Applied Soil Ecology 72 (2013) 215–224

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Ecotoxicological effects of different glyphosate formulations

M. Sihtmäe*, I. Blinova, K. Künnis-Beres, L. Kanarbik, M. Heinlaan, A. Kahru

National Institute of Chemical Physics and Biophysics, Laboratory of Environmental Toxicology, Akadeemia tee 23, 12618 Tallinn, Estonia

ARTICLE INFO

Article history:
Received 21 February 2013
Received in revised form 14 June 2013
Accepted 1 July 2013

Keywords:
Roundup
Soil self-remediation
Soil bacteria
Daphnia magna
Vibrio fischeri

ABSTRACT

Glyphosate is an active substance of the most used herbicides worldwide. Nevertheless, questions on safety of glyphosate-based herbicides are periodically raised and recent studies indicate that glyphosate may not be as safe as assumed, mostly due to the additives/surfactants in its formulations. The aim of this study was to evaluate the effects of isopropylamine salt of glyphosate and two glyphosate-based herbicides, Roundup Max™ (containing surfactant polyethoxylated tallow amine, POEA) and Roundup Quick™ (without POEA), on non-target species. Special focus was on the evaluation of long-term effects of high concentrations (simulating accidental pollution, e.g. transportation spills) of glyphosate formulations on soil health. Laboratory ecotoxicity testing was conducted with (i) two aquatic organisms – crustaceans *Daphnia magna* and marine bacteria *Vibrio fischeri*, (ii) five bacterial strains (*Escherichia coli* MG1655, *Pseudomonas putida* KT2440 and three bacterial isolates from the soil) and (iii) terrestrial plants *Raphanus sativus* and *Hordeum vulgare*. Laboratory toxicity results showed that among the non-target test species, *D. magna* and *V. fischeri* were the most sensitive to glyphosate formulations: acute EC50 values ranged from 4 to 49 mg L^{-1}. Direct relation between the toxicity of the tested formulations and the presence/absence of the surfactant POEA was not evident. Long-term outdoor experiments (April to September 2012) showed that the number of heterotrophic microbes in Roundup-spiked (up to 1000-fold the recommended field rate) soils during two months after the treatment were significantly higher than in the control soils, especially in case of Roundup Quick™. Residual toxicity of the treated soils to terrestrial plants decreased more rapidly in Roundup Quick-spiked soils. It was shown that in temperate climate conditions the recovery of soil health in case of (accidental) pollution by glyphosate formulations is slow and may even exceed the duration of the vegetation period. The mobility of glyphosate in the soils proved very low thus risks to aquatic ecosystems due to application of glyphosate-based herbicides may occur rather in case of direct contamination of surface water.

1. Introduction

Herbicides account for about 40% of the pesticide volume used worldwide. Glyphosate is one of the most common herbicides used in agriculture, but also in forestry and horticulture (including home use) (EPA, 2011). Moreover, glyphosate is one of the first herbicides against which crops (e.g. soy, maize, cotton) have been genetically modified (James and Krattiger, 1996; UK GM Science Review panel, 2003). Thus, it is expected that the expansion of glyphosate-resistant crops will further increase the use of this pesticide.

Glyphosate is a broad spectrum, post-emergent herbicide that inhibits the growth of plants through interfering with the biosynthetic pathway of the essential aromatic amino acids phenylalanine, tyrosine and tryptophan. Specifically, it is an inhibitor of the enzyme in shikimate pathway, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Vencill, 2002; Tomlin, 2006) which exists in plants and in some microorganisms but not in animals (Steinrücken and Amrhein, 1980). Due to its assumingly plant-specific mode of action, it is generally considered of low toxicity to animals (Giesy et al., 2000) and other non-target organisms (WHO, 1996). The manufacturers advertise the "low toxicity and environmental friendliness" of glyphosate-based herbicides (Monsanto, 2012). However, according to the information from the respective chemical safety data sheets, the glyphosate-based herbicides are classified as hazardous to the aquatic environment (toxic to aquatic life with long lasting effects).

Glyphosate (IUPAC name *N*-(phosphonomethyl)-glycine) as an active substance as well as glyphosate-based herbicides have been extensively studied for the properties to produce adverse effects on human health and ecosystems (U.S. EPA, 1993; Giesy et al., 2000; Williams et al., 2000; EC, 2002; Govindarajulu, 2008). Nevertheless, questions on safety of glyphosate-based herbicides are periodically raised and recent independent studies indicate that glyphosate may not be as safe as previously assumed (Paganelli et al., 2010; Guilherme et al., 2012; Koller et al., 2012; Moore et al., 2012). Moreover, only a small fraction of the applied herbicides reaches the

* Corresponding author. Tel.: +372 6398382; fax: +372 6398382.
E-mail address: mariliis.sihtmae@kbfi.ee (M. Sihtmäe).

0929-1393/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.apsoil.2013.07.005

target species (Pimentel, 1995) and the residual amount of herbicides in soil and water may pose hazard to human, animal and crop health.

In the European Union (EU), the marketing and use of pesticides is regulated by Regulation 1107/2009/EC (EC, 2009). Active substances (e.g. glyphosate) are approved at the EU level, while the plant protection products (e.g. Roundup) containing these substances are authorised at Member State level. Currently, glyphosate and its main degradation product aminomethylphosphonic acid (AMPA) are not included in the existing list of priority substances under the Water Framework Directive (EC, 2000) and there are no environmental quality standard (EQS) values set for these substances at the EU level. Some countries have proposed various EQS values for glyphosate in the surface water, ranging from 10 $\mu g\,L^{-1}$ to 27,000 $\mu g\,L^{-1}$ (Kreuger and Asp, 2005; CCME, 2012; UKTAG, 2012). This orders of magnitude range points to the uncertainties in the existing assessment of the environmental risks of glyphosate contamination.

When discussing the toxicity of pesticides to non-target species, the focus is mostly on the active substance. However, pesticides are formulated products and usually contain additives (e.g. surfactants) which enhance their effectiveness but at the same time may increase the toxicity to non-target biota (Tsui and Chu, 2003; Edginton et al., 2004). Glyphosate-based pesticides are commercialised in many different formulations such as Roundup, Rodeo, Aquamaster etc. Frequently, the information on the surfactants and additives is not clearly stated by the manufactures. As the toxicity of glyphosate-based products may differ from the toxicity of pure glyphosate, the information on toxicity and environmental fate of a specific glyphosate formulation is needed for relevant risk assessment.

In Estonia, glyphosate-based formulations are the most used pesticides since 2002 and this trend shows a clear increase (Estonian Agricultural Board, 2012). Currently, there are about 30 different glyphosate-based formulations registered in Estonia (Register of Plant Protection Products, 2012) and additional information on environmental hazard of different formulations of glyphosate-based herbicides in the local climatic conditions would be useful for relevant risk assessment.

In the current study the potential harmful effects of two glyphosate-based herbicides Roundup Quick™ and Roundup Max™ assigned for home and industrial use, respectively, were evaluated in both, short-term laboratory and long-term outdoor experiments. 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 high concentrations (e.g. as a result of an accident during transportation) of glyphosate-based herbicides on soil microbes in the northern (part of the) temperate climate zone.

2. Materials and methods

2.1. Test chemicals

Isopropylamine (IPA) salt of glyphosate (CAS no 38641-94-0), 40% (w/v) solution in water (29.6% acid equivalents, AE, by weight), was purchased from Sigma–Aldrich (Schnelldorf, Germany). Herbicide formulations, Roundup Quick™ (spray, 0.72% AE by weight) with the composition of IPA salt of glyphosate (CAS no 38641-94-0) 1%, water 94%, other additives 5% (not specified by the manufacturer) and Roundup Max™ (granulated, 68% AE by weight) consisting of ammonium salt of glyphosate (CAS no 114370-14-8) 75%, surfactant POEA (CAS no 61791-26-2) 21%, sodium sulphite 0.5%, other additives 3.5% (not specified by the manufacturer), were produced by Monsato Europe S.A. (Antwerpen, Belgium). For the experiments (Supplementary material, Fig. S1), stock solutions of IPA salt of glyphosate and Roundup formulations were prepared in MilliQ water, stored in the dark at room temperature and tested for toxicity within one week. pH of the initial glyphosate solutions was not adjusted in order to mimic real use conditions. The initial pH values of the stock solutions of IPA salt of glyphosate (6.8 g AE L^{-1}), Roundup Max™ (6.8 g AE L^{-1}) and Roundup Quick™ (7.2 g AE L^{-1}) were 4.6, 4.2 and 7.0, respectively.

2.2. Laboratory toxicity assays

2.2.1. Acute immobilization assay with crustacean Daphnia magna

Testing with the freshwater crustacean *D. magna* adhered to OECD 202 guideline (OECD, 2004). The neonates of *Daphnia* less than 24 h old, obtained by the hatching of ephippia, were exposed to different concentrations of herbicide solutions or soil extracts at 20 °C for 48 h in the dark. The ISO medium (OECD 202) was used as the test medium.

2.2.2. Acute luminescence inhibition assay with bacterium Vibrio fischeri

The test (exposure time 30-s and 30-min) was performed at 20 °C on automatic tube-luminometer 1251 (ThermoLabsystems, Finland), connected to a computer operated by Multiuse software (BioOrbit, Finland) following the Flash-assay protocol (ISO, 2010). The exact procedure is described in Põllumaa et al. (2000) and Mortimer et al. (2008) except the inhibition of bacterial bioluminescence that was calculated as percentage of the unaffected (negative) control (2% NaCl). Reconstituted *V. fischeri* Reagent (Aboatox, Turku, Finland) was used for testing. IPA salt of glyphosate and Roundup formulations were tested in 2% NaCl. Each test was performed three times, each in 5–7 replicate dilutions. Controls, both negative (2% NaCl) and positive (3,5-dichlorophenol; 3,5-DCP), were included in each measurement series. Samples were continuously automatically mixed during the recording of luminescence. pH values in *V. fischeri* tests were in the range of 5.3–6.0 in tests with IPA salt of glyphosate and Roundup Max™ and 6.5–7.0 with Roundup Quick™.

2.2.3. Bacterial growth inhibition assay

The effects of IPA salt of glyphosate and Roundup formulations on growth of bacteria during 26 h were studied. Bacterial biomass was evaluated by optical density ($OD_{600\,nm}$) of the bacterial suspensions. Altogether five bacterial strains were used: *Escherichia coli* MG1655, *Pseudomonas putida* KT2440 and three strains isolated by us from the control soil used in the outdoor experiments. One soil isolate was identified as *Bacillus mycoides* (gram-positive endospore forming aerobic bacilli), the other two were named soil bacterium M1 (gram-positive non-sporulating aerobic bacilli) and soil bacterium M2 (gram-negative aerobic coccobacilli), respectively (Supplementary material, Fig. S2). All these three bacterial isolates represented dominant viable strains in the untreated soil samples. Half-strength Luria-Bertani (LB) medium (trypton 5.0 g L^{-1}, yeast extract 2.5 g L^{-1}) was used for the cultivation of bacteria and dilution of chemicals. For the experiments bacterial cultures incubated at 24 °C overnight were adjusted to $OD_{600\,nm}$ 0.08–0.1 and an equal amount of the chemical or half-strength LB growth medium (control) was added. The tested concentrations of Roundup Quick™, Roundup Max™ and the IPA salt of glyphosate were 3600, 1800, 900, 450 and 225 mg AE L^{-1}. All the stock solutions were UV-sterilised (2 × 6 W Hg lamp) before testing. The tests were performed in sterile 24-well polypropylene microplates (Falcon) at room temperature (24 °C) on microplate shaker (Titramax 1000, Heidolph). Optical density (OD_{600}) was recorded by Multiskan Spectrum microplate spectrophotometer (Thermo Electron

Corporation, Finland) by interval of one hour during the first ten hours (soil isolates) or thirteen hours (*E. coli, P. putida*) and then after 22, 24 and 26 h. All the tests were performed in at least 3 replicates. Bacterial cultures were grown at 24 °C, optimal for the natural soil isolates and also acceptable for most of the mesophilic bacteria, including *E. coli, P. putida* and *B. mycoides*.

2.2.4. Bacterial viability assay (a 'spot'-test)

The 'spot'-test (described in detail by Kasemets et al., 2013) was used as an additional viability endpoint for the bacterial growth inhibition assays to test the ability of the toxicant-exposed bacteria to form colonies on nutrient agar after 26 h exposure to the tested chemicals. For that, 3 μl of the culture from each microplate well (treated and not-treated) was pipetted ('spotted') onto nutrient agar and incubated at 24 °C for 48–72 h. The growth of bacteria (formation of colonies) was evaluated visually on Difco™ Plate Count Agar (PCA) (formula as gram per litre: pancreatic digest of casein 5.0, yeast extract 2.5, dextrose 1.0, agar 15.0).

2.2.5. Analysis of the bacterial numbers from the Roundup-spiked soils

Colony-forming units (CFU) of heterotrophic bacteria (HB) from different time points of spiked and control soil samples were analysed on Difco™ Plate Count Agar (PCA) by spread plate technique. Briefly, 29 ml of sterile MilliQ water was mixed with 1 g of fresh soil in sterile plastic tube, shaken for 30 min on an orbital shaker and then vortexed for 30 s. The obtained slurry was used for preparing of the serial decimal dilutions in sterilised tap-water. Three replicate plates were inoculated from the three subsequent dilutions per sample. The inoculated plates were incubated at the room temperature (22–24 °C) in the dark and after 3 and 5 days incubation the CFU were counted. The results are presented as bacteria (CFU) per gram of dry soil.

For the microscopic enumeration of total number of bacteria in the soil, 1 g of fresh soil was mixed with 29 ml of sterile MilliQ water in 40 ml sterile screw cap cell culture tube and fixed with 37% formaldehyde (final concentration in sample 0.8–1.2%). The tubes were shaken on orbital shaker for 30 min. Then large soil particles were allowed to settle (5 min) and 0.5 ml of the upper layer containing bacterial suspension was filtered through 0.2 μm pore size black polycarbonate membrane filter (Nuclepore Track-Etch membrane Whatman, diameter 25 mm). For the enumeration of the total number of soil bacteria the acridine orange (AO) stained bacteria (Zimmermann, 1977) were counted using fluorescence microscope Olympus CX41.

2.2.6. Statistical methods

The toxicity values (EC50 – the median effective concentration of the toxicant that induces a designated effect in 50% of the test organisms after a specified exposure time) were determined from dose–response curves by the REGTOX software for Microsoft Excel (Vindimian, 2009) using the Log-normal model. One-way analysis of variance (ANOVA) followed by *t*-tests were used to determine statistical significance of the differences between toxic effects of the investigated compounds. The differences were considered significant, when $p < 0.05$.

2.3. Long-term outdoor experiments

Soil samples used for the long-term outdoor experiments were collected from the upper 5–20 cm layer of uncontaminated arable land not used during the past 10 years. Soil samples were air-dried at room temperature and sieved at 2 mm before use. Main characteristics of the soil: pH 7.0, C_{org} – 3.5%, soil texture – loam.

Tests were performed in containers with the total volume of the soil 6075 cm^3 and surface area of 486 cm^2. The containers were first filled with unpolluted fine sand (2 cm on the bottom) and then field-moist (initial water content about 23%) organic soil (10.5 cm) was added. The moisture content of the soil was determined by drying the soil samples at 105 °C for 24 h. All the results were calculated per gram of dry soil.

Soils were spiked with different doses (recommended for herbicidal use, 245 mg m^{-2}; 100-fold; 300-fold and 1000-fold rates) of two herbicide formulations, Roundup Quick™ and Roundup Max™, and were exposed outdoors during four months (from April to September 2012). 486 ml of the aqueous solutions of Roundup formulations of appropriate concentration was added to each container, while control containers received an equal amount of distilled water.

To evaluate the effects of the different concentrations of herbicides on soil microbes the number of viable heterotrophic bacteria (HB) was analysed after 10, 21, 45 and 108 days of spiking the soils. At the end of the experiment, after 108 days, the fluorescence microscopic counting of total number of the bacteria in treated and untreated (control) soils were performed in addition to viable plate counts.

Residual toxicity of the treated soils to non-target organisms was monitored using terrestrial (higher plants) and aquatic test species during several weeks after the spiking. In the plant assay, the inhibition of seed germination and shoot growth of the red radish *Raphanus sativus* and barley *Hordeum vulgare* in the treated soils were evaluated. The 48-h acute immobilization assay with crustacean *D. magna* and 30-min acute luminescence inhibition assay with bacterium *V. fischeri* were used for determining potential hazardous effects of leaching of herbicides from the treated soils into waterbodies. For that, aqueous extracts of the treated soils were prepared in ISO medium used in *D. magna* assay. The soil was added to ISO medium in 1:10 ratio and shaken at 200 rpm at 21 °C for 24 h. The suspension was clarified by centrifugation and supernatants were used in toxicity testing with crustacean *D. magna* and bacteria *V. fischeri*.

3. Results and discussion

The use of herbicides may pose hazard to non-target species. Commercial glyphosate formulations contain various additives, including surfactants. One of the most well-known additives is the surfactant polyoxyethyleneamine or polyethoxylated tallow amine, both abbreviated as POEA. Several studies have reported that the toxicity of glyphosate-based herbicides to aquatic organisms is largely due to this surfactant in the mixture (Folmar et al., 1979; Servizi et al., 1987; Buhl and Faerber, 1989; Mann and Bidwell, 1999; Tsui and Chu, 2003; Edginton et al., 2004; Moore et al., 2012). Specifically, commercial glyphosate formulations containing POEA were considerably more toxic to amphibians (Mann and Bidwell, 1999; Perkins et al., 2000; Edginton et al., 2004; Howe et al., 2004), to aquatic microalgae, protozoa and crustaceans (Tsui and Chu, 2003) than pure active substance or some glyphosate formulations with other surfactants (the identity and composition of these surfactants are often trademark protected).

In the current study, the short-term toxicity of glyphosate as an active substance (IPA salt of glyphosate) and two commercial glyphosate formulations with POEA (Roundup Max™) or without POEA (Roundup Quick™) to freshwater crustacean *D. magna*, laboratory test bacteria marine bacterium *V. fischeri*, soil bacterium *P. putida*, intestinal bacterium *E. coli* and three soil bacteria isolated by us from the local soil was studied. In addition, outdoor experiments were performed to assess/compare the long-term effects of different Roundup formulations on natural soil microbial numbers at recommended and elevated (simulating accidental pollution, e.g. spill during transportation) field application rates. Biological

methods were used to evaluate the residual toxicity of treated soils at different time points.

3.1. Laboratory studies

3.1.1. Toxicity to aquatic species

Crustaceans *D. magna* and bacteria *V. fischeri* are aquatic species widely used in ecotoxicology. The toxicity of IPA salt of glyphosate and both Roundup formulations to these species ranged from 4.2 to 48.9 mg AE L^{-1} (Table 1). In case of *D. magna*, IPA salt of glyphosate (48-h EC50 = 4.2 mg AE L^{-1}) was about 10-fold more toxic than the tested Roundup formulations and no statistically significant differences ($p > 0.05$) between toxicities of Roundup Max™ and Roundup Quick™ were found. In case of *V. fischeri* Roundup Quick™ (30-min EC50 = 5.4 mg AE L^{-1}) was slightly more toxic ($p < 0.05$) than IPA salt of glyphosate (EC50 = 7.5 mg AE L^{-1}) and Roundup Max™ (EC50 = 7.6 mg AE L^{-1}) (Table 1). Hence, *V. fischeri* 30-min luminescence inhibition assay was up to 9 fold more sensitive than 48 h *D. magna* assay towards Roundup formulations but slightly less sensitive towards IPA salt of glyphosate (Table 1). However, the ability of *V. fischeri* to yield colonies on agar after the exposure to Roundup Max™ (the 'spot'-test, Fig. S3) showed that *V. fischeri* minimal bactericidal concentration (MBC) values were higher than the respective EC50 values and quite similar for *D. magna* 48-h EC50 values (Table 1). Thus, these tests did not reveal any link between the toxicity and presence/absence of surfactant (POEA) in the studied glyphosate products.

The toxicity results for *D. magna* in the current study (Table 1) are comparable to the data of the Safety Data Sheets (SDS) (Monsato Europe S.A. 2000, 2010). In *D. magna* tests, the effect of pH on the test results can be excluded as there was only a slight difference in the pH of the test solutions (pH at EC50: IPA salt of glyphosate-7.2; Roundup Quick™-7.1 and Roundup Max™-6.7). It can therefore be concluded that both investigated glyphosate formulations were about 10-fold less toxic to crustaceans than IPA salt of glyphosate. This conclusion, however, differs from the results presented by Tsui and Chu (2003), who demonstrated that Roundup was 77 fold more toxic than IPA salt of glyphosate to another aquatic crustacean, *Ceriodaphnia dubia*. This controversy may be explained by different formulations of Roundup and/or different test species used.

As mentioned above, the toxicity of Roundup formulations and IPA salt of glyphosate for *V. fischeri* was approximately in the same range (30-min EC50 values from 5.4 to 7.6 mg AE L^{-1}), but Roundup Quick™ was slightly more toxic ($p < 0.05$) than IPA salt of glyphosate and Roundup Max™. For the visualisation of the changes of the bacterial bioluminescence immediately after the contact with pesticide, kinetic bioluminescence inhibition test *V. fischeri* was conducted. This assay is very rapid and sensitive: the effect of toxic organic compounds on bacterial bioluminescence is noticeable already in few seconds after the exposure (Mortimer et al., 2008; Kurvet et al., 2011). Comparing the pattern of the 30-s kinetics of the glyphosate products it can be concluded that the mechanism of toxic action of Roundup Quick™ to *V. fischeri* is different from that of IPA salt of glyphosate and Roundup Max™ (Fig. 1). Indeed, in case of Roundup Quick™ the most remarkable effect on bacterial luminescence occurred already within the first few seconds and the effect reached plateau after 10 s showing rapid deleterious effects on bacterial cell membrane. However, in case of Roundup Max™ and especially IPA salt of glyphosate the effects were not so rapid and by 30 seconds the effect had still not reached the plateau (Fig. 1). However, the final EC50 values after 30-min of exposure were comparable (Table 1).

The *V. fischeri* luminescence inhibition assay EC50 values of this study are in agreement with the data from Kahru et al. (1996), Chang et al. (1981) and McFeters et al. (1983) but up to 20 fold lower than those reported by Tsui and Chu (2003), Hernando et al. (2007) and Bonnet et al. (2007). Such variations could be explained by different composition of the tested Roundup formulations but also by different test conditions (temperature, exposure time) and/or bacterial preparations used.

Indeed, due to many factors that may influence the test results it is often difficult to compare data originating from different literature sources. According Giesy et al. (2000), data reported on the short-term toxicity of glyphosate and its formulated products for the aquatic organisms vary remarkably: (1) microorganisms (3–7 day EC50 0.64–590 mg AE L^{-1}); (2) macrophytes (7–14 day EC50 1.6–25.5 mg AE L^{-1}); (3) invertebrates (2–10 day EC50 7 – >1000 mg AE L^{-1}); (4) fish (2–4 day LC50 5.8 – >1000 mg AE L^{-1}). The large variation of toxicity values may be mostly explained by the wide variety of the tested glyphosate-based herbicides but also by different test organisms, test conditions (temperature, test media) and test designs. In addition, there is a number of different formulations with analogous brand name (e.g. Roundup), which exhibit varying degrees of toxicity (Nandula, 2010). The toxic effect is a resultant of all the components in the formulation, incl. so-called "other additives". The unknown additives (e.g. up to 5% of weight in case of Roundup Quick™ used in this study) may also modulate the toxicity of the main components (active compound and surfactant) of herbicide formulations to aquatic species. Moreover, it is often not clear whether the toxicity data of glyphosate-based herbicides in literature are presented as the toxicity of the whole formulation or expressed as the acid equivalent, complicating the comparison with the existing information on the toxicity.

Due to the extensive use of glyphosate-based herbicides, glyphosate and its major metabolite AMPA have frequently been detected in water bodies. Both glyphosate and AMPA are water soluble and can persist in aquatic environments for several weeks (Giesy et al., 2000). According to a review (WRc Plc, 2009), glyphosate was detected in about 30% and AMPA in 50% of samples collected across Europe in 1993–2009. The highest observed concentrations were: glyphosate – 50 μg L^{-1}, AMPA – 49 μg L^{-1}. AMPA has usually been detected in higher concentrations and in a larger proportion of samples than glyphosate. High concentrations of glyphosate (up to 328 μg L^{-1}) have also been recently reported in USA (Battaglin et al., 2009). As mentioned in the Introduction, the proposed environmental quality standards for freshwater ecosystems vary largely (10–27,000 μg L^{-1}). According to the Water Framework Directive (EC, 2000), the permitted level of chemicals in surface water may be evaluated using a safety factor 1000 for the lowest acute EC50 value from the applied test set. If we divide the lowest EC50 value (4.2 mg L^{-1}, Table 1) for glyphosate obtained in the current study by 1000, the non-hazardous concentration of glyphosate for aquatic ecosystems may be roughly estimated as 4.2 μg L^{-1}, which is 10 to 100-fold lower than the highest observed concentrations. In Estonia, the screening data on glyphosate and AMPA have recently been made available and in some cases the concentrations of glyphosate and AMPA have remained below the limits of quantification (BaltActHaz, 2011, 2012). However, the highest reported concentrations of glyphosate and AMPA in surface water were 0.29 and 0.93 μg L^{-1}, respectively (Maves, 2010).

3.1.2. Inhibition of the growth of different bacterial strains by studied Roundup formulations

Microbial community of natural soil normally contains both, rapidly growing and slowly growing heterotrophic bacteria. The ratio of these two groups of bacteria depends on several environmental factors of which the most important are temperature and the availability of biodegradable organic substrates. Therefore, freshly isolated soil bacterial strains with different growth characteristics (Table 2) – *B. mycoides*, soil bacterium M1 and

Table 1
The short-term toxic effect of Roundup Max™, Roundup Quick™ and IPA salt of glyphosate to crustacean *Daphnia magna* and bacteria *Vibrio fischeri*.

Toxicity	*Daphnia magna*	*Vibrio fischeri*	
	Immobilization of crustaceans 48-h EC50[a]	Inhibition of the luminescence 30-min EC50[a]	Inhibition of the further growth MBC[b]
Roundup Max™ (with POEA)	38.1 ± 6.7	7.6 ± 0.9	34
Roundup Quick™ (without POEA)	48.9 ± 5.5	5.4 ± 1.3*	Not tested
IPA salt of glyphosate	4.2 ± 1.8*	7.5 ± 1.4	Not tested

[a] EC50 – the median effective concentration, mg AE L^{-1}, of the toxicant that induces a designated effect in 50% of the test organisms upon specified exposure time.
[b] MBC – minimum bactericidal concentration, mg AE L^{-1}. The lowest tested concentration that completely inhibited the visible growth of bacteria on the agarized test medium at room temperature in the dark after 30 min of incubation to Roundup Max™ (Supplementary material, Fig. S3).
* Statistically significant ($p < 0.05$) difference from the other tested products in this toxicity test.

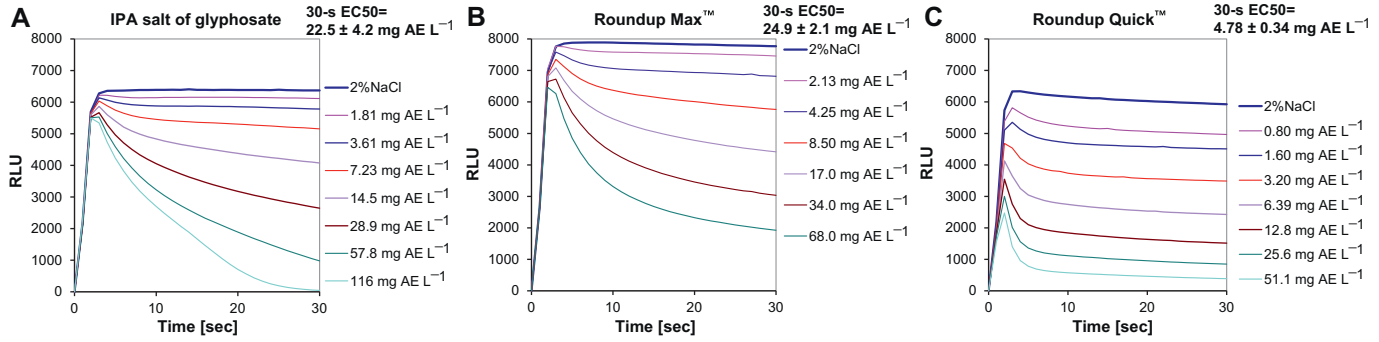

Fig. 1. The kinetic 30-s dose-effect curves of luminescence of *Vibrio fischeri* exposed to the different glyphosate products: A – IPA salt of glyphosate; B – Roundup Max™; C – Roundup Quick™. RLU – relative light units; 2% NaCl – negative control and diluent.

soil bacterium M2 – were used in addition to widely used laboratory test bacteria *E. coli* and *P. putida*, to study the toxic effect of glyphosate-based herbicides to various heterotrophic bacteria.

The results showed that toxicity of the tested glyphosate products to heterotrophic bacteria depended on the origin and growth characteristics of the tested bacterial strains. Despite the identical growth conditions, the growth pattern of the five tested bacterial strains (*E. coli*, *P. putida*, *B. mycoides*, soil bacteria M1 and M2) and their response to the glyphosate products varied largely (Table 2). The growth inhibition assay with the bacteria isolated from the soil demonstrated very different sensitivity of soil bacteria to the Roundup formulations and IPA salt of glyphosate. The results showed that the tested products of glyphosate totally inhibited the growth of indigenous gram-positive soil bacterial strains (*B. mycoides*, soil bacterium M1) already at the lowest test concentration (225 mg AE L^{-1} of glyphosate). However, glyphosate may also support the growth of some bacteria (e.g. *Pseudomonas* spp) (Gimsing et al., 2004). The results of the current study showed that the tested Roundup formulations were not toxic to gram-negative bacteria *P. putida, E. coli*, and soil bacterium M2 (Table 2): the EC50 values of tested products based on bacterial growth inhibition were 340–515, 506–706 and 845–1855 mg AE L^{-1}, respectively. Analogously to *V. fischeri* bioluminescence inhibition assay (Table 1), the EC50 values based on growth inhibition on five bacterial strains for three glyphosate products did not depend on the presence/absence of POEA.

In addition, a 'spot'-test was performed at the end of the growth inhibition tests (after 26-h incubation). Treated and non-treated bacterial cultures were examined for their ability to grow (form colonies) on the nutrient agar medium but also to verify the absence of the contamination of the test culture. The results of the 'spot'-test were coherent with the results of the growth inhibition test (Fig. 2 and Table 2).

Table 2
The effect of Roundup Max™, Roundup Quick™ and IPA salt of glyphosate on bacterial growth in laboratory conditions.

Test bacteria	Laboratory test strains				Bacterial strains isolated from the soil					
	Pseudomonas putida		*Escherichia coli*		Soil bacterium M2		Soil bacterium M1		*Bacillus mycoides*	
Duration of the lag phase (h)[a]	0.5		1		4.5		10		1	
μ_{max} (h^{-1})[b]	0.58		0.21		0.47		0.21		0.69	
	5h-EC50	MBC	5h-EC50	MBC	8h-EC50	MBC	16h-EC50	MBC	5h-EC50	MBC
Roundup Max™ (with POEA)	340	Not tested	548	Not tested	846	3600	<225	<225	<225	<225
Roundup Quick™ (without POEA)	515	Not tested	706	Not tested	1855	>3600	<225	<225	<225	<225
IPA salt of glyphosate	343	Not tested	506	Not tested	845	3600	<225	<225	<225	<225

[a] Duration of the lag phase – the period of time between the introduction of the control bacteria (i.e., no toxicants added) into the culture medium and the time it begins to increase exponentially.
[b] μ_{max} – maximum specific growth rate of the control bacteria.
EC50 – the concentration of the toxicant, mg AE L^{-1}, that inhibited bacterial growth by 50% after a specified exposure time.
MBC – minimal bactericidal concentration, mg AE L^{-1}. The lowest tested concentration that completely inhibited the visible growth of bacteria on the Difco™ Plate Count Agar (PCA) medium at room temperature in the dark after 26 h of incubation to the three glyphosate products (Roundup Quick™, Roundup Max™ and IPA salt of glyphosate). For more detail, see Section 2 and also Fig. 2.

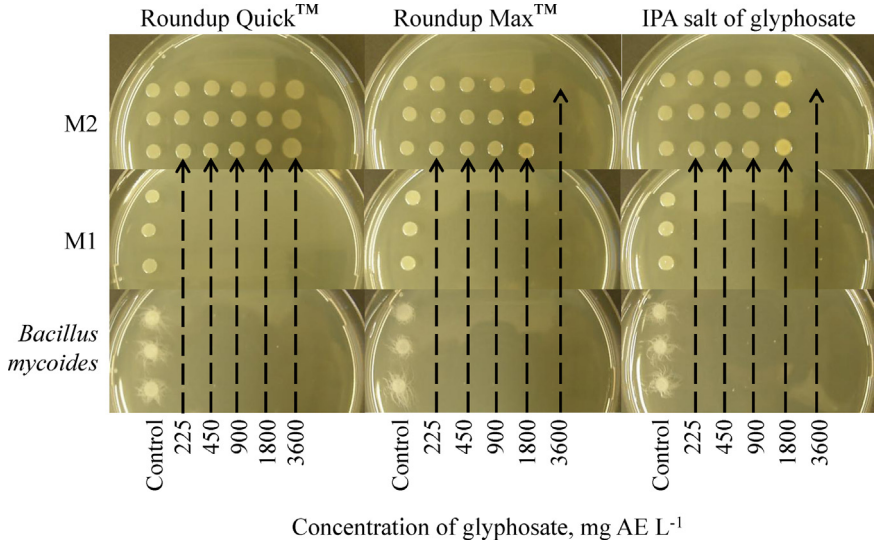

Fig. 2. Ability of bacterial strains isolated from the soil (M1, M2 and *Bacillus mycoides*) to yield colonies on the Difco™ Plate Count Agar (PCA) medium after 26 h of incubation at room temperature in the dark to different glyphosate concentrations of the three glyphosate products (Roundup Quick™, Roundup Max™ and IPA salt of glyphosate). For more details, see Section 2 and also Table 2 for minimal bactericidal concentration (MBC) values.

3.2. Long-term outdoor experiments

The persistence of glyphosate in the soil depends on soil type, climatic conditions and soil microbial activity. According to literature data, the half-life of glyphosate in field soils varies from 2 to 197 days (Giesy et al., 2000). The mean degradation half-life of glyphosate in the field soil has been suggested to be 30 days (Monsato, 2005). However, it should be mentioned that little research has been done on pesticide degradation in northern environmental conditions (temperate climate zone). Cold climate may influence glyphosate degradation in the soil and repeated applications could lead to accumulation in the soil (Dibyendu et al., 1989; Stenrød et al., 2005; Laitinen et al., 2009).

In the current study, the experiments were performed in climatic conditions typical for Estonia characterised by short and cool vegetative season (Supplementary material, Fig. S4). Soil samples were spiked with different doses (recommended, 100-fold, 300-fold and 1000-fold application rates) of Roundup formulations and exposed outdoors for four months. 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 on the specific formulation used (e.g. the recommended application rate for Roundup Max™ is 0.75–4.0 kg ha^{-1} and Roundup Quick™ 2.45 kg ha^{-1}). For soil experiments, the average treatment rate (2.45 kg ha^{-1}) recommended by the producer for weed control was chosen.

3.2.1. The impact of Roundup formulations on soil microbes

The fundamental role of the microorganisms in the biological and biochemical processes in the soil is generally acknowledged (Nielsen and Winding, 2002). In case of glyphosate contamination, the soil microbes are the main biological agents in bioremediation (Ermakova et al., 2010). Indeed, glyphosate degradation occurs quite rapidly in the presence of microorganisms but not in sterile conditions (Friestad and Brønstad, 1985; Torstensson, 1985; Giesy et al., 2000; Busse et al., 2001; Vereecken, 2005). At the recommended application rates and even at moderately increased concentrations (up to 100× field rate) glyphosate has generally been found harmless to soil microorganisms (Olson and Lindwall, 1991; Stratton and Stewart, 1992; Ratcliff et al., 2006; Zabaloy et al., 2012). Moreover, soil microorganisms can use glyphosate as an alternative phosphorus source (Borggaard and Gimsing, 2008).

In the current study, the approach based on the estimation of the number of viable aerobic heterotrophic bacteria (HB) by plate count method and by microscopic counting of total bacterial numbers (TBN) was used for the evaluation of the impact of Roundup formulations on soil microbes. The number of HB in the soils depended on the exposure time since treatment and on the applied Roundup formulation. The mean number of viable HB in non-treated (control) soil during the four month exposure (April 27 to August 21, 2012) was quite stable, ranging from $20{\cdot}10^6$ to $9{\cdot}10^6$ CFU g^{-1} dry soil (Table 3). The results are comparable to the data presented by other authors for unpolluted soils (Grayston et al., 2004; Lawlor et al., 2000; Black et al., 2003). The total heterotrophic bacterial numbers, evaluated by viable cell counts, had a tendency to increase in Roundup-exposed soils even in case of the highest application rates of the tested herbicide (Table 3). In case of the recommended field rate of Roundup formulations the number of HB in soils 10 days after the treatment was only slightly higher (2–4 times) than in the control soil. Remarkable increase in the number of HB (45 to 48-fold) was observed at the 100-fold and 300-fold recommended field rate of Roundup Quick™ at 10 and 21 days after the treatment, respectively. In the soil treated with Roundup Max™ the increase in the number of HB was maximum at the 1000× recommended field application rate after 10 days (4 fold) and at the 300-fold recommended field rate after 45 days (5 fold) when compared to the control soil (Table 3). Significantly higher number of HB (10 days after the exposure) in soil treated with Roundup Quick™ compared to Roundup Max™ indicates different impact of two formulations on soil microbial community (Supplementary material, Fig. S5). Thus, the impact of the glyphosate-based herbicides on the soil bacterial number depended not only on the concentration of glyphosate (the active substance), but even more on the additives, i.e. on the full composition of the herbicide formulations.

It should be mentioned that some microbial species involved in the glyphosate degradation are unable to grow *in vitro* and form visible colonies on the standard nutrient agar (Forlani et al., 1999). As a result, only part of soil bacteria can be enumerated by the colony forming units (CFU). In the current study, about 0.1% of the soil bacteria that were estimated by the direct microscopic counting were capable to grow/form colonies on the Difco™ Plate Count Agar (PCA) medium. Therefore, direct fluorescence microscopic counting of total bacteria number (TBN) was additionally used for the enumeration of the bacteria at the end of the experiment

Table 3
The effect of Roundup Max™ and Roundup Quick™ on the soil bacteria.

Spiked concentrations of Roundup		Number of viable aerobic heterotrophic bacteria, HB (CFU g^{-1})[a] 10^6 g^{-1} dry soil				Total number of bacteria, TBN (cells g^{-1})[b] 10^6 g^{-1} dry soil
		10 d[c]	21 d[c]	45 d[c]	108 d[c]	108 d[c]
Roundup Max™	As recommended (245 mg m^{-2})	33.8 ± 2.8*	14.0 ± 0.1	27.6 ± 2.1*	8.6 ± 2.1	27.3 ± 2.5
	100× as recommended	39.9 ± 3.7*	26.9 ± 1.3*	48.4 ± 2.8*	8.6 ± 0.4	25.7 ± 1.7*
	300× as recommended	37.9 ± 1.4*	25.6 ± 1.1*	76.4 ± 3.1*	7.6 ± 0.3	24.0 ± 1.1*
	1000× as recommended	77.3 ± 1.5*	21.3 ± 1.4	52.6 ± 0.9*	7.1 ± 0.1	22.7 ± 0.9*
Roundup Quick™	As recommended (245 mg m^{-2})	85.1 ± 5.6*	20.0 ± 1.4	28.7 ± 0.8*	9.3 ± 0.2	22.4 ± 1.4*
	100× as recommended	953.0 ± 56*	256.2 ± 14*	91.0 ± 4.8*	15.2 ± 0.1*	24.3 ± 1.5*
	300× as recommended	360.8 ± 28*	708.6 ± 35*	134.6 ± 11*	24.1 ± 3.2*	22.5 ± 0.8*
Control	–	19.8 ± 2.8	15.8 ± 3.2	14.0 ± 3.7	8.5 ± 2.2	34.8 ± 3.6

[a] The number of viable aerobic heterotrophic bacteria (HB) in the soil was obtained by plate counts using Difco™ Plate Count Agar (PCA) and designated as colony forming units (CFU).
[b] The total number of bacteria in the soil (TBN) was analysed by fluorescence microscopy.
[c] Days passed from the spiking of the soil with indicated Roundup formulations.
* Statistically significant ($p < 0.05$) difference from the control.

(108 days after the spiking, Table 3). The microscopically evaluated total bacterial numbers in treated soils were slightly lower than in the control soil after 108 days of the soil treatment with the Roundup formulations.

3.2.2. *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*). In addition to shoot growth inhibition, visible changes in morphological traits (colour and shape) of the plants were evaluated (data not shown). According to the producer's information, seeding of vegetables should take place 21 days after application of the glyphosate-based formulation (Monsato, 2002), therefore plant tests were performed 24, 47, 68, 82 and 110 days after soil treatment with the Roundup formulations.

Our experiments showed that barley was slightly more sensitive to Roundup formulations than red radish (Figs. 3 and 4). Therefore, the tests with radish were performed only twice (24 and 47 days after the spiking of soil with Roundup formulations) and further effects of soil treatment with Roundup formulations were evaluated with barley.

Results of the plant tests demonstrated that the recommended dose of the Roundup formulations did not affect the plant growth of either test species from already 24 days after the soil treatment. However, residual toxicity of soils treated with 100-fold and 300-fold doses disappeared only after 68 days and the toxic effect of the highest concentration of Roundup Max™ (1000-fold the recommended application rate) to barley after 110 days. In general, toxicity of Roundup Quick-spiked soils to barley decreased more rapidly than toxicity of Roundup Max-spiked soils (Fig. 4). The more rapid 'detoxification' of the Roundup-Quick treated soil compared with Roundup-Max was coherent with the higher bacterial numbers in the Roundup-Quick treated soil (Table 3).

In the view of the aforesaid, it could be concluded that in case of high (up to 1000-fold of recommended dose) contamination, the time needed for the decrease of glyphosate content in the soils to safe level may exceed the duration of vegetative period in climatic (temperate) conditions typical for Estonia. This conclusion is in agreement with the data on the effects of glyphosate on the soil microbes presented above.

In addition, as the two tested plant species (*R. sativus* and *H. vulgare*) showed different sensitivity to Roundup Max™ and Roundup Quick™ (Figs. 3 and 4), it is advisable to use at least two species from different families to monitor the remediation process at the contaminated sites.

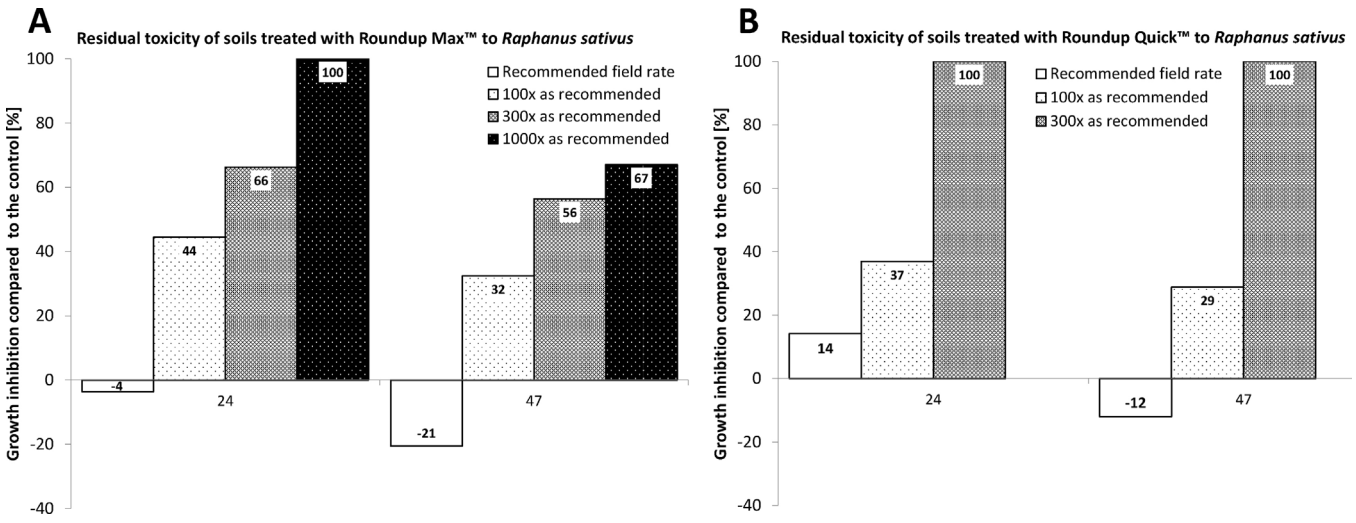

Fig. 3. Residual toxicity of soils treated with Roundup Max™ (A) and Roundup Quick™ (B) to red radish *Raphanus sativus*. Seeds of red radish were sown 24 and 47 days after the application of Roundup formulations.

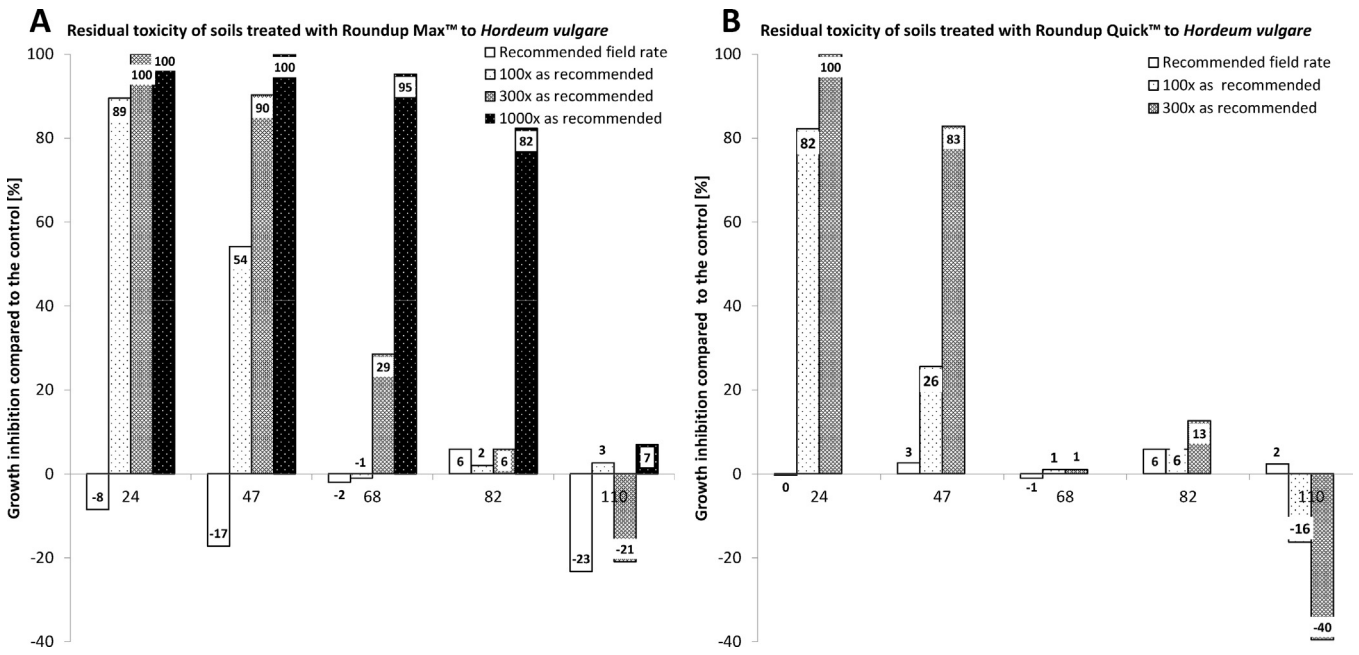

Fig. 4. Residual toxicity of soils treated with Roundup Max™ (A) and Roundup Quick™ (B) to barley *Hordeum vulgare*. Seeds of barley were sown 24, 47, 68, 82 and 110 days after the application of Roundup formulations.

The toxicity assessment of water leachates from soils helps to predict the risk of contamination of ground- and surface waters as well as to provide information on the potential hazard to organisms which may come into contact with soil water. 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, *D. magna* and *V. fischeri*. This shows that mobility of the glyphosate-based herbicides in soils with comparatively high content of organic matter is very low even in the case of high contamination. This is in agreement with the recent 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; Shipitalo et al., 2008).

4. Conclusions

The results of the current study demonstrated that the toxicity of the glyphosate-based herbicides to non-target aquatic (crustaceans and bacteria) and terrestrial organisms (soil bacteria and plants) vary within a wide range.

Short-term toxicity tests showed that non-target aquatic species were much more sensitive to glyphosate formulations than the tested soil microbial strains. The results from the tests with the three natural bacterial strains, isolated from soil, showed that the indigenous gram-positive soil bacteria are seemingly more sensitive to glyphosate based herbicides than the gram-negative bacteria.

The long-term outside experiments revealed the different effects of the two tested glyphosate formulations (Roundup Quick™ and Roundup Max™) on the soil bacteria that are the main biological detoxifiers of glyphosate in the soil.

Direct relation between the toxicity of the tested formulations and the presence/absence of surfactant POEA was not evident as tested species showed different effects. Roundup Quick™ (without POEA) was more toxic to aquatic bacteria *V. fischeri* but less toxic to soil bacteria strains and terrestrial plants than Roundup Max™ containing POEA. Thus, the difference in toxicity of the two investigated glyphosate products (Roundup Max™ and Roundup Quick™) depended not only on POEA addition but also on other additives used in the specific formulation. Therefore, when reporting results of toxicity testing of glyphosate formulation it is very important to provide the complete name of the tested product and all the possible information on its chemical composition.

In typical Estonian climatic conditions, characterised by short vegetative period and relatively long cold period, the time needed for self-remediation of the soils in case of accidental pollution by glyphosate formulations (e.g. more than 100-fold the recommended field rate) may exceed the duration of the vegetative period. The mobility of the glyphosate in soils proved very low. Thus, application of glyphosate-based herbicides may pose risk to aquatic ecosystem stability mostly in case of direct contamination of surface water.

Acknowledgements

This research is supported by the Central Baltic INTERREG IV A Finnish-Estonian project: Risk Management and Remediation of Chemical Accidents (RIMA), Estonian targeted funding project (SF0690063s08) and basic funding of the National Institute of Chemical Physics and Biophysics.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2013.07.005.

References

BaltActHaz, 2011. Report on hazardous substances screening results in the aquatic environment of Estonia. In: Completed within the project LIFE07 ENV/EE/000122 "Baltic Actions for Reduction of Pollution of the Baltic Sea from Priority Hazardous Substances". BaltActHAz, Tallinn, Estonia.

BaltActHaz, 2012. Proposals for the Estonian State Monitoring Programme 2012. In: Completed within the project LIFE07 ENV/EE/000122 "Baltic Actions for Reduction of Pollution of the Baltic Sea from Priority Hazardous Substances" (BaltActHAz). Estonian Environmental Research Centre, Tallinn, Estonia.

Battaglin, W.A., Rice, K.C., Focazio, M.J., Salmons, S., Barry, R.X., 2009. The occurrence of glyphosate, atrazine, and other pesticides in vernal pools and adjacent streams in Washington, DC, Maryland, Iowa, and Wyoming, 2005–2006. Environ. Monit. Assess. 155, 281–307.

Black, H.I.J., Parekh, N.R., Chaplow, J.S., Monson, F., Watkins, J., Creamer, R., Potter, E.D., Poskitt, J.M., Rowland, P., Ainsworth, G., Hornung, M., 2003. Assessing soil biodiversity across Great Britain: national trends in the occurrence of heterotrophic bacteria and invertebrates in soil. J. Environ. Manage. 67, 255–266.

Bonnet, J.L., Bonnemoy, F., Dusser, M., Bohatier, J., 2007. Assessment of the potential toxicity of herbicides and their degradation products to non-target cells using two microorganisms, the bacteria *Vibrio fischeri* and the ciliate *Tetrahymena pyriformis*. Environ. Toxicol. 22, 78–91.

Borggaard, O.K., Gimsing, L., 2008. Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. Pest Manag. Sci. 64, 441–456.

Buhl, K.J., Faerber, N.L., 1989. Acute toxicity of selected herbicides and surfactants to larvae of the midge *Chironomus riparius*. Arch. Environ. Contam. Toxicol. 18, 530–536.

Busse, M.D., Ratcliff, A.W., Shestak, C.J., Powers, R.F., 2001. Glyphosate toxicity and the effects of long-term vegetation control on soil microbial communities. Soil Biol. Biochem. 33, 1777–1789.

CCME, 2012. Canadian water quality guidelines: glyphosate. In: Scientific Criteria Document. Canadian Council of Ministers of the Environment, Winnipeg. ISBN 978-1-896997-83-4 PDF, Available at: http://www.ccme.ca/assets/pdf/pn_1469_cwqg_glyphosate_scd_1.0.pdf (accessed 17.12.12).

Chang, J.C., Taylor, P.B., Leach, F.R., 1981. Use of the Microtox assay system for environmental samples. Bull. Environ. Contam. Toxicol. 26, 150–156.

Dibyendu, N.R., Samir, K.K., Satinath, B., Douglas, A.C., Dean, G.T., Raj, P., 1989. Persistence, movement and degradation of glyphosate in selected Canadian boreal forest soils. J. Agric. Food Chem. 37, 437–440.

EC, 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for the Community action in the field of water policy. In: Official Journal of the European Union L 327. European Commission, Brussels, Belgium.

EC, 2002. Review report for the active substance glyphosate, 6511/VI/99-final, 21 January 2002. European Commission, Health & Consumer Protection Directorate-General, Brussels, Belgium.

EC, 2009. Regulation No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. In: Official Journal of the European Union L 309/1. European Commission, Brussels, Belgium.

Edginton, A.N., Sheridan, P.M., Boermans, H.J., Thompson, D.G., Holt, J.D., Stephenson, G.R., 2004. A comparison of two factorial designs, a complete 3×3 factorial and a central composite rotatable design, for use in binomial response experiments in aquatic toxicology. Arch. Environ. Contam. Toxicol. 46, 216–223.

EPA, 2011. Pesticides Industry Sales and Usage: 2006 and 2007 Market Estimates. In: Grube, Arthur, Donaldson, Davis, Kiely, Timothy, Wu, La (Eds.), Biological and Economic Analysis Division, Office of Pesticide Programs, Office of Chemical Safety and Pollution Prevention. U.S. Environmental Protection Agency, Washington, DC, USA (February).

Ermakova, I.T., Kiseleva, N.I., Shushkova, T., Zharikov, M., Zharikov, G.A., Leontievsky, A.A., 2010. Bioremediation of glyphosate-contaminated soils. Appl. Microbiol. Biotechnol. 88, 585–594.

Estonian Agricultural Board, 2012. Sales of Plant Protection Products in Estonia, Available at: http://www.pma.agri.ee/index.php?id=104&sub=132&sub2=247 (accessed 17.12.12).

Folmar, L.C., Sanders, H.O., Julin, A.M., 1979. Toxicity of the herbicide glyphosate and several of its formulations to fish and aquatic invertebrates. Arch. Environ. Contam. Toxicol. 8, 269–278.

Forlani, G., Mangiagalli, A., Nielsen, E., Suardi, C.M., 1999. Degradation of the phosphonate herbicide glyphosate in soil: evidence for a possible involvement of unculturable microorganisms. Soil Biol. Biochem. 31, 991–997.

Friestad, H.O., Brønstad, J.O., 1985. Improved polarographic method for determination of glyphosate herbicide in crops, soil, and water. J. Assoc. Off. Anal. Chem. 68, 76–79.

Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological risk assessment for roundup herbicide. Rev. Environ. Contam. Toxicol. 167, 35–120.

Gimsing, A.L., Borggaard, O.K., Jacobsen, O.S., Aamand, J., Sørensen, J., 2004. Chemical and microbiological soil characteristics controlling glyphosate mineralisation in Danish surface soils. Appl. Soil Ecol. 27, 233–242.

Govindarajulu, P.P., 2008. Literature Review of Impacts of Glyphosate Herbicide on Amphibians: What Risks can the Silvicultural Use of this Herbicide Pose for Amphibians in B.C.? B.C. Ministry of Environment, Victoria, BC (Wildlife Report No. R-28).

Grayston, S.J., Campbell, C.D., Bardgett, R.D., Mawdsley, J.L., Clegg, C.D., Ritz, K., Griffiths, B.S., Rodwell, J.S., Edwards, S.J., Davies, W.J., Elston, D.J., 2004. Assessing shifts in soil microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. Appl. Soil Ecol. 25, 63–84.

Guilherme, S., Santos, M.A., Barroso, C., Gaivão, I., Pacheco, M., 2012. Differential genotoxicity of Roundup® formulation and its constituents in blood cells of fish (*Anguilla anguilla*): considerations on chemical interactions and DNA damaging mechanisms. Ecotoxicology 21, 1381–1390.

Hernando, M.D., De Vettori, S., Martínez Bueno, M.J., Fernández-Alba, A.R., 2007. Toxicity evaluation with *Vibrio fischeri* test of organic chemicals used in aquaculture. Chemosphere 68, 724–730.

Howe, C.M., Berrill, M., Pauli, B.D., Helbing, C.C., Werry, K., Veldhoen, N., 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ. Toxicol. Chem. 23, 1928–1938.

ISO, 2010. Water quality—Kinetic determination of the inhibitory effects of sediment, other solids and coloured samples on the light emission of *Vibrio fischeri* (kinetic luminescent bacteria test). International Organization for Standardization, Geneva, Switzerland (ISO 21338:2010).

James, C., Krattiger, A.F., 1996. Global review of the field testing and commercialization of transgenic plants 1986 to 1995: the first decade of crop biotechnology. In: International Service for the Acquisition of Agri-biotech Applications (ISAAA) Briefs No. 1. ISAAA, Ithaca, NY, pp. 31, Available at: http://www.isaaa.org/kc/Publications/pdfs/isaaabriefs/Briefs%201.pdf (accessed 17.12.12).

Kahru, A., Tomson, K., Pall, T., Külm, I., 1996. Study of toxicity of pesticides using luminescent bacteria *Photobacterium phosphoreum*. Water Sci. Technol. 33, 147–154.

Kasemets, K., Suppi, S., Künnis-Beres, K., Kahru, A., 2013. Toxicity of CuO nanoparticles to yeast *Saccharomyces cerevisiae* BY4741 wild-type and its nine isogenic single-gene deletion mutants. Chem. Res. Toxicol. 26, 356–367.

Koller, V.J., Fürhacker, M., Nersesyan, A., Mišík, M., Eisenbauer, M., Knasmueller, S., 2012. Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells. Arch. Toxicol. 86, 805–813.

Kreuger, J., Asp, J., 2005. Environmental Quality Standards (EQS) for pesticides. In: Seminar on Glyphosate and Water. Dept. Soil Sciences, Div. Water Quality Management, SLU, Uppsala, Sweden (30.09.05, Brussels).

Kurvet, I., Ivask, A., Bondarenko, O., Sihtmäe, M., Kahru, A., 2011. LuxCDABE—Transformed constitutively bioluminescent *Escherichia coli* for toxicity screening: comparison with naturally luminous *Vibrio fischeri*. Sensors 11, 7865–7878.

Laitinen, P., Rämö, S., Nikunen, U., Jauhiainen, L., Siimes, K., Turtola, E., 2009. Glyphosate and phosphorus leaching and residues in boreal sandy soil. Plant Soil 323, 267–283.

Laitinen, P., Siimes, K., Eronen, L., Rämö, S., Welling, L., Oinonen, S., Mattsoff, L., Ruohonen-Lehto, M., 2006. Fate of the herbicide glyphosate, glufosinateammonium, phenmedipham, ethofumesate and metamitron in two Finnish arable soils. Pest Manag. Sci. 62, 473–491.

Lawlor, K., Knight, B.P., Barbosa-Jefferson, V.L., Lane, P.W., Lilley, A.K., Patond, G.I., McGrath, S.P., O'Flaherty, S.M., Hirsch, P.R., 2000. Comparison of methods to investigate microbial populations in soils under different agricultural management. FEMS Microbiol. Ecol. 33, 129–137.

Mann, R.M., Bidwell, J.R., 1999. The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. Arch. Environ. Contam. Toxicol. 36, 193–199.

Maves, 2010. Euroopa Parlamendi ja Nõukogu 6. detsembri 2008 direktiivi 2008/105/EÜ nõuete täitmiseks prioriteetsete ainete inventuur ning seire korralduse analüüs (Koostaja Tamm, I.). MAVES, Tallinn, Töö nr 10028. Available at: http://www.envir.ee/../Direktiivi+2008_105_EÜ+prioriteetsete+ainete+inventuur.pdf (accessed 17.12.12).

McFeters, G.A., Bond, P.J., Olson, S.B., Tchan, Y.T., 1983. A comparison of microbial bioassays for the detection of aquatic toxicants. Water Res. 17, 1757–1762.

Monsato Europe S.A., 2000. Roundup Quick, Safety Data Sheet, Commercial Product.

Monsato Europe S.A., 2010. Roundup Max, Safety Data Sheet, Commercial Product.

Monsato, 2002. Roundup Max: Quick Guide to Weed Control, Available at: http://www.monsanto.co.za/af/content/products/labels/glyphosates/roundupmax.pdf (accessed 17.12.12).

Monsato, 2005. Backgrounder: Glyphosate Half-Life in Soil, Available at: http://www.monsanto.com/products/Documents/glyphosate-background-materials/gly_halflife_bkg.pdf (accessed 09.01.13).

2012. Monsato, Available at: http://www.monsanto.com/products/Pages/roundup-safety-background-materials.aspx (accessed 17.12.12).

Moore, L.J., Fuentes, L., Rodgers Jr., J.H., Bowerman, W.W., Yarrow, G.K., Chao, W.Y., Bridges Jr., W.C., 2012. Relative toxicity of the components of the original formulation of Roundup to five North American anurans. Ecotoxicol. Environ. Saf. 78, 128–133.

Mortimer, M., Kasemets, K., Kurvet, I., Heinlaan, M., Kahru, A., 2008. Kinetic *Vibrio fischeri* bioluminescence inhibition assay for study of toxic effects of nanoparticles and colored/turbid samples. Toxicol. In Vitro 22, 1412–1417.

Nandula, V.K., 2010. Glyphosate Resistance in Crops and Weeds: History, Development, and Management. John Wiley & Sons, Inc., Hoboken, New Jersey.

Nielsen, M.N., Winding, A., 2002. Microorganisms as Indicators of Soil Health. National Environmental Research Institute, Denmark, Technical Report No. 388. Available at: http://www2.dmu.dk/1_viden/2_publikationer/3_fagrapporter/rapporter/fr388.pdf (accessed 20.12.12).

OECD, 2004. Guideline for testing of chemicals. In: Test No. 202: Daphnia sp. Acute Immobilisation Test. Organisation for Economic Co-operation and Development, Paris, France.

Olson, B.M., Lindwall, C.W., 1991. Soil microbial activity under chemical fallow conditions: effects of 2,4-D and glyphosate. Soil Biol. Biochem. 23, 1071–1075.

Paganelli, A., Gnazzo, V., Acosta, H., López, S.L., Carrasco, A.E., 2010. Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling. Chem. Res. Toxicol. 23, 1586–1595.

Perkins, P.J., Boemans, H.J., Stephenson, G.T., 2000. Toxicity of glyphosate and triclopyr using the frog embryo teratogenesis assay – *Xenopus*. Environ. Toxicol. Chem. 19, 940–945.

Pimentel, D., 1995. Amounts of pesticides reaching target pests: environmental impacts and ethics. J. Agr. Environ. Ethic. 8, 17–29.
Põllumaa, L., Kahru, A., Eisenträger, A., Reiman, R., Maloveryan, A., Rätsep, A., 2000. Toxicological investigation of soil with the solid-phase flash assay: comparison with other ecotoxicological tests. ATLA 28, 461–472.
Ratcliff, A.W., Busse, M.D., Shestak, C.J., 2006. Changes in microbial community structure following herbicide (glyphosate) additions to forest soils. Appl. Soil Ecol. 34, 114–124.
Register of Plant Protection Products, 2012. Public Registers of Agricultural Board: Register of Plant Protection Products (TKV), Available at: http://saku.pma.agri.ee:22001/jisweb/forms/mainframe.htm (accessed 17.12.12).
Servizi, J.A., Gordon, R.W., Martens, D.W., 1987. Acute toxicity of Garlon 4 and Roundup herbicides to salmon, Daphnia and trout. Bull. Environ. Contam. Toxicol. 39, 15–22.
Shipitalo, M.J., Malone, R.W., Owens, L.B., 2008. Impact of glyphosate-tolerant soybean and glufosinate-tolerant corn production on herbicide losses in surface runoff. J. Environ. Qual. 37, 401–408.
Steinrücken, H.C., Amrhein, N., 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic acid-3-phosphate synthase. Biochem. Biophys. Res. Commun. 94, 1207–1212.
Stenrød, M., Eklo, O.M., Charnay, M.P., Benoit, P., 2005. Effect of freezing and thawing on microbial activity and glyphosate degradation in two Norwegian soils. Pest Manag. Sci. 61, 887–898.
Stratton, G.W., Stewart, K.E., 1992. Glyphosate effects on microbial biomass in a coniferous forest soil. Environ. Toxicol. Water Qual. 7, 223–236.
Tomlin, C.D.S., 2006. The Pesticide Manual: A World Compendium, 14th ed. British Crop Protection Council, Hampshire, UK.
Torstensson, L., 1985. Behavior of glyphosate in soils and its degradation. In: The Herbicide Glyphosate. Swedish University of Agricultural Sciences, Uppsala, Sweden, pp. 137–150 (chap. 9).
Tsui, M.T., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52, 1189–1197.
U.S. EPA, 1993. Re-registration Eligibility Decision (RED): Glyphosate, EPA-738-F-93-011, September 1993. United States Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Washington, DC, USA.
UK GM Science Review panel, 2003. GM Science Review First Report. Prepared by the UK GM Science Review panel. Chairman Professor Sir David King, Chief Scientific Advisor to the UK Government, pp. 9.
UKTAG, 2012. UK Technical Advisory Group on the Water Framework Directive. Proposals for further environmental quality standards for specific pollutants, Available at: http://www.wfduk.org/sites/default/files/Media/UKTAG%20Specific%20Pollutants_final_011412.pdf (accessed 17.12.12).
Vencill, W.K. (Ed.), 2002. Herbicide Handbook. , 8th ed. Weed Science Society of America, Lawrence, KS, USA.
Vereecken, H., 2005. Mobility and leaching of glyphosate: a review. Pest Manag. Sci. 61, 1139–1151.
Vindimian, E., 2009. MSExcel macro REGTOX_EV7.0.6.xls, Available at: http://www.normalesup.org/~vindimian/(accessed 01.02.13).
WHO, 1996. Data Sheets on Pesticides: Glyphosate; International Programme on Chemical Safety. World Health Organization, Food and Agriculture Organization, Geneva, Switzerland.
Williams, G.M., Kroes, R., Munro, I.C., 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul. Toxicol. Pharmacol. 31, 117–165.
WRc Plc, 2009. Survey of glyphosate and AMPA in groundwaters and surface waters in Europe. In: Horth, H., Blackmore, K. (Eds.), A Review has Undertaken on Behalf of Monsanto Europe. WRc Plc, Swindon, England (Report No. UC8073.02. November 2009).
Zabaloy, M.C., Gómez, E., Garland, J.L., Gómez, M.A., 2012. Assessment of microbial community function and structure in soil microcosms exposed to glyphosate. Appl. Soil Ecol. 61, 333–339.
Zimmermann, R., 1977. Estimation of bacterial number and biomass by epifluorescence microscopy and scanning electron microscopy. In: Rheinheimer, G. (Ed.), Microbial Ecology of the Brackish Water Environment. Ecol. Stud. 25, Berlin, pp. 103–120.