The toxicity of glyphosate-based herbicides to soil bacteria and freshwater crustaceans

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Glyphosate (N-[phosphonomethyl]glycine) is the most widely used herbicide globally in terms of treated area as well as total amount used (Coupe et al., 2012). It is the active compound of RoundUp and being applied in agriculture as weed control agent. Glyphosate (RoundUp) is also used to treat winter cover crops in order to obtain a better establishment of the subsequent spring crop and to artificially accelerate and synchronize ripening of various crops (Duke and Powles, 2009; Helander et al., 2012; Landbrugsrådgivning Syd, 2012). In addition, global glyphosate market includes applications in non-agricultural areas, such as in home and garden, industrial and railroads (Woodburn, 2000).
Introduction

In Estonia *glyphosate-based formulations* are the most used among all pesticides since 2002 (*284 tonnes of glyphosate-based formulations were sold in 2010*) and their usage shows clear increase (*Estonian Agricultural Board, 2012*).

Currently, there are around *34 different glyphosate-based formulations registered for using in Estonia*, e.g. *Agro-Glyfo, Barclay Barbarian, Dalgis, Dominator, Glyfos, Glyphomax, Jablo, Ranger, Rodeo, Rosate, Taifun B, Roundup Quick, Roundup Gold, Roundup Max, Roundup Bio*, etc.
The use of pesticides in Estonia
The study aimed

(i) to compare the potential harmful effects of different commercial formulations of glyphosate to non-target species;

(ii) to evaluate the influence of glyphosate-based herbicides on the soil bacteria and the whole microbial community in the northern temperate climate zone.

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This research was a part of the European Union Central Baltic INTERREG IVA programme 2007-2013 project: Risk Management and Remediation of Chemical Accidents (RIMA).


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Tested herbitcides:

A - Isopropylamine (IPA) salt of glyphosate (*Sigma-Aldrich*)

B - *Roundup® Quick*, isopropylamine (IPA) salt of glyphosate, 72g/L, (spray; *MONSANTO Europe S.A.*)

C - *Roundup® Max*, ammonium salt of glyphosate - 75%, surfactant -21% (granulated; *MONSANTO Europe S.A.*)

➢ B and C have been the most used glyphosate products in Estonia.
Scheme of the whole experiment

40% (w/v) solution in water (Sigma-Aldrich)

IPA salt of glyphosate

spray: 1% IPA salt of glyphosate, 94% water, 5% other additives (Monsanto)

Roundup Quick™

granulated: 75% ammonium salt of glyphosate, 21% surfactant (POEA), 0.5% sodium sulphite, 3.5% other additives (Monsanto)

Roundup Max™

**Short-term effects**

→ 48-h immobilization assay with *Daphnia magna*

→ 30-s/30-min luminescence inhibition assay with *Vibrio fischeri*

*Photo of V. fischeri colonies is taken in the dark.*

→ 26-h bacterial growth inhibition assay with *Escherichia coli, Pseudomonas putida* and 3 strains isolated from the soil used in the outdoor experiments

**Long-term effects**

*Natural soil samples treated with different concentrations of Roundup formulations were exposed outdoors for 4 months*

Roundup Quick™, recommended field rate, 100- and 300-fold rates)

Roundup Max™, recommended field rate, 100-, 300- and 1000-fold rates

Soils were tested at different time points

Microbial community

Terrestrial plants

Colonies of soil bacteria growing on Plate Count Agar at 24°C 72-h

Seed germination and shoot growth of *Raphanus sativus* and *Hordeum vulgare*
The toxicity of glyphosate and its formulations was tested using:

1) freshwater crustacean *Daphnia magna*
2) marine laboratory test bacterium *Vibrio fischeri*
3) environmental bacterium *Pseudomonas putida* (Gram-)
4) intestinal bacterium *Escherichia coli* (Gram-)
5) three bacterial strains isolated from the field soil:
   • *Bacillus mycoides* (Gram+)
   • *Arthrobacter sp.* (oligotrophic, Gram+)
   • unidentified strain M2 (saprotrophic, Gram-)
The bacterial strains isolated from the field soil

Arthrobacter sp.  Bacillus mycoides  Soil bacterium M2

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The toxicity assays:

• **48 h acute immobilization assay** with *Daphnia magna* (OECD Guideline 202)

• **30-min acute luminescence inhibition assay** with *Vibrio fischeri*

• **24-h growth inhibition tests** based on optical density detection with *Pseudomonas putida* (incubation at 30° C), *Escherichia coli* (30° C), with freshly isolated from field soil bacterial strains (24° C)

• **“spot”-test** – additional **viability endpoint test** for the bacterial growth inhibition assays to clarify the ability of the toxicant-exposed bacteria to form colonies.

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“Spot”-test with glyphosate and *Vibrio fishery*

**METHOD description**

For that, 3-5 µl of the culture mixed with the tested chemical and the control culture was pipetted (‘spotted’) onto nutrient agar and incubated.

The appearance of a **spot** – **growth** was evaluated visually.

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The residual toxicity of treated soils

As plants are target organisms for the herbicides, the residual contamination of the treated soils was evaluated with two common crop species belonging to different plant orders and commonly used in Estonia: horticultural crop red radish (*Raphanus sativus*) and agricultural crop, field-grown barley (*Hordeum vulgare*). According to the producer's information, seeding of vegetables should take place 21 days after application of the glyphosate-based formulation (*Monsato, 2002*).
RESULTS (1)

<table>
<thead>
<tr>
<th></th>
<th>EC50 [mg/L]</th>
<th>EC50 [g/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Growth inhibition test, 24 h</td>
</tr>
<tr>
<td></td>
<td><em>D. magna</em> Immobilization assay, 48 h</td>
<td><em>V. fischeri</em> flash assay, 30 min</td>
</tr>
<tr>
<td>Roundup Max</td>
<td>38,1 ±6,7</td>
<td>7,57 ±0,90</td>
</tr>
<tr>
<td>Roundup Quick</td>
<td>48,9 ±5,5</td>
<td>5,43 ±1,31</td>
</tr>
<tr>
<td>IPA salt</td>
<td>4,2 ±1,8</td>
<td>7,47 ±1,37</td>
</tr>
</tbody>
</table>

Among tested species the aquatic organisms - bacterium *V. fischeri* and crustacean *D. magna* were much more (up to 1000 times) sensitive to glyphosate than the bacteria from bacterial culture collection.

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Bacterial growth inhibition assays

Despite of identical growth conditions the response to the glyphosate-based products of the tested bacteria (E. coli, P. putida, isolated from soil bacteria: B. mycoides, Arthrobacter sp. and strain M2) varied largely.

The indigenous Gram-positive soil microbes (Bacillus mycoides and Arthrobacter sp.) were much more sensitive to glyphosate based herbicides than the Gram-negative bacteria (Pseudomonas putida, E. coli).

RESULTS (2a)

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RESULTS (2b)

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The results of the spot-tests with the soil bacterial strains (saprotrophic strain M2, oligotrophic strain Arthrobacter sp and Bacillus mycoides).

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WHO is *Bacillus mycoides*?

- **B. mycoides isolates (BmJ)** has been used in a foliar spray preparation as a fungicide for disease prevention in plants.
- **B. mycoides** has demonstrated disease control potential when applied to sugar beets, potatoes, tomatoes, peppers, and can control growth of fungal pathogens, *Phythium* in ornamental plants and *Botrytis gray mold* in tomatoes.

*Bacillus mycoides* isolates has been used to control plant diseases, e.g. *Cercospora leaf spot* (*Cercospora beticola* Sacc.) of sugar beet, in both glasshouse and field experiments. Disease control is attributed to the bacterium's ability to induce systemic resistance (*Bargabus et al*, 2002).

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Outdoor long-term soil experiment

In practice, the application rate of glyphosate varies depending on the target species, the growth stage of the weed, the application method as well as the specific formulation used.

The recommended application rates are:

- for Roundup Max™ - 0.75-4.0 kg ha⁻¹
- for Roundup Quick™ - 2.45 kg ha⁻¹

For soils experiments the average treatment rate 2.45 kg ha⁻¹ recommended by the producer to control the weed was taken.

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The experiments were performed in climatic conditions typical for Estonia - characterised by short and cool growing season.

The time period of the outdoor experiment was 108 days, stating from May.

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The impact of Roundup formulations on the soil microbial community (2)

The estimation of growth dynamic of aerobic heterotrophic bacteria (HB) and total bacteria number (TBN) was used for evaluation of the impact of glyphosate-based herbicides on soil microbial community.

The mean number of culturable heterotrophic bacteria (HB) in non-treated field soil (control) varied from $9 \cdot 10^6$ to $20 \cdot 10^6$ CFU g$^{-1}$ dry soil during the four months exposure.

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## RESULTS (4a)

<table>
<thead>
<tr>
<th>Supplied herbicide concentrations</th>
<th>Number of HB (CFU)*</th>
<th>TBN (cells)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^6 g⁻¹ dry soil</td>
<td>10^9 g⁻¹ dry soil</td>
</tr>
<tr>
<td></td>
<td>10 d#</td>
<td>21 d#</td>
</tr>
<tr>
<td>Roundup Max</td>
<td>34</td>
<td>14</td>
</tr>
<tr>
<td>As recommended (245 mg/m²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100x as recommended</td>
<td>40</td>
<td>27</td>
</tr>
<tr>
<td>300x as recommended</td>
<td>38</td>
<td>26</td>
</tr>
<tr>
<td>1000x as recommended</td>
<td>77</td>
<td>21</td>
</tr>
<tr>
<td>Roundup Quick</td>
<td>85</td>
<td>20</td>
</tr>
<tr>
<td>As recommended (245 mg/m²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100x as recommended</td>
<td>953</td>
<td>256</td>
</tr>
<tr>
<td>300x as recommended</td>
<td>361</td>
<td>709</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>20</td>
</tr>
</tbody>
</table>

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RESULTS (4b)

The evaluation of the soil bacterial community diversity by phenotypic characteristics on nutrient agar growing bacterial colonies showed that Roundup Quick™ had stronger selective effect on the composition of natural HB community than Roundup Max™.
Glyphosate \((N\text{-phosphonomethylglycine})\)
Mobility of the glyphosate-based herbicides in soils

The aqueous extracts (1:10) of the soils sampled 10 days after spiking with even the highest dose (1000-fold the recommended) of herbicide were not toxic (compared to the control, the toxic effect did not exceed 10%) to aquatic species, *Daphnia magna* and *Vibrio fischeri* (!?). This shows that mobility of the glyphosate-based herbicides in soils with high content of organic matter is very low.

*This is in agreement with the studies on the mobility of glyphosate in soil showing that the loss rate of glyphosate from agricultural fields is lower than for other herbicides (Laitinen et al., 2006 and Shipitalo et al., 2008).*
The results of laboratory inhibition/immobilization tests:

- The toxicity of the glyphosate-based herbicides to non-target aquatic and terrestrial organisms vary within a wide range.
- Non-target aquatic species were much more sensitive to glyphosate formulations than the model and soil bacteria;
- Different glyphosate formulations (Roundup Quick, Roundup Max™) can have different effects on the soil bacteria. The difference in toxicity may be due to different additives used in the specific formulations.
- The indigenous Gram-positive soil bacteria (B. mycoides and Arthrobacter sp.) were more sensitive to glyphosate based herbicides than the Gram-negative bacteria (P. putida, E. coli).

CONCLUSIONS (1)

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The long-term outdoor experiment demonstrated that

- the community level end points, like **bacterial total number** or the **total colony count** failed to **detect changes** that were found at **lower levels of microbial organization**.
- high concentrations of Roundup Quick™ **increased the heterotrophic bacteria number** in soil, but also **decreased the diversity of indigenous bacterial community**
- the **bioavailable fraction** (toxicity) of glyphosate based herbicides is controlled by **soil properties**, in particular by **organic matter content**
- In **nordic regions** (short and cool vegetation period), the **soil microbial communities** (soil health) **may not recover** in case of regular (intensive) use of glyphosate based herbicides
Cold climate may influence glyphosate degradation in soil and repeated applications could lead to accumulation in soil also after early season applications (Dibyendu et al., 1989; Stenrød et al., 2005; Laitinen et al., 2009).

Australian scientists Philip Mercurio et al (2014) quantified the biodegradation of glyphosate using the native bacterial populations and coastal seawater. The half-life for glyphosate at 25 °C in low-light was 47 days, extending to 267 days in the dark at 25 °C and 315 days in the dark at 31 °C.

This study demonstrates glyphosate is moderately persistent in the marine water under low light conditions and is highly persistent in the dark.


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What can happened?

• the changes in microbial community composition would have been more pronounced if the pesticide additions had been repeated.

• If the microbial populations of the less dominant functional groups become insufficient to effectively perform soil biological processes, those processes will be adversely affected.

• the shifts of microbial community structure in soil can lead to successions that could have long-term effects on soil food webs and soil biochemical processes.

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Thank you for your attention!