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RIMA

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

Kai Künnis-Beres

Tallinn Technical University, Marine Systems Institute

Akadeemia tee 15A, Tallinn, Estonia

kai.kunnis-beres@msi.ttu.ee

Workshop on pesticide fate in soil and water in the northern zone
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INTRODUCTION

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*).

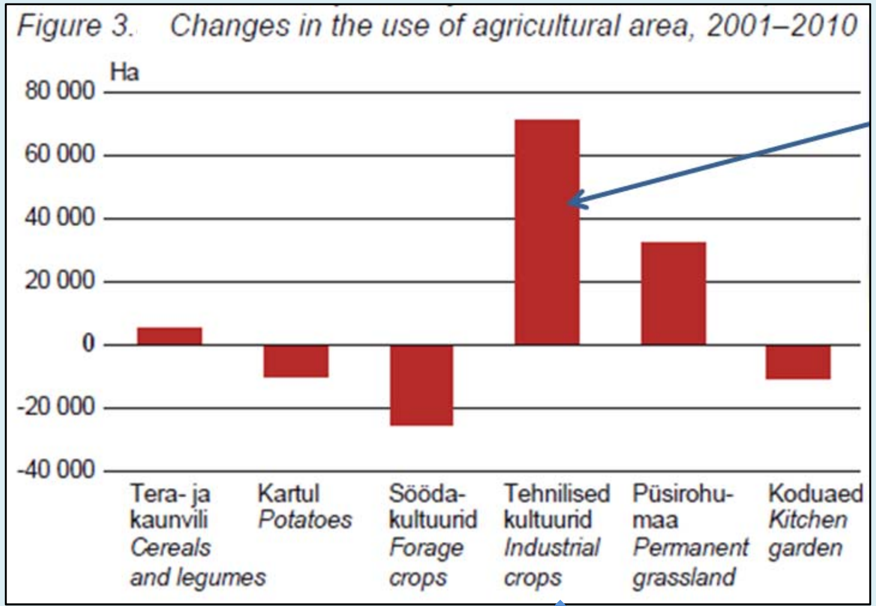
In addition, **global glyphosate market includes applications in non-agricultural areas**, such as in **home** and **garden**, **industrial** and **railroads** (*Woodburn, 2000*).

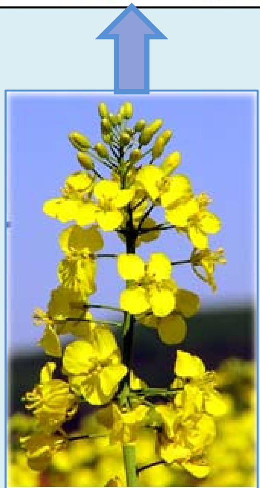
Introduction

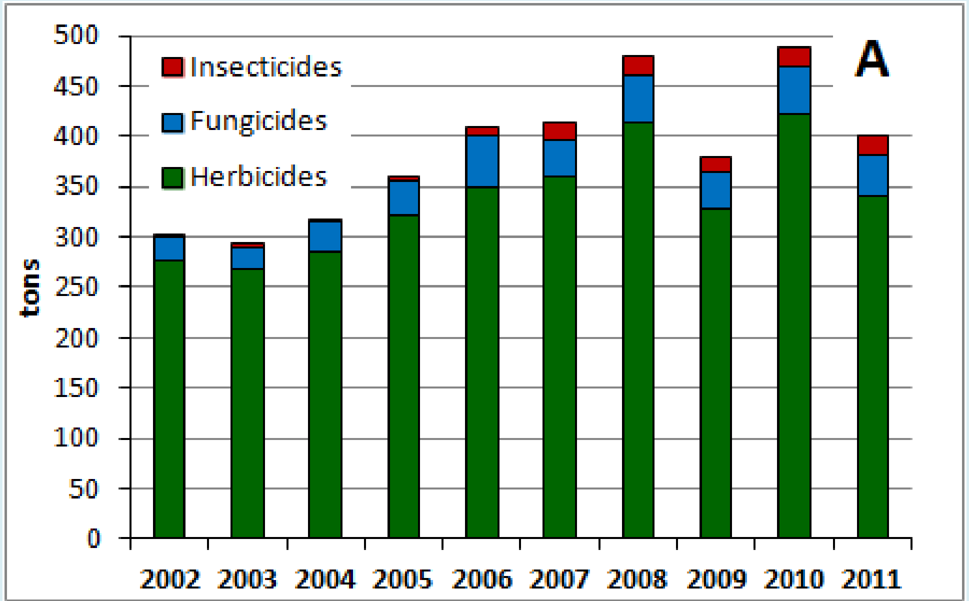
In Estonia *glyphosate-based formulations* are the most used among all pesticides since 2002 (*284 tonnes of glyphosate-based formulations were sold in 2010*) and their usage shows clear increase (*Estonian Agricultural Board, 2012*).

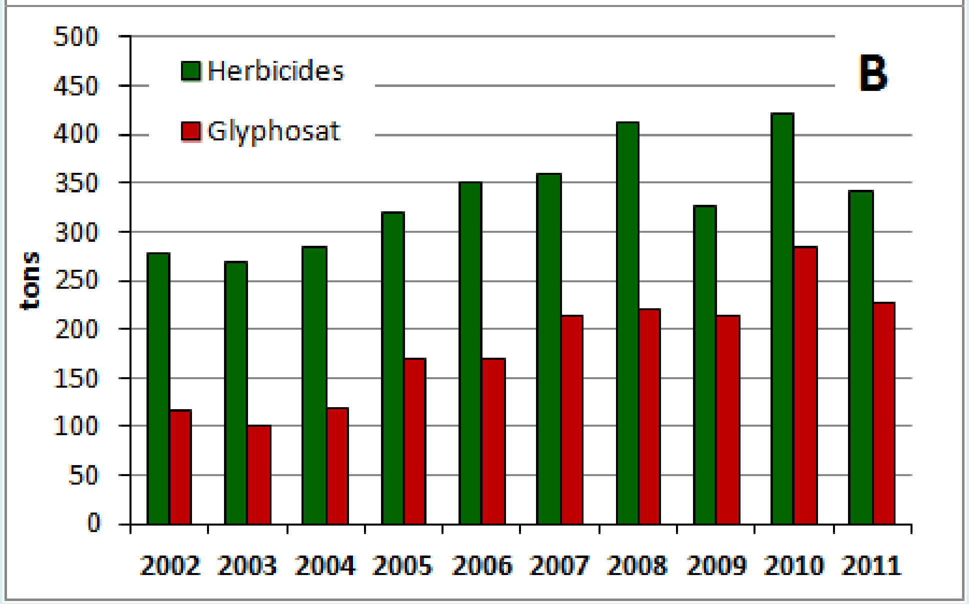
Currently, there are around *34 different glyphosate-based formulations* registered for using in Estonia, e.g. *Agro-Glyfo, Barclay Barbarian, Dalgis, Dominator, Glyfos, Glyphomax, Jablo, Ranger, Rodeo, Rosate, Taifun B, Roundup Quick, Roundup Gold, Roundup Max, Roundup Bio*, etc.

The use of pecticides 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.

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).

The main results of the project have been published:

M.Sihtmäe, I.Blinova, K.Künnis-Beres, L.Kanarbik, M.Heinlaan, A.Kahru. ***Ecotoxicological effects of different glyphosate formulations***. *Aquatic Soil Ecology 72 (2013) 215-224*.

Uppsala, 7-8. September 2016

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.*)

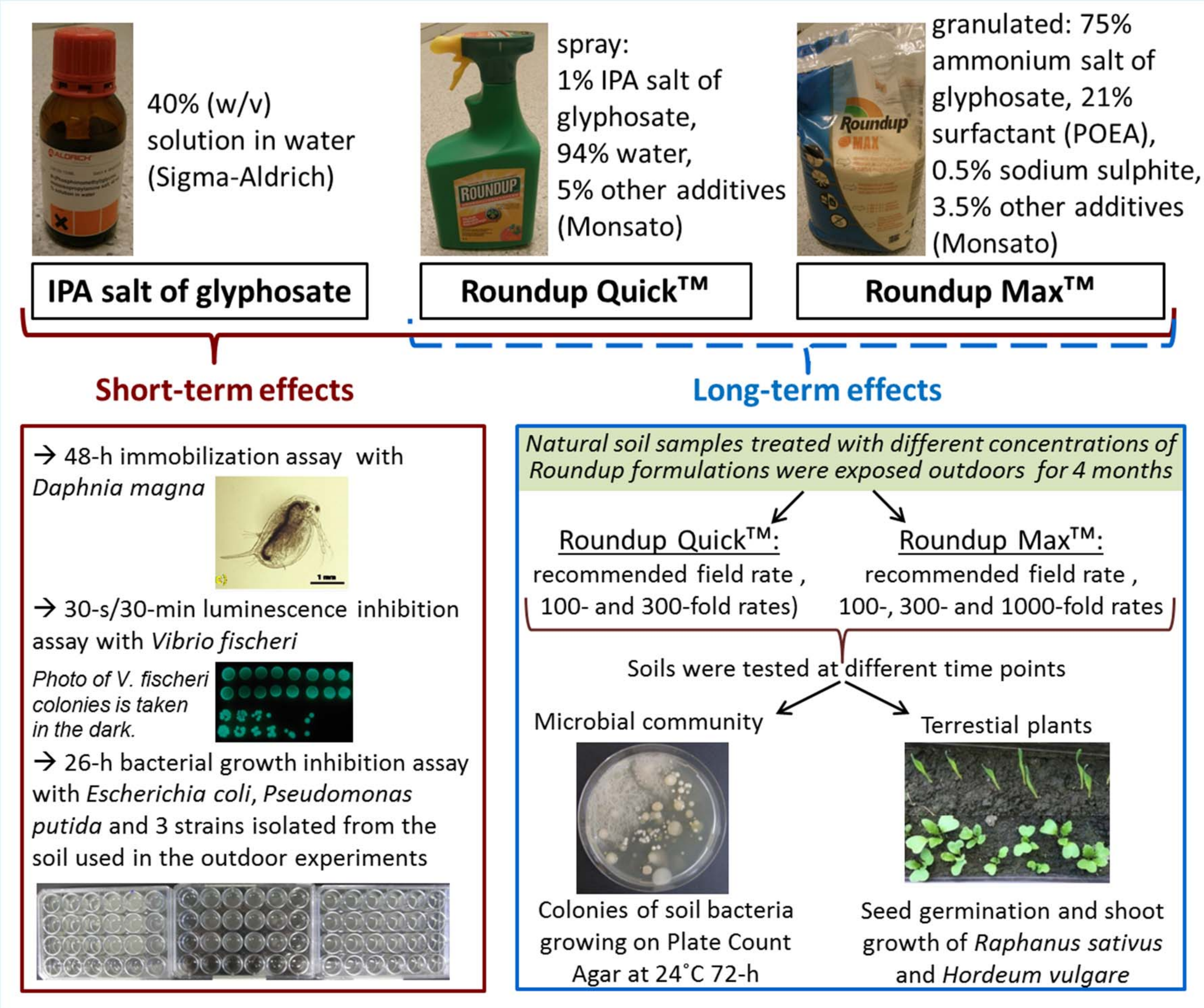
A

B

C

- ➢ **B** and **C** have been the most used glyphosate products in Estonia.

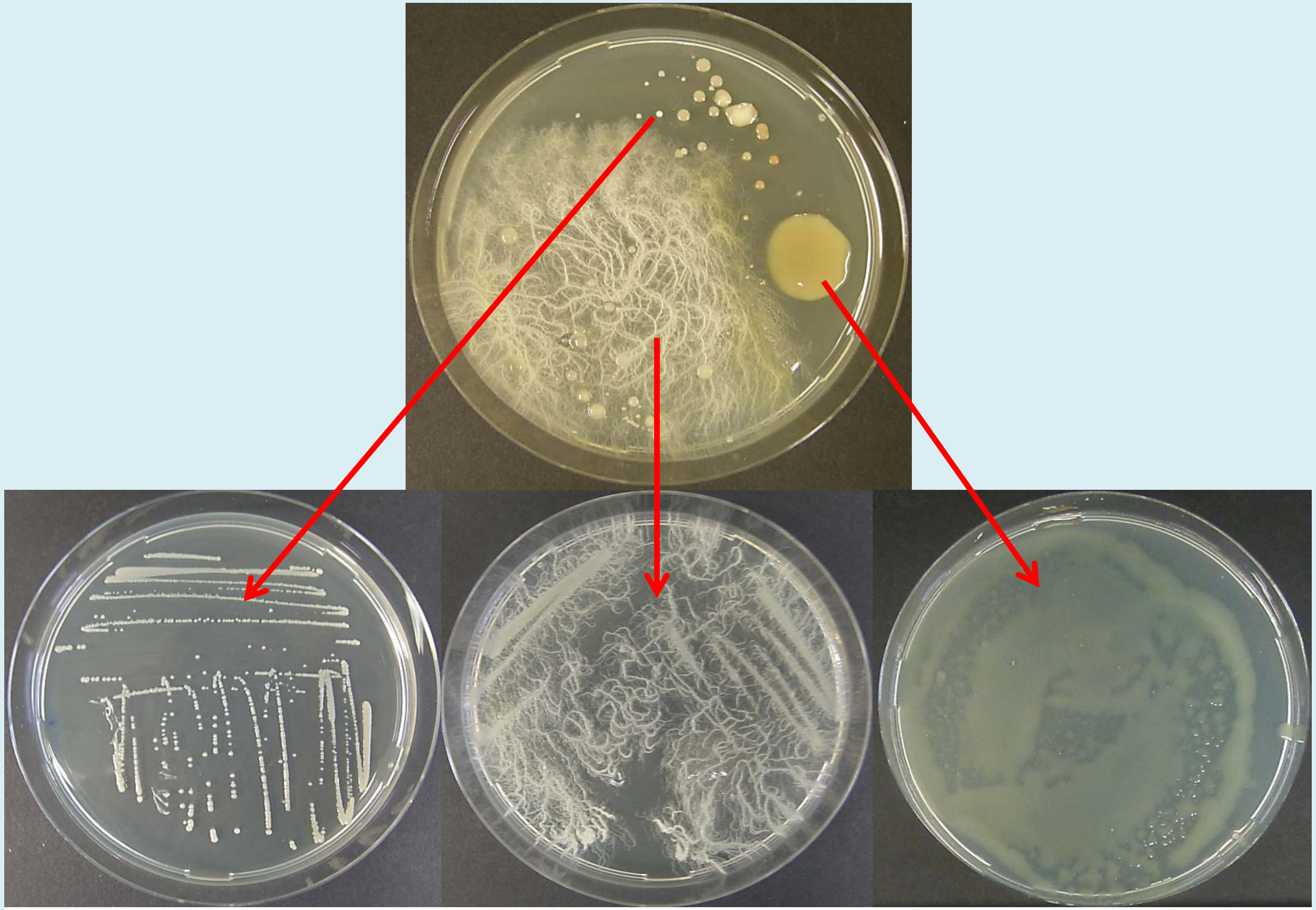
Scheme of the whole experiment



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

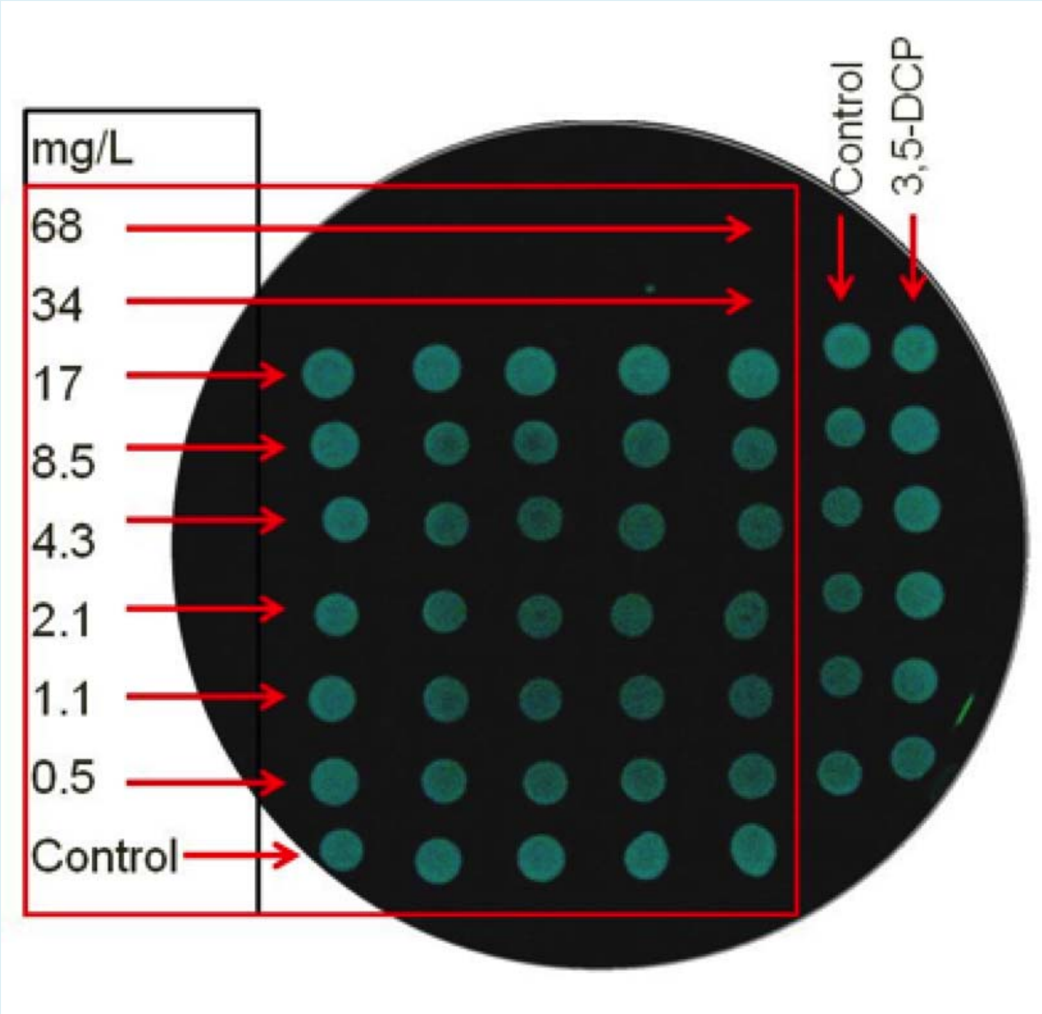


Arthrobacter sp. *Bacillus mycoides* Soil bacterium M2

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* (incubabation 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**.

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



METHOD description

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

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

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)

	EC50 [mg/L]		EC50 [g/L]	
	D. magna Immobilization assay, 48 h	*V. fischeri flash assay*, 30 min	Growth inhibition test, 24 h	
			P. putida	*E. coli*
Roundup Max	38,1 ±6,7	7,57 ±0,90	4,0 ±0,3	4,25 ±0,5
Roundup Quick	48,9 ±5,5	5,43 ±1,31	no inhib.	1,8 ±0,7
IPA salt	4,2 ±1,8	7,47 ±1,37	3,7 ±0,2	3,7 ±0,2

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.

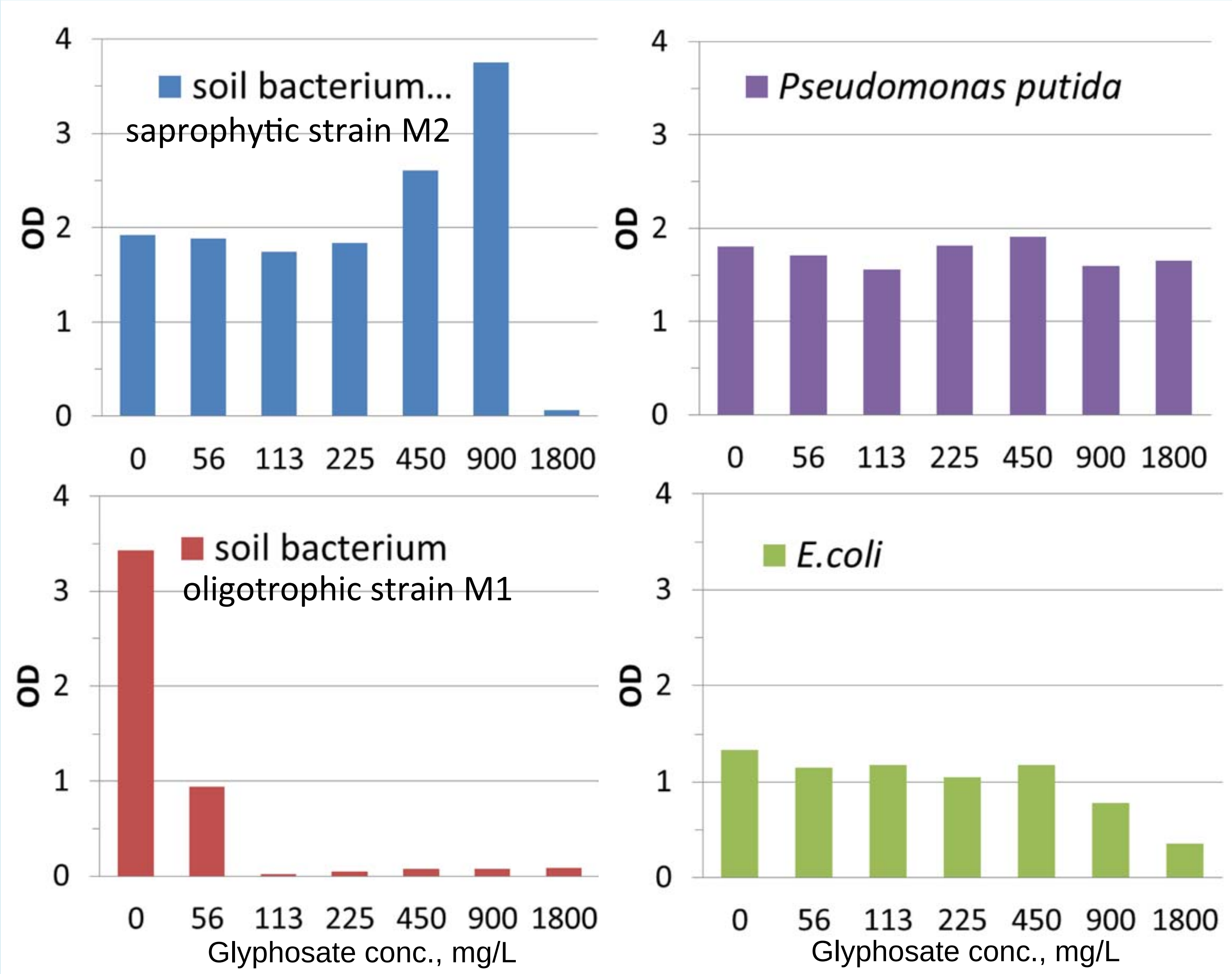
RESULTS (2a)

Bacterial growth inhibition assays

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

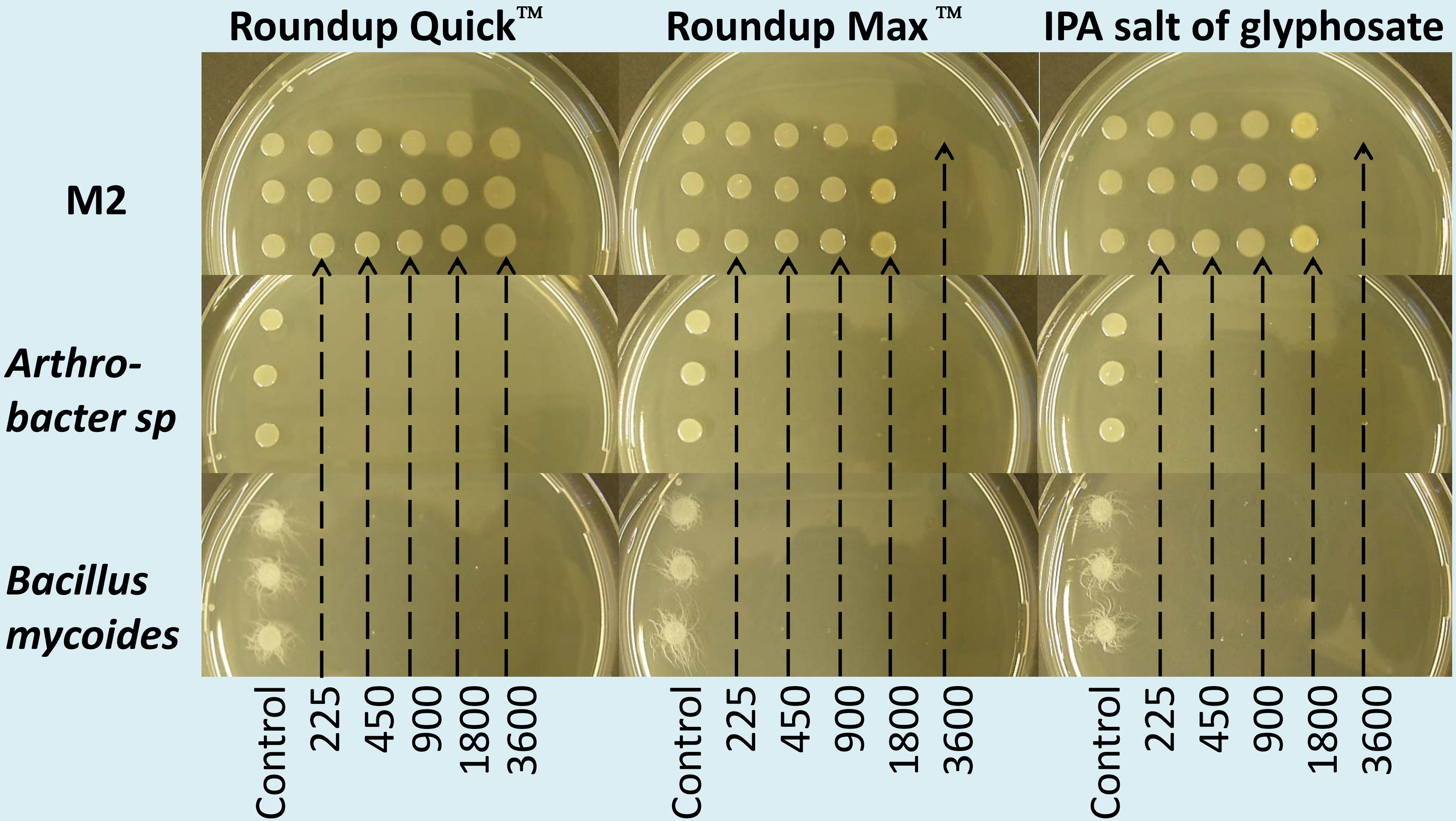
The **indigenous Gram-positive soil microbes** (*Bacillus mycoides and Arthrobacter sp.*) were **much more sensitive** to glyphosate based herbicides than the **Gram-negative bacteria** (*Pseudomonas putida, E. coli*).

RESULTS (2b)



soil bacterium...
saprophytic strain M2
Pseudomonas putida
soil bacterium
oligotrophic strain M1
E.coli
OD
0
1
2
3
4
0 56 113 225 450 900 1800
Glyphosate conc., mg/L

RESULTS (3)



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

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*).

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.

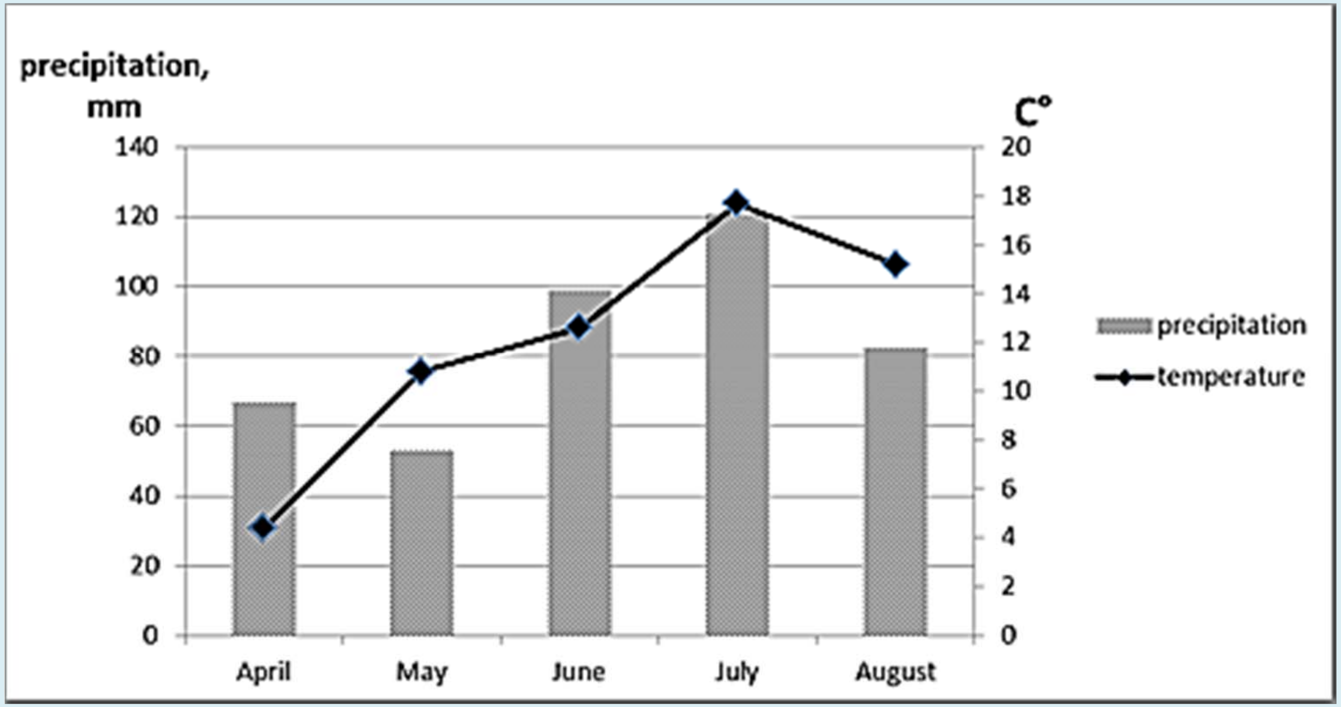
The recommended application rates are :

- 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.

The impact of Roundup formulations on the soil microbial community (1)

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**.

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.

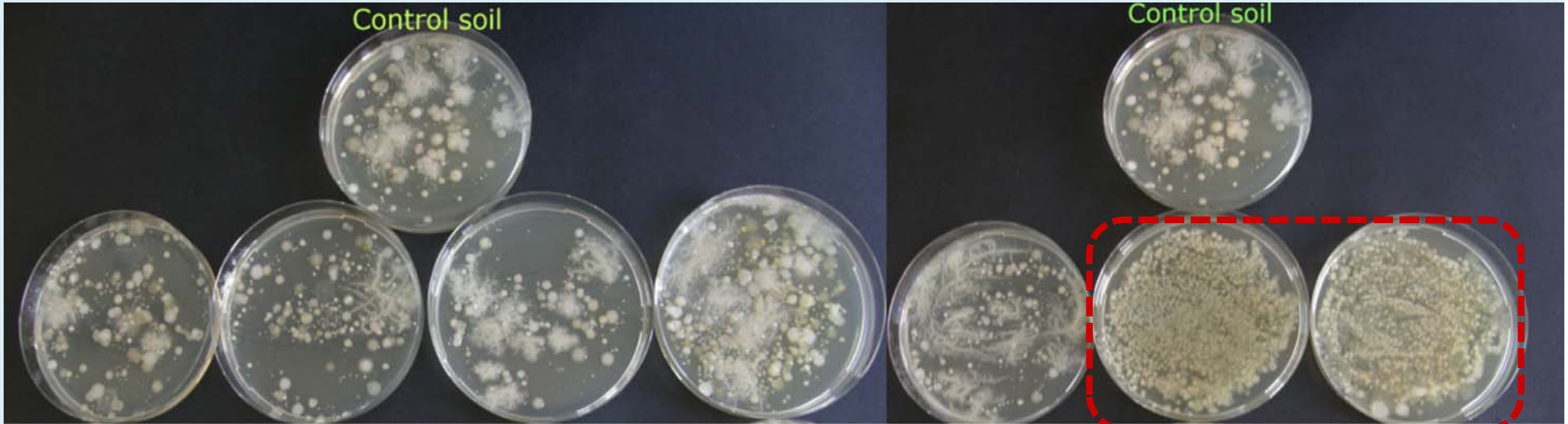
The mean number of culturable heterotrophic bacteria (HB) in non-treated field soil (control) varied from $9 \cdot 10^6$ to $20 \cdot 10^6$ CFU g^{-1} dry soil during the four months exposure.

RESULTS (4a)

Supplied herbicide concentrations		Number of HB (CFU)* 10^{6} g^{-1} dry soil				TBN (cells)** 10^{9} g^{-1} dry soil
		10 d#	21 d#	45 d#	108 d#	108 d#
Roundup Max	As recommended (245 mg/m^2)	34	14	28	9	27
	100x as recommended	40	27	48	9	26
	300x as recommended	38	26	76	8	24
	1000x as recommended	77	21	53	7	22
Roundup Quick	As recommended (245 mg/m^2)	85	20	29	9	22
	100x as recommended	953	256	91	15	24
	300x as recommended	361	709	135	24	23
Control	-	**20**	**16**	**14**	**9**	**35**

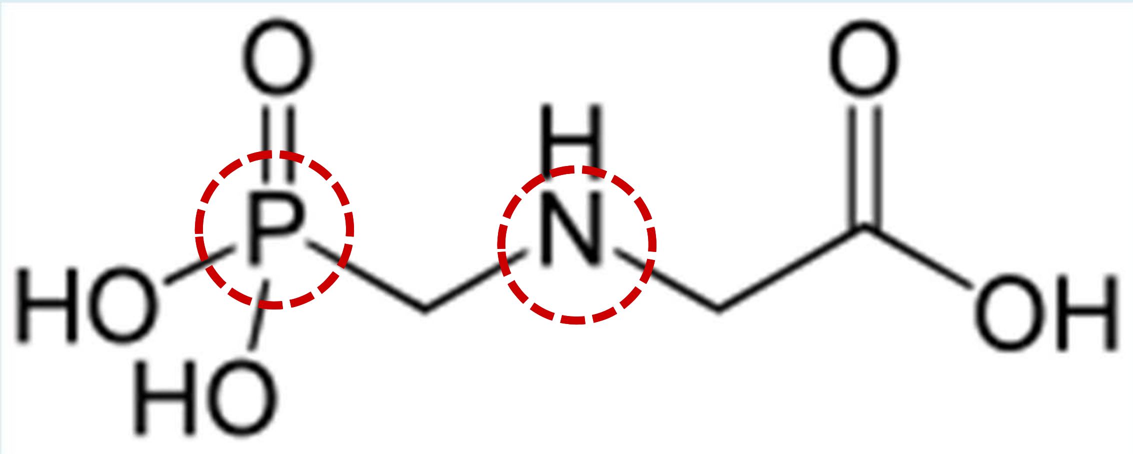
RESULTS (4b)

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™



Roundup Max glyph. 245 mg/m2 (recommended conc. for on field use)	Roundup Max glyph. 24,48 g/m2 (100x recommended concentration)	Roundup Max glyph. 73,44 g/m2 (300x recommended concentration)	Roundup Max glyph. 244,8 g/m2 (1000x recommend. concentration)	Roundup Quick glyph. 240 mg/m2 (recommended conc. for in garden use)	Roundup Quick glyph. 24,0 g/m2 (100x recommended concentration)	Roundup Quick glyph. 72,0 g/m2 (300x recommended concentration)

Glyphosate (*N-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).

CONCLUSIONS (1)

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 (2)

<u>The long-term outdoor experiment demonstrated that</u>

- ✓ 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 indigeous 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**.

__Mercurio__, P., Flores, F., Mueller, J.F., Carter, S., Negri, A.P. (2014) __Glyphosate persistence in seawater.__ Marine Pollution Bulletin 85:385–390

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**.


Thank you for your attention!