



Does application of the herbicide glyphosate promote priming effects in soil?

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Abstract

Priming effects have gotten revived interest in the last decade due to the concerns of global warming. Priming are defined as an increase in decomposition of soil organic matter due to the addition of organic or mineral substances. The worldwide used herbicide glyphosate is rapidly degraded by microorganisms. Hence, it might give rise to priming effects in soils. This was tested by adding glyphosate in three different concentrations (0.5, 5 and 50 $\mu\text{g/ g}$ soil) as well as one control (no addition) to a grassland soil. The soil was partly amended with cellulose to obtain observations in both C- and N-limited soils. The SOM mineralization rate was found to increase significantly at the concentration of 5 μg glyphosate/ g soil. The relative increase compared to control was 26% in the C-limited soil and 9% and 12% in the two N-limited soils. Priming was only observable during the first 24 hours. Based on these findings, glyphosate seem to be able to cause priming effects in soils. The relative increase in SOM mineralization was not remarkably high and the effects were found to be short term. Nevertheless, the herbicide is believed to have a possible significance on the CO_2 -flux from soil to the atmosphere. The mineralization rate of N in the soil organic matter was not found to increase by glyphosate addition. In contrast, a trend for decreasing mineralization rate was found in the C-limited soil.

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1. Introduction

Soil contains the largest carbon pool in terrestrial environments (Thiessen et al. 2013) and provides microorganisms with a substantial energy source. Soils are able to serve as either a sink or a source for atmospheric CO₂ (Fontaine et al. 2003), meaning that there is either a net amount of CO₂ sequestered to or released from the soil. According to Thiessen et al. (2013) the concentration of atmospheric CO₂ could change dramatically by a slight turnover of soil organic matter (SOM). If the SOM decomposition increases this could result in a depletion of soil C stock, while if the decomposition decreases it could instead result in an increasing C stock (Thiessen et al. 2013) However, an increase in SOM decomposition could also result in the release of plant available N, which could promote plant growth and C sequestration (Bengtson et al. 2012), lowering the atmospheric carbon. Additional mineralization could also bring about gaseous N losses and leaching of NO₃⁻, if N is abundant in the soil (Kuzyakov et al. 2000).

Soil contains a mix of easily biodegradable and recalcitrant organic structures. Fresh organic matter (FOM), e.g. plant litter and root exudates are considered rather easily biodegradable and among the more recalcitrant material is soil with no longer distinguishable components, referred to as SOM (Thiessen et al. 2013). Microbial respiration is responsible for a large part of the C flux from the biosphere to the atmosphere (Schlesinger and Andrews 2000). It is believed that bacteria are restrained when it comes to degrading SOM and that they need access to fresh C or N sources to be able to deal with the SOM (Fontaine et al. 2007, Bengtson et al. 2012, and Kuzyakov et al. 2000). Many studies have shown that the decomposition of SOM increases when such easily available material are added to soil. These, so called priming effects, were e.g. found by; Hamer and Marschner (2005a) after fructose and alanine addition to soil, Thiessen et al. (2013) after litter addition, Lou et al. (2011) after biochar addition and Bengtson et al. (2012), who found that root exudation results in priming. It is important to distinguish between real and apparent priming effects. Real priming involves the degradation of SOM while apparent priming only is a result of a higher microbial turnover rate, due to an increased C and N mineralization (Blagodatskaya and Kuzyakov 2008, Bengtson et al. 2012). Another distinction that has to be clarified is that of positive and negative priming. Positive priming is when the SOM decomposition is accelerated by the addition of different easily available C and N structures and negative priming is when the decomposition of SOM is reduced (Kuzyakov et al. 2000). The reduction could be explained by different causes, e.g. that; the added substance has a toxic impact on the microorganisms, it becomes unavailable due to adsorption on soil particles or that the microorganisms prefers the more easily available substance over SOM (Hamer and Marschner 2005a).

The exact mechanisms of priming are not fully unraveled. There are, however, two different mechanisms that are more commonly brought up in the literature, when trying to explain the phenomena. The first mechanism has to do with co-metabolism of SOM. This means that microorganisms use the energy from more easily degradable compounds to increase their growth and enzyme production, and with the use of these enzymes they are able to degrade more recalcitrant SOM structures (Kuzyakov and Blagodatskaya 2008, Hamer and Marschner

2005a). The second mechanism is elaborated by Fontaine et al. (2003) who argue that priming effects is caused by the competition for energy and nutrients between r- and k-strategic microorganisms. The r-strategists dominates at the initial stage, when FOM is abundant, but as that easily available energy source decline, the k-strategists become predominant that is more specialized in decomposing more recalcitrant material.

The objective of this study is to determine if the herbicide glyphosate causes priming of SOM. Glyphosate (N-(phosphonomethyl)glycine), with the trade name Roundup, is a foliar herbicide used on crops for weed management (Simonsen et al. 2008). It was introduced on the market in 1974 (Duke and Powles 2008) and has since become the most commonly used herbicide worldwide (Helander et al. 2012, Duke et al. 2012). The wide use is due to several reasons such as; glyphosate being active on a broad range of plant species (Duke and Powles 2008), the development of an extensive adoption of glyphosate resistant (GR) crops (Helander et al. 2012) and the fact that it is considered fairly environmental friendly (Duke et al. 2012). The reason for the latter is that glyphosate does not persist long in the environment, the activity of the substance in soil is very low because of its strong binding to soil particles, and it has shown a low toxicity to mammals (Duke et al. 2012). The mode of action of glyphosate is that it causes the inhibition of an enzyme of the shikimate metabolic pathway which only exists in green plants and certain fungal and bacterial strains, wherefore, glyphosate is considered harmless to non-target organisms, with the few exceptions of the two latter mentioned groups (Helander et al. 2012, Duke et al. 2012).

The common use rate of glyphosate per application is somewhere between 0.5 and 2.0 kg/ha, with often repeated application during a growing season (Duke et al. 2012). Since glyphosate is sprayed on the leaves of the plants, a considerable amount of the substance does not reach the soil. However, the soil will also be exposed of glyphosate through foliage wash off during rain or irrigation (Mamy and Barriuso 2005), exudation via plant roots and the release from decomposing plant tissue (Duke et al. 2012). The distribution of glyphosate in soil does not become uniform, but is more concentrated in the top soil (Duke et al. 2012). When glyphosate reaches the soil, a substantial fraction may bind to soil particles, resulting in low leaching potential of the herbicide (Simonsen et al. 2008, Duke et al. 2012). The adsorption increases with decreasing pH and is negatively correlated to the phosphate content in the soil, because glyphosate compete with phosphate for the binding sites on soil particles, as it is the phosphonic part of the glyphosate molecule that is adsorbed (Mamy and Barriuso 2005, Duke et al. 2012). The copper content, as well as the iron and aluminum oxides content, also increase glyphosate adsorption (Mamy and Barriuso 2005). In a study by Mamy and Barriuso (2005) the adsorption was most prominent in the first two hours and stabilized within 24 hours. After 24 hours the adsorption decreased, which was ascribed to the degradation of glyphosate to aminomethylphosphonic acid (AMPA), the main degradation product, which was assumed to be a weaker adsorbent.

Rueppel et al. (1997) found that microorganisms are responsible for the degradation of glyphosate and that mechanical degradation could be considered as insignificant. Both aerobic and anaerobic bacteria are able to degrade glyphosate and it is shown to be a rather rapid

process (Rueppel et al. 1997). Some strains of bacteria have been found to be able to grow on phosphonates as the sole source of carbon, nitrogen and phosphorus (Krzysko-Lupika and Orlik 1997). It has also been shown that glyphosate can be degraded by some strains of fungi. AMPA as well as other degradation products, e.g. glyoxylate and sarcosine, have also been found to promote growth of microorganisms (Duke et al. 2012). In ^{14}C -labeled glyphosate fate studies it has been found that the microbial respiration begins directly after glyphosate application to soil, i.e. there is no lag phase, indicating that most soil contain microorganisms that are able to degrade the herbicide (Duke et al. 2012). The metabolic degradation pathway for glyphosate is presented in figure 1 (from Duke et al. 2012).

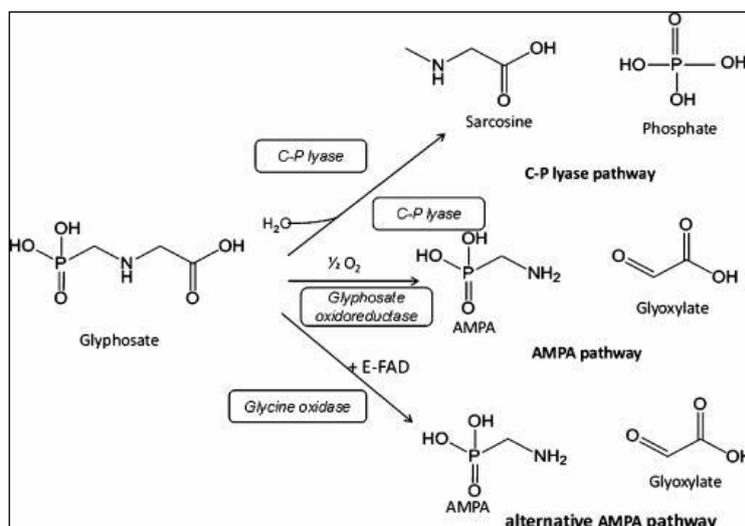


Figure 1: Metabolic degradation pathway for glyphosate (Duke et al. 2012)

The estimated half-life (DT_{50}) of glyphosate and its metabolite AMPA varies somewhat between different studies. Simonsen et al. (2008) found a DT_{50} of glyphosate of 9 days and for AMPA 32 days, according to IPCS (1996) the DT_{50} of glyphosate is 60 days and Bergström et al. (2011) found it to be as long as 110 to 151 days for glyphosate and 35 to 98 days for AMPA, in samples from a clay soil. However, Bergström et al. (2011) found it to be considerably shorter in a sandy soil, wherefore, they ascribed the longer degradation to adsorption, which is stronger in clayey soils. Other factors, which always control microbial degradation, are temperature and soil moisture (Thiessen et al. 2013). Even if most part of glyphosate degrades rather rapid, trace amounts of glyphosate have been found in soil after more than two years from application (Simonsen et al. 2008).

Since glyphosate appears to be a rather easily available C and nutrient source for microorganisms it has the potential to induce priming effects in soil. Glyphosate is used extensively and all over the world which could result in considerable consequences if it is found to promote priming. An elevation of the atmospheric CO_2 is one of the concerns. Another aspect is that agricultural fields could be depleted of organic matter, which would lower the quality of the soil and possibly reduce crop yield. There could also be a problem of leaching of NO_3^- since these fields most often are fertilized, with N and P, resulting in a surplus of N which may not be taken up by plants.

The aim of this study was to find out if glyphosate promote priming in soils, thus, increase the decomposition of SOM. Priming effects have been found to increase in soils with low N availability, due to that microorganisms utilize SOM for N requirements (Sullivan and Hart 2013). Priming have also been found to increase with increasing additions of easily available C and nutrient sources (Paterson and Sim 2013). Therefore, it was hypothesized that if the soil was initially scarce in N the priming effects would be more prominent and the higher the addition of glyphosate the higher were the priming effects expected to be. The hypotheses were tested by adding glyphosate at different concentrations to C and N limited soils.

2. Materials and methods

2.1. Soil sampling and preparation

Soil was collected in February 2013 from a site in the south of Sweden, located near the Lake Bysjön in the community of Vomb. Sampling was made from the upper 20 centimeters of the soil, which is classified as a sandy grassland soil. The annual mean temperature at the site is approximately 7°C and the annual mean precipitation is around 700 mm (SMHI 2011). Stones and larger roots were removed from the collected soil by hand, whereupon, the soil was stored, in the dark at 4°C, until the start of the experiments.

2.2. Experiment design

Laboratory experiments were performed on soil microcosms. Sixty grams of soil was added to cans with volumes of about 1 dm³. The microcosms were then divided in three groups. The first group received 5 mg cellulose/ g soil, the second group 10 mg cellulose/ g soil and the last group was left untreated. The soils receiving cellulose were assumed to become N limited, with greater limitation ascribed to the higher dose, and the soil with no amendment was assumed to be C limited. In the following experiments, cellulose was considered a part of SOM. After addition of cellulose, the soil samples were kept in dark at room temperature (about 20°C) for incubation during two weeks. When necessary, water was added to the soils to keep the water content at 50% of the water holding capacity (WHC). After two weeks, glyphosate was added at three concentrations (see below). There were four replicates of all treatments. Soil property measurements were performed before the start of the actual experiments and on unagitated soil with four replicates per treatment.

2.3. Soil properties

2.3.1. pH

Mixtures of 20 ml of distilled water and 10 g of soil were mixed on a shaker board for three hours. The pH of the soil slurry was measured with a PHM92 Lab pH meter. The pH was 6.7.

2.3.2. Soil moisture and organic matter content

Fifteen g of soil was added to ceramic cups of known weight. The filled cups were weighed and put in oven, at 105°C for three hours, to eliminate water from the soil. The cups were weighed once more and then put in oven, at 600°C for four hours, to eliminate the SOM content after which a final weighing followed. The soil moisture could be calculated by taking the difference in weight between the untreated soil and soil that had been dried at 105°C. The

SOM content of the soil was calculated by taking the difference in weight between when dried at 105°C and dried at 600°C. The soil moisture was estimated to 13.0% and the SOM to 3.0%.

2.3.3. Water holding capacity

Funnels were prepared by adding filters and attaching plastic tubes, with sealed openings, to the narrow end of the funnels. An amount of 50 g of soil was added to the filters as well as approximately 50 ml of water, for saturation of the soil. The samples were left for 30 minutes to allow the soil to become completely saturated. The sealing was then opened and the samples were drained for 60 minutes. The moist soil were transferred to pre-weighed ceramic cups and weighed additional before going into oven to dry, at 105°C. The samples were left in oven over night and weighed again the next day. The WHC was estimated according to equation 1:

$$\frac{m_{\text{water in saturated soil}}}{m_{\text{saturated soil}}} \times 100 = WHC (\%) \quad (1)$$

where m is the mass weight, in grams. The WHC was calculated to 26.7%.

2.4. Addition of glyphosate

After the two week stabilization period, ¹³C-labeled glyphosate (5.3 atom% ¹³C) of three concentrations (0.5, 5 and 50 µg glyphosate/ g soil) were added to the microcosms. The lower concentration was supposed to resemble the recommended spraying dose, the intermediate dose a possible scenario if glyphosate persisted in soil between applications and the highest dose was chosen to see if glyphosate at all is able to promote priming in soil.

2.5. Soil respiration & priming effects

Soil respiration was measured at three occasions. The first measurement was performed immediately after addition of glyphosate, and the other two 3 and 6 days after the addition.

Approximately one gram of soil was added to 20 ml vials. Each vial was aerated with compressed air, for around 10 seconds, and sealed with a gas tight cap. The samples were left for incubation, overnight at 20°C. After incubation one ml of the air was transferred, using a syringe, to 12 ml exetainer vials filled with helium gas. The concentration and ¹³C/¹²C ratio of CO₂ in the exetainers were measured on a GasBench II connected to a Delta V Plus isotope-ratio mass spectrometer (Thermo Scientific Inc., Bremen Germany), at the stable isotope facility, Department of Biology, Lund University. The respiration rate was estimated by equation 2:

$$\frac{m_{CO_2}}{m_{soil} \times t} = \text{Respiration rate } (\mu\text{g CO}_2 / (\text{g soil} \times \text{days})) \quad (2)$$

where m_{CO₂} is the mass (µg) of CO₂, calculated from the CO₂ peaks obtained from the gas chromatograph, m_{soil} is the wet weight of used soil (g) and t is the time (days) of incubation.

To determine what fractions of respired CO₂ that originates from SOM and what part originates from glyphosate, an isotopic mixing model was used (equation 3):

$$\frac{(At\%^{13}C_{glyCO_2} - At\%^{13}C_{contrCO_2})}{(At\%^{13}C_{gly} - At\%^{13}C_{contrCO_2})} = f_{gly} \quad (3)$$

where f_{gly} is the fraction of soil CO₂ efflux derived from glyphosate, $At\%^{13}C_{glyCO_2}$ is the measured atom% ¹³C of soil CO₂ efflux from soils receiving glyphosate additions, $AT\%^{13}C_{contrCO_2}$ is the atom% ¹³C of CO₂ from soils not receiving glyphosate additions (SOM-derived), and $At\%^{13}C_{gly}$ is the atom% ¹³C of the applied glyphosate.

To estimate the level of priming, a relative SOM mineralization was calculated for all treatments by comparing the SOM mineralization rate for treatments receiving glyphosate with the controls. Since all of the respired CO₂ originates from SOM in the controls, divergence from this rate indicate a change in mineralization of SOM which is assumed to be induced by the treatment. A higher relative SOM mineralization indicates a positive priming effect and a lower mineralization a negative priming effect.

2.6. Gross N-transformation

To estimate the gross N-transformation rate the ¹⁵N-pool dilution technique was used, as described by IAEA (2001). In short, the technique is carried out by labeling the NH₄⁺-pool with a ¹⁵N-solution. The proportion of ¹⁵N and ¹⁴N in the pool is then determined, both directly after labeling and after a 24 h incubation. As the microorganisms mineralize organic N unlabeled ¹⁴NH₄⁺ will be formed and the proportion of the two isotopes in the NH₄⁺-pool will be altered. This alteration gives an estimation of the gross mineralization rate.

A duplicate set of cans (48x2) were filled with 15 g of soil. A ¹⁵N-solution (¹⁵NH₄Cl, 109 mg/L 99 atom% ¹⁵N) was added to each can in the amount of 0.5 ml, whereupon, the soil was mixed with help of a spoon. The first set was extracted immediately after the addition of the N-solution and the second set on the following day. Both sets of samples underwent the procedures described in the next two paragraphs.

Extraction of inorganic N was performed by filling each can with 50 ml of 1M KCl. The samples were put on a horizontal shaker, for 30 minutes, to allow NH₄⁺ and NO₃⁻, to move out into the solution. The extract was then filtered through a Whatman GF/F-filter and the filtrate collected in a new set of cans.

The first step for isolation of NH₄⁺ and NO₃⁻ from the soil extract was to make NH₄⁺ traps. Round filter pieces, about 5mm in diameter, were cut out using a paper punch. The filters were put on a strip of PTFE tape. To each filter piece, 10 µl of 2.5M KHSO₄ was added. An additional strip of PTFE tape was placed on top of the filters. The traps were sealed, without touching the filters, by help of the wide end of a 1 ml pipette tip. One trap and 0.2 g of MgO were added to the cans containing the KCl-extract. Lids were put on and the cans were incubated at 20°C for 6 days. After incubation, the filters were dried and then transferred to 5x8 mm tin cups, with help of a sterile pincer, for further analyses. The cans were left open for one week, to let NH₃ evaporate, followed by the addition of a new set of NH₄⁺ traps. To the cans were also added 0.2 g of MgO and 0.2 g of Devarda's alloy, which was used to convert the NO₃⁻ into NH₄⁺. The cans were closed and incubated, at 20°C for four days, after which the filters were transferred to tin cups as described above. The filters were analyzed for

total N concentration as well as $^{15}\text{N}/^{14}\text{N}$ ratios at the stable isotope facility at the Department of Biology, Lund University. Samples were flash-combusted in a Flash 2000 elemental analyzer, and the isotopic ratios determined by a Delta V Plus isotope-ratio mass spectrometer connected to the elemental analyzer via the ConFlow IV interface (Thermo Scientific Inc., Bremen Germany). Isotopic ratios of the samples were calibrated against standards of known isotopic composition (six replicates of each). The analytical precision obtained for the standards was $<0.2\%$. The gross N mineralization and nitrification were calculated using Fluaz (Mary et al. 1998).

2.7. Bacterial growth

Bacterial growth was measured by using the homogenization-centrifugation technique followed by incorporation of labeled ^3H -leucine into bacterial proteins (Bååth 1994). Due to the substance being radioactive, the protein synthesis could be measured which gave an estimation of bacterial growth. The bacterial growth was estimated at three times, 1, 3 and 6 days after addition of glyphosate.

The initial step was to extract bacteria from the soil. Approximately one gram of soil was added to centrifuge tubes and mixed with 20 ml of distilled water. The soil slurry was mixed by vortex, for three minutes at 2000rpm, followed by centrifugation for ten minutes at 3000rpm (1000 x g). The mixing of the soil slurry caused the bacteria to move out into the soil solution. A supernatant was formed, containing the bacteria, from which 1.5 ml was transferred to eppendorf-vials.

After the extraction, the leucine incorporation step followed. To each eppendorf-vial an amount of 20 μl of [^3H]-leucine was added. This was mixed by vortex, for about five seconds, after which the vials were left for incubation, at 20°C for two hours. When incubation was finished, 75 μl of 100% trichloroacetic acid (TCA) was added to each vial. The samples were mixed by vortex, followed by centrifugation for eight minutes at 13 000rpm. The supernatant was removed with help of a pasteur-pipette connected to the aspiration system. Seventy five μl of 5% TCA was then added to each vial after which they were mixed by vortex for 5 seconds. The vials were centrifuged and the supernatant removed from the vials, as described above. An amount of 1.5 ml 80% ethanol was added to the vials followed by mixing through vortex, for 5 seconds, and centrifugation, for 8 minutes at 13 000rpm. The formed supernatant was removed once more. Furthermore, 0.2 ml of 1M NaOH was added to the vials. The samples were mixed by vortex until the formed pellet had dissolved and were then placed in oven, for 30 minutes at 90°C. To cool the samples, they were put in freezer for 10 minutes. A scintillation cocktail was added to each vial, in an amount of one milliliter, and mixed through vortex until the solution became clear. Finally, the samples were placed in scintillation vials for the determination of [^3H]-radioactivity which was measured by a Beckman LS6500 Multipurpose Scintillation Counter.

In equation 4, the rate of leucine uptake by bacteria is given (expressed as the amount pmol leucine per gram of soil and hour) and this represents the bacterial growth rate:

$$\frac{DPM \times 9.4764e^{-5}}{V_{suspension} / V_{initial} \times m_{soil} \times t} = \text{Bacterial growth (pmol leucine / (g}_{soil} \times h)) \quad (4)$$

where DPM (disintegrations per minute) is the measurement of [³H]-radioactivity, 9.4764e⁻⁵ is a conversion constant, V_{suspension} is the volume (ml) of the supernatant transferred from the centrifuge tubes, V_{initial} is the volume (ml) of the soil slurry in the centrifuge tubes, m_{soil} is the wet weight (g) of added soil and t is the time (h) of incubation.

2.8. Statistical analyses

For statistical analyses the software program IBM SPSS Statistics 21 was used. Significant differences of means between treatments were analyzed for bacterial growth and soil respiration by an analysis of variance test (ANOVA). For an ANOVA to be reliable the variance of the means should preferably be equal between treatments. Since the data did not fulfill this assumption, the values were log₁₀-transformed which fitted the assumption better, even though, Levene's test of equality of error variance was still not fulfilled (P<0.05). However, the means were not correlated with the variances, and ANOVA's are considered to be robust in such conditions even if the variances are not identical (Lindman 1974). Fischer LSD was used for post hoc tests. To test the relationship between glyphosate additions and gross N mineralization linear regression was used. Before the analysis the glyphosate concentrations were (log₁₀+1)-transformed.

3. Results

3.1. Total soil respiration

The total rate of respired CO₂, stemmed from both SOM and glyphosate mineralization, was found to differ significantly between treatments with different glyphosate additions (Fig. 2) (ANOVA, P<0.05). The rate was considerably higher in soils treated with glyphosate at the concentration of 5 µg/g soil compared to 0.5 µg/ g soil and the control (Fischer LSD, P<0.01). Treatments receiving the highest concentration (50 µg/ g soil) showed no significant difference in total respiration from the other treatments, only a tendency towards being lower compared to those receiving 5 µg/ g soil. The respiration rate was significantly higher day 1 compared to day 3 and 6 (Fischer LSD, P<0.05).

A significant difference in respiration was found between soils with different cellulose amendments (Fig. 2) (ANOVA, P<0.001). Soils receiving cellulose in either concentrations of 5 and 10 mg/ g soil showed an increase in soil respiration compared to soil with no cellulose amendment (Fischer LSD, P<0.001). No difference was found between the two cellulose concentrations.

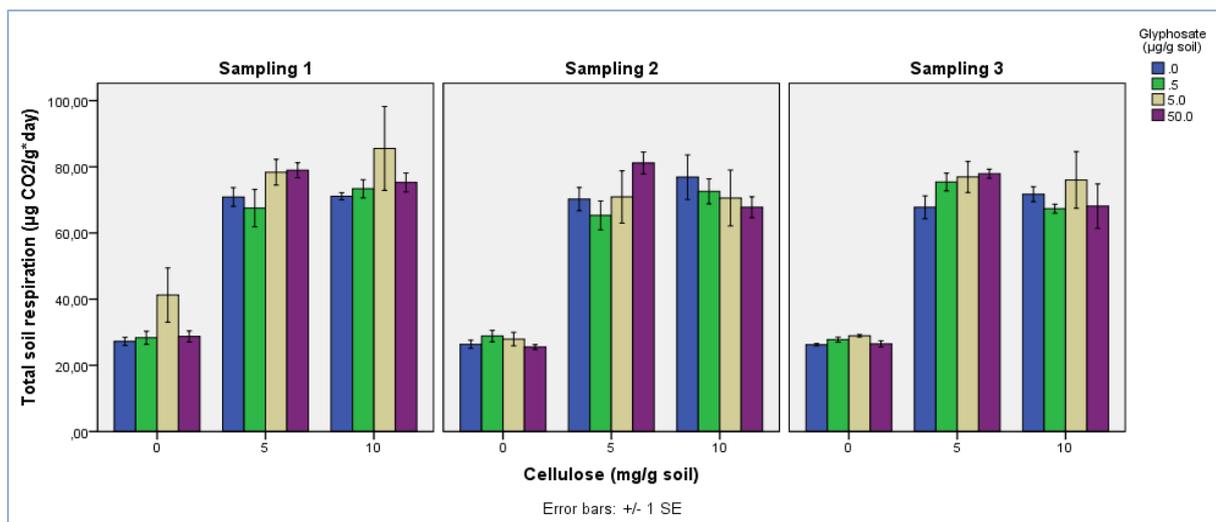


Figure 2: Mean total respiration rate estimated in soils with; three different cellulose amendments (0, 5 and 10 mg/ g soil), at three sampling periods (day 1, 3 and 6) and with all treatments receiving either of three concentrations of glyphosate (0.5, 5 or 50 µg/ g soil) or the control (no glyphosate addition). The total respiration rate includes both SOM and glyphosate mineralization rate. (n=4, for all treatments).

3.2. SOM mineralization and priming effects

When the total respiration was partitioned into SOM mineralization and glyphosate mineralization it was revealed that glyphosate additions caused a significant difference in the SOM mineralization rate (Fig. 3) (ANOVA, $P < 0.05$). The rate was significantly higher in treatments that received 5 µg/ g soil compared to treatments receiving any of the other concentrations, including the control (Fischer LSD, $P < 0.05$). This increased mineralization, in soils with 5 µg glyphosate/g soil, indicates that positive priming has occurred. The effect was prominent independently of the cellulose amendment, but was only detectable at the first sampling. The relative increase, compared to the control, was 26% in the soil with no cellulose amendment and 9% and 12% in the soils with 5 respective 10 mg/ g soil. Since the priming effects were only noticeable at the first sampling period it appears to be a short term effect.

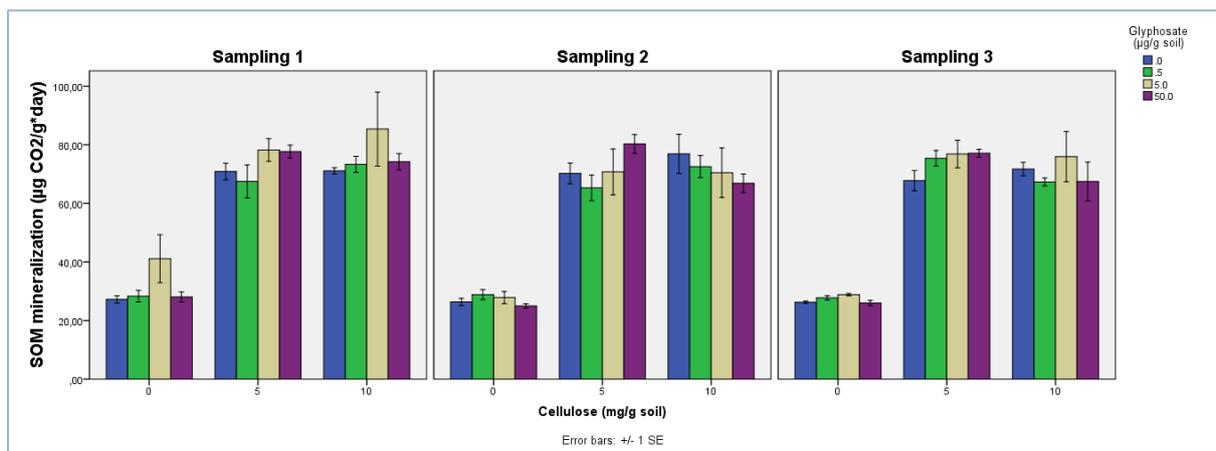


Figure 3: Mean SOM mineralization rate estimated in soils with; three different cellulose amendments (0, 5 and 10 mg/ g soil), at three sampling periods (day 1, 3 and 6) and with all treatments receiving either of three concentrations of glyphosate (0.5, 5 or 50 µg/ g soil) or the control (no glyphosate addition). (n=4, for all treatments)

3.3. Glyphosate mineralization

The glyphosate mineralization rate differed significantly between different glyphosate additions, cellulose additions and sampling periods (Fig. 4) (ANOVA, $P < 0.001$, for all three factors). The mineralization of glyphosate increased with increasing concentration. Highest rate was found at the first sampling period and then it decreased with each sampling (Fischer LSD, $P < 0.05$). A significantly increased mineralization rate was found when cellulose was added (Fischer LSD, $P < 0.001$), with no difference between the two concentrations (5 and 10 mg/ g soil).

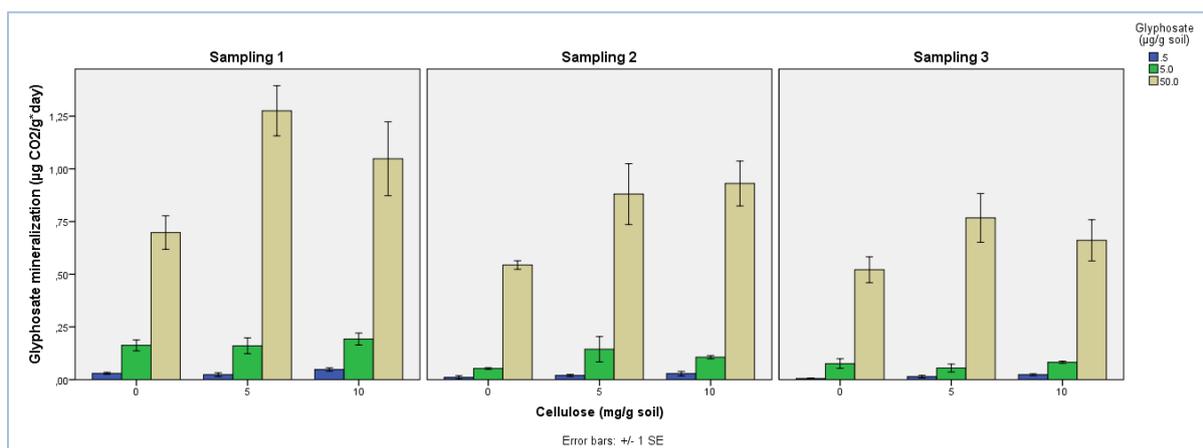


Figure 4: Mean glyphosate mineralization rate estimated in soils with; three different cellulose amendments (0, 5 and 10 mg/ g soil), at three sampling periods (day 1, 3 and 6) and with all treatments receiving either of three concentrations of glyphosate (0.5, 5 or 50 µg/ g soil) (n=4, for all treatments).

3.4. Gross N-transformation

A trend of decreasing mineralization rate with increasing glyphosate concentration was found in the soil with no cellulose amendment (Table. 1) (Linear regression, $P < 0.01$, $R^2 = 0.994$). In the two soils receiving cellulose (5 respective 10 mg cellulose/ g soil) the trend was less obvious, however, there seemed to be a decreased rate at the highest glyphosate concentration. Nitrification was detected solely in the soil with no cellulose additions and not in the remaining two soils. No trend in the nitrification rate was found.

Table 1: The mineralization and nitrification rate estimated in three types of soil (0, 5 and 10 mg cellulose/ g soil) receiving either of three concentrations of glyphosate (0.5, 5 or 50 µg/ g soil) or the control (no glyphosate addition). Values between brackets represent the standard errors (n=4). N.D. denotes not detected.

Cellulose (mg/ g soil)	Glyphosate (µg/ g soil)	Mineralization (mg N/ kg soil * day)	Nitrification (mg N/ kg soil * day)
0	0	0.58 (0.04)	0.36 (0.07)
0	0.5	0.53 (0.03)	0.49 (0.02)
0	5	0.42 (0.09)	0.44 (0.06)
0	50	0.27 (0.07)	0.41 (0.05)
5	0	1.16 (0.62)	N.D.
5	0.5	1.54 (0.91)	N.D.
5	5	1.21 (0.25)	N.D.
5	50	1.00 (0.37)	N.D.
10	0	1.86 (0.74)	N.D.
10	0.5	2.09 (2.04)	N.D.
10	5	1.75 (1.21)	N.D.
10	50	1.69 (1.01)	N.D.

3.5. Bacterial growth

The bacterial growth differed significantly between samples with different cellulose additions (Fig. 5) (ANOVA, $P < 0.001$). When cellulose was added the growth rate significantly increased (Fischer LSD, $P < 0.001$). No difference was found between the two added amounts (5 and 10 mg/ g soil).

The bacterial growth also differed significantly between different glyphosate additions (ANOVA, $P < 0.001$), with a significantly lower growth rate in soils that received glyphosate in the concentration of 50 $\mu\text{g/ g}$ soil compared to 0.5 $\mu\text{g/ g}$ and the control (Fischer LSD, $P < 0.001$). There was also a significantly lower growth rate with glyphosate of 5 $\mu\text{g/ g}$ soil compared to 0.5 $\mu\text{g/ g}$ (Fischer LSD, $P < 0.05$), at the second sampling, three days after the addition of glyphosate.

A significant difference was also found between sampling periods (ANOVA, $P < 0.001$). The bacterial growth was significantly higher in sampling 1 than in the other two samplings (Fischer LSD, $P < 0.001$).

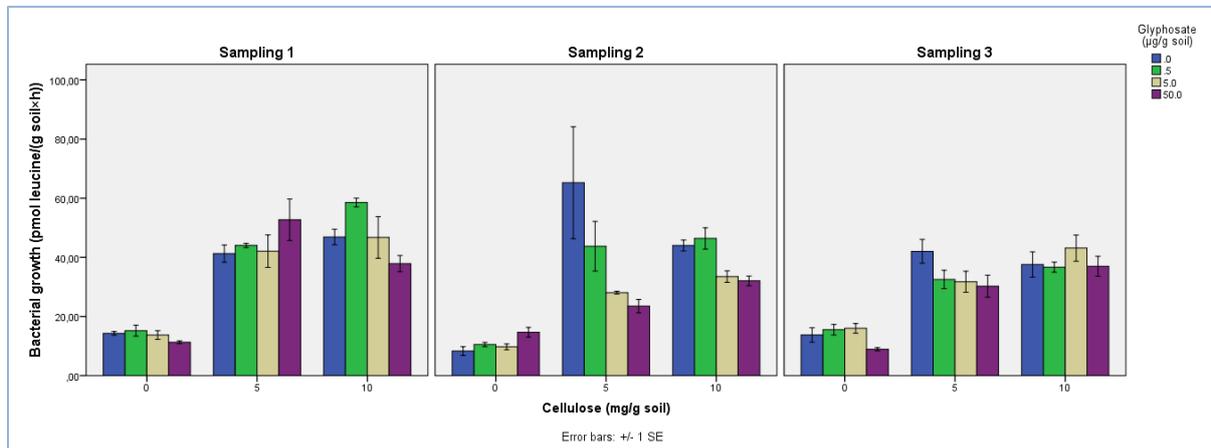


Figure 5: Bacterial growth rate measured in soils with; three different cellulose amendments (0, 5 and 10 mg/ g soil), at three sampling periods (day 1, 3 and 6) and with all treatments receiving either of three concentrations of glyphosate (0.5, 5 or 50 $\mu\text{g/ g}$ soil) or the control (no glyphosate addition). ($n=4$, for all treatments)

4. Discussion

The aim of this study was to find out if the herbicide glyphosate promotes priming effects in soil. Indications of that positive priming had occurred was found from measurements of SOM mineralization the first day after addition of glyphosate, where the relative SOM mineralization increased at the concentration of 5 $\mu\text{g/ g}$ soil. The priming effects were not found to be highest at the maximum concentration (50 μg glyphosate/ g soil) as was hypothesized. One reason for this could be that glyphosate has a toxic effect on the microorganisms. As was stated by Helander et al. (2012), the bacteria might be affected by glyphosate through blockage of the shikimate metabolic pathway. In this study, the bacterial growth rate was found to be lowest at the highest glyphosate concentration (50 $\mu\text{g/ g}$ soil) and this could be due to that the substance, in such a high dose, becomes toxic. However, since the growth rate did not decline by half at the highest glyphosate concentration, as did the mineralization rate, this cannot be the sole explanation. Another reason why priming would

not be highest at 50 µg glyphosate/ g soil could be that the microorganisms get sufficient energy and nutrients from degrading glyphosate and, therefore, do not need to degrade the more recalcitrant SOM.

The priming effects seemed to be fairly short term since positive priming were only observable the first day. According to Fontaine et al. (2007), a reason for decreasing priming effects could be the exhaustion of the easily available nutrient source. The glyphosate mineralization rate decreased within only a few days, which is most likely due to that glyphosate degrades rather rapidly (Rueppel et al. 1997). The degradation is assumed to follow a pseudo-first order kinetics, meaning it is only the concentration of glyphosate that controls the mineralization rate and the microorganisms are thought to exist in surplus. A lower concentration of glyphosate would, therefore, decrease the mineralization rate. Accordingly, the glyphosate seemed to be mineralized at a rate that coincides with the relative occurrence of the herbicide in the soil. The amount of glyphosate therefore decreased after the first sampling and the explanation for the short term priming effect seems to comport with this case. Another explanation for the decreasing priming effects after the first day could be that a considerable amount of the glyphosate has adsorbed to soil particles at the second and third sampling and thereby has become non-bioavailable for microbial degradation. According to Duke et al. (2012), glyphosate strongly binds to soil particles, why, this explanation might also be reasonable.

The method used for testing priming effects does not differentiate real from apparent priming. However, the bacterial growth did not significantly differ between the control and the addition of 5 µg glyphosate/ g soil and this is an indication of that the increased soil respiration was not caused by apparent priming, i.e. an increased turnover of the microbial biomass (Bengtson et al. 2012). If apparent priming had occurred, a relatively higher bacterial growth would be expected. An increased growth leads to a more rapid respiration and an elevated number of bacteria performing the respiration, which would explain the additional CO₂. Since no increased growth was detected, the increased SOM mineralization is believed to have been caused by real priming.

Priming was only observed at glyphosate addition of 5 µg/ g soil, which is somewhat above the recommended spraying dose. This might imply that the dosage normally used is too small to cause any priming. However, even though glyphosate degrades rapidly traces of it have been found some years after application (Simonsen et al. 2008). Because of the fact that some of the herbicide can persist, and since spraying can occur several times per year (Duke et al. 2012), it might be possible that levels as high as 5 µg/ g soil could occur in soils.

The magnitude of priming found in this study, with an increased SOM mineralization of 26%, were rather low in comparison with what has been found in other studies. Hamer and Marschner (2005b) found positive priming effects that increased SOM mineralization with 127% after combined addition of fructose and alanine to a forest soil. The same substances were tested separately on an arable soil, which gave an increased SOM mineralization of between 10 and 63% (Hamer and Marschner 2005a). Nottingham et al. (2009) found an increase of 169% after sucrose addition to a forest soil and Bengtson et al. (2012) an increase

of between 56 and 244% when examining priming effects of root exudates. An increased SOM mineralization of 26% may not seem much but, as was mentioned earlier, soil respiration is responsible for a large part of the C flux from the biosphere to the atmosphere (Schlesinger and Andrews 2000). Thus, additional CO₂ released by glyphosate addition might be relevant to consider, especially since glyphosate is extensively used globally. However, the short term effects of the priming lower the relevance somewhat. Regarding the risk for depletion of the organic matter in soil, caused by an increased SOM mineralization of 26%, it could be estimated by comparing the increase in mineralization rate with the sequestration rate of C. If the estimated C sequestration is several times higher, depletion of soil C due to glyphosate addition is not likely.

The increase in bacterial growth rate and soil respiration rate when cellulose was added suggest that the bacteria initially were C-limited, as was assumed. No additional stimulation of growth was found at the highest cellulose addition indicating that microorganisms became limited by N. However, the results indicated that priming effects can occur regardless of C or N limitation. The strongest priming were, nonetheless, found in the soil with no cellulose amendment (the C limited soil), which might imply a stronger tendency for priming in these soils. The relative increase, when 5 µg glyphosate/ g soil was added, was 26% in this soil compared to 9% and 12% in the two soils with cellulose amendment (5 and 10 mg/ g soil respectively). Other authors have suggested that C limited microorganisms are a necessity for priming to occur (Bengtson et al. 2012), and that priming are more common in nutrient poor soils (Fontaine et al. 2003). In contrast, Hamer and Marschner (2005a) investigated priming effects in different soil types but could not find any relationship between soil properties and priming effects. However, they did find that positive priming are most prominent in forest soils that contain recalcitrant SOM. It is possible that glyphosate would also cause higher priming in forest soils. The soil used in the present study was a grassland soil which is more similar to agricultural soils, with regards to primarily pH and organic content. Since glyphosate is not only used in agriculture but also in forestry (IPCS 1996) this might be relevant to investigate. It is also possible that priming on agricultural fields would be lower than what was found in this study. If the herbicide is applied close in time after fertilizers have been used, that might reduce the priming effect if nutrient limitation is a prerequisite for priming to occur.

Estimations of gross N-transformation indicated that there was a negative correlation between mineralization rate and glyphosate concentration. There are two potential explanations for such a relationship. The first is that glyphosate might be toxic to the microorganism, which would impair their ability for mineralization and the second explanation is that the microorganisms get sufficient amounts of nitrogen from degrading glyphosate and, therefore, do not need to acquire N from SOM degradation which probably is more energy demanding. Nitrification was not detected after measurements in the two cellulose amended soils. A reason for that could be that the additional cellulose input made the soils N limited (as was also the intention) which lead to that the heterotrophic bacteria outcompeted the nitrifying bacteria for NH₄⁺ (Verhagen and Laanbroek 1991). Thus, the N became immobilized through uptake by heterotrophic bacteria.

5. Conclusion

Based on the findings from this study, glyphosate seem to be able to cause priming effects in soils. The relative increase in SOM mineralization of 26% was not remarkably high and the effects were found to be short term. Nevertheless, glyphosate addition seems to have a possible significance on the CO₂ flux from soil to the atmosphere which warrants further investigation. Measurements of gross N-transformation indicated that glyphosate did not increase mineralization of N in the soil organic matter. In fact, high amounts of the herbicide seemed to decrease the mineralization rate. The risk of NO₃⁻-leaching from soil after application of glyphosate appeared low, since no change in nitrification rate was found compared to the control (no glyphosate addition).

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