ECOFUN-MICROBIODIV: an FP7 European project for developing and evaluating innovative tools for assessing the impact of pesticides toxicity on soil microbial diversity and functions

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ECOFUN-MICROBIODIV: an FP7 European project for developing and evaluating innovative tools for assessing the impact of pesticides toxicity on soil microbial diversity and functions

http://www4.inra.fr/ecofun_microbiodiv

http://fabricemarti8.wix.com/ecofun-microbiodiv

ECOFUN-MICROBIODIV

Development and evaluation of innovative tools to estimate the ecotoxicological impact of low dose pesticide application in agriculture on soil functional microbial biodiversity

The ECOFUN-MICROBIODIV project aimed at developing and evaluating innovative tools to estimate the ecotoxicological impact of low dose pesticides used in agriculture on soil functional microbial biodiversity. It proposed to estimate the impact of sulfonylurea herbicide on soil microbial biodiversity and functioning.

Consortium

This program is coordinated by F. Martin-Laurent (INRA, France).

This program will be done by a scientific consortium constituted of five European laboratories:
- Institute Rudjer Boskovic, Croatia
- INRA, Dijon, France
- University of Hohenheim, Germany
- University of Novi Sad, Serbia
- University of Thessaly, Greece

Pesticide Behaviour in Soils, Water and Air, 2-4 September 2013, York, UK
Despite the pivotal role of microorganisms in ecosystem functioning the assessment of pesticides soil microbial toxicity is lagging behind the recent methodological advances in soil microbiology.
Context: EU level pesticide regulation

- **European Soil Framework Directive** – identifies agricultural practices as a major threat for soil biodiversity

**Estimation of the impact of pesticides on soil microbiota**

- Modified Sturm test: estimation of pesticide biodegradability and of pesticide on the biodegradation of a reference substrate [OECD 301B]
- Carbon mineralization test [OECD 217]
- Nitrogen mineralization test [OECD 216]

These global tests do not provide a comprehensive assessment of pesticides impact on the soil microbial communities and do not reveal consequences on soil ecosystem services

**ECOFUN-MICROBIODIV project aimed at**

Develop and evaluate innovative tools to estimate the impact of low-dose herbicides **sulphonylureas** on the function and population dynamics of broad microbial groups and selected microbial taxa
Scheme: ECOFUN-MICROBIODIV organized in 6 WP

- WP1 Management, coordination and process review
- WP2 Dissemination - Stakeholder Oriented and Policy Implications
- WP3 Set up of field and greenhouse experiments
- WP4 Agronomical monitoring
- WP5 Pesticide monitoring
- WP6 Microbial monitoring
Set-up of the field experiment

- Novi Sad, Serbia, June to October 2011.
- Full randomized block pattern (6m x 5m)
- 3 treatments: x1, x2, x5 of the recommended agronomic dose (80 g a.i. ha\(^{-1}\))
- Control plots
- 4 replicate plots per treatment
- Tier II toxicity assessment: representing realistic exposure scenario

**Test compound – sulfonylurea herbicide Nicosulfuron**

- Used for the post-emergence control of annual grass and broad-leaf weeds in maize
- Application rate 10-1000 times lower than conventional herbicides
- Inhibition of the *actohydroxyacid synthase* (AHAS) \(\rightarrow\) biosynthesis of valine, leucine and isoleucine (branched chain amino acids)
Set-up of the greenhouse experiment

- Dijon, France, under controlled conditions
- 5 repeats per treatment
- 3 treatments: x10, x100, x1000 of the recommended agronomic dose (80 g a.i. ha\(^{-1}\))
- control pots
- Tier I toxicity assessment – under extreme long-term exposure scheme
- 5 culture cycles
WP1 Management, coordination and process review

WP2 Dissemination - Stakeholder Oriented and Policy Implications

WP3 Set up of field and greenhouse experiments

WP4 Agronomical monitoring

WP5 Pesticide monitoring

WP 6 Microbial monitoring
Agronomic monitoring: efficiency of herbicide

- Crop yield measurement and weed development
  (weeds identification using morphological traits and seeds collected to test for nicosulfuron resistance)

Pesticide monitoring: persistence of herbicide in soil

- Developing of the method for monitoring low-dose herbicide in soil
  1) Extraction (accelerated solvent extraction, ASE*)
  2) Sample concentration and cleanup (soild phase extraction, SPE**)
  3) Residue analysis (HPLC*** equipped with a UV diode array detector system; 245 nm)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0.2% Acetic acid in H₂O v/%</th>
<th>Acetonitrile v/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>96</td>
</tr>
</tbody>
</table>

*ASE 200; Dionex, Sunnyvale, CA, USA;
** Strata-X, Strata-NH₂
*** Varian, Walnut Creek, CA, USA
Scheme

WP1 Management, coordination and process review

WP2 Dissemination - Stakeholder Oriented and Policy Implications

WP3 Set up of field and greenhouse experiments

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WP 6 Microbial monitoring
Testing pertinence of the existing standard methods

Develop, test and propose new methods for standardization

- methods aimed at studying abundance (ISO14240:2), diversity (ISO/TS29843-1) and activity (ISO/TS 22939) of the soil microflora

- methods based on direct soil DNA extraction (ISO 11063) and further PCR
  - qPCR analysis assessing dynamics of the most abundant microbial taxa involved in key ecosystem services (NWI 17601*)
  - Arbuscular mycorrhizal fungi as bioindicators – changes in their diversity and community structure

The combined application of different tools will provide a comprehensive assessment of the effects of herbicide on soil microbial communities
### Microbail monitoring - methodology

**Estimating impact of nicosulfuron on:**

1. **Soil microbial diversity**
   - (structure and abundance)
   - **Methodology:**
     - A-RISA, qPCR, PLFA, enzyme activities, arbuscular mycorrhiza fungal colonization, DGGE, sequencing

2. **Soil microbial function**
   - Targeting microbial groups participating in important geochemical cycles
   - **Methodology:**
     - qPCR \((amoA_a, amoA_b)\), PLFA

3. **Herbicide impact on bacterial populations**
   - Harboring AHAS gene
   - **Methodology:**
     - A-RISA, qPCR, pyrosequencing

4. **Estimating adaptation of soil microbes on nicosulfuron**
   - **Methodology:**
     - Isolation of nicosulfuron-resistant and nicosulfuron-degrading populations, RFLP, sequencing
Synthesis of the results

Field experiment: Dissipation

- x1 dose rate
- x2 dose rate
- x5 dose rate

% of initial recovery

Days

Greenhouse: Accumulation

% relative to NS concentration at Cycle 1

x10 dose rate
x100 dose rate
x1000 dose rate

Field exp.
Tier II toxicity
+/- - - - - - - +

Greenhouse exp.
Tier I toxicity
+ + + + + + + + +

- = non significantly affected; + = significantly affected as compared with control

Resistant microbial population

Pesticide Behaviour in Soils, Water and Air, 2-4 September 2013, York, UK
Repeated treatment in greenhouse experiment yielded in a significant decrease of total bacterial community; Gram-negative and Gram-positive bacterial populations.

Impact of nicosulfuron on the structure of soil microbial community by means of PLFA

* p<0.05
Functional impact of nicosulfuron on microbial community by enzyme activity measurements

Measurement of a wide range of enzymes involved in C, N and P cycling covering important ecosystemic services

C cycle
β-glucosidase
β-xylosidase

N cycle
N-acetyl-β-glucosaminidase

P cycle
phosphatase

☑️ all enzymes were negatively affected by repeated exposure to the highest dose rates of nicosulfuron
Impact of nicosulfuron on structure and abundance of bacterial and fungal community by A-RISA and qPCR

### Bacteria community

<table>
<thead>
<tr>
<th></th>
<th>Greenhouse exp.</th>
<th>Field exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>No impact</td>
<td>No impact</td>
</tr>
</tbody>
</table>
| Abundance | Impact (X100, x1000 dose) | Impact (x10 dose)

**B-Proteobacteria, Planctomycetes, Actinobacteria, Firmicutes**

### Fungal community

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<thead>
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<td>Structure</td>
<td>Impact (x100, x1000)</td>
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<td>Abundance</td>
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**β-Proteobacteria, Acidobacteria, α-Proteobacteria, Actinobacteria, Bacteroidetes, Planctomycetes, Verrucomicrobiales, Firmicutes, Gemmatimonadetes, Crenarchaeota**

* p<0.05, ** p<0.01, *** p<0.001
High responsiveness of AM fungi to pollutant exposure and their key role on plant diversity and functioning of above ground ecosystems: ideal bio-indicators for assessing the soil microbial ecotoxicity of pollutants*

Gradual build up of nicosulfuron residues in the greenhouse exp. induced drastic changes in plant growth, establishment of symbiosis and the diversity and community structure of AM fungi

- Nicosulfuron impaired (x100 dose) or entirely halted maize growth (x1000 dose) from cycle 2 onwards - mycorrhizal colonization showing similar response

* Establishment of ISO10832 'Effects of pollutants on mycorrhizal fungi'
DGGE fingerprinting analysis of the intraradical mycorrhizal community in maize roots

- structure of the AM fungal community was clearly affected by nicosulfuron: higher dose - more significant changes
- clear reduction of the community diversity for x100 dose from cycle 2

- maize roots were mostly colonized by different members of Glomus group
- some members showed tolerance or even a positive response to nicosulfuron while others were clearly sensitive to nicosulfuron
Phylogenetic diversity of the NS-resistant populations developed under different exposure schemes

- resistant mutants = harboring AHAS gene non-sensitive to nicosulfuron
- difference in resistant community structure under low and high pressure

Some genera showed more tolerance to higher doses of nicosulfuron:
- **Bacillus** - most abundant population (65% of the isolates)

Appearance of specific nicosulfuron resistant sub-populations:
- **Sphingobium, Burkholderia, Cupriavidus & Sphingobacterium**

**Greenhouse exp.**

- Bacillus & Arthrobacter - most abundant populations
- Specific isolates: **Flexibacteraceaee, Chitinophaga & Rhizobium**

**Field exp.**
The combined application of well-standardized tools of different resolution level can provide a comprehensive assessment of the effects of pesticides on soil microbial functions and population dynamics.

Proposing new method to International Standard Organisation

Standardization = a 6 steps process

Acceptance of the new work item (ISO 17601) ‘Estimation of abundance of selected microbial gene sequences by qPCR from DNA directly extracted from soil’ (ISO TC4/WG4)
- Intermediary meeting of ISO (Berlin, Mar 2012)
- Annual meeting of ISO (Helsinki, Sep 2012)

Organization of the International interaboratory ring test
- first call for ring test published by ISO the 11 December 2012.


Thank you for your attention

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