affects their potential toxicity on soil microorganisms. Fungicides are expected to be inherently more toxic to soil fungi, unlike herbicides and insecticides that are not expected to be toxic to the soil microbiota [25–27]. However, deviations from this general rule are often reported. Previous studies have suggested that herbicides which act by inactivating plant enzymes like acetolacate synthase (EC 2.2.1.6) or 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27), that have homologues in other living organisms including soil microorganisms, might exert toxicity onto soil microorganisms [28, 29]. Along this line, Thiour-Mauprivez *et al.* [30] proposed a mode-of-action-based assessment of the toxicity of herbicides (possibly applicable for other pesticide groups) on the soil biota by considering the enzymes targeted by each pesticide group as molecular biomarkers.

The famous statement of Paracelsus "*All things are poison and nothing is without poison. Solely the dose determines that a thing is not a poison*" largely applies to the interaction of pesticide with soil microorganisms where higher dose rates often induce higher inhibitory effects on the abundance and activity of soil microorganisms [31]. However, in certain studies effects of pesticides on soil microorganisms were evident only upon increase of the dose rates at levels not relevant for agricultural use [32, 33]. Exceptions to this dose-dependent response of microorganisms to pesticides have been also reported [34]. The phenomenon of hormesis, the stimulation of microbial growth upon exposure to low dose rates of pesticides, has been often responsible for deviation from dose-dependent responses [35].

Soil-applied pesticides are expected to be more potent for toxicity on soil microorganisms based on their intentional application in soil which results in higher exposure levels. Conversely, foliage applied compounds are largely intercepted by plant foliage, with the level of interception varying from 20 to 80 % according to the growth stage of the crop, thereby reducing the amount of pesticide reaching the soil and hence the exposure of soil microbiota. However, fully systematic applied chemicals like glyphosate could translocate from leaves to roots and eventually to soil contributing to the exposure of soil to pesticides [36]. Still, we should consider that the true extent of exposure of the soil microbiota to any pesticide compound, no matter the application regime followed, is strongly affected by its persistence and bioavailability in soil.

3 Key elements of a risk assessment scheme

A robust and comprehensive assessment of the risk of pesticides exerting undesirable effects on the soil microbiota should encompass the following elements: (a) an accurate estimation of the level and the duration of the exposure; (b) the use of high resolution, sensitive and, most importantly, standardized methods; (c) identification of soil microbial groups that could be used as indicators of the toxicity of pesticides on the soil microbiota; (d) a revised and novel tiered risk assessment scheme. The content of these elements will be discussed further below.

3.1 Pesticide exposure estimation

Determination of the level and the duration of the exposure should be an indispensable part of any toxicity assay. Temporal measurement of the dissipation of pesticides during any assay would define the level and the duration of exposure of the soil microbial community to the pesticide in question. Furthermore, detection and quantitation of pesticide TPs formed in soil would provide information on the potential involvement of TPs on toxicity effects observed. This is already done in the current regulatory framework, although the selection of TPs, relevant for inclusion in risk assessment, relies solely on the quantity of the TP formed (more than 10 % of the parent), excluding TPs that do not meet the 10 % rule but show high potency due to their chemical structure or mode of action. 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 or a combination of both). Such an example is offered by the study of Vasileiadis *et al.* [31], who identified via correlation testing and a series of soil and *in vitro* tests, 3,5-dichloroaniline, the main TP of the fungicide iprodione (3-(3,5-dichlorophenyl)-*N*-(propan-2-yl)-2,4-dioxoimidazolidine-1-carboxamide) in soil, as the main driver of the toxicity observed on a range of functional microbial attributes.

3.2 Standardization of methods in soil microbial ecology

The recent methodological advances in soil microbial ecology and microbial ecotoxicology revealed a previously unprecedented microbial complexity and diversity and highlighted the multifaceted role of soil microorganisms in ecosystem functioning [35]. The methods available could be classified into: (a) **functional methods,** that determine the activity of enzymes, rates of microbial processes or the activity of microbial groups controlling key functions in terrestrial ecosystems; (b) **diversity methods,** which determine the composition of the soil microbiota operating at varying resolution from phylogenetically distinct (*e.g.* bacteria, archaea, fungi) to functionally distinct microbial groups (i.e. AOM, AMF, methane-oxidizing microorganisms, nitrogen-fixing bacteria, denitrifying bacteria). A key step for the implementation of these established and advanced methods in environmental risk assessment is their standardization. Standardization of functional methods is well advanced compared to the diversity methods.

Functional methods include measurements of (i) the activity of soil microbial exoenzymes involved in carbon, nitrogen, phosphorus and sulfur cycling, and (ii) the rates of microbial processes like soil microbial respiration, potential nitrification or denitrification. These methods have been used in several studies for assessing the toxicity of pesticides on soil microorganisms [25, 33, 34, 38, 39]. For these methods, relevant ISO standards are available (Table 1), while at the same time high throughput approaches facilitating their rapid regulatory uptake have been developed for measuring soil microbial exoenzymes [40] and soil microbial respiration through MicroResp® [41]. However, the frequent lack of consistency in the response of these methods to pesticide exposure, appearing as effects deviating from the expected dose-dependent pattern, might be a plausible explanation for their exclusion from environmental risk assessment [42, 43].

Nucleic acid-based methods determining the abundance (DNA) or the activity (RNA) of key functional microbial groups via quantitative Polymerase Chain Reaction (q-PCR) and Reverse Transcription-q-PCR (RT-q-PCR), respectively, could be also considered as tools for measuring microbial functions, although they were not considered in environmental risk assessment due to lack of standardization [43]. Relatively recent advances with the development of ISO standards for direct soil DNA extraction (ISO11063-2012 replaced by ISO11063-2020) [44] and determination of the abundance of soil microbial groups via q-PCR (ISO17601) [45] addressed these concerns and paved the way for their implementation in pesticide environmental risk assessment. Several soil studies have determined effects of pesticides on the abundance of phylogenetically distinct microbial groups like bacteria, fungi or archaea or the abundance of functional microbial groups like AOM (ammonia-oxidizing microorganisms) [31, 46, 47], sulfur-oxidizing bacteria [34] and degraders of biogenic aromatic compounds [48] using q-PCR standardized protocols. Besides mere determination of microbial abundance in soils, RT-q-PCR approaches based on soil RNA offer a more sensitive measurement of the potential effects of pesticides on microbial activity, by measuring the number of mRNA transcripts of microbial genes involved in key soil microbial functions. However, only a few studies have used RT-q-PCR approaches to determine pesticide effects on soil microbial activity, mostly targeting AOM [42]. This most probably reflects the sensitivity of soil RNA to lysis during laboratory handling which often results in low recovery of gene transcripts.

Diversity methods include Phospholipid Fatty Acids Analysis (PLFAs), molecular PCR-based fingerprinting like Denaturating Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphism (TRFLP), and the more recent and powerful amplicon sequencing approaches. Phospholipid Fatty Acids Analysis is a well standardized method (ISO/TS29843-1 and -2) [49, 50], which provides information about the overall size and the composition of the living soil microbial community, however at a rather low phylogenetic resolution [51]. Conversely, PCR-based molecular fingerprinting or sequencing methods could provide robust phylogenetic information at family, genus or even species level, but are faced with limited standardization. The depth of phylogenetic analysis of the soil microbial community offered by these methods has increased from the lower resolution of earlier fingerprinting methods like TRFLP and DGGE [52, 53], to the higher resolution amplicon sequencing approaches or so called meta-taxonomics [54]. Denaturating Gradient Gel Electrophoresis and TRFLP, either as stand-alone approaches or in combination with clone libraries, have been heavily used in the period of 2000–2015 to determine effects of pesticides on the diversity of phylogenetically and functionally distinct microbial groups [55, 56]. However, they could not provide quantitative information for less abundant members of the soil microbiota. Since 2015, several studies have used meta-taxonomic approaches to identify effects of pesticides on the diversity of bacteria, fungi [57, 58] and functional microbial groups like AOM [31]. Benchmarking protocols for the preparation and setup of meta-taxonomic 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 and have been largely adopted by most recent pesticide soil ecotoxicity studies making a major step towards their formal standardization. However, what we are still missing is the standardization of the bioinformatic pipeline for the analysis of amplicon sequencing data and the way to make use of these multivariate data in a regulatory context. These issues and ways to resolve them are further discussed in Sections 4.5 and 5.1 respectively.

Thiele-Bruhn *et al.* [59] classified the different methods available in soil microbial ecology according to the Ecosystem Services Approach, in line with the recommendation of EFSA [19], that functional endpoints are more attainable and ready-to-implement compared to diversity endpoints. In this approach, methods, traditional and new ones, were classified according to their relevance for a list of eight ecosystem services, namely: (a) biodiversity, genetic resources, cultural services; (b) food web support; (c) biodegradation of pollutants; (d) nutrient cycling; (e) pest control and plant growth promotion; (f) carbon cycling; (g) greenhouse gas emissions; (h) soil structure formation. Most of the above-mentioned services are emanated from the same basic functions. The authors highlighted the potential of q-PCR-based methods to define, in a standardized way, pesticide effects on microbial groups performing key soil functions involved in soil fertility (nitrogen and phosphorus transformation, plant growth promotion) and environmental quality (greenhouse gas emissions, natural attenuation of pollutants). All these point towards the use of endpoints which represent a microbial function until we will be able to make use of the wealth of data derived from meta-taxonomic analysis in a standardized way and in a regulatory context.

3.3 Soil microbial indicators

A first assessment of the potential toxicity of pesticides on aquatic and terrestrial macro-organisms is performed through tests on specific organisms from different trophic levels identified as bioindicators (single species tests). A cornerstone organism for pesticide ecotoxicology is *Daphnia magna*, used as an indicator of aquatic invertebrates, while *Onchorhynchus mykiss*, *Eisenia foetida*, *Selenastrum capricornutum* and *Lemna minor* are used as toxicity indicators for fishes, earthworms, algae and aquatic plants, respectively [19, 60]. These bioindicators were selected based on the following criteria: (i) they have a key ecological role; (ii) they are characterized by high sensitivity to pesticides, compared to other species in the same group of organisms; (iii) they show a clear ecotoxicological response to pesticides; (iv) we have a very good knowledge of their life cycle; and (v) there are standardized tests available for the determination of their response to pesticides.

If the same logical path is to be followed for the establishment of a new framework to assess the toxicity of pesticides on soil microorganisms, new microbial groups which fulfil the above criteria should be identified to serve as candidate bioindicators. Previous studies have pointed to AOM [61], AMF [19, 33], nitrogen-fixing bacteria [62], protists [63], microalgae [64] and cyanobacteria [65], all of which are involved in key soil microbial functions as shown in Fig. 2. In the following sections we will try to describe the pros and cons of these potential bioindicator microbial groups with relevant examples from the literature.

3.3.1 Arbuscular mycorrhizal fungi

The responsiveness of arbuscular mycorrhizal fungi (AMF) to pesticide exposure was evident in earlier studies [26, 66] leading to the development of the ISO-10832 standard measuring the impact of toxicants on AMF spore germination [67] (Table 1). Karpouzas *et al.* [33] used molecular tools to assess the toxicity of pesticides on AMF and suggested their use as potential bioindicators for assessing the toxicity of pesticides on soil microorganisms. It was only in 2017 when EFSA also suggested the inclusion of AMF as soil microbial indicators for pesticide toxicity [19]. Arbuscular mycorrhizal fungi are the most ubiquitous plant symbiotic microbes on earth with up to 80 % of higher plants colonized by obligate biotrophic fungi of the phylum Glomeromycota [68]. They colonize plant roots and increase the capacity of the plants to acquire nutrients, especially P, and water from soil, offering plant tolerance to biotic and abiotic stress [69]. In exchange for all these benefits provided to plants, AMF derive plant photosynthates. Besides improving plant fitness, AMF also contribute to the formation and stabilization of soil aggregation [16], improve soil carbon stocks [70], contribute to nutrient exchanges between plants connected *via* a network of AMF mycelia, and control the composition and productivity of plant communities [71].

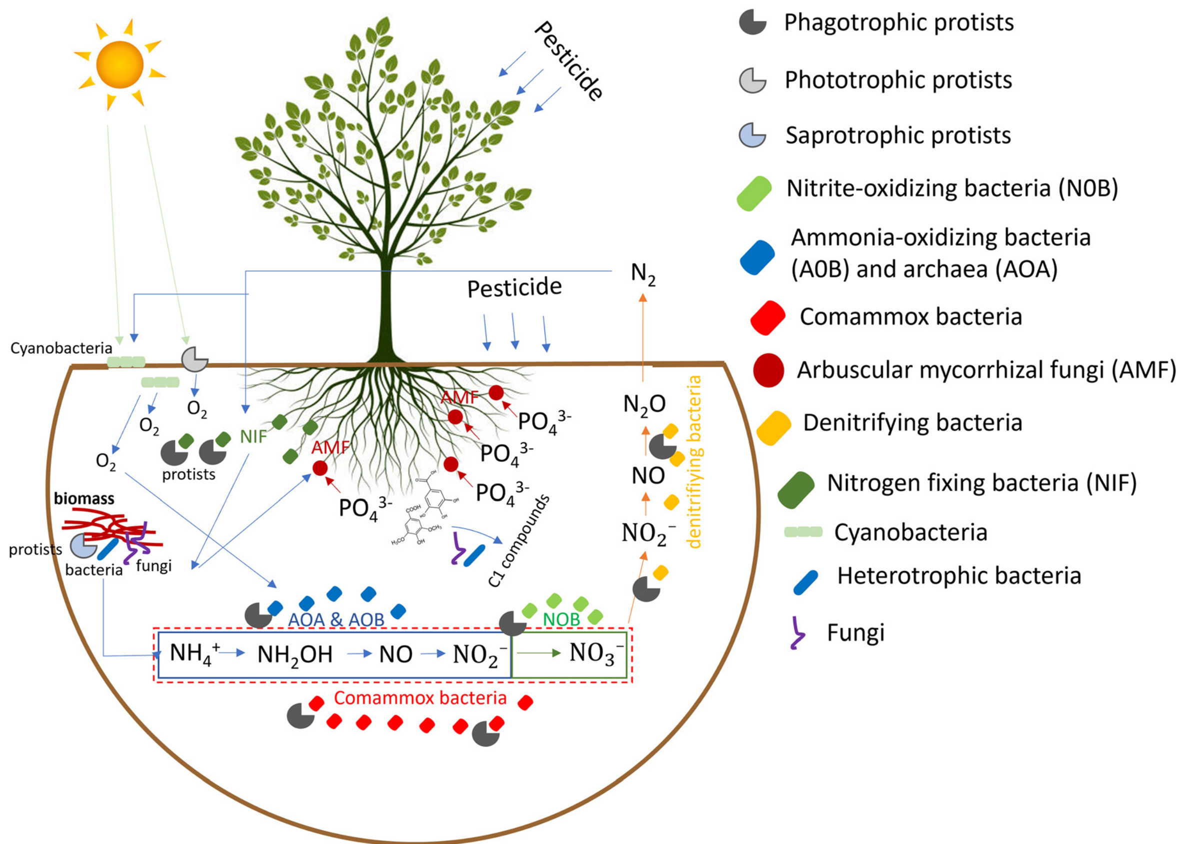

Fig. 2: The different functional microbial groups that could be used as indicators of the soil microbial toxicity of pesticides and their ecological role in soil. Upon their application, either on the foliage or directly in soil, pesticides come into contact with different microbial groups performing significant soil functions like (a) Arbuscular mycorrhizal fungi (AMF) that contribute to enhanced PO_4^{3-} uptake by plants; (b) nitrogen-fixing bacteria (NIF), which fix atmospheric N_2 to NH_4^+, operating as free-living or as symbionts in the roots of leguminous plants; (c) ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), comammox bacteria and nitrite-oxidizing bacteria (NOB) collectively transforming NH_4^+ to NO_3^- (d) denitrifying bacteria that transform NO_3^- to N_2 through the intermediate production of deleterious gases like NO and N_2O (e) cyanobacteria on surface soil that photosynthesize and release O_2 in soil (f) heterotrophic bacteria and fungi that decompose soil organic matter and release organic nitrogen that could be further hydrolyzed to NH_4^+ feeding into the nitrification process (g) protists that server a range of soil functions acting as saprotrophs, phototrophs and phagotrophs feeding on bacteria, fungi and small eukaryotes.

Several previous studies have tested the toxicity of pesticides on AMF at various experimental scales [27, 33, 72, 73]. However, certain characteristics of their lifestyle such as (i) their obligatory symbiotic nature and (ii) the succession of intraradical and extraradical life stages makes essential the use of complementary experimental approaches to be able to distinguish between direct toxicity effects on AMF *vs* indirect effects driven by the potential phytotoxicity of pesticides. The former is expected by fungicides, whereas indirect toxicity effects on AMF are expected by herbicides. Giovannetti *et al.* [66] tested 14 pesticides for their effects on spore germination and pre-symbiotic mycelial growth. They reported a higher toxicity of fungicides compared to the other pesticide groups tested. Another important issue that needs extra consideration is the differential exposure of the different life stages of AMF to pesticides. Extraradical AMF life stages are prone to pesticide exposure, compared to intraradical stages which deviate pesticide exposure, especially when non-systemic pesticides are tested. The standard ISO10832 «*Effects of pollutants on mycorrhizal fungi- germination test*» is using the germination of spores of *Funeliformis mosseae*, one of the most well studied AMF, as a relevant toxicity endpoint [67]. Mallmann *et al.* [74] proposed an optimization of this ISO test with the use of *Gigagspora albida* and *Rhizophagus clarus*, to cover a wider diversity of AMF, and boric acid as negative control instead

of cadmium nitrate, which was initially chosen as the specific ISO standard was originally designed to assess the ecotoxicity of metals to AMF.

The toxicity of pesticides on natural assemblages of AMF has been determined in planted pot and field studies with mycorrhizal colonization of roots, phosphorus content and other plant physiological attributes (root and shoot biomass), utilized as ecotoxicological endpoints [72, 75]. These studies introduce realism and complexity in the toxicity assessment, but their outcome is affected by confounding pesticide- and AMF-related factors. This was demonstrated in a study by Karpouzas *et al.* [33] who observed a major decrease in the level of mycorrhizal colonization of maize roots upon repeated applications of nicosulfuron (2-{[(4,6-dimethoxypyrimidin-2-yl)carbamoyl]sulfamoyl}-*N*,*N*-dimethylpyridin-3-carboxamide) at rates higher than 10 times the recommended dose. However, it was still not possible to determine with this experimental set up the nature of the effects on AMF; direct on AMF or indirect via a possible toxicity of the herbicide nicosulfuron to the plant host. In this respect, the complementary use of *in vitro* tests could provide insights into the potential toxicity of pesticides on the different life stages of AMF, and hence clarify the nature of the effects observed.

Although AMF are obligatory symbionts, they could be cultivated *in vitro* on self-propagating mycorrhized *Agrobacterium tumefaciens* transfer-DNA transformed roots of *Daucus carota* or *Medicago truncatula* growing in sterilized minimal medium [76–78]. Furthermore, the flexible compartmentalization of these *in vitro* cultivation systems (mono-, bi- or tri-compartmental systems) allows the examination of the toxicity of pesticides at the different life stages of AMF and could provide insights into the toxicity mechanisms. In its simpler form, the mono-compartmental axenic culture system of AMF has been used to assess the toxicity of pesticides at the symbiotic phase, however the potential phytotoxicity of pesticides (*e.g.* herbicides) on the transformed roots cannot be excluded and might complicate results interpretation. Using this system, Wan *et al.* [76] first calculated Inhibitory Concentration 50 % (IC_{50}) values for a range of pesticides using reduction in extraradical mycelium sporulation as a conservative endpoint. Benomyl (methyl [1-(butylcarbamoyl)-1*H*-benzimidazol-2-yl]carbamate), chlorothalonil (benzene-2,4,5,6-tetrachloro-1,3-dicarbonitrile) and glyphosate (*N*-(phosphonomethyl)glycine) were the most toxic pesticides (IC_{50} < 1 mg/L) compared to AMPA (2-amino-3-(5-methyl-3-oxo-2,3-dihydro-1,2-oxazol-4-yl)propanoic acid) (IC_{50} = 4.2 mg/L), the major TP of glyphosate, and the botanical pesticide azadirachtin (dimethyl (2a*R*,2a^1*R*,3*S*,4*S*,4a*R*,5*S*,7a*S*,8*S*,10*R*,10a*S*)-10-(acetyloxy)-3,5-dihydroxy-4-methyl-4-[(1a*R*,2*S*,3a*S*,6a*S*,7*S*,7a*S*)-6a-hydroxy-7a-methyl-3a,6a,7,7a-tetrahydro-2,7-methanofuro[2,3-*b*]oxireno [2,3-e] oxepin-1a(2*H*)-yl]-8-{[(2*E*)-2-methylbut-2-enoyl]oxy}octahydro-1*H*,7*H*-naphtho[1,8-*bc*:4,4a-*c'*]difuran-5,10a(8*H*)-dicarboxylate) (IC_{50} = 230 mg/L). Zocco *et al.* [77] first used a bi-compartmental system, composed of a root compartment and hyphae compartment, to determine the toxicity of the fungicides fenpropimorph ((2*R*,6*S*)-4-[(*RS*)-3-(4-*tert*-butylphenyl)-2-methylpropyl]-2,6-dimethylmorpholine) and fenhexamid (*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexane-1-carboxamide) on the symbiotic phase, the hyphae and the spores at the post-symbiotic phase. Eventually, Dupre de Boulois *et al.* [79] proposed the use of a three-compartment *in vitro* system, composed of the root compartment and the hyphae compartment mentioned above, plus a shoot compartment where a plantlet was allowed to grow. These systems were used to test the toxicity of fenpropimorph and fenhexamid on the capacity of extraradical hyphae and spores to colonize roots, while they offer the opportunity to determine effects on phosphorus uptake using ^{33}P [77]. These tri-compartmental *in vitro* systems could be further modified to include a second shoot compartment associated with the hyphae compartment addressing the effect of pesticides on the capacity of extraradical hyphae to sporulate and colonize plant crops where the pesticide tested is destined for use [73]. Besides determination of the toxicity of pesticides on AMF, Campagnac *et al.* [80] used the bi-compartmental assay to disentangle the pesticide toxicity mechanisms on AMF. They showed that the toxicity of fenpropimorph on AMF was the result of its inhibitory effect on the sterol biosynthesis of mycorrhized plantlets. Similarly, Zocco *et al.* [78] used the tri-compartmental system to define the toxicity mechanism of the same two fungicides on the phosphorus uptake machinery.

The delayed implementation of PCR-based molecular methods in AMF research, changed dramatically the taxonomy of Glomeromycota [81]. To date, a limited number of studies have used molecular approaches to disentangle the effects of pesticides on the diversity of AMF, with the focus being on the intraradical rather

than on the soil reservoir of Glomeromycota. Karpouzas *et al.* [33] used a combinatory DGGE – cloning approach to study the effects of nicosulfuron on the intraradical AMF community and noted a dramatic decrease in the diversity of AMF in maize roots when the herbicide was applied at levels which were approximating ×10 the recommended dose. A more recent study explored the effect of pesticide mixtures (fenhexamid, folpet (2-trichloromethylsulfanyl)-1*H*-isoindole-1,3(2*H*)-dione) and deltamethrin ((*S*)-cyano (3-phenoxy)methyl (1*R*,3*R*)-3-(2,2-bromothenyl)-2,2-dimethylcyclopropane-1-carboxylate) on the soil diversity of AMF via clone library with taxon-specific primers [27]. They observed a reduction of AMF soil diversity with increasing dose rates. Jin *et al.* [75] was the first and only study to date that investigated the effects of pesticides on the intraradical AMF diversity using meta-taxonomics. They noted pesticide-specific effects, with *Gigaspora hoi* and *Acalauspora uera* showing increasing sensitivity to fludioxonil in pea and chickpea respectively.

Overall, AMF could be considered as potential microbial indicators for the soil microbial toxicity of pesticides based on (i) their key role in soil ecosystem functions, (ii) our good knowledge of their life cycle and biology, (iii) their overall high sensitivity to pesticides, and (iv) the availability of standardized tools to define effects of pesticides on their growth, although efforts are in progress to improve the existing ISO-10832 method and/or to develop new standards for AMF toxicity testing. Still their obligate symbiotic nature stresses the need for use of combinatory approaches including both *in vitro* and soil-plant tests to define in a comprehensive way the true extent of pesticide toxicity. Despite their high responsiveness to pesticides, we are still missing studies assessing the sensitivity of AMF to pesticides comparatively with other microbial groups characterized as potent bioindicators. Such studies will be particularly useful in the quest for identifying optimum microbial indicators for assessing the soil microbial toxicity of pesticides. A recent paper provides an industry perspective for the use of AMF in pesticide risk assessment; it highlights limitations of currently available methods for AMF (from *in vitro* to field testing) and proposes adjustments to improve clarity in the interpretation of the results, and highlights knowledge gaps that should be addressed for a meaningful AMF-based assessment of the toxicity of pesticides on soil microorganisms [82].

3.3.2 Ammonia-oxidizers and other nitrogen cycling microorganisms

Nitrification annual contribution to soil N is particularly high and has been estimated to reach 330 Tg (or $330 \cdot 10^{12}$ g) of N [83]. Hence perturbations in its operation would adversely affect N balance in soil. Ammonia-oxidizing microorganisms control the rate limiting step of nitrification, which involves the oxidation of ammonia to hydroxylamine [83]. This is further transformed to nitrite through the intermediate formation of nitrogen monoxide (NO) [84]. Subsequently, dioxidonitrate(1-) (nitrite) is further oxidized to trioxidonitrate(1-) (nitrate) by nitrite-oxidizing bacteria (NOB) [85]. Apart from their direct involvement in ammonia oxidation, AOM was relatively recently shown to contribute to the production of dinitrogen oxide (N_2O), a major greenhouse gas, through a process called nitrifiers denitrification [86]. Ammonia oxidation is performed by a phylogenetically narrow group of microorganisms with new players discovered in the last 20 years like: (i) ammonia-oxidizing bacteria (AOB), mostly belonging to β-proteobacteria and specifically to *Nitrosomonas* and *Nitrosospira* [87]; (ii) ammonia-oxidizing archaea (AOA) belonging to the phylum Thaumarcheota with lineages *Nitrososphaerales* and *Nitrosotaleales,* dominating in neutral to alkaline and acidic soils, respectively [88]; and (iii) comammox bacteria which perform the full nitrification process in a single cell [89]. The latter group is abundant in terrestrial ecosystems; however, their active participation in ammonia oxidation in soil was only recently proven [90]. Still representatives of this group are yet to be isolated from soil. Soil physicochemical properties, like pH [91] and ammonia concentration levels, exert a strong niche specialization effect on AOM. Ammonia-oxidizing archaea dominate in acidic soils, whereas AOB become dominant in neutral to alkaline soils [92]. Similarly, high ammonia concentrations in soil generally favor AOB, while AOA and comammox bacteria prefer oligotrophic ammonia conditions [93]. All autotrophic AOM possess a periplasmic enzyme called ammonia monooxygenase which is responsible for the oxidation of ammonia to hydroxylamine [83]. The gene encoding the alpha subunit of ammonia monooxygenase (*amoA*) has been used as a gene marker for the phylogenetic classification of AOM [85, 86].

This gene has been used for the design of group-specific primers that have been utilized for the measurement of the abundance, activity and diversity of AOA, AOB and comammox bacteria in soil [31, 94–96].

Domsch [97] first reported the high sensitivity of *Nitrosomonas* and *Nitrobacter* to pesticides. This initial observation was verified by recent studies which reinforced the potential high sensitivity of AOM to abiotic stressors, including pesticides [59]. Several studies have explored the response of AOM to pesticide exposure by measuring their abundance (q-PCR) [98–100], their activity (potential nitrification or inorganic nitrogen pools) [42], and their diversity *via* DGGE, TRFLP and meta-taxonomic analysis of the *amoA* gene [31, 32, 101]. Hund-Rinke *et al.* [102] 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 [103]), MicroResp®, and exoenzymes activity. All tests showed a similar sensitivity towards the tested nano-pesticide. However, potential nitrification showed the most consistent concentration–effect response, compared to the other two methods tested were results were more variable and weaker concentration-effect relationships were observed. Similar studies have also reported the sensitivity of potential nitrification to pesticide exposure [104]. Potential nitrification is a measure of the rate of the microbial transformation of ammonia to nitrite, in contrast to the nitrogen mineralization test which is a combined measurement of ammonification and nitrification compromising its sensitivity to abiotic stressors. As all potential rate assays, potential nitrification has limited ecological relevance and it is inherently biased towards specific microbial groups (*e.g.* AOB); hence, its use in studying the ecology of AOM is severely restricted [105]. However, when it is used in a purely ecotoxicological context, it remains a valuable tool, especially its ISO standard (ISO-15685) [103], which could be readily included in the battery of tests used to assess the soil microbial toxicity of pesticides.

Besides the effects of pesticides on the abundance of AOM, only a few studies have looked at effects at the transcription level. Papadopoulou *et al.* [42] showed that ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline), an antioxidant used for the control of apple scald in fruit-packaging plants, had a temporal inhibitory effect on AOM which became evident only when *amoA* transcripts were monitored. Considering the high turnover rates of RNA in soil, RNA-based approaches provide a more in-depth analysis of the effects of pesticides on functional microbial groups like AOM. However, the labile nature of RNA has precluded its wider use in pesticide microbial ecotoxicological studies and maybe its use could be considered when refinement of the ecotoxicological part of the risk assessment is requested. Overall, AOM constitute very appealing bioindicators for the assessment of the toxicity of pesticides on soil microorganisms as they (i) control a very significant function in soil nitrogen cycling, (ii) are sensitive to pesticide exposure providing an ecotoxicologically relevant response, (iii) are well studied regarding their life cycle, ecology, biochemistry and physiology, and (iv) could be monitored at abundance and activity levels using already available standardized methods. In addition, previous studies have also indicated that AOM measured endpoints like potential nitrification and AOM abundance were the most sensitive endpoints amongst several tested [34, 102, 104].

Other nitrogen cycling microbial groups, besides AOM, have been considered responsive to pesticide exposure like denitrifying bacteria [106] and nitrogen-fixing bacteria [62]. Both these groups are involved in greenhouse gas emissions and nitrogen soil sequestration, hence their perturbation by pesticides might affect key ecosystem services [59]. Several recent studies have monitored the effects of pesticides on the abundance of denitrifying bacteria through q-PCR analysis of bacterial genes involved in the different steps of denitrification, however no clear ecotoxicological response to pesticide exposure was evident [34, 106, 107]. Pesticide effects on nitrogen-fixing bacteria have been also explored, via *nifH*-based q-PCR, with the results varying from no inhibition by trifluralin (2,6-dinitro-*N,N*-dipropyl-4-(trifluoromethyl)aniline) [108], to transient inhibition by chlorothalonil [109], and to strong inhibition by 1,3-dichloroprop-1-ene [110].

3.3.3 Protists

Protists are the most ubiquitous soil eukaryotes spanning the entire eukaryotic tree of life [111], and encompass a range of lifestyles including phagotrophs, phototrophs, saprotrophs and symbionts [112]. The abundance

of protists in soil varies from 10^4 to 10^8 g^{-1} of soil [113] and their community composition is mostly driven by soil moisture [114] and to a lesser extent by the prokaryotic microbial composition, in line with the bacterivore feeding regime of certain soil protists. Other confounding soil factors that seem to act as major drivers of the prokaryotic communities, like pH [13], are not particularly important for soil protists, except for parasitic whose composition is driven by the pH preferences of their hosts [115].

Heterotrophic soil protists, traditionally called protozoa, have attracted attention as potential indicators of soil pollution. They are categorized into five morphologically distinct groups: naked and testate amoebae, flagellates, ciliates and parasitic Sporozoa [63]. They were previously considered as solely bacterivores, however recent studies have shown that they are omnivores feeding on fungi [116], algae [117], other protists [118] and even small size animals like nematodes and rotifers [119]. High-throughput sequencing analysis of the composition of the soil protists suggested that consumers (i.e. predatory protists) constitute the dominant functional group in most soils around the globe [114]. The capacity of soil protists to prey on a wide range of soil prokaryotic and eukaryotic organisms leads to the efficient turn-over of nutrients from microbial biomass [120]. This enriches soil with previously unattainable nutrients like nitrogen and phosphorus that could be taken up by plants supporting enhanced plant growth [112].

Foissner [63], in a first review, proposed the use of heterotrophic soil protists as indicators of environmental perturbations in the soil ecosystem. This suggestion was based on certain characteristics which are considered desirable for such bioindicators: (i) they constitute ubiquitous microorganisms in soil, (ii) they have key ecological roles in nutrients recycling and in the control of other prokaryotic and eukaryotic soil organisms, (iii) they are characterized by rapid growth which ensures their rapid reaction to environmental perturbations. On the other hand there are restrictive factors for their potential use as bioindicators: (i) testing of heterotrophic soil protists requires the presence of relevant prey organisms, while effects of pollutants on the prey and/or the predator might complicate the interpretation of the results; (ii) enumeration using non molecular tools is not well established and still relies on time consuming dilution culture techniques; (iii) there are no standardized methods to assess the effects of pollutants on protists; (iv) taxonomic classification until recently relied on morphological features of protists which is a daunting task considering their enormous morphological variation and the limited number of specialized taxonomists. The recent development and optimization of high-throughput amplicon sequencing approaches for protists revealed an enormous diversity, similar to prokaryotic microorganisms, in soil [121–123].

Only a few *in vitro* and soil microcosm studies have explored pesticide effects on soil protists. Schreiber and Brink [124] used an *in vitro* assay with the ciliates *Colpoda cucullus* and *Blepharisma undulans* and the flagellate *Oikomonas termo* fed on *Enterobacter aerogenes* to determine the toxicity of MCPA (4-chloro-2-methylphenoxy)acetic acid), dichloprop ((2*R*)-(2,4-dichlorophenoxy)propanoic acid), clorpyralid (3,6-dichloropyridine-2-carboxylic acid), benomyl (methyl [1-(butylcarbamoyl)-1*H*-benzimidazol-2-yl]carbamate) and fenvalerate ((*RS*)-cyano(3-phenoxyphenyl)methyl (*RS*)-2-(4-chlorophenyl)-3-methylbutyrate) in axenic and non-axenic cultures. They calculated 9h-Lethal Concentration 50 % (LC_{50}) and Lethal Concentration 10 % (LC_{10}) based on the reduction in the turbidity of the culture and noted that some of the chemicals could be toxic to soil protists at rates similar to the recommended dose rates. More recently, Amacker *et al.* [65] devised an *in vitro* assay for testing the toxicity of pesticides on the testate amoeba *Euglypha rotunda* growing in co-culture with *Escherichia coli*. They validated this assay with the herbicide metolachlor (chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-[(2*RS*)-1-methoxypropan-2-yl]acetamide) and observed non-linear effects on the tested protist, a problem often encountered in pesticide ecotoxicity studies that prevents the calculation of toxicity units (*e.g.* EC_{50}). A common feature in all *in vitro* tests is the inclusion of controls testing the potential toxicity of pesticides on the co-cultured bacteria. This is essential to distinguish between direct toxic effects of the pesticides on the protist or indirect effects stemming from the lack of feed (toxic to the bacterial prey). Ekelund [125] tested, in small soil microcosms (5 g), the toxicity of the fungicide fenpropimorph on soil protists *via* enumeration of flagellates, naked amoeba, ciliates and other amoeba. They noted a feeding-specific effect of the fungicide on soil protists with bacterivorous flagellates and naked amoeba being sensitive

to fenpropimorph, unlike ciliates and other amoeba that were not affected. The lack of any effect on fungal hyphae provided a further proof of direct toxicity of the fungicide to soil protists.

In recent years, the effects of pesticides on soil protist diversity could be studied using amplicon sequencing with primers targeting soil eukaryotes [116]. Still only a few ecotoxicological studies have used this approach. Carley *et al.* [126] tested the toxicity of $Cu(OH)_2$ nanopesticide on the microbial community in soil and water/sediment mesocosms. They observed no significant effects of the copper nanopesticide on the soil microbiota (bacteria, fungi, microeukaryotes), while individual protist groups like Cercozoa were negatively affected by the $Cu(OH)_2$ nanopesticide. Fournier *et al.* [127] tested the effect of a synthetic (propamocarb (propyl 3-(dimethylamino)propylcarbamate) + fosetyl Aluminium (aluminium tris(ethyl phosphonate)) and of a microbial pesticide (*Clonostachys rosea* f. *catenulate*) on the composition of the soil bacterial, fungal and protist community. They noted, *via* network co-occurrence analysis, that the synthetic pesticide affected keystone fungal and protist ASVs suggesting a broader effect on the biotic interactions between these two microbial groups. These studies suggest that the effects of pesticides on the diversity of protists should be always examined in comparison with interacting microbial (bacteria, fungi) and animal (nematodes) soil populations. Using this approach results of pesticide toxicity could be investigated at the soil-food web level, moving away from the single microbial group perspective, the focus of most soil ecotoxicity assays. Overall, soil protists could be good potential candidate bio-indicators of the soil microbial toxicity of pesticides. Still *in vitro* and in soil experimental protocols should be developed and standardized before their use and implementation in the pesticide regulatory framework.

3.3.4 Other microbial groups as potential bioindicators

Photosynthetic microorganisms like micro-algae and cyanobacteria have been often used as indicators of the toxicity of pesticides in aquatic ecosystems [128]. However, the value of these microorganisms in soil ecosystems has been overlooked. Micro-algae and cyanobacteria are ubiquitous colonizers of topsoil [129] that contribute to several soil ecosystem services like (i) support to soil fertility through N_2 and CO_2 fixation [130] and (ii) stabilization of soil aggregates [131], which results in reduced soil erosion and limited losses of water and nutrients [132]. Despite their major functional role in soil, their use in the assessment of the soil microbial ecotoxicity of pesticides was not explored. Pipe [133], after reviewing studies looking at the effects of pesticides on soil cyanobacteria and micro-algae, suggested that herbicides, especially those which act on the photosynthetic machinery of plants, are more toxic to these microbial groups compared to insecticides and fungicides.

Considering the main role of cyanobacteria on nitrogen fixation in rice paddy fields [134], the effect of rice pesticides on cyanobacteria has attracted attention [135]. Beyond rice cultivation, little is known about the potential toxicity of pesticides on cyanobacteria in other agricultural settings. Crouzet *et al.* [136] investigated the dose–response effects of the herbicide mesotrione (*rac*-2-[4-(methanesulfonyl)-2-nitrobenzoyl]cyclo-hexane-1,3-dione), acting as a competitive inhibitor of 4-hydroxyphenyl-pyruvate-dioxygenase involved in the biosynthesis of photosynthetic pigments, on soil photosynthetic microbial communities (particularly cyano-bacteria) using a microcosm approach. Mesotrione induced significant decreases on the photosynthetic biomass at dose rates 10 times the recommended, while structural effects on the cyanobacterial community were evident only at higher dose rates. More recently, Crouzet *et al.* [137] studied the effects of isoproturon (*N*′-[4-(propanol-2-yl)phenyl]-*N*,*N*-dimethylurea), a substituted phenylurea herbicide known to be a photo-synthesis inhibitor, on soil microalgae and cyanobacteria and their soil aggregation capacity. They noted that the herbicide negatively impacted the soil microalgal community, resulting in a significant decrease of the size of the conventional soil aggregates. These studies put forward these functional microbial groups (algae and cyanobacteria) as promising bioindicators for deciphering the impacts of pesticides on soil functions (*e.g.* soil aggregation) in agroecosystems.

3.4 A new risk assessment scheme and tests required for the determination of the toxicity of pesticides on soil microorganisms

3.4.1 A new risk assessment scheme proposed

Environmental risk assessment of pesticides relies on a tiered system composed of tiers of increasing complexity and realism. Tier I is based on the assessment of the toxicity using highly conservative assays. If risk assessment indicates potential undesirable effects at this step of the process, then assessment is performed at Tiers II and III, which are characterized by less conservative and more realistic assays at both exposure and toxicity level. This tiered scheme is the backbone of the current environmental risk assessment of pesticides, but this is not yet applicable to soil microorganisms. Earlier studies in the late 70s provided the first experimental guidelines on how to determine the toxicity of pesticides on soil microorganisms [25, 138]. They suggested that if significant inhibitory effects are observed at lab scale, the toxicity of pesticides on soil microorganisms should be further explored at field scale. Following the same philosophy, Karpouzas *et al.* [139] proposed a similar tiered system for the assessment of the soil microbial toxicity of pesticides. Their approach was composed of three tiers of increasing complexity based on the ecotoxicological response of key soil functional groups like AOM and AMF or others which remain to be defined (Fig. 3): (i) a Tier I *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; (ii) a Tier II toxicity assessment in lab soil microcosms (or planted pot studies when AMF are considered) against natural assemblages of AOM and AMF;

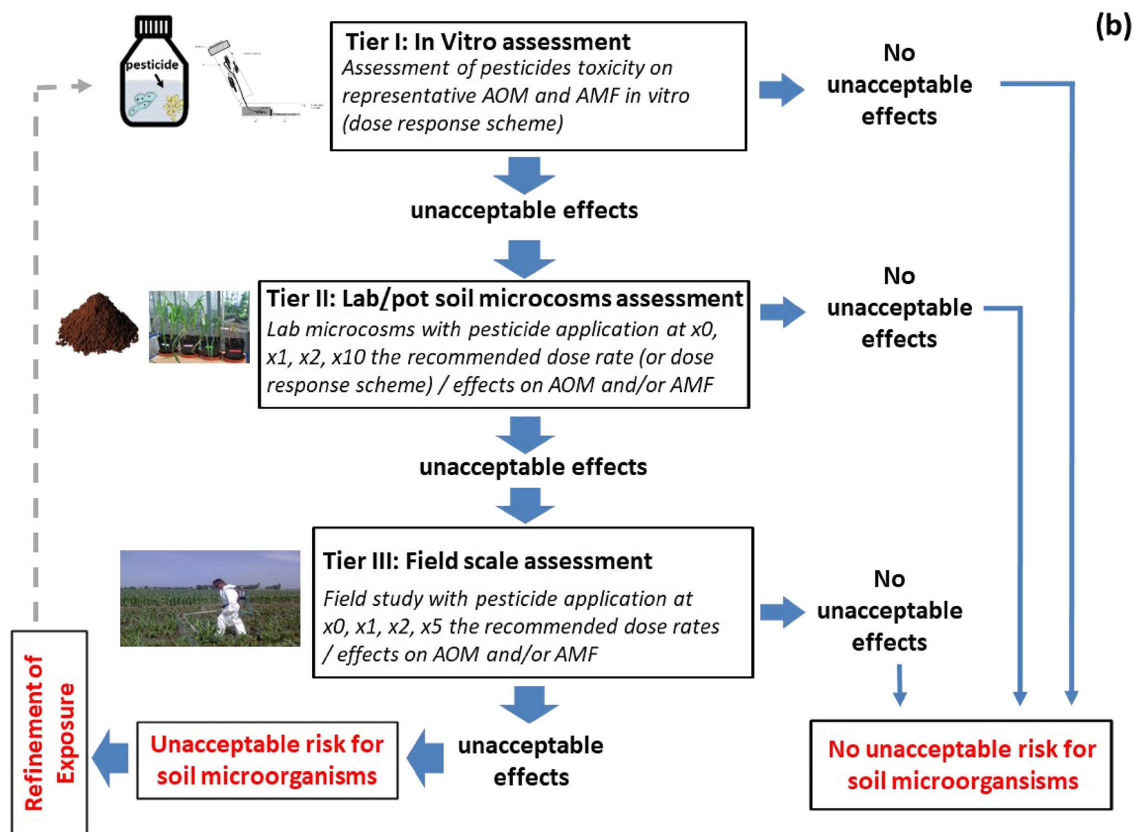

Fig. 3: A three-tier risk assessment scheme proposed for assessing the risk associated with the use of pesticides for soil microorganisms. AOM: Ammonia-oxidizing microorganisms; AMF: Arbuscular mycorrhizal fungi (adopted upon permission by Karpouzas *et al.* [129]).

and (iii) a Tier III toxicity assessment at field scale (under cropping conditions) against natural assemblages of AOM and AMF. In cases where an unacceptable risk for soil microorganisms is still evident, refinement of exposure could be an option to minimize risk.

3.4.2 *In vitro* tests

Single species tests constitute the first and most conservative step in the assessment of the toxicity of pesticides on soil organisms. *In vitro* testing of the toxicity of pesticides on soil microorganisms could be an equivalent first tier assay against bioindicator organisms like AMF and AOM. However, this approach has not been used in soil microbial ecotoxicology most probably due to: (a) the bacterial species concept which remains elusive due to their different evolution rates driven by horizontal gene transfer and high rates of homologous recombination [140]; (b) the complexity of soil microbiota which prevented until recently the identification of ecotoxicologically relevant soil microbial species: and (c) the poor culturability of the majority of soil microorganisms in currently available growth media. *In vitro* assays have been utilized to determine the toxicity mechanism and calculate toxicity endpoints for AMF, as has been described earlier in this review [77, 78, 80].

Unlike AMF, *in vitro* testing has not been largely explored for other soil functional microbial groups like AOM which have shown sensitivity to pesticides. *In vitro* cultivation of soil derived AOB and AOA is a rather difficult task. This is mirrored in the very limited number of currently available pure cultures [88]. *In vitro* assays with soil derived AOB and AOA have been mostly used to determine the activity of synthetic [141–143] and biological nitrification inhibitors [144, 145]. Such studies allow the calculation of Effective Concentration 50 % (EC_{50}) values based on the measurement of NO_2 production or growth of AOM by q-PCR. However, in most studies to date, growth and activity measurements seem to concur [142, 146], suggesting that inhibition levels on AOM could be determined just by simply measuring NO_2 formation. Vasileiadis *et al.* [31] first used *in vitro* assays to test the toxicity of iprodione and its main TP, 3,5-dichloroaniline on (i) two AOA isolates *Candidatus* Nitrosocosmicus frankladianus and *Candidatus* Nitrosotalea sinensis occupying contrasting ecological niches and representing widely distributed neutrophilic and acidophilic AOA lineages, respectively [147, 148], (ii) an AOB *Nitrosospira multiformis*, with members of *Nitrospira* sp. being dominant among the AOB community in terrestrial ecosystems [149]. More recently Papadopoulou *et al.* [142] used *in vitro* assays to determine the effect of ethoxyquin, its two main TPs quinone imine (4-iminocyclohexa-2,5-dien-1-one) and dimethyl-ethoxyquinoline (6-ethoxy-2,4-dimethylquinoline), and of other nitrification inhibitors on the same AOM isolates plus *Nitrosomonas europaea*. Based on the calculation of EC_{50} values, it was demonstrated that there is a high inhibitory activity of ethoxyquin and quinone imine on AOA but not on AOB. This *in vitro* approach could be adopted for assessing the toxicity of pesticides on other functional soil microbial guilds like nitrogen-fixing bacteria and NOB.

In vitro tests are overall fast track, simple to undertake and readily adjusted to high-throughput versions. Still certain aspects of these test should be standardized with the most important being the selection of soil-derived strains per functional microbial guild that would be included in pesticide testing. The strains included in such ecotoxicity assays should fulfill certain criteria like (a) they should show an ecotoxicologically relevant response to pesticide exposure (dose-dependent response) and (b) they should cover a range of ecotypes like r- and k-strategists (i.e. AMF) or acidophilic and neutrophilic/alkaliphilic AOM or AOM favored under contrasting ammonium soil levels. Preliminary tests with selected members of these microbial guilds will pave the way for the standardization of these tests. It should be mentioned here that *in vitro* microbial testing, as all Tier I tests, should be considered as a simplified tests of low ecological relevance but it is still useful to get a first assessment of the potential toxicity of pesticides on the soil microbiota.

3.4.3 In soil tests with natural or inoculated microbial assemblages

In cases where *in vitro* assays indicate that toxicity levels exceed threshold values, further soil microcosm or mesocosm tests should be employed to determine toxicity under more realistic conditions. Soil laboratory

tests could be of variable size, spanning from a small mass of soil (5 to 100 g) to pot studies where plants are cultivated under controlled conditions (2 to 5 kg soil). The presence of plants is indispensable in studies looking at pesticide effects on AMF. The dose rates tested could be selected based on the OECD 216 test (*e.g.* 1 time, 2 times, 10 times the recommended dose rate) or a dose-dependent application scheme could be followed.

Effects of pesticide at microcosm or mesocosm tests could be determined at the level of natural microbial assemblages using measurements of abundance, activity or diversity endpoints. Alternatively, pesticide effects could be examined in more controlled soil systems that have been sterilized with mild sterilization methods (i.e. γ-irradiation or fumigation) and the microbial community has been reinstated either with inoculation of mock microbial communities of known composition and complexity [56] or with more complex microbial inocula derived from serial dilutions of the microbiota collected from the original soil before its sterilization [150]. The composition of the diluted soil microbiota could be determined via amplicon sequencing [151] and further manipulated i.e. by selectively removing fractions of the soil microbiota through various treatments like the application of antibiotics or filtration to determine pesticide effects on a specific soil microbial guild without confounding microbial interactions. Such approaches could serve as potential refinement assays to examine the toxicity of pesticides on specific components of the microbial community without having the limitations of *in vitro* assays and the overall complexity of soil microcosms.

In case an unacceptable toxicity effect of pesticides on soil microorganisms is identified at lab microcosm or pot studies, toxicity is evaluated at field level. At these experimental scales pesticide toxicity applies solely on natural microbial assemblages. Regardless of the experimental scale considered (microcosm, pot or field) pesticide dissipation and transformation should be determined on a temporal basis enabling the identification of the toxicant (parent vs TP).

4 Open issues and knowledge gaps

4.1 Low-risk pesticides

The growing public concern about the effects of synthetic pesticides on environmental quality and soil health, has shifted attention to low-risk pesticides. However, their introduction in pesticide market has been hampered by the stringency of the current EU regulatory framework which treats low-risk pesticides in the same way as synthetic ones [152, 153]. This is reflected in the much lower number of low-risk pesticides reaching the market in Europe compared to USA, China, Brazil and India [154]. Low-risk pesticides is a broad group which encompasses (a) **microbials,** where the active agent is a microorganism that protects crops from fungal and insect infestations; (b) **natural products or biochemicals or botanicals and peptides,** that are biogenic compounds, products of the secondary metabolism of plants and microorganisms, with strong biocidal activity; (c) **semiochemicals, including pheromones and allelochemicals,** that are emitted by animals, plants and other organisms and impose a physiological or behavioral response in individuals of the same species or other species respectively [155]; and (d) **double stranded RNA (dsRNA)-based pesticides** that work through the natural gene silencing RNA interference (RNAi) mechanism of several living organisms [156]. Their mode of action is based on gene-specific small interfering RNA (siRNA) or microRNA (miRNA) molecules that bind on mRNA with complementary nucleotide sequences and trigger their lysis, hence preventing protein production [157]. Prevention of the biosynthesis of proteins involved in the pathogenesis of pests or pathogens could lead to plant protection.

As their name indicates, low-risk pesticides are characterized as safe and environmentally friendly solutions, mostly due to their biological origin. However, hard evidence to verify their low-risk character is scarce. Amongst natural products, azadirachtin is the hallmark compound of this group. It is one of the most studied low-risk pesticide regarding its off-target toxicity to soil microorganisms and the results obtained to date do not support a low-risk profile. Azadirachtin showed low *in vitro* toxicity towards AMF, compared to synthetic pesticides [76]. This was not verified in soil studies, where the recommended dose of azadirachtin

negatively affected the abundance and the transcriptional activity of AOM, nitrogen-fixing and denitrifying bacteria [98, 158] and reduced the diversity of bacteria, fungi and AMF [56, 99]. In the same study, azadirachtin showed equivalent or stronger inhibitory effects than the synthetic pesticides tested. When looking at semiochemicals, little is known regarding the off-target toxicity of pheromones to the soil microbiota, as expected due to their volatile nature that precludes exposure of soil microorganisms. On the other hand, there are a few studies looking at the effects of allelochemicals acting as herbicides on the soil microbiota. Hence, Romdhane *et al.* [29] compared the effects of the allelochemical β-triketone herbicide leptospermone (2,2,4,4-tetramethyl-6-(3-methylbutanoyl) cyclohexane-1,3,5-trione) and its synthetic derivative sulcotrione (2-[2-chloro-4-(methanesulfonyl)benzoyl]cyclohexane-1,3,5-trione) on the soil microbial community. In line with the results of azadirachtin, leptospermone induced stronger changes in the soil bacterial community composition than sulcotrione. These studies certainly challenge the general perception that natural products are characterized by lower off-target toxicity compared to synthetic pesticides.

In accordance with the other low-risk pesticide groups, little is known regarding the potential effects of microbial pesticides on the soil microbiota. 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 *Paracoccus lilacinus* strain PL251, which did not have a direct inhibitory effect on AOM [101]. In contrast Yu *et al.* [159], 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 [160–162]. Still the potential of a microbial pesticide to adversely affect the soil microbiota should be determined at the strain level taking into consideration its capacity to produce bioactive metabolites [163] or its cargo of transferable antibiotic resistance genes [164]. Such information could be initially deduced by the genomic data of the microbial strain and further verified experimentally.

The dsRNA-based pesticides have not reached the market yet, but they are expected to become available in the forthcoming years. Although studies regarding their efficacy [165], mode of application [166], persistence [167] and toxicity on non-target organisms [168] are available, we still have no idea about their potential effects on the soil microbiota. The highly specific mode of action of the RNAi mechanism on target mRNA suggests limited off-target toxicity effects. Bioinformatic analysis focusing on the design of dsRNA molecules with minimum homology to mRNA of non-target organisms, coupled with biological experimentation starting with phylogenetically close organisms should be considered in the assessment of the soil microbial ecotoxicity of dsRNA pesticides [169]. However, differences in the RNAi machinery of different organisms and variable uptake and internal distribution of dsRNA in different organisms are factors that might strongly influence the efficiency but also the ecotoxicity of ds-RNA pesticides [170], including effects on the soil microbiota.

According to the registration framework in Europe, low-risk pesticides undergo the same registration process as synthetic pesticides. We argue that the risk assessment procedure for the different types of low-risk pesticides should be adjusted to account for the characteristics, properties and needs of these products. This should certainly include the implementation of tools for monitoring the environmental fate and persistence of low-risk pesticides in soil.

4.2 Nano-pesticides

Nano-pesticides are plant protection products which are composed of nanoparticles, particulate substances which have one dimension less than 100 nm, that are used to increase the efficacy and reduce the environmental footprint of pesticide active ingredients [171]. They could be classified into two broad categories: (i) organic pesticide active ingredients that are complexed onto nanocarriers (soft nanoparticles like polymers,

solid lipid or hard nanoparticles like silica or carbon nanotubes), (ii) inorganic active ingredients without a nanocarrier that are used directly (*e.g.* CuO, $Cu(OH)_2$) [172, 173].

Nano-pesticides of the first group (organic pesticides complexed onto nanocarriers) show increasing efficacy (by 10 to 20 %), and persistence, whereas we know very little about their ecotoxicity compared to their corresponding conventional formulation and the pure active ingredients. For now, there are only a few studies looking at the off-target toxicity, including the soil microbiota, of organic nano-pesticides, their conventional formulations and the active ingredients. For example, Maruyama *et al.* [174] assessed the impact of the herbicides imazapic (*rac*-2-[4-methyl-5-oxo-4-(propan-2-yl)-4,5-dihydro-1*H*-imidazol-2-yl]-5-methylpyridine-3-carboxylic acid) and imazapyr (*rac*-2-[4-methyl-5-oxo-4-(propan-2-yl)-4,5-dihydro-1*H*-imidazol-2-yl] pyridine-3-carboxylic acid) encapsulated in alginate/chitosan and chitosan/tripolyphosphate nanoparticles on soil microorganisms involved in nitrogen cycling, but they did not include in their study any conventional formulation of the two herbicides. They found that encapsulated herbicides were less toxic compared to the non-encapsulated compounds.

Regarding nano-pesticides of the second group (inorganic active ingredients without a nanocarrier), we have got several studies that have investigated their effects on the soil microbiota. In a few studies, the authors examined the toxicity of nano-pesticide formulations comparatively to the inorganic active ingredient to be able to distinguish the contribution of the two components, inorganics or nanocarriers, in the toxicity observed [175, 176]. In the most comprehensive of these studies, Swart *et al.* [175] analyzed separately the effects of the formulated silver and CuO nanoparticles, their ionic counterparts $CuCl_2$ and $AgNO_3$, and their inert carriers on the soil microbiota at different concentration levels. They noted a dose-dependent effect on the soil bacterial community by the formulated products and their ionic controls but not by the nanocarriers, suggesting that the inorganics are the main drivers of the effects on soil microbiota. In support of this, Zheng *et al.* [177] demonstrated, via *in vitro* testing, that the toxic effect of silver nanoparticles on *Paracoccus denitrificans*, a model denitrifying bacterium, was mostly driven by silver ions decreasing the expression of denitrification and glucose assimilation enzymes and increasing polyhydroxybutyrate biosynthesis. Several other studies have reported the effects of inorganic nano-pesticides on the soil microbiota with the measured endpoints varying. Sillen *et al.* [178] studied the impact of silver nanoparticles on the activity of the rhizosphere microbiota *via* transcriptomic analysis. They noted an increased transcription of genes involved in copper resistance (*e.g.* efflux pumps of Ag/Cu) and a decreased transcription of ammonia monooxygenase and urease of AOA but not of AOB. In a similar study, Simonin *et al.* [179] showed that $Cu(OH)_2$ nano-pesticides, applied to soil under three different fertilization regimes (ambient, low, high), did not induce any significant effect on AMF and nitrogen-fixing bacteria. Similarly, Xu *et al.* [180] tested the effects of TiO_2 and CuO nano-pesticides on the soil microbiota and showed that the latter exhibited higher toxicity, as denoted by the significant reduction of microbial biomass carbon, total PLFAs, urease, phosphatase and dehydrogenase activity.

Overall, the small size and high surface/volume ratio of nano-pesticides coupled with the high toxicity of their inorganic active ingredients, favor their strong interaction with soil microorganisms and increase the possibility of effects on the soil microbiota. Future studies should focus on the assessment of the effects of organic nano-pesticides on the soil microbiota, comparatively to their conventional formulation and active ingredients.

4.3 Pesticide mixtures (and co-formulants)

Formulated pesticides are composed of the active ingredients and several other co-formulants that aim to maximize active ingredients efficiency on the target. The identity of these co-formulants in pesticide commercial formulations is not disclosed due to intellectual property rights. However, they could be responsible for or enhance the toxicity (*e.g.* by increasing the soil bioavailability) of pesticide compounds on soil microorganisms. Ecotoxicological studies exploring the toxicity of active ingredients and the corresponding commercial formulation should be employed to distinguish the contribution of the different

components of pesticide formulations on the soil microbial toxicity. In such a study, Crouzet *et al.* [136] showed that the commercial formulation of the herbicide mesotrione, when applied at 10 times the recommended dose, induced stronger effects on the structure of the soil cyanobacteria community compared to the same levels of the pure pesticide compound. In a similar study, Rousidou *et al.* [101] showed that co-formulants of the formulation of the bionematicide BIOACT® (glucose or skimmed milk), which contains spores of the nematode parasitic fungus *P. lilacinus*, were responsible for the transient inhibition of AOM upon soil application of BIOACT®.

Besides additives, several of the commercial pesticide formulations contain more than a single active substance [181], hence releasing mixtures of pesticides in the environment. This might trigger effects deviating from those seen when applied individually. To date, most studies available on the toxicity of pesticide mixtures have focused on aquatic toxicity endpoints [182]. They suggested that, for mixtures composed of active ingredients with the same or different mode of action, the concentration addition (CA) or the independent action (IA) models, respectively, could predict toxicity [183]. On the other hand, we still know little about the potential toxicity of pesticide mixtures on soil biota and microbiota. Evaluating the applicability of CA and IA models for assessing the toxicity of pesticides on the soil microbiota or designing new models, more relevant for soil ecosystems, could be a new frontier in pesticide soil microbial ecotoxicology.

4.4 Transformation products (TPs)

Environmental risk assessment should go beyond parent compounds and address also the potential toxicity of pertinent pesticide TPs. In a benchmarking study, Sinclair and Boxall *et al.* [184] explored via meta-analysis the toxicity of 89 transformation products (TPs) obtained from 37 parent compounds. They showed that 70 % of the pesticide TPs have either similar or lower toxicity to aquatic organisms compared to their parent compounds. The remaining 30 % of the TPs tested were more toxic than the parent compound. This list included TPs that (i) carried the pesticide toxicophore moiety (i.e. oxime carbamate compounds) (ii) were the active compounds formed from a pro-pesticide (i.e. organophosphate compounds) or (iii) had a more potent mode of action compared to the parent compound (i.e. carbamate and organotic compounds). The EU regulatory framework still uses an exposure-driven assessment approach whereby TPs that are formed at levels exceeding 10 % of the applied parent compound are considered as relevant for environmental risk assessment [185]. This approach has often been criticized as it excludes from risk assessment a large number of TPs formed at lower amounts than the cutoff value of 10 %: the rational for defining a relevant TP should rather be based on their toxicophore moieties or mode of action [186, 187]. A reflection of these decisions was the late (20 or 30 years after their market introduction) identification of toxicologically relevant TPs like the carcinogenic N-nitroso-dimethylamine (*N*,*N*-dimethylnitrous amide), a TP of the fungicide tolylfluanid (*N*-[dichloro(fluoro)methanesulfanyl]-*N′*,*N′*-dimethyl-*N*-(4-methylphenyl)sulfuric diamide) [188]. The pitfalls of this exposure-driven *a priori* toxicological characterization of pesticide TPs were highlighted by Storck *et al.* [189] who proposed the implementation of *a posteriori* characterization of the toxicological relevance of TPs.

To date, there are several examples in the literature where TPs rather than the parent compounds are incriminated for toxicity on the soil microbiota. Papadopoulou *et al.* [42] identified quinone imine, a TP of the fruit preservative ethoxyquin, as the main driver of the significant reduction in the abundance and diversity of AOM in soils treated with ethoxyquin. More recently Vasileiadis *et al.* [31] showed 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. In both these studies the toxicity of TPs was tested at levels relevant to their rate of formation by the parent compound, an issue that should be always considered in relevant soil studies. One of the better illustrated examples of toxicity of pesticide TPs comes from chlorothalonil. Using a conservative exposure scenario, the repeated application (4 times) of chlorothalonil in soil at dose rates 5 times the recommended led to the production of levels of 2,4,5-trichloro-6-hydroxybenzene-1,3-dicarbonitrile which induced strong inhibitory effects on the soil microbiota [190, 191].

In silico screening of the potential toxicity of pesticides on the soil microbiota could be an invaluable tool for avoiding the market introduction of compounds whose application could lead to devastating effects on the soil microbiota. A database and prediction tool for the biotransformation of organic contaminants (EnviPath) [192], 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 [193]. Complementary tools (i.e., QSAR) enabling the prediction of the soil microbial toxicity of TPs, such as TyPoL, could allow for a targeted investigation of the TPs toxicity. Further validation of these *in silico* prediction tools with *in vitro* and in soil experimental data will enable their use in the prediction of soil microbial ecotoxicity of pesticides in the frame of environmental risk assessment.

4.5 Diversity analysis

The introduction of amplicon sequencing approaches has revolutionized our view of microbial diversity across all environmental compartments [194], including soil, which is considered the most complex environmental matrix [195]. Amplicon sequencing could monitor, at high resolution level, the composition of bacterial, archaeal, fungal and eukaryotic soil communities [196]. In addition, specific protocols allow the analysis of the diversity of key functional microbial groups like AOA, AOB [31, 197], and AMF [198], although the depth and quality of the analysis of the functional microbial diversity could be compromised by the lack of high quality or updated databases (i.e. Abell *et al.* [87], for AOB). Sequencing data generation is followed by *in silico* analysis relying on online databases (Silva, UNITE, etc.) and the use of freeware bioinformatic tools. As all DNA-based PCR methods, amplicon sequencing analysis could be affected by the presence of relic DNA from dead soil microorganisms complicating the interpretation of the data [199]. DNA relics can be removed from soil samples through treatment with DNAses [200]. Alternatively, soil RNA (and thereof cDNA) being unstable and leaving no relics upon cell lysis, could be used as a target matrix for amplicon sequencing providing insights into the active fraction of the soil microbiome [201].

The different steps of the process (PCR amplification, Sequencing, Bioinformatic Analysis) show variable levels of standardization with PCR amplification steps being more standardized than data analysis, where several issues remain open [202]. The Earth Microbiome Project, a concerted action started in 2010 [203], provided standardized amplicon sequencing protocols for studying soil microbial diversity using validated barcoded primer sets and thermocycling conditions for the amplification of the 16S rRNA gene of bacteria and archaea [204], the ITS region of fungi [205] and the 18S rRNA gene of soil eukaryotes [206] (more details available at https://earthmicrobiome.org/). These protocols have been acknowledged by the scientific community and peers which encourage all groups working on soil microbiomes to utilize the Earth Microbiome Project protocols in relevant studies to ensure comparability and high quality of the data produced [207].

The analysis of amplicon sequencing data involves several steps like: (i) merging of amplicons, removal of primers and barcodes, quality control (ii) development of the data matrix by clustering into Operational Taxonomic Units (OTUs) or using Amplicon Sequence Variants (ASVs) (iii) production of the taxonomic table which will be used for further analysis [208]. In all these steps decisions made and parameters selected are known to strongly affect the outcome of the analysis. For example, the use of ASVs instead of OTUs have often a marked effect on the output of community diversity analysis, with the ASV method outperforming the OTU method when the sequencing depth is adequate [202]. The latter reflects the sequencing effort efficiency to capture the existing microbial diversity and could be determined via rarefaction analysis [209]. In a first effort to contribute to a standardized platform of analysis, the Earth Microbiome Project has proposed QIIME as a tool for bioinformatic processing of the sequencing data [210], while other software like DADA2 [211], and USEARCH [212] could be used for the same purpose. It is beyond the purpose of this paper to provide a detailed technical guide on amplicon sequencing data analysis. The readers could refer to excellent reviews [213–216] and new perspectives in this topic [217]. Overall, the lack of standardization in amplicon sequencing data processing and analysis hampers their use in a regulatory context. We believe that the on-going effort by the

scientific community to set such standards will pave the way for their inclusion in the regulatory scheme in a near future.

Today, several studies have used amplicon sequencing to study the effects of pesticides on the soil microbial diversity [31, 57, 110, 218]. Most of these studies were aligned with the amplicon sequencing protocols of the Earth Microbiome Project, enabling the meta-analysis of the global dataset. In this context, Rocca *et al.* [219] presented a first meta-analysis platform called the Microbiome Stress Project, which was based on amplicon sequencing datasets obtained from experiments looking on the effects of a range of stressors on microbial diversity. This platform could be used as a base for a future systematic meta-analysis of the effects of pesticides on the soil microbiota.

Besides the lack of standardization, soil microbial ecotoxicology lacks a conceptual framework on how to translate the enormous volume of information generated by amplicon sequencing to a realistic toxicity assessment for soil microorganisms. It is now rather well-documented that, despite functional redundancy, soil microbial diversity losses often lead to reciprocal negative effects on soil microbial functioning [150, 220]. However, we still need to define the normal operating range for the different microbial endpoints as determined by amplicon sequencing. This will enable us to establish threshold values beyond which observed microbial diversity losses will have critical ecotoxicological effects. For example, we still have no answer to the question "What is the level of soil microbial diversity reduction that we could consider as imposing no unacceptable risk for the soil microbiota?". Aquatic ecotoxicologists have identified Hazard Concentration 5 % (HC5) as a threshold value for environmental concentrations of pesticides at which, if exceeded, an unacceptable risk to the aquatic biota occurs [221]. Forthcoming research efforts should define normal operating range values per microbial endpoint that can be used by soil microbial ecotoxicologists to define relevant environmental thresholds that will preserve soil microbial diversity and functions.

5 Future research perspectives

5.1 Introducing advanced statistical approaches in soil microbial ecotoxicology

Aquatic ecotoxicology was a pioneer in the development and implementation of advanced statistical approaches like species sensitivity distribution (SSDs) [222] or principal response curves (PRCs) [223]. The former has been used as a refinement tool to determine threshold environmental pesticide concentrations that have no unacceptable effects on 95 % of the population of a trophic level, relying on data from single species tests [224]. On the other hand, PRC is a multivariate ordination method used for the analysis of data derived from complex aquatic mesocosm studies. It provides an overview of the response of the community of a given group of organisms (i.e. arthropods) on a range of toxicant concentrations, while at the same time it gives information of the response of the individual species to the toxicant [225]. Webster *et al.* [226] first suggested the potential use of SSDs in soil microbial ecotoxicology. The construction of SSDs could be based on EC_{50} values calculated from amplicon sequencing data for ASVs, whose abundance show a dose-dependent response (Fig. 4a). Species Sensitivity Distributions could be constructed for a whole microbial domain (i.e. bacteria, fungi, protists), distinct bacterial or fungal phyla or classes (i.e. Proteobacteria, Actinobacteria, etc.), but also for functionally distinct microbial groups like AOB, where phylogeny could provide direct functional assignments. Regarding functional microbial groups, *in vitro* toxicity tests using representative strains (equivalent to single species tests used in aquatic ecotoxicology) could be used to calculate EC_{50} used as input data for the construction of SSDs per microbial function. These functional endpoints could be used for the construction of Functional Species Distribution providing pesticide environmental concentration levels that could have no unacceptable effects to 90 or 95 % of the key soil microbial functions (Fig. 4a). Similarly, amplicon sequencing data derived from soil microcosm or mesocosm studies could be used for the construction of PRCs for the whole prokaryotic, fungal or protist community providing a domain-specific assessment of their response to pesticide exposure while also pointing to specific microorganisms that exhibit differential response to the general pattern (Fig. 4a).

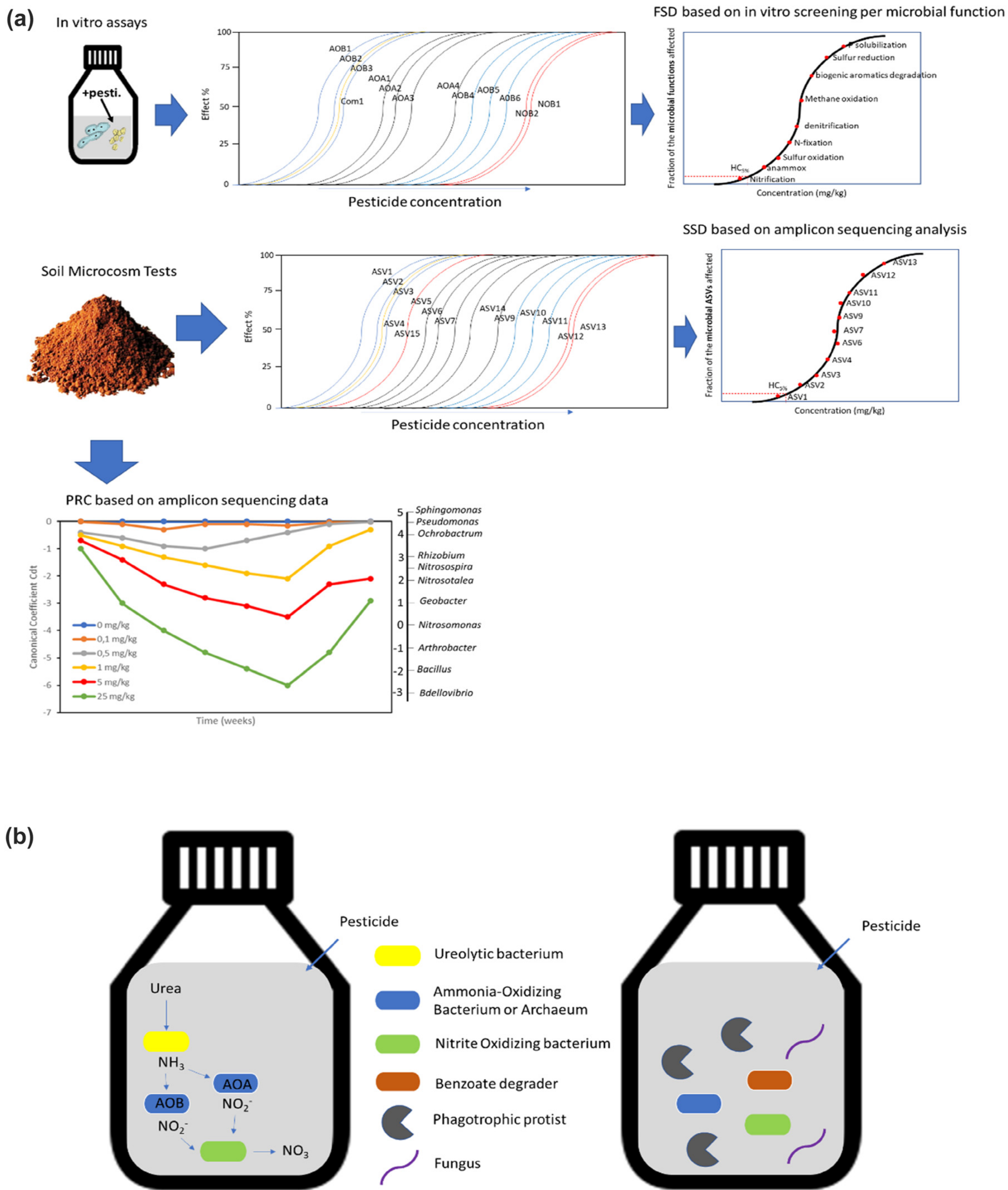

Fig. 4: A glance in novel tools that could be used to advance the assessment of the toxicity of pesticides on soil microorganisms. (a) The construction of species sensitivity distributions (SSDs), Function Sensitivity distributions (adapted by Webster *et al.* [202]) and principal response curves (PRCs) using data from *in vitro* assays or amplicon sequencing datasets from soil studies; (b) synthetic microbial communities performing a collaborative function like nitrification (left bottle) composed of an ureolytic bacterium, an ammonia-oxidizing bacterium (AOB), an ammonia-oxidizing archaeum (AOA) and a nitrite-oxidizing bacterium (NOB) or simulating soil food-web (right bottle) composed of phagotrophic protists and bacteria or fungi whose function can be monitored like benzoate degraders, AOA and AOB.

The concept of SSDs was only recently employed to assess the toxicity of nano-pesticides on the soil microbial community. Hund-Rinke *et al.* [102] determined the effects of silver nanoparticles applied at five dose rates on the soil bacterial community using amplicon sequencing. They calculated EC_{50} values for different bacterial orders and verified the sensitivity of *Nitrosomonadales* to silver nanoparticles, as derived from potential ammonia oxidation studies. More recently, Swart *et al.* [175] tested the toxicity of silver and copper nanoparticles on the soil and gut microbiota of earthworms. Based on bacterial amplicon sequencing data, they calculated ASV specific EC_{50} values which were then used for the construction of SSDs and the calculation of HC_5, which indicated high risk for the soil microbiota by CuO nanoparticles.

The use of SSDs but also PRCs could make feasible the implementation of amplicon sequencing data in pesticide risk assessment. Alternatively, SSDs derived from (i) meta-analysis of already available amplicon sequencing datasets from studies that meet certain experimental criteria of quality and (ii) novel soil microcosm studies (including control and at least four dose rates) testing the toxicity of a range of pesticides on soil microorganisms using amplicon sequencing measurements, could be used to identify responsive and sensitive microbial groups. These could be further promoted as microbial indicators in soil microbial ecotoxicity testing.

5.2 Ecosystem level assessment

Most soil studies to date have assessed the toxicity of pesticides on individual taxa or functional groups separately [62] or in the context of a biochemical pathway (*e.g.* denitrification), ignoring the ecological dimension of the effects observed [106]. In nature, microorganisms are assembled into complex and diverse communities where subpopulations are intertwined by metabolic links or other types of interactions [227], potentially important for ecosystem functioning. The 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 [228], or to functional complementarity of network members, resulting in better exploitation of resources and elevated resistance to stress [229]. Ammonia-oxidizing microorganisms and NOB constitute one of the most well studied functional microbial networks whereby the nitrite produced by AOM through ammonia oxidation is used as a substrate by NOB to produce nitrate [83]. Recent studies suggested that the interactions between AOM and NOB could be even more complex where the latter hydrolyze urea to NH_3, which is then taken up by urease-negative AOB that, in turn, produce the NO_2 that is consumed by NOB [230]. These functional microbial networks operating within nitrogen cycling could be the focus of future research on the effects of pesticides. This is possible due to (i) our deep knowledge of the physiology, diversity and functioning of the microbial groups participating in these networks, (ii) the availability of relevant monitoring methods and (iii) the tight association of microbial groups participating in the different steps of the process. Studies exploring the potential resistance and resilience of such microbial networks to pesticide exposure will provide an ecosystem level look at the impact of pesticides on soil microbial functioning.

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 [231]. Predation by protists influences bacterial diversity and productivity with consequences on the flux of organic nutrients into biomass at higher trophic levels [232]. The diversity of both protists and bacteria interactively determines the performance of the predator [233]. External perturbations (i.e. pesticides) could affect diversity at both trophic levels with possible effects on ecosystem functioning [234], an aspect overlooked so far in pesticide research. Considering the power and the resolution of amplicon sequencing tools, we posit that soil studies should study bacterial, fungal and protistan communities and identify, via appropriate co-occurrence network analysis, pesticide-driven interactions between protists and bacteria/fungi.

Synthetic microbial communities are currently at the forefront of microbial ecology and biotechnology with applications in the biosynthesis of molecules of industrial interest or in the biodegradation of pollutants [235]. Recently, Karkaria *et al.* [236] proposed the use of computational methods for building stable synthetic

microbial communities. The concept of synthetic microbial communities could be used to study the effects of pesticides on nitrifying microbial networks composed of ureolytic bacteria, AOB, AOA and NOB [237]. Multitrophic synthetic microbial communities composed of microorganisms from different trophic levels exhibiting predator–prey interdependencies could be also used in soil microbial ecotoxicology to verify pesticide-driven effects on protists and their prey (bacteria or fungi) (Fig. 4b). This approach could be utilized as a refinement option when Tier I *in vitro* tests suggest unacceptable risks for the soil microbiota.

5.3 Toxicity of pesticides on the soil interactome (plant microbiome and gut microbiome of terrestrial organisms)

Beyond soil, pesticides could directly or indirectly affect the composition of the microbial communities colonizing internal (endophytic) or external (epiphytic like in phyllosphere or carposphere) plant tissues composing all together the plant microbiome whose importance for agricultural production and plant communities is immense [238]. Recent evidence suggests that pesticide effects go beyond plants in agricultural systems and reach the symbiome of insects and earthworms with reciprocal implications for ecosystem functioning [239].

Pesticide applied directly on plant foliage could directly affect epiphytic microbial communities. On the other hand, pesticides applied via soil drenching, besides affecting the seed and rhizosphere microbiome, could be taken up by plants and could impose strong effects on the endophytic microbiome. Schaeffer *et al.* [240] showed, using amplicon sequencing, that fungicides could impose detrimental effects on yeasts inhabiting the nectar of almond trees like *Metschnikowia* which are important players in pollination. Katsoula *et al.* [58] showed that repeated applications of iprodione on the foliage and in the rhizosphere of pepper plants induced significant alterations in the epiphytic and soil microbiota and led to an adaptation of both microbial communities, rhizospheric and epiphytic, to enhanced biodegradation of iprodione. In contrast, other studies have suggested limited effects of pesticides on the epiphytic microbial communities [241, 242].

The effects of pesticides extend to the symbiome of earthworms and arthropods exposed to pesticides through soil or through feeding on plants. Recent studies investigated the effects of nano-pesticides on the gut microbiome of earthworms and showed contrasting results [175, 243]. Interestingly Swart *et al.* [175] reported that CuO nanoparticles negatively affected bacteria considered as common residents of the gut of earthworms like *Candidatus* Lumbricincola, which are expected to have serious implications for host vigor. On the other hand, the regular exposure of insects to pesticides like atrazine (6-chloro-N^2-ethyl-N^4-(propan-2-yl)-1,3,5-triazine-2,4-diamine), trichlorfon (dimethyl *rac*-2,2,2-trichloro-1-hydroxyethyl phosphonate), or fenitrothion (*O,O*-dimethyl *O*-(4-nitro-3-methylphenyl) phosphorothioate) could select in the insect symbiome for microorganisms that carry catabolic genes like *atz* which encode for enzyme catalyzing the degradation and detoxification of atrazine [244–246].

The soil microbiome is the main reservoir for plant and insect microbiomes [247]. Considering the effect of pesticides on the soil microbiota and the direct and indirect exposure of plants and insects to pesticides, we posit that effects of pesticides might be better examined at the ecosystem level where the microbiome of all biotic interactors will be studied in parallel.

5.4 Interactive effects of pesticides with other pollutants

Pesticides often encounter other organic pollutants in agricultural soils like veterinary drugs (antibiotics and anthelmintics), which end up in agricultural soil mainly through the application of manure from livestock units [248, 249]. Kurenbach *et al.* [250] first showed that treating bacterial pathogenic strains with herbicides like glyphosate ([(phosphonomethyl)amino]acetic acid) and dicamba (3,6-dichloro-2-methoxybenzoic acid) could induce a multiple antibiotic resistance phenotype associated with increasing upregulation of efflux pumps. This

was further verified in soil where the application of glyphosate, glufosinate (ammonium *rac*-2-amino-4-[hydroxy(methylphosphonoyl)] butanoate), and dicamba increase the prevalence of antibiotic resistance genes and mobile genetic elements in soil microbiomes [251]. These findings suggest that the application of herbicides in agricultural soils might exert a selection pressure favoring the co-selection of antibiotic resistance thereby contributing to the global antimicrobial resistance problem. However, the full extent of this phenomenon at microbiome level is not known (i.e. this is common among all pesticide groups), while the combined effects of veterinary drugs and pesticides, beyond antibiotic resistance gene dispersal, should be investigated in an ecotoxicological context.

Another group of emerging pollutants in agricultural soils are microplastics. They are plastic fragments of size smaller than 5 μm formed by the disintegration of plastic fragments released in soils and their levels in certain agricultural soils could reach up to 6.7 % [252]. A few studies in recent years have highlighted the potential adverse effects of microplastics on the soil microbiota [253, 254]. Microplastics are known to interact with pesticides in agricultural soils offering sites for their adsorption [255, 256]. Although this might suggest that microplastics might act as sinks for pesticides limiting their bioavailability and hence reducing their adverse effects on the soil microbiota, we still lack data to support this hypothesis.

Another aspect which was only recently acknowledged is the ecological role of plastisphere, the interface between soil and plastic surfaces. The composition of the microbial communities colonizing the plastisphere is only just starting to be explored compared to aquatic ecosystems [257]. Recently, Zhu *et al.* [258] showed that the plastisphere selects for microbial communities that are involved in diverse metabolic pathways including an enrichment in antibiotic resistance genes and bacterial pathogens compared to the surrounding soil. In this framework, the adsorption of pesticides on microplastic surfaces could favor the selection of microorganisms with tolerance or increased biodegradation capacities against pesticides. Studies trying to explore these interactions will strongly promote our knowledge on the interactive effects of pesticides with microplastics on the soil microbiota and will explore microbial evolutionary aspects that have not yet been investigated before.

5.5 Beyond amplicon sequencing: looking at pesticide effects using shotgun meta-omic approaches

Amplicon sequencing analysis could provide insights into the phylogenetic identity of the soil microbial responders to pesticides, but it falls short in providing solid functional assignments to the members of soil microbial communities. Such information could be derived through the application of shotgun meta-genomic and meta-transcriptomic approaches. To date the application of these meta-omic tools in soil microbial ecotoxicology is rare. Wu *et al.* [259] used a combination of amplicon sequencing and shotgun meta-genomics to look at the effects of thiamethoxam ((*EZ*)-{3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl-1,3,5-oxadiazinan-4-ylidene}nitramide) on the soil microbiota. Beyond effects on bacterial diversity and abundance which were temporal and recovery was observed, they noted negative effects of thiamethoxam on (i) the relative abundance of nitrogen cycling genes (ii) Carbon metabolism genes involved in glycolysis, and Krebs cycle (iii) genes involved in signal regulating pathways like ABC transporters. The use of shotgun meta-genomic and meta-transcriptomic analysis in soil microbial ecotoxicology would go beyond descriptive phylogenetic information on pesticide microbial responders and look at effects at the metabolic potential and functional level while at the same time acquiring strong phylogenetic signals.

6 Conclusions

The effects of pesticides on soil microorganisms have been long overlooked at the regulatory level, despite the pivotal role of microorganisms in soil ecosystem functioning. We posit that the time is right for advancing pesticide risk assessment on soil microorganisms. This should be based on a revised tiered risk assessment

scheme characterized by (i) analytical tiers with increasing level of microbial complexity (from single microorganisms at *in vitro* to natural microbial assemblages in soil), (ii) the use of currently available standardized methods, as a start to this revision effort (iii) defined functional microbial groups as key indicators of the soil microbial toxicity of pesticides. Such microbial indicators have been already identified and their relevance in pesticide soil microbial ecotoxicology could be further validated in studies looking at their comparative response under the same pesticide exposure scenarios. Ongoing standardization of amplicon sequencing approaches along with the implementation of advanced ecotoxicological analytical tools (*e.g.* SSDs, PRCs), synthetic microbial ecology approaches (synthetic microbial communities) and meta-analysis of the currently available and continuously produced amplicon sequencing data will provide the needed data for defining the normal operating range, threshold values for these microbial endpoints and identify or verify potential microbial indicator groups, all considered as essential elements of an advanced assessment of the potential risk of pesticides for the soil microbiota. The further upsurge of meta-omic tools in soil microbial ecology will shed light into the mechanisms of toxicity of pesticides on soil microorganisms and will eventually shift the focus to an ecosystem level assessment of the soil microbial toxicity of pesticides.

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Abbreviations

AMPA	2-Amino-3-(5-methyl-3-oxo-2,3-dihydro-1,2-oxazol-4-yl)propanoic acid
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
AOM	Ammonia-oxidizing microorganisms
ASV	Amplicon Sequence Variant
AMF	Arbuscular mycorrhizal fungi
CA	Concentration addition
DGGE	Denaturating Gradient Gel Electrophoresis
dsRNA	Double Stranded RNA
EC_{50}	Effective Concentration 50 %
ERA	Environmental Risk Assessment
EFSA	European Food Safety Authority
EU	European Union

HC5	Hazard Concentration 5 %
IA	Independent action
IC_{50}	Inhibitory Concentration 50 %
ISO	International Organization for Standardization
LC_{10}	Lethal Concentration 10 %
LC_{50}	Lethal Concentration 50 %
miRNA	MicroRNA
NOB	Nitrite-oxidizing bacteria
OECD	Organization for Economic Co-operation and Development
OTU	Operational Taxonomic Unit
PLFAs	Phospholipid Fatty Acids Analysis
PRCs	Principal Response Curves
q-PCR	quantitative Polymerase Chain Reaction
QSAR	quantitative structure-activity relationship
RT-q-PCR	Reverse Transcription q-PCR
siRNA	Small Interfering RNA
SSDs	Species Sensitivity Distribution
TRFLP	Terminal Restriction Fragment Length Polymorphism
TPs	Transformation Products

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References

[1] J. Bælum, T. Henriksen, H. C. B. Hansen, C. S. Jacobsen. *Appl. Environ. Microbiol.* **72**, 1476 (2006).
[2] B. Horemans, K. Bers, E. Ruiz Romero, E. Pose Juan, V. Dunon, R. De Mot, D. Springael. *Appl. Environ. Microbiol.* **82**, 2843 (2016).
[3] R. K. Osborn, S. G. Edwards, A. Wilcox, P. P. Haydock. *Pest Manag. Sci.* **66**, 253 (2010).
[4] E. S. Papadopoulou, S. Lagos, F. Spentza, E. Vidiadakis, P. A. Karas, T. Klitsinaris, D. G. Karpouzas. *Pest Manag. Sci.* **72**, 1040 (2016).
[5] C. Rousidou, D. Karaiskos, D. Myti, E. Karanasios, P. A. Karas, M. Tourna, E. A. Tzortzakakis, D. G. Karpouzas. *FEMS Microbiol. Ecol.* **93**, fiw219 (2017).
[6] L. J. Krutz, D. L. Shaner, M. A. Weaver, R. M. Webb, R. M. Zablotowicz, K. N. Reddy, Y. Huang, S. J. Thompson. *Pest Manag. Sci.* **66**, 461 (2010).
[7] A. Walker, P. Brown, A. R. Entwistle. *Pestic. Sci.* **17**, 183 (1986).
[8] T. El-Sebai, B. Lagacherie, J. F. Cooper, G. Soulas, F. Martin-Laurent. *Agron. Sustain. Dev.* **25**, 271 (2006).
[9] EFSA. *EFSA J.* **17**, 5743 (2019).
[10] R. Loos, B. M. Gawlik, G. Locoro, E. Rimaviciute, S. Continue, G. Bidoglio. *Environ. Pollut.* **157**, 561 (2009).
[11] V. Silva, H. G. J. Mol, P. Zomer, M. Tienstra, C. J. Ritsema, V. Geissen. *Sci. Total Environ.* **653**, 1532 (2019).
[12] R. B. Schafer, M. Liess, R. Altenburger, J. Filser, H. Hollert, M. Rob-Nickoll, A. Schaffer, M. Scheringer. *Environ. Sci. Eur.* **31**, 21 (2019).
[13] N. Fierer. *Nat. Rev. Microbiol.* **15**, 579 (2017).
[14] P. Trivedi, J. E. Leach, S. G. Tringe, T. Sa, B. K. Singh. *Nat. Rev. Microbiol.* **18**, 607 (2020).
[15] P. Lemanceau, M. Blouin, D. Muller, Y. Moenne-Loccoz. *Trends Plant Sci.* **22**, 583 (2017).
[16] M. C. Rillig, C. A. Aguilar-Trigueros, J. Bergmann, E. Verbruggen, S. D. Veresoglou, A. Lehmann. *New Phytol.* **205**, 1385 (2015).
[17] EFSA. *EFSA J.* **8**, 1821 (2010).
[18] *OECD Guidelines for the Testing of Chemicals, Section 2*, OECD Publishing, Paris (2000).
[19] EFSA PPR Panel. *EFSA J.* **15**, 4690 (2017).
[20] Q. Jia, T. Liu, F. Yan, Q. Wang. *Environ. Toxicol. Chem.* **39**, 352 (2020).
[21] J. Roy, P. K. Ojha, E. Carnesecchi, A. Lombardo, K. Roy, E. Benfenati. *J. Hazard Mater.* **386**, 121660 (2020).
[22] J. J. Villaverde, B. Sevilla-Morán, C. López-Goti, J. L. Alonso-Prados, P. Sandín-España. *SAR QSAR Environ. Res.* **31**, 49 (2020).

[23] H. Traore, O. Crouzet, L. Mamy, C. Sireyjol, V. Rossard, R. Servien, E. Latrille, F. Martin-Laurent, D. Patureau, P. Benoit. *Environ. Sci. Pollut. Res.* **23**, 4320 (2018).
[24] A. Hardy, D. Benford, T. Halldorsson, M. J. Jeger, H. K. Knutsen, S. More, H. Naegeli, H. Noteborn, C. Ockleford, A. Ricci, G. Rychen, J. R. Schlatter, V. Silano, R. Solecki, D. Turck, E. Benfenati, Q. M. Chaudhry, P. Craig, G. Frampton, M. Greiner, A. Hart, C. Hogstrand, C. Lambre, R. Luttik, D. Makowski, A. Siani, H. Wahlstroem, J. Aguilera, J.-L. Dorne, A. F. T. Dumon, M. Hempen, S. Valtena Martınez, I. Martino, C. Smeraldi, A. Terron, N. Georgiadis, M. Younes. *EFSA J.* **15**, e04971 (2017).
[25] R. M. Atlas, D. Pramer, R. Bartha. *Soil Biol. Biochem.* **10**, 231 (1978).
[26] M. T. Wan, J. R. Rahe. *Environ. Toxicol. Chem.* **17**, 1041 (1998).
[27] F. Rivera-Becerril, D. van Tuinen, O. Chatagnier, N. Rouard, J. Beguet, C. Kuszala, G. Soulas, V. Gianinazzi-Pearson, F. Martin-Laurent. *Sci. Total Environ.* **577**, 84 (2017).
[28] I. Petric, D. G. Karpouzas, D. Bru, N. Udikovic-Kolic, E. Kandeler, S. Djuric, F. Martin-Laurent. *Environ. Sci. Pollut. Res.* **23**, 4320 (2016).
[29] S. Romdhane, M. Devers-Lamrani, J. Beguet, C. Bertrand, C. Calvayrac, M.-V. Salvia, A. Ben Jrad, F. E. Dayan, A. Spor, L. Barthelmebs, F. Martin-Laurent. *Sci. Total Environ.* **651**, 241 (2019).
[30] C. Thiour-Mauprivez, F. Martin-Laurent, C. Calvayrac, L. Barthelmebs. *Sci. Total Environ.* **684**, 314 (2019).
[31] S. Vasileiadis, E. Puglisi, E. S. Papadopoulou, G. Pertile, N. Suciu, A. Papolla, M. Tourna, P. A. Karas, F. Papadimitriou, A. Kasiotakis, N. Ipsilanti, A. Ferrarini, S. Sulowic, F. Fornasier, G. W. Nicol, M. Trevisan, D. G. Karpouzas. *Appl. Environ. Microbiol.* **84**, e01536-18 (2018).
[32] M. Hernández, Z. Jia, R. Conrad, M. Seeger. *FEMS Microbiol. Ecol.* **78**, 511 (2011).
[33] D. G. Karpouzas, E. S. Papadopoulou, I. Ipsilantis, I. Friedel, I. Petric, N. Udikovic-Kolic, S.E. DjuricKandeler, U. Menkissoglu-Spiroudi, F. Martin-Laurent. *Ecol. Indicat.* **39**, 44 (2014).
[34] P. A. Karas, C. Baguelin, G. Pertile, E. S. Papadopoulou, S. Nikolaki, V. Storck, F. Ferrari, M. Trevisan, A. Ferrarini, F. Fornasier, S. Vasileiadis, G. Tsiamis, F. Martin-Laurent, D. G. Karpouzas. *Sci. Total Environ.* **637–638**, 636 (2018).
[35] I. L. M. Jakobsen Murmann, S. Rosendahl. *Soil Biol. Biochem.* **159**, 108299 (2021).
[36] P. Laitinen, S. Ramo, K. Siimes. *Plant Soil* **300**, 51 (2007).
[37] W. H. Lewis, G. Tahon, P. Geesink, D. Z. Sousa, T. J. G. Ettema. *Nat. Rev. Microbiol.* **19**, 225 (2021).
[38] D. G. Karpouzas, E. Kandeler, D. Bru, I. Friedel, Y. Auer, S. Kramer, S. Vasileiadis, I. Petric, N. Udikovic-Kolic, S. Djuric, F. Martin-Laurent. *Soil Biol. Biochem.* **75**, 282 (2014b).
[39] C. Zhang, T. Zhou, L. Zhu, Z. Du, B. Li, J. Wang, J. Wang, Y. Sun. *Sci. Total Environ.* **666**, 89 (2019).
[40] C. W. Bell, B. E. Fricks, J. D. Rocca, J. M. Steinweg, S. K. McMahon, M. D. Wallenstein. *J. Vis. Exp.*, **81**, e50961 (2013).
[41] S. Wakelin, E. Lombi, E. Donner, L. Macdonald, A. Black, M. O'Callaghan. *Environ. Pollut.* **179**, 177 (2019).
[42] E. S. Papadopoulou, P. Tsachidou, S. Sulowic, U. Menkissoglu-Spiroudi, D. G. Karpouzas. *Appl. Environ. Microbiol.* **82**, 747 (2016).
[43] F. Martin-Laurent, E. Kandeler, I. Pertic, S. Djuric, D. G. Karpouzas. *Environ. Sci. Pollut. Res.* **20**, 1203 (2013).
[44] ISO. ISO 11063:2020 Soil quality—Direct extraction of soil DNA, 2nd ed., pp. 1–10 (2020).
[45] ISO. ISO 17601:2016 Soil quality—Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil, 1st ed., pp. 1–31 (2016).
[46] L. Feld, M. H. Hjelmsø, M. S. Nielsen, A. D. Jacobsen, R. Rønn, F. Ekelund, P. H. Krogh, B. W. Strobel, C. S. Jacobsen. *PLoS One* **10**, e0126080 (2015).
[47] W. Fang, D. Yan, X. Wang, B. Huang, X. Wang, J. Liu, X. Liu, Y. Li, C. Ouyang, Q. Wang, A. Cao. *Front. Microbiol.* **9**, 2529 (2018).
[48] N. El Azhari, E. Dermou, R. L. Barnard, V. Storck, T. Tourna, J. Beguet, P. Karas, L. Lucini, N. Rouard, L. Botteri, F. Ferrari, M. Trevisan, D. G. Karpouzas, F. Martin-Laurent. *Sci. Total Environ.* **637–638**, 892 (2019).
[49] ISO/TS 29843-1:2010 Soil quality—Determination of soil microbial diversity—Part 1: Method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis, 1st ed., pp. 1–9 (2010).
[50] ISO/TS 29843-2:2021 Soil quality—Determination of soil microbial diversity—Part 2: Method by phospholipid fatty acid analysis (PLFA) using the simple PLFA extraction method, 2nd ed., pp. 1–7 (2021).
[51] A. Fostegard, A. Tunlid, E. Baath. *Soil Biol. Biochem.* **43**, 1621 (2011).
[52] B. G. Clement, L. E. Kehl, K. L. DeBord, C. L. Kitts. *J. Microbiol. Methods* **31**, 135 (1998).
[53] G. Muyzer, K. Smalla. *Ant. van Leeuwen* **73**, 127 (1998).
[54] J. K. Harris, J. W. Sahl, T. A. Castoe, B. D. Wagner, D. D. Pollock, J. R. Spear. *Appl. Environ. Microbiol.* **76**, 3863 (2010).
[55] X. Li, H. Zhang, M. Wu, Z. Su, C. Zhang. *J. Environ. Sci.* **20**, 1126 (2008).
[56] I. Ipsilantis, C. Samourelis, D. G. Karpouzas. *Soil Biol. Biochem.* **45**, 147 (2012).
[57] V. Storck, S. Nikolaki, C. Perruchon, C. Chabanis, A. Sacchi, G. Pertile, C. Baguelin, P. A. Karas, A. Spor, M. Devers, E. S. Papadopoulou, O. Sibourg, C. Malandain, M. Trevisan, F. Ferrari, D. G. Karpouzas, G. Tsiamis, F. Martin-Laurent. *Front. Microbiol.* **9**, 1412 (2018).
[58] A. Katsoula, S. Vasileiadis, M. Sapountzi, D. G. Karpouzas. *FEMS Microbiol. Ecol.*, **96**, fiaa056 (2020).
[59] S. Thiele-Bruhn, M. Schloter, M. B-Wilke, L. A. Beaudette, F. Martin-Laurent, N. Cheviron, C. Mougin, J. Rombke. *Soils* **6**, 17 (2020).
[60] EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues). *EFSA J.* **11**, 3290 (2013).

[61] E. Wessén, S. Hallin. *Ecol. Indicat.* **11**, 1696 (2011).
[62] C. Lu, Z. Yang, J. Liu, Q. Liao, W. Ling, M. G. Waigi, E. S. Odinga. *Chemosphere* **256**, 127098 (2020).
[63] W. Foissner. *Agric. Ecosyst. Environ.* **74**, 95 (1998).
[64] V. Dupraz, S. Stachowski-Haberkorn, J. Wicquart, N. Tapie, H. Butzinski, F. Ackha. *Chemosphere* **221**, 278 (2019).
[65] N. Amacker, E. A. D. Mitchell, B. J. D. Ferrari, N. Cherve. *Chemosphere* **201**, 351 (2018).
[66] M. Giovannetti, A. Turrini, P. Strani, C. Sbrana, L. Avio, B. Pietrangeli. *Prevention Today* **2**, 47 (2006).
[67] ISO/TS 10832:2009 Soil quality—Effects of pollutants on mycorrhizal fungi—Spore germination test, 1st ed., pp. 1–19 (2010).
[68] T. E. Kiers, M. Duhamel, Y. Beesetty, J. A. Mensah, O. Franken, E. Verbruggen, C. R. Fellbaum, G. A. Kowalchuk, M. A. Hart, A. Bago, T. M. Palmer, S. A. West, P. Vanderkoornhuyse, J. Jansa, H. Bucking. *Science* **333**, 880 (2011).
[69] S. D. Veresoglou, M. C. Rillig. *Biol. Lett.* **8**, 214 (2012).
[70] C. W. Fernandez, P. G. Kennedy. *New Phytol.* **205**, 1378 (2015).
[71] M. G. van der Heijden, R. D. Bardgett, N. M. van Straalen. *Ecol. Lett.* **11**, 296 (2008).
[72] M. Druille, M. N. Cabello, M. Omacini, R. A. Golluscio. *Appl. Soil Ecol.* **64**, 99 (2013).
[73] C. Buysens, H. D. de Boulois, S. Declerck. *Mycorrhiza* **25**, 277 (2015).
[74] G. C. Mallmann, J. Paulo Sousa, I. Sundh, S. Pieper, M. Arena, S. P. da Cruz, O. Klauberg-Filho. *Ecotoxicology* **27**, 809 (2018).
[75] H. Jin, J. J. Germida, F. L. Walley. *Appl. Soil Ecol.* **72**, 22 (2013).
[76] M. T. Wan, J. E. Rahe, R. G. Watts. *Environ. Toxicol. Chem.* **17**, 1421 (1998).
[77] D. Zocco, J. Fontaine, E. Lozanova, L. Renard, C. Bivort, R. Durand, A. Grandmougin-Ferjani, S. Declerck. *Mycol. Res.* **112**, 592 (2008).
[78] D. Zocco, I. M. Van Aarle, E. Oger, L. Lanfranco, S. Declerck. *Mycorrhiza* **21**, 363 (2011).
[79] H. Dupré de Boulois, L. Voets, B. Delvaux, I. Jackobsen, S. Declerck. *Environ. Microbiol.* **8**, 1926 (2006).
[80] E. Campagnac, J. Fontaine, A. L. H. Sahraoui, F. Laruelle, R. Durand, A. Grandmougin-Ferjani. *Phytochemistry* **69**, 2912 (2008).
[81] Z. Oehl, E. Sieverding, J. Palenzuela, K. Ineichen, G. Alves da Silva. *IMA Fungus* **2**, 191 (2011).
[82] C. J. Sweeney, M. Bottoms, S. Ellis, G. Emst, S. Kimmel, S. Loutseti, A. Schimera, L. S. C. Carniel, A. Sharples, F. Staab, M. T. Marx. *Environ. Toxicol. Chem.* **41**, 1808 (2022).
[83] M. M. M. Kuypers, H. K. Marchant, B. Kartal. *Nat. Rev. Microbiol.* **320**, 889 (2018).
[84] J. D. Caranto, K. M. Lancaster. *Proc. Nat. Acad. Sci. USA* **114**, 8217 (2017).
[85] M. Pester, F. Maixner, D. Berry, T. Rattei, H. Koch, S. Lucker, B. Nowka, A. Richter, E. Spieck, E. Lebedeva, A. Loy, M. Wagner, H. Daims. *Environ. Microbiol.* **16**, 3055 (2014).
[86] N. Wrage-Monnig, M. A. Horn, R. Well, C. Muller, G. Velthof, O. Oenema. *Soil Biol. Biochem.* **123**, A3 (2018).
[87] G. C. J. Abell, S. S. Robert, D. M. F. Frampton, J. K. Volkman, F. Rizwi, J. Csontos, L. Bodrossy. *PLoS One* **7**, e51542 (2012).
[88] R. J. E. Alves, B. Q. Minh, T. Urich, A. von Haeseler, C. Schleper. *Nat. Commun.* **9**, 1517 (2018).
[89] H. Daims, E. V. Lebedeva, P. Pjevac, P. Han, G. Herbold, M. Albertsen, N. Jehmlich, M. Palatinszky, A. Bulaev, R. H. Kirkegaard, M. von Bergen, T. Rattei, B. Bendinger, P. H. Nielsen, M. Wagner. *Nature* **528**, 504 (2015).
[90] Z. Wang, Y. Cao, X. Zhu-Barker, G. W. Nicol, A. L. Wright, Z. Jia, X. Jiang. *Soil Biol. Biochem.* **135**, 392 (2019).
[91] G. W. Nicol, S. Leininger, C. Schleper, J. I. Prosser. *Environ. Microbiol.* **10**, 2966 (2008).
[92] R. Hatzenpichler. *Appl. Environ. Microbiol.* **78**, 7501 (2012).
[93] K. D. Kits, C. J. Sedlacek, E. V. Lebedeva, P. Han, A. Bulaev, P. Pjevac, A. Daebeler, S. Romano, M. Albertsen, L. Y. Stein, H. Daims, M. Wagner. *Nature* **549**, 269 (2017).
[94] J. H. Rotthauwe, K. P. Witzel, W. Liesack. *Appl. Environ. Microbiol.* **63**, 4704 (1997).
[95] M. Tourna, T. E. Freitag, G. W. Nicol, J. I. Prosser. *Environ. Microbiol.* **10**, 1357 (2008).
[96] P. Pjevac, C. Schauberger, L. Poghosyan, C. W. Herbold, M. A. H. J. van Kessel, A. Daebeler, M. Steinberger, M. S. M. Jetten, S. Lucker, M. Wagner, H. Daims. *Front. Microbiol.* **8**, 1508 (2017).
[97] K. H. Domsch. *Symp. Biol. Hung.* **11**, 337 (1972).
[98] S. Singh, R. Gupta, M. Kumari, S. Sharma. *Environ. Sci. Pollut. Res.* **22**, 11290 (2015).
[99] S. Singh, R. Gupta, S. Sharma. *J. Hazard Mater.* **291**, 102 (2015).
[100] W. Fang, X. Wang, B. Huang, D. Zhang, J. Liu, D. Yan, Q. Wan, A. Cao, Q. Han. *Ecotoxicol. Environ. Saf.* **187**, 109850 (2020).
[101] C. Rousidou, E. S. Papadopoulou, M. Kortsinidou, I. O. Giannakou, B. K. Singh, U. Menkissoglu-Spiroudi, D. G. Karpouzas. *Soil Biol. Biochem.* **67**, 98 (2013).
[102] K. Hund-Rinke, A. Hummler, R. Schlinkert, F. Wege, G. Broll. *Environ. Sci. Eur.* **31**, 86 (2019).
[103] ISO 15685:2012 Soil quality—Determination of potential nitrification and inhibition of nitrification—Rapid test by ammonium oxidation, 2nd ed., pp. 1–11 (2012).
[104] O. Crouzet, P. Besse-Hogan, I. Batisson, F. Bonnemoy, J. Bohatier, C. Mallet. *Proceedings of the Symposium on Pesticide Behaviour in Soils, Water and Air, York*, pp. 12–13, United Kingdom (2009).
[105] C. Hazard, J. Prosser, G. W. Nicol. *Soil Biol. Biochem.* **157**, 108242 (2021).
[106] X. Hu, Y. Wang, X. Su, Y. Chen. *Sci. Total Environ.* **636**, 1408 (2018).
[107] X. Su, Y. Wang, G. Peng, Q. He. *Environ. Sci. Pollut. Res.* **27**, 17370 (2020).
[108] P. Du, X. Wu, J. Xu, F. Dong, X. Liu, Y. Zheng. *J. Hazard Mater.* **353**, 204 (2018).
[109] M. Zhang, Z. Xu, Y. Teng, P. Christie, J. Wang, W. Ren, Y. Luo, Z. Li. *Sci. Total Environ.* **543**, 636 (2016).

[110] W. Fang, D. Yan, Q. Wang, B. Huang, Z. Ren, X. Wang, X. Wang, Y. Li, C. Ouyang, Q. Migheli, A. Cao. *Sci. Total Environ.* **650**, 44 (2019).
[111] S. M. Adl, A. G. B. Simpson, C. E. Lane, J. Lukes, D. Bass, S. S. Bowser, M. W. Brown, F. Burki, M. Dunthorn, V. Hampl, A. Heiss, M. Hoppenrath, E. Lara, L. le Gall, D. H. Lynn, H. McManus, E. A. D. Mitchell, S. E. Mozley-Stanridge, L. W. Parfrey, J. Pawlowski, S. Rueckert, L. Shadwick, C. L. Schoch, A. Smirnov, F. W. Spiegel. *J. Eukaryot. Microbiol.* **59**, 429 (2012).
[112] S. Geisen, E. A. D. Mitchell, S. Adl, M. Bonkowski, M. Dunthorn, F. Ekelund, L. D. Fernández, A. Jousset, V. Krashevska, D. Singer, F. W. Spiegel, J. Walochnik, E. Lara. *FEMS Microbiol. Rev.* **42**, 293 (2018).
[113] S. M. Adl, D. C. Coleman. *Biol. Fertil. Soils* **42**, 168 (2005).
[114] A. M. Oliverio, S. Geisen, M. Delgado-Baquerizo, F. T. Maestre, B. L. Turner, N. Fierer. *Sci. Adv.* **6**, eaax8787 (2020).
[115] A. Dupont, R. I. Griffiths, T. Bell, D. Bass. *Environ. Microbiol.* **18**, 2010 (2016).
[116] S. Geisen. *Environ. Microbiol.* **18**, 1669 (2016).
[117] A. Busch, S. Hess. *Protist* **168**, 12 (2017).
[118] C. V. W. Seppey, D. Singer, K. Dumack, B. Fournier, L. Belbahri, E. A. D. Mitchell, E. Lara. *Soil Biol. Biochem.* **112**, 68 (2017).
[119] D. Gilbert, C. Amblard, G. Bourdier, A. J. Francez, E. A. D. Mitchell. *L'Annee Biologique* **39**, 57 (2000).
[120] M. Bonkowski, M. Clarholm. *Acta Protozool.* **51**, 237 (2012).
[121] S. T. Bates, J. C. Clemente, G. E. Flores, W. A. Walters, L. W. Parfery, R. Knight, N. Fierer. *ISME J.* **7**, 652 (2013).
[122] L. Grossmann, M. Jensen, D. Heider, S. Jost, E. Glucksman, H. Hartikainen, S. S. Machamdallie, M. Gardner, D. Hoffmann, D. Bass, J. Boenigk. *ISME J.* **10**, 2269 (2016).
[123] Z.-B. Zhao, J.-Z. He, Z. Quan, C.-F. Wu, R. Sheng, L.-M. Zhang, S. Geisen. *Soil Biol. Biochem.* **148**, 107863 (2020).
[124] B. Schreiber, B. Brink. *Biol. Fertil. Soils* **7**, 289 (1989).
[125] F. Ekelund. *J. Appl. Ecol.* **36**, 233 (1999).
[126] L. N. Carley, R. Panchagavi, X. Song, S. Davenport, C. M. Bergemann, A. W. McCumber, C. K. Gunsch, M. Simonin. *Environ. Sci. Technol.* **54**, 8878 (2020).
[127] B. Fournier, S. P. D. Santos, J. A. Gustavsen, G. Imfeld, F. Lamy, E. A. D. Mitchell, M. Mota, D. Noll, C. Plantchamp, T. J. Heger. *Sci. Total Environ.* **738**, 139635 (2020).
[128] T. Lu, Q. Zhang, M. Lavoie, Y. Zhu, Y. Ye, J. Yang, H. W. Paerl, H. Qian, Y. G. Zhu. *Microbiome* **7**, 128 (2019).
[129] B. Metting. *Bot. Rev.* **47**, 195 (1981).
[130] R. M. Veluci, D. Neher, R. Weicht. *Microb. Ecol.* **51**, 189 (2006).
[131] G. Z. De Caire, M. S. de Cano, M. C. Z. der Mulé, R. M. Palma, K. Colombo. *J. Appl. Phycol.* **9**, 249 (1997).
[132] X. Peng, M. A. Bruns. *Front. Environ. Sci.* **6**, 156 (2019).
[133] A. E. Pipe. *Rev. Environ. Contam. Toxicol.* **127**, 95 (1992).
[134] M. H. Mian, W. D. P. Stewart. *Plant Soil* **83**, 363 (1985).
[135] M. Debnath, N. C. Mandal, S. Ray. *Indian J. Microbiol.* **52**, 275 (2012).
[136] O. Crouzet, J. Wizniowski, F. Donnadieu, F. Bonnemoy, J. Bohatier, C. Mallet. *Arch. Environ. Contam. Toxicol.* **64**, 23 (2013).
[137] O. Crouzet, L. Consentino, J. P. Petraud, C. Marrauld, J.-P. Aguer, S. Bureau, C. Le Bourvellec, L. Touloumet, A. Berrard. *Front. Microbiol.* **10**, 1319 (2019).
[138] B. G. Johnen, E. A. Drew. *Soil Sci.* **123**, 319 (1977).
[139] D. G. Karpouzas, G. Tsiamis, M. Trevisan, F. Ferrari, C. Malandain, O. Sibourg, F. Martin-Laurent. *Environ. Sci. Pollut. Res.* **23**, 18947 (2016).
[140] C. Fraser, E. J. Alm, M. F. Polz, B. G. Spratt, W. P. Hanage. *Science* **323**, 741 (2009).
[141] A. E. Taylor, K. Taylor, B. Tennigkeit, M. Palatinszky, M. Stieglmeier, D. D. Myrold, C. Schleper, M. Wagner, P. J. Bottomley. *Appl. Environ. Microbiol.* **81**, 1942 (2015).
[142] E. S. Papadopoulou, E. Bachtsevani, E. Lampronikou, E. Adamou, A. Katsaouni, S. Vasileiadis, C. Thion, U. Menkissoglou-Spiroudi, G. W. Nicol, D. G. Karpouzas. *Front. Microbiol.* **11**, 2795 (2020).
[143] C. L. Wright, A. Schatteman, A. T. Crombie, J. C. Murrell, L. E. Lehtovirta-Morley. *Appl. Environ. Microbiol.* **86**, e02388 (2020).e02319.
[144] J. Zhao, M. O. Bello, Y. Meng, J. I. Prosser, C. Gubry-Rangin. *Soil Biol. Biochem.* **141**, 107673 (2020).
[145] J. Kaur-Bhambra, D. L. R. Wardak, J. I. Prosser, C. Gubry-Rangin. *Biol. Fertil. Soils* **58**, 241 (2022).249.
[146] L. E. Lehtovirta-Morley, D. T. Verhamme, G. W. Nicol, J. I. Prosser. *Soil Biol. Biochem.* **62**, 129 (2013).
[147] L. E. Lehtovirta-Morley, C. Ge, J. Ross, H. Yao, G. W. Nicol, J. I. Prosser. *FEMS Microbiol. Ecol.* **89**, 542 (2014).
[148] L. E. Lehtovirta-Morley, J. Ross, L. Hink, E. B. Weber, C. Gubry-Rangin, C. Thion, C., J. I. Prosser, G. W. Nicol. *FEMS Microbiol. Ecol.* **92**, fiw057 (2016).
[149] W. Xia, C. Zhang, X. Zeng, Y. Feng, J. Weng, X. Lin, J. Zhu, Z. Xiong, J. Xu, Z. Cai, Z. Jia. *ISME J.* **5**, 1226 (2011).
[150] L. Philippot, A. Spor, C. Henault, D. Bru, F. Bizouard, C. M. Jones, A. Sarr, P.-A. Maron. *ISME J.* **7**, 1609 (2013).
[151] Y. Yan, E. E. Kuramae, P. G. L. Klinkhamer, J. A. van Veen. *Appl. Environ. Microbiol.* **81**, 4246 (2015).
[152] C. Frederiks, J. H. H. Wesseler. *Pest Manag. Sci.* **75**, 87 (2018).
[153] M. C. Vekemans, P. A. Marchand. *Environ. Sci. Pollut. Res.* **27**, 39879 (2020).
[154] A. Balog, T. Hartel, H. D. Loxdale, K. Wilson. *Pest Manag. Sci.* **73**, 2203 (2017).

[155] OECD. Guidance for Registration for Pheromones and Other Semiochemicals used for arthropod pest control. Series on Pesticides No.12 (2002).
[156] D. Baulcombe. *Nature* **431**, 356 (20042004).
[157] OECD. Considerations for the Environmental Risk Assessment of the application of sprayed or externally applied ds-RNA-based pesticide. Series on Pesticides No. 104 (2020).
[158] M. Gopal, A. Gupta, V. Arunachalam, S. P. Magu. *Bioresour. Technol.* **98**, 3154 (2007).
[159] Z. Yu, Y. Zhang, W. Luo, Y. Wang. *Biol. Fertil. Soils* **51**, 343 (2015).
[160] M. Krober, D. Wibberg, R. Grosch, F. Eikmeyer, B. Verwaaijen, S. P. Chowdhury, A. Hartmann, A. Puhler, A. Schluter. *Front. Microbiol.* **5**, 252 (2014).
[161] J. Mayerhofer, S. Eckard, M. Hartmann, G. Grabenweger, F. Widmer, A. Leuchtmann, J. Enkerli. *FEMS Microbiol. Ecol.* **93**, fix117 (2017).
[162] J. Zimmermann, M. K. Musyoki, G. Cadisch, F. Rasche. *Fungal Ecol* **23**, 1 (2016).
[163] European Commission. Guidance on the risk assessment of metabolites produced by microorganisms used as plant protection active substances. SANCO/2020/12258, pp. 1–42 (2020).
[164] European Commission. Guidance on the approval and low-risk criteria linked to “antimicrobial resistance” applicable to microorganisms used for plant protection in accordance with regulation (EC) No 1107/2009. SANTE/2020/12260, pp. 1–14 (2020).
[165] M. Wang, A. Weiberg, F. M. Lin, B. P. Thomma, H. D. Huang, H. Jin. *Nat. Plants* **2**, 16151 (2016).
[166] A. Dalakouras, W. Jarausch, G. Buchholz, A. Bassler, M. Braun, T. Manthey, G. Krczal, M. Wassenegger. *Front. Plant Sci.* **9**, 1253 (2018).
[167] K. M. Parker, V. Barragán Borrero, D. M. Van Leeuwen, M. A. Lever, B. Mateescu, M. Sander. *Environ. Sci. Technol.* **53**, 3027 (2019).
[168] J. Romeis, E. Widmer. *Front. Plant Sci.* **11**, 679 (2020).
[169] A. Dietz-Pfeilstetter, M. Mendelsohn, A. Gathmann, D. Klinkenbuß. *Front. Plant Sci.* **12**, 682387 (2021).
[170] A. M. W. Cooper, K. Silver, J. Zhang, Y. Park, K. Y. Zhu. *Pest Manag. Sci.* **75**, 18 (2019).
[171] R. S. Kookana, A. B. A. Boxall, R. T. Reeves, R. Ashauer, S. Beulke, Q. Chaudhry, G. Cornelis, T. F. Fernandes, J. Gan, M. Kah, I. Lynch, J. Ranville, C. Sinclair, D. Spurgeon, K. Teide, P. J. Van den Brink. *J. Agric. Food Chem.* **62**, 4227 (2014).
[172] L. Li, Z. Xu, M. Kah, D. Lin, J. Filser. *Environ. Sci. Technol.* **53**, 7923 (2019).
[173] M. Kah, R. S. Kookana, A. Gogos, T. D. Bucheli. *Nat. Biotechnol.* **13**, 677 (2018).
[174] C. R. Maruyama, M. Guilger, M. Pascoli, N. Bileshy-José, P. C. Abhilash, L. F. Fraceto, R. de Lima. *Sci. Rep.* **6**, 1 (2016).
[175] E. Swart, T. Goodall, P. Kille, D. J. Spurgeon, C. Svendsen. *Environ. Pollut.* **267**, 1156233 (2020).
[176] S. Peixoto, I. Henriques, S. Loureiro. *Environ. Pollut.* **269**, 116113 (2021).
[177] X. Zheng, J. Wang, Y. Chen, Y. Wei. *J. Hazard Mater.* **344**, 291 (2018).
[178] W. M. A. Sillen, S. Thijs, G. R. Abbamondi, R. R. De La Torre, N. Weyens, J. C. White, J. Vangrosveld. *Microbiome* **8**, 127 (2020).
[179] M. Simonin, B. P. Colman, W. Tang, J. D. Judy, S. M. Anderson, C. M. Bergemann, J. D. Rocca, J. M. Unrine, N. Cassar, E. S. Bernhardt. *Front. Microbiol.* **9**, 1769 (2018).
[180] C. Xu, C. Peng, L. Sun, S. Zhang, H. Huang, Y. Chen, J. Shi. *Soil Biol. Biochem.* **86**, 24 (2015).
[181] A. Coors, T. Frische. *Environ. Sci. Eur.* **23**, 22 (2011).
[182] N. H. Nowell, J. E. Norman, P. W. Moran, J. D. Martin, W. W. Stone. *Sci. Total Environ.* **476–477**, 144 (2014).
[183] J. B. Belden, R. J. Gilliom, M. J. Lydy. *Integrated Environ. Assess. Manag.* **3**, 364 (2007).
[184] C. J. Sinclair, A. B. A. Boxall. *Environ. Sci. Technol.* **37**, 4617 (2003).
[185] B. I. Escher, K. Fenner. *Environ. Sci. Technol.* **45**, 3835 (2011).
[186] V. Storck, L. Lucini, L. Mamy, F. Ferrari, E. S. Papadopoulou, S. Nikolaki, P. A. Karas, R. Servien, D. G. Karpouzas, M. Trevisan, P. Benoit, F. Martin-Laurent. *Environ. Pollut.* **208**, 537 (2016).
[187] C. Ji, Q. Song, Y. Che, Z. Zhou, P. Wang, J. Liu, Z. Sun, M. Zhao. *Environ. Int.* **137**, 105490 (2020).
[188] K. Fenner, S. Canonica, L. P. Wackett, M. Elsner. *Science* **341**, 752 (2013).
[189] V. Storck, D. G. Karpouzas, F. Martin-Laurent. *Sci. Total Environ.* **575**, 1027 (2017).
[190] X. Wu, Y. Yin, S. Wang, Y. Yu. *Environ. Sci. Pollut. Res.* **21**, 3452 (2014).
[191] Y. Teng, M. Zhang, G. Yang, J. Wang, P. Christie, Y. Luo. *Environ. Sci. Pollut. Res.* **24**, 3562 (2017).
[192] J. Wicker, T. Lorsbach, M. Gutlein, E. Schmid, D. Latino, S. Kramer, K. Fenner. *Nucl. Acids Res.* **44**, D502 (2016).
[193] D. A. R. S. Latino, J. Wicker, M. Gutlein, E. Schmid, S. Kramer, K. Fenner. *Environ. Sci. Proces. Implem.* **19**, 449 (2017).
[194] L. V. Alteio, J. Seneca, A. Canarini, R. Angel, J. Jansa, K. Guseva, C. Kaiser, A. Richter, H. Schmidt. *Soil Biol. Biochem.* **160**, 108357 (2021).
[195] S. Bickel, D. Or. *Nat. Commun.* **11**, 116 (2020).
[196] A. Scholer, S. Jacquiod, G. Vestergaard, S. Schulz, M. Schloter. *Biol. Fertil. Soils* **53**, 485 (2017).
[197] C. Lin, H. Xu, W.S. QinXu, X. Tang, L. Kuang, X. Wang, B. Jiang, J. Chen, J. Shan, J. Adams, H. Qin, B. Wang. *Front. Microbiol.* **11**, 560942 (2020).
[198] M. Tsiknia, V. Skiada, I. Ipsilantis, S. Vasileiadis, N. Kavroulakis, S. Genitsaris, K. Papadopoulou, M. Hart, J. Klironomos, D. G. Karpouzas, C. Ehaliotis. *FEMS Microbiol. Ecol.* **97**, fiab10 (2021).

[199] P. Carini, M. Delgado-Baquerizo, E.-L. S. Hinckley, H. Holland-Moritz, T. E. Brewer, G. Rue, C. Vanderburgh, D. McKnight, N. Fierer. *mBio* **11**, e02776 (2020).19.
[200] P. Carini, P. J. Marsden, J. W. Leff, E. E. Morgan, M. S. Strickland, N. Fierer. *Nat. Microbiol.* **2**, 16242 (2017).
[201] S. Vieira, J. Sikorski, S. Dietz, K. Herz, M. Schrumpf, H. Bruelheide, D. Scheel, M. W. Friedrich, J. Overmann. *ISME J.* **14**, 463 (2019).
[202] L. Joos, S. Beirinckx, A. Haegeman, J. Debode, B. Vandercasteele, S. Baeyen, S. Goormachtig, L. Clement, C. De Tender. *BMC Genom.* **21**, 733 (2020).
[203] J. A. Gilbert, J. K. Jansson, R. Knight. *BMC Biol.* **12**, 69 (2014).
[204] A. E. Parada, D. M. Needham, J. A. Fuhrman. *Environ. Microbiol.* **18**, 1403 (2016).
[205] N. A. Bokulich, D. A. Mills. *Appl. Environ. Microbiol.* **79**, 2519 (2013).
[206] L. A. Amaral-Zettler, E. A. McCliment, H. W. Ducklow, S. M. Huse. *PLoS One* **4**, e6372 (2009).
[207] P. Nannipieri, C. R. Penton, W. Purahong, M. Schloter, J. D. van Elsas. *Biol. Fertil. Soils* **55**, 765 (2019).
[208] Y.-X. Liu, Y. Qin, T. Chen, M. Lu, X. Qian, X. Guo, Y. Bai. *Prot. Cell* **12**, 315 (2021).
[209] A. Scholer, S. Jacquiod, G. Vestegaard, S. Schulz, M. Schloter. *Biol. Fertil. Soils* **53**, 485 (2017).
[210] E. Bolyen, J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Galich, H. Alexander, E. J. Alm, M. Arumugam, F. Asnicar, Y. Bai, J. E. Bisanz, K. Bittinger, A. Brenjrod, C. J. Brislawn, C. T. Brown, B. J. Callahan, A. M. Caraballo-Rodriguez, J. Chase, E. K. Cope, R. Da Silva, C. Diener, P. C. Dorrestein, G. M. Douglas, D. M. Durall, C. Duvallet, C. F. Edwardson, M. Ernst, M. Estaki, J. Fouquier, J. M. Gauglitz, S. M. Gibbons D. L. Gibson, A. Gonzalez, K. Gorlick, J. Guo, B. Hillmann, S. Holmes, H. Holste, C. Huttenhower, G. A. Huttley, S. Janssen, A. K. Jarmusch, L. Jiang, B. D. Kaehler, K. B. Kang, C. R. Keefe, P. Keim, S. T. Kelley, D. Knights, I. Koester, T. Kosciolek, J. Kreps, M. G. I. Langille, J. Lee, R. Ley, Y.-X. Liu, E. Loftfield, C. Lozupone, M. Maher, C. Marotz, B. D. Martin, D. McDonald, L. J. McIver, A. V. Melnik, J. L. Metcalf, S. C. Morgan, J. M. Morton, A. T. Naimey, A. J. Navas-Molina, L. F. Nothias, S. B. Orchanian, T. Pearson, S. L. Peoples, D. Petras, M. L. Preuss, E. Pruesse, L. B. Rasmussen, A. Rivers, M. S. Roberson II, P. Rosenthal, N. Segata, M. Schaffer, A. Shiffer, R. Sinha, S. J. Song, J. R. Spear, A. D. Swafford, L. R. Thompson, P. J. Torres, P. Trinh, A. Tripanti, P. J. Turnbaugh, S. Ul-Hasan, J. J. J. van der Hooft, F. Vargas, Y. Vázquez-Baeza, E. Vogtmann, M. von Hippel, W. Walters, Y. Wan, M. Wang, J. Warren, K. C. Weber, C. H. D. Williamson, A. D. Willis, Z. Z. Xu, J. R. Zaneveld, Y. Zhang, Q. Zhu, R. Knight, J. G. Caporaso. *Nat. Biotechnol.* **37**, 852 (2019).
[211] B. J. Callahan, P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. Johnson, S. P. Holmes. *Nat. Methods* **13**, 581 (2016).
[212] R. C. Edgar. *Bioinformatics* **26**, 2460 (2010).
[213] A. Scholer, S. Jacquiod, G. Vestegaard, S. Schulz, M. Schloter. *Biol. Fertil. Soils* **53**, 485 (2017).
[214] L. V. Alteiro, J. Seneca, A. Canarini, R. Angel, J. Jansa, K. Guseva, C. Kaiser, A. Richter, H. Schmidt. *Soil Biol. Biochem.* **160**, 108357 (2021).
[215] N. Bokulich, S. Subramanian, J. J. Faith, D. Gevers, J. I. Gordon, R. Knight, D. A. Mills, J. G. Caporaso. *Nat. Methods* **10**, 57 (2013).
[216] L. Tedersoo, M. Bahram, L. Zinger, R. H. Nilsson, P. G. Kennedy, T. Yang, S. Aslan, V. Mikryukov. *Mol. Ecol.* **31**, 2769 (2022).
[217] Z. Kolarikova, R. Slavikova, C. Kruger, M. Kruger, P. Kohout. *New Phytol.* **231**, 490 (2021).
[218] S. Fang, H. Nan, D. Lv, X. You, J. Chen, C. Li, J. Zhang. *Chemosphere* **267**, 129248 (2021).
[219] J. D. Rocca, M. Simonin, J. R. Blaszczak, J. G. Ernakovich, S. M. Gibbons, F. S. Midani, A. D. Washburne. *Front. Microbiol.* **9**, 3272 (2019).
[220] C. Wagg, S. F. Bender, F. Widmer, M. G. A. van der Heijden. *Proc. Nat. Acad. Sci. USA* **111**, 5266 (2014).
[221] T. C. M. Brock, G. H. P. Arts, L. Maltby, P. J. Van den Brink. *Integr. Environ. Assess. Manag.* **2**, e20 (2006).
[222] G. K. Frampton, S. Jansch, J. J. Scott-Fordsmand, J. Roembke, P. J. van den Brink. *Environ. Toxicol. Chem.* **25**, 2480 (2005).
[223] P. J. Van den Brink, C. J. F. Ter Braak. *Aquat. Ecol.* **32**, 163 (1998).
[224] S. Belanger, M. Barron, P. Craig, S. Dyer, M. Galay-Burgos, M. Hamer, S. Marshall, L. Posthuma, S. Raimondo, P. Whitehouse. *Integr. Environ. Assess. Manag.* **13**, 664 (2017).
[225] P. J. Van den Brink, P. J. den Besten, A. de Vaate, C. J. F. ter Braak. *Environ. Monit. Assess.* **152**, 271 (2009).
[226] N. S. Webster, M. Wagner, A. P. Negri. *Environ. Microbiol.* **20**, 1925 (2018).
[227] M. T. Mee, J. J. Collins, G. M. Church, H. H. Wang. *Proc. Nat. Acad. Sci. USA* **111**, E2149 (2014).
[228] M. Loreau, P. Naeem, P. Inchausti, J. Bengtsson, J. P. Grime, A. Hector, D. U. Hooper, M. A. Huston, D. Rafaelli, B. Schmid, D. Tilman, D. A. Wardle. *Science* **294**, 804 (2001).
[229] V. M. Savage, C. T. Webb, J. Norberg. *J. Theor. Biol.* **247**, 213 (2007).
[230] H. Koch, S. Lücker, M. Albertsen, K. Kitzinger, C. Herold, E. Spieck, E., P. H. Nielsen, M. Wagner, H. Daims. *Proc. Nat. Acad. Sci. USA* **112**, 11371 (2015).
[231] M. P. Thakur, S. Geisen. *Trends Microbiol.* **27**, 771 (2019).
[232] E. Glücksman, T. Bell, R. I. Griffiths, D. Bass. *Environ. Microbiol.* **12**, 3105 (2010).
[233] M. Saleem, I. Fetzer, H. Harms, A. Chatzinotas. *ISME J.* **7**, 1912 (2013).
[234] B. Karimi, J.-Y. Cahurel, L. Gontier, L. Charlier, M. Chovelon, H. Mahe, L. Ranjard. *Environ. Chem. Lett.* **18**, 1947 (2017).
[235] S. Che, Y. Men. *J. Ind. Microbiol. Biotechnol.* **46**, 1343 (2019).
[236] B. D. Karkaria, A. J. H. Fedorec, C. P. Barnes. *Nat. Commun.* **12**, 672 (2021).

[237] M. E. R. Christiaens, J. De Paepe, C. Ilgrande, J. De Vrieze, J. Barys, P. Teirlink, K. Meerbergen, B. Lievens, N. Boon, P. Clauwaert, S. E. Vlaeminck. *Syst. Appl. Microbiol.* **42**, 126021 (2019).
[238] G. Berg, D. Rybakova, M. Grube, M. Köberl. *J. Exp. Bot.* **67**, 995 (2016).
[239] E. V. S. Motta, K. Raymann, N. A. Moran. *Proc. Nat. Acad. Sci. USA* **115**, 10305 (2018).
[240] R. N. Schaeffer, R. L. Vannette, C. Brittain, N. M. Williams, T. Fukami. *Environ. Microbiol. Rep.* **9**, 79 (2017).
[241] A. R. Ottesen, S. Gorham, J. B. Pettengill, S. Rideout, P. Evans, E. Brown. *J. Sci. Food Agric.* **95**, 1116 (2015).
[242] M. Perazzolli, L. Antonielli, M. Storari, M. G. Puopolo, M. Pancher, O. Giovannini, M. Pindo, I. Pertot. *Appl. Environ. Microbiol.* **80**, 3585 (2014).
[243] D. Zhu, J. Ma, G. Li, M. C. Rillig, Y.-G. Zhu. *ISME J.* **16**, 521 (2022).
[244] G.-H. Wang, B. M. Berdy, O. Velasquez, N. Jovanovic, S. Alkhalifa, K. P. C. Minbiole, R. M. Brucker. *Cell Host Microbe* **27**, 1 (2020).
[245] D. Cheng, Z. Guo, M. Riegler, Z. Xi, G. Liang, Y. Xu. *Microbiome* **5**, 13 (2017).
[246] H. Itoh, T. Hori, Y. Sato, A. Nagayama, K. Tago, M. Hayatsu, Y. Kikuchi. *ISME J.* **12**, 909 (2018).
[247] I. Zarraonaindia, S. M. Owens, P. Weisenhorn, K. West, J. Hampton-Marcell, S. Lax, N. A. Bokulich, D. A. Mills, G. Martin, S. Taghavi, D. van der Lelie, J.A. Gilbert. *mBio* **6**, e02527 (2015).14.
[248] L. Prchal, R. Podlipna, J. Lamka, T. Dedkova, L. Skalova, I. Vokral, L. Lecova, T. Vanek, B. Szotakova. *Environ. Sci. Pollut. Res.* **23**, 13015 (2016).
[249] M. Pan, L. M. Chu. *Environ. Pollut.* **231**, 829 (2017).
[250] B. Kurenbach, D. Marjoshi, C. F. Amábile-Cuevas, G. C. Ferguson, W. Godsoe, P. Gibson, J. A. Heinemann. *mBio* **6**, e00009 (2015).
[251] H. Liao, X. Li, Q. Yang, Y. Bai, P. Cui, C. Wen, C. Liu, Z. Chen, J. Tang, J. Che, Z. Yu, S. Geisen, S. Zhou, V.-P. Friman, Y.-G. Zhu. *Mol. Biol. Evol.* **38**, 2337 (2021).
[252] S. Fuller, A. Gautam. *Environ. Sci. Technol.* **50**, 5774 (2016).
[253] Y. Huang, Y. Zhao, J. Wang, M. Zhang, W. Jia, X. Qin. *Environ. Pollut.* **254**, 112983 (2019).
[254] D. Lin, G. Yang, P. Dou, S. Qian, L. Zhao, Y. Yang, N. Fanin. *Proc. R. Soc. B* **287**, 20201268 (2020).
[255] T. Wang, C. Yu, Q. Chu, F. Wang, T. Lan, J. Wang. *Chemosphere* **244**, 125491 (2020).
[256] U. Sunta, F. Prosenc, P. Trebse, T. G. Bulc, M. B. Kralj. *Chemosphere* **261**, 127762 (2020).
[257] R. J. Wright, M. G. I. Langille, T. R. Walker. *ISME J.* **15**, 789 (2021).
[258] G. Zhu, R. Du, D. Du, R. Qian, M. Ye. *Environ. Pollut.* **283**, 117095 (2021).
[259] C. Wu, Z. Wang, Y. Ma, J. Luo, X. Gao, J. Ning, X. Mei, D. She. *J. Haz. Mat.* **405**, 124275 (2021).