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Agricultural soils, pesticides and microbial diversity

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Pesticide effects on microbial community structure and activity in soil are reviewed, showing that methodological developments within the past few years have generated new possibilities for assessing pesticide effects. The first example is the use of mRNA quantification showing that nitrification processes are indeed very susceptible to some pesticides, and that there is correlation between the mRNA transcript quantity and the nitrification rate. The second example is devoted to pesticides influencing microbial community structures. The emergence of high throughput sequencing techniques now allows a more detailed analysis of which bacterial species are influenced.

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Introduction

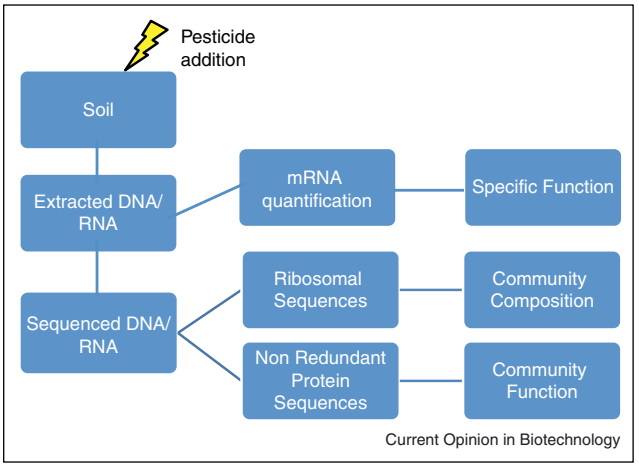
Soil microbes are responsible for many ecosystem services, such as litter degradation — reviewed in Schneider *et al.* [1], the promotion of plant growth as reviewed in Hayat *et al.* [2], nutrient cycling [3] and the degradation of pollutants and pesticides [4,5]. All of these functions are of great importance to both the farmer and society and therefore, it is of great importance to establish if any of these soil ecosystem services are hampered by the addition of pesticides (Fig. 1).

The importance of knowing the bioavailability of the pesticides tested in studies of pesticide effects on soil microbial diversity and function cannot be overly stressed. For unknown reasons, effect studies and biodegradation study are often not linked — even in the literature from the last four years, as we have done in this review. The understanding of the changes in function and diversity are indeed very dependent on the bioavailability of the compounds under study. Thus, in a recent study by Feld *et al.* (2013, unpublished data), the effect of a fumigant was immediate and after less than four days, the fumigant had evaporated. Thus, after this period, the compound ceased to exist and the microbial communities could start to recover. If in this case no knowledge had been available on the disappearance of the compound from the soil systems, it would have been difficult to explain the dynamic of the system that had been found. This is, however, highly dependent on which compound is being studied, and [6] applied copper to soil in different concentrations and after five years, most of the metal was still present in the soil. Albeit only a small fraction was bioavailable (as measured as the $CaCl_2$ extractable fraction), the presence of copper leads to increased abundance of Firmicutes [6]. Since a large variation of sorption and degradation has been found for the same pesticide between different soils [7], it is recommended to always perform fate studies on the same systems as the side effect studies are being carried out. In studies comparing the effects of ten very different pesticides on soil microbial functional diversity and enzyme activity, the experiments were carried out over a period of 12 months, likely allowing for huge differences of the pesticide bioavailability — either due to different sorption or degradation in the soil [8•]. Thus, the changes seen during the 12-month incubation might not reflect an universal found effect.

Pesticide effects on microbial activity in soil

The current legislation system for pesticides only demands a few tests in relation to soil microbiology, that is, carbon utilization and nitrification. In general, no effect is seen from the carbon utilization test, and the nitrification test is the only test that sometimes shows in the official pesticide legislative application that a pesticide will harm microbial driven soil processes. The success of the nitrification test as a pesticide side effect test is likely due to the fact that no soil fungi are known to be involved in the nitrification process — and that only few species of bacteria are able to perform the process. The involved species from both archaeal and bacterial domains are relatively well studied [9•] and recently, the expressed functional genes (amoA and amoB) involved in the nitrification process have been used to quantify the effect of pesticides on nitrification (unpublished data Feld *et al.*). The authors found a strong influence of the fumigant Basamid (which transforms into an isothiocyante-like

Figure 1



Conceptual workflow of future studies of pesticide effects on soil bacteria using next generation sequencing and qPCR on specific functional genes.

compound) on the bacterial ammonium-oxidizer activity, while the fungicide Tridex did not cause any significant effect on the amoA mRNA level (unpublished data Feld *et al.*).

One other microbial-driven ecosystem function that has been found to be affected by pesticide use is the degradation of pesticides by increasing the pesticide degrading populations [10,11]. Bælum, *et al.* [10] found using quantitative measurements of soil DNA and RNA, that repeated application of the soil herbicide MCPA resulted in an increased population of microorganisms that could degrade the compound. The same observation was found by Lancaster et al. [11] who applied five rounds of the herbicide glyphosate to soil and found that the microbial biomass incorporated the herbicide faster after four rounds of applications.

Soil enzyme activity measurements were found to be more than Biolog EcoPlates sensitive in describing changes in the functional diversity of soil microbial communities following soil treatment with high amounts of ten different pesticides [8•]. They found that phenol oxidase enzyme activity in soil was the best overall measurement for the short term (two months) effects of pesticides while arylamidase and b-glucosidase could be used to evaluate the resilience of the soil microbial communities [8•]. In another study, Biolog Ecolog was applied to effect studies of two preseed herbicides on microbial communities; however, the Biolog was found to give unclear responses [12]. Ecolog studies only test the functionality of the culturable part of the microbial community which might only constitute a small part of the total soil bacteria.

The overall idea of testing pesticide's influence on functional diversity has been questioned by the 'Everything is everywhere' hypothesis [13], indicating that changes to microbial communities are of minor influence, since fast adaptation in the microbial communities can be expected. In a study where the mineralization of three different kinds of litter was investigated in three different soils previously influenced by these three types of litter, they found that — despite the immediate low level of difference between the soils — litter from Rhodondendrum mineralized the fastest in the soil that usually received this type of litter and vice versa [14]. This finding is interesting since it might implicate that the bacterial community structure in the soil may have implications for the soil's service function in the ecosystem.

Pesticide effects on bacterial diversity

The soil ecosystem is a complex matrix typically inhabited by billions of bacteria, ten thousand protozoa, an intricate web of fungal hyphae and numerous other organisms including plants, nematodes and microarthropods [15]. The interactions within and between these groups make it very difficult to establish the direct and/or indirect effects of pesticide additions on the microbial community composition [16•]. In addition, the majority of microbial species living in soil have yet to be studied [17] and we often have little to no idea of their role and function in the soil's ecosystem [18]. In a few cases, the links between phylogeny and function are well established, with the Archaea and Bacteria's capability of ammonia-oxidation being a good example [19]. An increased assignment of the functional roles to specific bacterial taxonomic groups will greatly enhance the evaluation potential in diversity changes seen in pesticide-treated soil.

The influence of microbial diversity on soil functions is largely unclear. Some have argued that a relative decrease in species richness has little effect on soil functions [20] because of the huge number of species, and transient functional redundancy present in the soil ecosystem. Experimental studies have shown this to be true for soil functions such as carbon mineralization, denitrification and nitrification [21]. The influence of number of species on the mineralization of simple carbon sources was reviewed in [22], suggesting a limited influence of species richness since many studies show a functional saturation when adding more than ten species to the system. Most of these studies were done with culturable bacteria in a lab environment which is probably a poor representative of the soil ecosystem, so these studies are suggestive at best. In contrast, other functions such as resistance to invasion by pathogenic bacteria have been shown to decline with decreasing species richness [23•]. This could be of great importance since pathogenic bacteria from manure can contaminate crops, ground and surface water [24]. In addition, a positive effect of bacterial diversity was found

on ecosystem multi-functionality [25] and degradation of more specific compounds [26].

The effect of pesticides on microbial diversity is mainly affected by the type of pesticide used (Table 1). In general, the strongest effects are seen from the soil fumigants [27]. Analysis of the phospholipid fatty acid (PLFA) profiles in soils fumigated by the active ingredient methyl isothiocyanate showed an increase in the Gram positive bacteria [27] and a decrease in the Gram negative bacteria and fungi [28]. A recent study using a similar fumigant and 454 16S amplicon sequencing showed a marked increase in the relative abundance of Bacillus and Burkholderia species (Hjelmsoe *et al*., 2013, unpublished data). Common to all the studies were that the observed shifts in community structures were quite prolonged and lasted between one and three months, depending on the experimental setup [28].

Other pesticide types not targeting soil bacteria have also been shown to affect the soil's bacterial diversity: for example, for herbicides, the reduction of growth-promoting bacteria in rhizosphere by glyphosate [29] and the increase of Gram negative bacteria following treatment with naproamide [30]. Effects have also been seen for insecticides [31,32] and fungicides [33]. Specific pesticide effects are reviewed in [34,35]. Often though, the results are difficult to compare because of differences in experimental setups, dose concentrations and methods. The creation of a new standard for bacterial health ensuring the complete protection/evaluation of bacterial services would be a great help for future pesticide effect studies.

Current methodology in pesticide effect research

The huge diversity of bacteria in the soil ecosystem and the limited knowledge of their interactions, make

Table 1

Effects of pesticides on bacterial community composition

Pesticide	Target	Type	Effect	Method	Reference
Diuron or Linuron	Herbicide	Phenyl Urea	Removal of dominant *acidobacterium*	16S rDNA DGGE	El Fantroussi *et al*. (1999)
Glyphosate	Herbicide	Glycine	Increased relative abundance of β-*Proteobacteria* (*Burkholderia*)	16S rDNA clone library	Lancaster *et al*. (2010)
Napropramide	Herbicide	Amide	Initial decrease in bacterial and fungal abundance (day 1) followed by an increase in abundance of Gram negative bacteria and fungi (day 28)	Phospholipid fatty acid (PLFA)	Cycoń *et al*. (2013)
Metam sodium	Soil Fumigant	Dithiocarbamate	Dose dependent shift in community structure (after 5 weeks)	Soil Fatty acid methyl ester (FAME) profiles	Macalady *et al*. (1998)
Methyl Bromide	Soil fumigant	Organobromide	Increased abundance of gram positive bacteria	Phospholipid fatty acid (PLFA)	Ibekwe *et al*. (2001)
Methyl isothiocyanate	Soil fumigant	Organosulfur	Increased abundance of gram positive bacteria	Phospholipid fatty acid (PLFA)	Ibekwe *et al*. (2001)
Metam sodium	Soil Fumigant	Dithiocarbamate	Inhibitory effect on gram negative bacteria and fungi in both field and laboratory studies	Phospholipid fatty acid (PLFA)	Spyrou *et al*. (2009)
Cobber	Fungicide	Metal	Bioavailable Cu positively correlated with relative abundances of phylums *Acidobacteria* and negatively correlated with the phylums *Proteobacteria* and *Bacteroidetes*	Pyrosequencing of 16S rDNA amplicons	Berg *et al*. (2012)
Cobber	Fungicide	Metal	Decrease in abundance of acidobacteria and increase of Firmicutes. Bacillus community highly resistant to high cobber concentrations.	Denaturating gradient gel electrophoresis (DGGE) and 16S clone library	Wakelin *et al*. (2010)
cypermethrin	Insecticide	Synthetic pyrethroid	Increase in Gram-negative bacteria and decrease in firmicutes	PLFA and DGGE	Zhang *et al*. (2009)

Table 2

Effects of pesticides on microbial driven soil functions

Pesticide	Target	Type	Effect	Method	Reference
MCPA	Herbicide	Phenoxy acid	Increased expression of functional genes (tfdA) involved in MCPA degradation	mRNA quantification by RT-Q-PCR	Bælum *et al*. [10]
Dazomet	Soil Fumigant	Methyl isocyanite	Shut down of expression of functional genes (amoA) involved in nitrification	mRNA quantification by RT-Q-PCR	Feld *et al*. (unpublished data)
2.4-D	Herbicide	Phenoxy acid	Best general activity across the tested enzyme assays was a decreased phenol oxidase activity following pesticides application	Enzyme assays and Biolog Ecoplates	Floch *et al*. [8•]
Carbaryl	Herbicide	Carbamate			
Mancozeb	Fungicide	Carbamate			
Glyphosate	Herbicide	Organophosphate			
Parathionmethyl	Insecticide	Organophosphate			
Atrazin	Herbicide	Triazine			
Prometryne	Herbicide	Triazine			
Diuron	Herbicide	Urea			
Linuron	Herbicide	Urea			
2,4-D and Glyphosate	Herbicide Herbicide	Phenoxy acid Organophosphate	Combined addition decreased the functional diversity	Biolog Ecoplates	Lupwayi *et al*. [12]

evaluating pesticide effects quite challenging. Many of the studies done on pesticide effects on bacterial diversity use culture dependent methods such as Biolog EcoPlate [8•] or classical plate counting [30] (Table 2). However, the culturable part of the bacterial community may be a poor representative of the community inhabiting the soil [36]. Of the culture independent methods, most use the 16S rRNA gene as a molecular marker. These include DGGE [6,31,37,38], T-RFLP [39,40], RAPD [41] and 16S clone libraries [11,42]. While these methods are well established, their ability to describe changes in the bacterial community composition are, in terms of resolution, inferior to modern next generation sequencing (NGS) techniques. In recent years, NGS have been used to characterize the bacterial community composition in everything from ant colonies [43] to polluted soils [44,45]. Especially the possibility of using extracted RNA in NGS analysis, coupling bacterial community structure and function [46••], possibly in combination with auto sampling [47], seems to be a very promising tool to study pesticide effects on soil bacteria.

Conclusion

We anticipate that studies involving mRNA and rRNA directly extracted from agricultural soils and quantified using RT-PCR will be extended to other functional genes of interest in the future. However, the potential of using this quantitative analysis of specific transcripts is limited to those processes that have been thoroughly described on the gene level and validated in soil systems. In addition, new NGS methods could be used to establish key species for maintaining ecosystem services and to accurately determine effects of pesticides on soil bacterial diversity.

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