

Assessing the soil microbial ecotoxicity of pesticides: New risk assessment scheme using bioindicator microbial groups and standardized molecular tools

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Introduction

Soil microorganisms have a pivotal role in ecosystem functioning. Despite that little attention has been given to date on the toxicity of pesticides on soil microorganisms. This is mirrored in the pesticide regulatory framework (EU Regulation 1107/2009) which still relies on a dated and insensitive test (N mineralization OECD 216). This test fails to identify effects on key microbial functions and on the soil microbial diversity. This regulatory gap was identified by EFSA (2010) which named soil microorganisms as a 'specific protection goal' for environmental risk assessment (ERA). **In 2017 EFSA issued an opinion on 'the ERA of pesticides for in-soil organisms' where it stressed the need for novel tests to assess the toxicity of pesticides on soil microorganisms and proposed arbuscular mycorrhizal fungi (AMF) as a potential bioindicator microbial group. However it was still argued that the N mineralization test should be maintained as the core test in risk assessment.**

Is this EFSA Opinion a step forward in the ERA of pesticides?

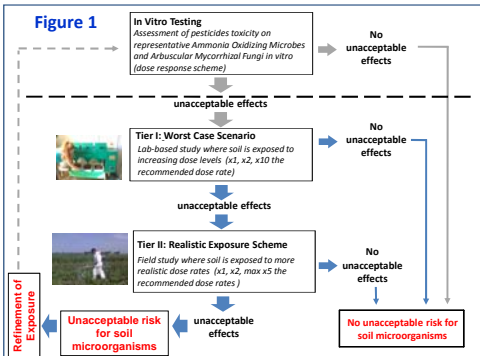
YES, since the regulatory gap is acknowledged and a microbial group (AMF) is proposed as a bioindicator for assessing the soil microbial toxicity of pesticides

But is it enough? What is it still missing?

No several more steps are required for a robust risk assessment

- A well-described **tiered risk assessment scheme**
- Use of **advanced & standardized methods**, available in soil microbial ecology
- Use of alternative bioindicator microbial groups to complement AMF

Tiered – risk assessment scheme



In the frame of two EU-funded projects, the SEE.ERAnet **ECOFUN - MICROBIODIV** and the IAPP-FP7-MSCA **LOVE-TO-HATE** we developed a novel tiered risk assessment scheme for assessing the soil microbial toxicity of pesticides (Fig. 1). This is based on two tiers involving pesticide testing against natural microbial assemblages in microcosm and field scale.

An *in vitro* assessment of the pesticides toxicity on **ammonia-oxidizing microbes (AOM)** and **AMF** could act as an initial conservative tier I. However, certain aspects of *in vitro* tests should be standardized (i.e. selection of "the most sensitive" representative per functional group) before their implementation in pesticide ERA

Implementation of advanced and standardized methods in ERA

Recent methodological advances in soil microbial ecology allowed the in-depth study of the effects of stressors (i.e. pesticides) on the structure and function of the soil microbial community. To date several of these tools are ISO standardized (Table 1) and ready to be implemented in the ERA of pesticides

Year	Method	ISO
2009	Effects of pollutants on mycorrhizal fungi-germination test	ISO10832
2010	Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates	ISO22839
2010	Determination of soil microbial by phospholipid fatty acid analysis (PLFA) - Part 1 and 2	ISO29843-1 ISO29843-2
2012	Method to directly extract DNA from soil samples	ISO11063
2012	Determination of potential nitrification and inhibition of nitrification—rapid test by ammonium oxidation	ISO15685
2013	Estimation of abundance of selected microbial gene sequences by quantitative realtime PCR from DNA directly extracted from soil	ISO17601

Use of complementary microbial indicator groups (i.e. AOM)

Mounting evidence (Puglisi et al., 2012, Feld et al., 2015, Papadopoulou et al., 2016) suggest that **AOM could also serve as bioindicators** since (a) they are sensitive and responsive to stressors, (b) they mediate the rate-limiting step of N cycle and (c) there are ISO standardized tools available to measure their activity and abundance

Conclusions and the way forward.....

- A tiered assessment approach using the advanced standardized methods available will ensure a robust ERA for pesticides
- *In vitro* tests with AMF and AOM should be explored further as a conservative tier I like in all other trophic levels
- Multilevel standardization of amplicon sequencing approaches will be a powerful tool for assessing pesticide effects on soil microbial diversity

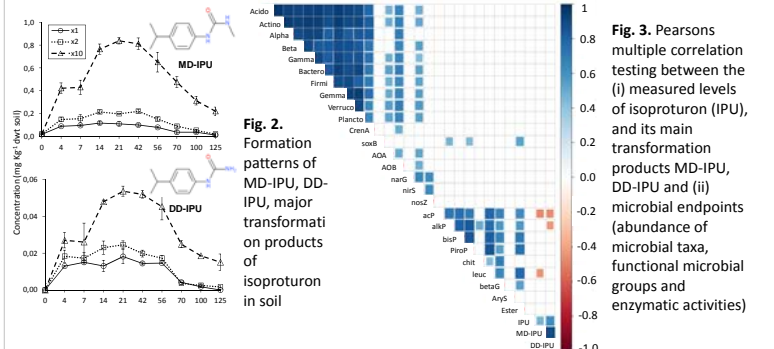
Tips for assessing the soil microbial toxicity of pesticides

Effects of pesticides on soil microbial functions

Parallel determination of pesticide dissipation and metabolism is essential

- Gives a measure of the level and the duration of the exposure of the soil microbial community to the pesticide in question
- Allows the identification of the causal agent of effects observed (parent compound or transformation product?)

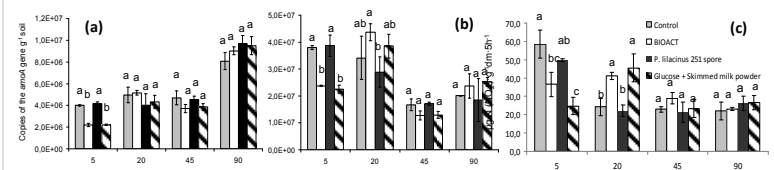
Case Study 1: We studied the dissipation and transformation of isoproturon (Fig. 2), applied at various dose rates, and its impact on different microbial endpoints (Fig. 3)



The reduced activity of acid and alkaline phosphatase and leucine aminopeptidase correlated negatively with MD-IPU & DD-IPU and not with isoproturon (Fig. 3)

The duration of the test should allow ample time for recovery

Case Study 2: In a microcosm study we assessed the toxicity of the biological nematicide BIOACT® containing spores of the fungus *Paecilomyces lilacinus* on the soil microbial community and in particular on Ammonia-oxidizing microbes (AOM)



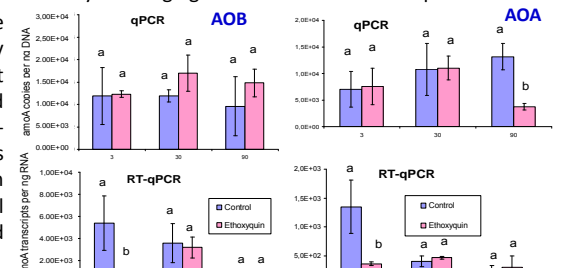
The impact of BIOACT®, *P. lilacinus* spores and Glucose + skimmed milk (co-formulants of BIOACT®) on the abundance of ammonia-oxidizing bacteria (a), ammonia-oxidizing archaea (b) and potential nitrification (c).

Temporal effects by BIOACT® on the function and abundance of AOM were observed at day 5 after application but they were alleviated from day 20 onwards (Fig. 4)

RNA-based methods allows identification of false negatives

- Application of RNA-based methods could be used as a higher-tier approach when conflicting evidence from biochemical and DNA-based assays were obtained
- Working with RNA is technically challenging but is the most accurate proof of effects

Case Study 3: We assessed the toxicity of the antioxidant ethoxyquin, contained in effluents from fruit-packaging plants discharged in soil, on the soil microbial community and particularly on AOM

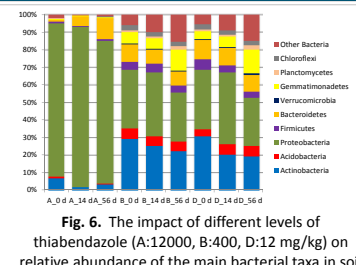


RNA-based methods (RT-q-PCR) identified a transient but significant effect on AOM by ethoxyquin which was not identified by DNA-based methods (q-PCR) (Fig. 5)

How about effects on the soil microbial diversity?

New tools based on amplicon Next-Generation sequencing enable the identification of effects even at rare members of complex microbial communities but at the moment they are not standardized (Fig. 6)

Proteobacteria dominated the soil bacterial community at high levels of thiabendazole while Actino-, Acido-bacteria, Firmicutes and Gemmatimonadetes were eliminated (Fig. 6)



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