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# Dynamics of microbial activity, community composition and degraders in soil microcosms treated with glyphosate and its three degradation products

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## Abstract

Widely-used glyphosate may produce AMPA, glycine and sarcosine degradation products. To date, little is known about effects of these degradation products on soil microorganisms and their potential degraders. Here, we incubated a soil spiked either with $^{13}$C-labeled glyphosate, AMPA, sarcosine or glycine for 75 days. During this period, soil respiration rates ($CO_2$) and mineralization rates of the compound ($^{13}CO_2$) were estimated in addition to phospholipid fatty acids (PLFAs and $^{13}$C-PLFAs) as biomarkers to identify major groups of microorganisms in soil. 16S/ITS rRNA amplicon sequencing was also conducted to identify the microbial community structures at the phylum and genus level. Soil respiration, mineralization rates, microbial biomass (PLFAs) as well as incorporation of $^{13}$C into PLFAs were highest for



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glycine and lowest for AMPA. Results demonstrated that Gram negative degraders were major degraders of each compound, whereas Gram-positive bacteria, actinobacteria and fungi (Ascomycota phylum) were decomposers of the primary degraders. Based on similar time-dependent shifts in ($^{13}$C-)PLFAs and abundances of 16S rRNA genera, we deduced that glyphosate was mainly degraded to glycine. However, stable isotope metagenomics or proteomics investigating the capability of specific bacterial/fungal genera to degrade glyphosate to either glycine or to AMPA product are still needed.

**Keywords**

Biodegradation; Sarcosine; Glycine; AMPA; Stable isotope probing; PLFA, DNA Metabarcoding

**Statement of environmental implication**

Glyphosate is most globally used herbicide of environmental and toxicological concern. This herbicide can be degraded to three different products: AMPA, glycine and sarcosine. Numerous studies reported the effect of glyphosate on microorganisms and its potential degraders; however, such studies with regard to the three degradation products of glyphosate are still elusive. The effects of glyphosate addition on soil microorganisms might not be caused directly by the glyphosate but indirectly by its degradation product(s). This is the first report comparing the impact of glyphosate and its degradation products on soil microbial activity, community composition as well as degraders.



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# 1. Introduction

Glyphosate [N-(phosphonomethyl) glycine] has emerged as one of the most widely used herbicides (by volume and land area treated) worldwide because of its great efficacy against large number of weeds (Battaglin et al., 2014; Benbrook, 2016; Wang et al., 2014). Such widespread addition of glyphosate to soils may affect soil microbiome. For example, glyphosate stimulated microbial respiration of soil (Aslam et al., 2014; Lane et al., 2012) especially with its increasing concentration (Nguyen et al., 2016). In contrast, microbial community composition as shown by phospholipid fatty acid (PLFA) analysis, namely the proportions of Gram-positive & Gram-negative bacteria, actinobacteria and fungi in soils amended with glyphosate were unchanged (Lane et al., 2012; Nguyen et al., 2018). However, shifts in bacterial community composition at the phylum level towards Bacteroidetes, Proteobacteria, Actinobacteria and Acidobacteria, and at the family level towards Flammeovirgaceae and Saprospiraceae (Sphingobacteriales spp.) in response to glyphosate application to soils were noticed by Guijarro et al. (2018) who applied 16S rRNA gene amplicon sequencing method.

Four groups of microbial degraders contributed to $^{13}C_3$-glyphosate degradation in soil as indicated by $^{13}$C-PLFA approach (Muskus et al., 2022). The Gram-negative bacteria predominated the initial phases of the $^{13}C_3$-glyphosate degradation, whereas the Gram-positive bacteria, actinobacteria and fungi prevailed the later phases in the study by Muskus et al. (2022). The following soil or sludge isolates of Gram-negative bacteria genera *Achromobacter* (Ermakova et al., 2017; Sviridov et al., 2012)*, Agrobacterium* (Wackett et al., 1987), *Comamonas* (Firdous et al., 2020), *Flavobacterium* (Balthazor and Hallas, 1986), *Ochrobactrum* (Hadi et al., 2013; Sviridov et al., 2012) and *Pseudomonas* (Dick and Quinn, 1995) were reported to utilize glyphosate as a P source. The Gram-positive bacterium *Bacillus* (Fan et al., 2012) and actinobacterium *Streptomycetes* (Obojska et al., 1999) which were



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isolated from soil and raw sludge, respectively; also were capable to degrade glyphosate. Furthermore, several genera of fungal glyphosate degraders were isolated from soils, e.g. *Aspergillus, Mucor, Scopulariopsis, Trichoderma* (Krzyśko-Łupicka & Orlik, 1997) and *Penicillium* (Klimek et al., 2001; Krzyśko-Łupicka and Orlik, 1997).

Glyphosate can be degraded to persistent aminomethylphosphonic acid (AMPA) and to either easily biodegradable sarcosine or glycine (Aslam et al., 2023; Carretta et al., 2021; Li et al., 2018; Pérez Rodríguez et al., 2019; Sun et al., 2019; S. Wang et al., 2016; Zhan et al., 2018). Not directly glyphosate, but its degradation product(s) also may indirectly change soil microbial activity and community composition as well as define microbial degraders. Previous studies investigated either the impact of glyphosate addition to soils on microbial activity and community composition (Lane et al., 2012; Nguyen et al., 2016) or the culturable bacterial and fungal glyphosate degraders (Zhan et al., 2018). To date, such studies with three glyphosate degradation products are scarce and these could complete the glyphosate degradation study. To address this current gap of research, we performed a comparative assessment of microbial activity, community composition and degraders using PLFA+$^{13}$C-PLFA and 16S/ITS rRNA analyses in response to glyphosate, AMPA, glycine or sarcosine addition to soil.

Our recent study suggested that glyphosate was mainly degraded via the sarcosine/glycine pathway yielding the glycine or sarcosine degradation product (Aslam et al., 2023). Therefore, we hypothesized that microbial activity, shifts in microbial community composition as well as degraders in the soil incubated with glyphosate would be similar to that of in soils treated with either glycine or sarcosine but different from the one which received AMPA. Therefore, objectives of this study were to compare: (i) respiration as a proxy for microbial activity, (ii) microbial community composition based on PLFA and



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16S/ITS rRNA analyses, (iii) mineralization and (iv) microbial degraders based on $^{13}$C-PLFA of soil spiked with 2-$^{13}$C-glyphosate with that of $^{13}$C-AMPA, $^{13}C_2$-glycine and $^{13}C_3$-sarcosine.

## 2. Materials and Methods

### 2.1. Reference soil

The reference soil was a Haplic Chernozem collected from the topsoil of the Static Fertilization Experiment in Bad Lauchstädt (Saxony-Anhalt, Germany). The soil received farmyard manure (30 t ha$^{-1}$) every second year and had a previous history of glyphosate application. The soil had 21% of clay, 68% of silt, and 11% of sand, and it is classified as a silty loam based on US Department of Agriculture soil classification system. The total nitrogen was 0.17%, total organic carbon (TOC) 2.1%, pH 6.6 (Muskus et al., 2019) and maximum water holding capacity was 47±1.9% (based on our measurements in the laboratory). Soil was sieved through a 2 mm sieve, and it was stored in a cold room at 4°C until start of incubation experiments. Soil moisture was adjusted to 60% of maximum water holding capacity.

### 2.2. Chemicals and reagents

The unlabeled glyphosate (99% purity), sarcosine (98% purity) and glycine (99.7% purity) were obtained from Sigma-Aldrich, Germany. The unlabeled AMPA (99% purity) was purchased from Alfa Aesar, Thermo Fisher (Kandel) GmbH. Labeled 2-$^{13}$C-glyphosate (isotopic purity for $^{13}$C: 99%) was obtained from Sigma-Aldrich, Germany. Labeled degradation products of glyphosate: $^{13}C_3$-sarcosine ($^{13}$C: 99%) and $^{13}C_2$-glycine ($^{13}$C: 99%) were purchased from Cambridge Isotope Laboratories, Inc. USA, whereas labeled $^{13}$C-AMPA ($^{13}$C: 99%) from Toronto Research Chemicals, Canada. All labeled chemicals had a chemical purity of 98%. All the other chemicals were obtained from Carl Roth (Karlsruhe, Germany) or



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VWR/Merck (Darmstadt, Germany). All solutions of labeled and unlabeled chemicals were prepared in milli-Q water at 50 mg $kg^{-1}$ soil.

## 2.3. Experimental setup

### 2.3.1. Incubation

We carried out 75-day long soil incubation experiment to investigate the dynamics of respiration, compound mineralization, microbial community composition and groups of microbial degraders of glyphosate and its three degradation products glycine, sarcosine and AMPA. A reference soil was spiked with either unlabeled (control) or 2-$^{13}$C-labeled glyphosate, $^{13}$C-AMPA, $^{13}C_2$-glycine, $^{13}C_3$-sarcosine at 50 mg $kg^{-1}$ soil and placed into 500-mL Schott bottles. Unlabeled controls were used to correct for $^{13}$C natural abundance in labeled counterparts. We also incubated a blank soil without compound in parallel to test the effect of the compound addition on soil respiration and microbial community composition. All soils were incubated at 20°C in dark as per OECD 307 guidelines (OECD, 2002). During the incubation, total $CO_2$ (soil respiration) and $^{13}CO_2$ (compound mineralization) was trapped in 2M NaOH which was replaced regularly. After 2, 4, 10, 18, 24, 32, 46, 61 and 75 days of incubation, the NaOH traps were taken for analysis of total $CO_2$ and $^{13}CO_2$. In addition, soil was sampled at 0, 2, 4, 18, 32 and 75 days to analyze PLFAs for groups of microbial community composition (total PLFAs; $PLFAs_{tot}$) and compound's degraders ($^{13}$C-PLFAs) characterisations. We also took soil samples for DNA metabarcoding analyses (16S/ITS rRNA) in order to complete $PLFAs_{tot}$ analyses with detailed microbial community composition (bacteria and fungi) analysis after 2/4 and 75 days.

### 2.3.2. Soil respiration and compound mineralization

The total amount of $CO_2$ in NaOH traps was determined using a total organic carbon analyzer (Multi N/C 21005, Jena, Germany). The isotopic composition of $CO_2$ ($^{13}C/^{12}C$ at%) was



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measured with gas chromatography-isotope ratio mass spectrometry (GC-IRMS; Finnigan MAT 252, Thermo Electron, Bremen, Germany, coupled to Hewlett Packard 6890 GC; Agilent Technologies, Germany) after separation on a BPX-5 column (50 m × 0.32 mm × 5 µm). The temperature parameters of GC-irMS had been previously described by Girardi et al. (2013). The mineralization of each compound ($^{13}CO_2$) was calculated from the total amount of $CO_2$ and its isotopic composition ($^{13}C/^{12}C$ at%) (Girardi et al., 2013).

### 2.3.3. Phospholipid Fatty acids (PLFAs)

The extraction, purification and derivatization procedures for PLFAs were described in detail by Nowak et al. (2011). Briefly, the PLFAs were extracted from soil with a mixture of phosphate buffer/methanol/chloroform, purified over silica gel and then derivatized with methanol/trimethylchlorosilane (MeOH/TMCS) to fatty acid methyl esters (FAMEs). The total amounts of PLFAs were determined as FAMEs using gas chromatography-mass spectrometry (GC-MS; HP 6890, Agilent) equipped with a BPX-5 column (30 m × 0.25m × 0.25 µm). The isotopic composition of FAMEs ($^{13}C/^{12}C$ at%) was measured with GC-IRMS. The temperature and other parameters for FAMEs separation using GC-MS and GC-IRMS were described by Nowak et al. (2011).

The PLFA biomarker is used to estimate the activity of the following four groups of microorganisms in the environment (Zelles, 1999): I: Gram-positive bacteria (iso- and anteiso-branched PLFAs), II: actinobacteria (sub-group of Gram-positive bacteria; 10-methyl branched PLFAs), III: Gram-negative bacteria (monounsaturated PLFAs) and IV: fungi (polyunsaturated PLFAs). In addition, the starvation of Gram-negative bacteria also can be elucidated based on cyclopropyl PLFAs (Kaur et al., 2005). Saturated straight-chain PLFAs which do not indicate any microbial group were included into the quantitation of $PLFAs_{tot}$ and $^{13}C$-PLFAs.



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### 2.3.4. DNA metabarcoding

DNA metabarcoding technique was used to investigate the abundance of microbial populations based on family, genetic and phyla in soils. Briefly, DNA from soil samples was extracted using soil kit and sent to the company for 16S and ITS rRNA sequencing.

#### 2.3.4.1. DNA extraction

DNA was extracted and purified from 0.25 g of soil with the DNeasy PowerSoil Kit (Qiagen, Benelux BV) according to the specifications of the company. The final volume of the DNA was 100 µL, and its concentration was monitored by a Qubit 3.0 fluorometer (Invitrogen, Life technologies) using the Qubit dsDNA HS (high sensitivity) kit (Thermo Fisher Scientific). DNA samples were stored at -80°C until they were transported to the commercial company Allgenetics (Spain) for PCR amplification and sequencing.

#### 2.3.4.2. Amplicon sequencing

The DNA concentration in the samples was adjusted to 0.01 ng $\mu L^{-1}$ and distributed in a 96-well plate for PCR amplification of the 16S rRNA gene. For library preparation, a fragment of the bacterial 16S rRNA region of ~300–350 bp was amplified using the primers 515F (5' GTG YCA GCM GCC GCG GTA A 3') (Parada et al., 2016) and 806R (5' GGA CTA CNV GGG TWT CTA AT 3') (Apprill et al., 2015). For fungi, the complete fungal ITS2 region of ~300 bp was amplified using the primers ITS86F (5' GTG AAT CATCGA ATC TTT GAA 3') (Y. et al., 1999) and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White et al., 1990). PCRs were carried out in a final volume of 25 µL, containing 2.5 µL of template DNA, 0.5 µM of the primers, 6.5 µL of Supreme NZYTaq 2x Green Master Mix (NZYTech), and ultrapure water up to 25 µL. The reaction mixture was incubated as follows: an initial denaturation at 95ºC for 5 min, followed by 25 cycles of 95ºC for 30 s, 50ºC for 45 s, 72ºC for



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45 s, and a final extension step at 72ºC for 7 min. The oligonucleotide indices which are required for multiplexing different libraries in the same sequencing pool were attached in a second PCR round with identical conditions but only 5 cycles and 60ºC as the annealing temperature. A negative control that contained no DNA (BPCR) was included in every PCR round to check for contamination during library preparation. The libraries were run on a 2% agarose gel stained with GreenSafe (NZYTech) and imaged under UV light to verify the library size. Libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), following the instructions provided by the manufacturer. Then, they were pooled in equimolar amounts according to the quantification data provided by the Qubit dsDNA HS Assay (Thermo Fisher Scientific). The pool was sequenced in a fraction of a MiSeq PE300 run (Illumina).

**Quality control and processing of sequencing data.** Illumina paired-end raw files consisted of forward and reversed reads sorted by library and their quality scores. The indices and sequencing primers were trimmed during the demultiplexing step. The quality of the FASTQ files was checked using the software FastQC (Andrews, 2010) and summarized using MultiQC (Ewels et al., 2016).

The obtained 16S/ITS amplicon reads were processed using QIIME2 (release 2020.8). DADA2 (Callahan et al., 2016), implemented in QIIME2, was used to: remove the PCR primers, filter the reads according to their quality, denoise, merge the reads, remove the chimaeric reads, and cluster the resulting sequences into amplicon sequence variants (ASVs). Due to the fact that the sequencing reads were longer than the 16S/ITS amplicons, non-biological DNA (primers, indices, and sequencing adapters) can appear at the reads ends. Therefore, we used cut-adapt (Martin, 2011) as a primer/adapter removal tool to trim these regions. For visualization, AMPVIS2 package was used in R to plot diversity analyses and



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heatmaps. All sequences were submitted to the NCBI GenBank Sequence Read Archive under the study accession number PRJNA951409.

### 2.4. Data analysis

All incubation treatments were carried out with three repetitions and all results were presented as averages and standard deviations, except for 16S/ITS rRNA which were shown as a pooled single measurement of triplicates. Respiration of soil ($CO_{2\ tot}$ $g^{-1}$ $day^{-1}$) was based on the quantitation of total $CO_2$ ($^{12}C+^{13}C$) rate per day. Respiration was used as a proxy for microbial activity following the addition of the respective compound to soil. By contrast, the $^{13}CO_2$ daily rates referred to $CO_2$ released from the mineralization of the compound. The total concentration of PLFAs ($^{12}C+^{13}C$; $PLFAs_{tot}$) indicated changes in the microbial biomass quantity and the shifts between microbial groups (four groups mentioned in section **2.3.3**) over 75 days of incubation. The amounts of $^{13}C$-PLFAs enabled time-dependent quantification of $^{13}C$-label incorporation into microbial biomass (microbial group of degraders) from the $^{13}C$-labeled compound and the shifts between the groups of microbial degraders.

The calculation of amounts of $^{13}C$-label in $^{13}CO_2$ and in $^{13}C$-PLFAs were based on the quantitation of their total carbon pools ($^{12}C+^{13}C$) and the determination of the $^{13}C$ excess over the controls as described previously (Wang et al., 2016). The amounts of $^{13}C$ in the respective fractions were expressed as percentages of the initially applied $^{13}C$ equivalents. The analytical uncertainty of the total carbon pool in each fraction was <5%, whereas the uncertainty of at% $^{13}C$ isotope signatures was <2% and <5% (of at% $^{13}C$) for unlabeled and labeled samples, respectively. A One-Way ANOVA was performed to test for a significant difference in respiration and mineralization rates and microbial biomass/groups using R software; the differences were considered significant when $p<0.05$. The time-dependent shifts between the



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four groups of microorganisms ($PLFAs_{tot}$ and $^{13}C$-PLFAs) and the microbial populations (16S/ITS rRNA) based on the genus level were visualized in heat maps using R software.

## 3. Results and Discussion

### 3.1. Soil respiration rate and microbial biomass

An addition of glycine, sarcosine and glyphosate to soil enhanced respiration accordingly by 91% (1.09±0.003 µmol $CO_{2\ tot}$ $g^{-1}$ $day^{-1}$), 56% (0.89±0.07 µmol $CO_{2\ tot}$ $g^{-1}$ $day^{-1}$) and 18% (0.67±0.01 µmol $CO_{2\ tot}$ $g^{-1}$ $day^{-1}$) as compared to the control without any compound (0.57±0.04 µmol $CO_{2\ tot}$ $g^{-1}$ $day^{-1}$) on day 2 (see **Figure 1**). In contrast, the respiration of soil treated with AMPA (0.53±0.05 µmol $CO_{2\ tot}$ $g^{-1}$ $day^{-1}$) was similar to that of the control ($p>0.05$). For all compounds, the respiration rate was highest on day 2 ($p<0.05$), then it gradually decreased towards the end (0.15±0.02 – 0.33±0.08 µmol $CO_{2\ tot}$ $g^{-1}$ $day^{-1}$on day 75). Only the soil respiration with sarcosine (0.33±0.08 µmol $CO_{2\ tot}$ $g^{-1}$ $day^{-1}$) was slightly higher than for other compounds (0.16±0.018 µmol $CO_{2\ tot}$ $g^{-1}$ $day^{-1}$) on day 75.

Evolution of microbial biomass based on $PLFAs_{tot}$ analysis in soils of four treatments was similar to the trends of respiration rates. The $PLFAs_{tot}$ were highest in soil spiked with glycine (0.054±0.002 µmol $g^{-1}$) or glyphosate (0.049±0.0022 µmol $g^{-1}$) on day 4 (**Figure 2**). Lowest magnitude of $PLFAs_{tot}$ were observed in AMPA and sarcosine treatments, as well as in control ($p<0.05$) which were at least two times lower than those in glycine and glyphosate treatments. We also observed that the $PLFAs_{tot}$ in both glycine and glyphosate treatments were nearly constant throughout the incubation period. In contrast, the $PLFAs_{tot}$ decreased progressively on day 75 in soils spiked with AMPA or sarcosine as well as in control.

Highest amounts of microbial biomass as well as respiration of soil spiked with glycine or glyphosate suggest that these two compounds enhanced microbial activity, and especially



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glycine. These findings are in a good agreement with previous studies with glycine (Apostel et al., 2013; McFarland et al., 2010; Xue et al., 2022; Zhang et al., 2019) and glyphosate (Lane et al., 2012; Nguyen et al., 2016). Comparable microbial biomass and respiration of soil spiked with AMPA with that of the control indicate no noticeable effect of the AMPA addition to soil on microbial activity, despite the fact that AMPA is persistent (Aslam et al., 2023; Battaglin et al., 2014; Domínguez et al., 2016). Although the respiration of soil spiked with sarcosine was higher than in control, $PLFAs_{tot}$ data however suggest lower magnitude of microbial biomass in soil. This result is difficult to explain; and it could be due to a low sarcosine use efficiency by microorganisms as a C-substrate (Oliver et al., 2021).

### 3.2. Microbial community composition based on $PLFAs_{tot}$ and 16S/ITS rRNA

Gram-negative bacteria were the most abundant group of microorganisms within the soil microbial community in all treatments (**Figure 3**), which were followed by Gram-positive bacteria and then by actinobacteria and fungi. Higher bacterial species richness was noticed for glyphosate (day 4: 1465, day 75: 1775, see **Figure S1** in supplementary material), glycine (day 2: 1550), sarcosine (day 2: 1625, day 75: 1475) and AMPA (day 4: 1420, day 75: 1460) treatments as compared to control (day 4: 1305, day 75: 1430). Bacterial species abundances were also higher for soils with the compound, i.e., glyphosate (day 4: 6.68, day 75: 6.79), glycine (day 2: 6.69), sarcosine (day 2: 6.77, day 75: 6.65) and AMPA (day 4: 6.68, day 75: 6.61) than in the control (day 4: 6.56, day 75: 6.65, **Figure S2**). These results suggest that addition of tested compounds to soil increased the diversity of bacterial community as well as their abundances. However, this effect was more apparent for glyphosate, glycine and sarcosine treatments and least in AMPA treatment.

The addition of glyphosate, glycine or sarcosine to soil increased the relative abundances of Gram-negative bacteria on day 4 as compared to control and especially in soils spiked with



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glycine and glyphosate (**Figure 3**). Similar observations were reported in other degradation studies with readily available substrates like glyphosate (Muskus et al., 2022) and amino acids (Apostel et al., 2013) in soils. It is noteworthy here that the abundance of Gram-negative bacteria was highest on day 4 in all treatments including the control set-up without a compound. Mixing of soil with water during the preparation of incubation vessels seems to have increased an availability of C and other nutrients in the soil to Gram-negative bacteria explaining their initially highest abundance in all soils (Moore-Kucera and Dick, 2008). After day 4 sampling, contribution of Gram-negative bacteria decreased in all treatments except for AMPA. The observed decrease in the abundance of Gram-negative bacteria is in line with an increasing abundance of the Gram-negative bacteria starvation marker (**Figure 3**).

The Gram-negative bacteria were dominated by two phyla Proteobacteria and Acidobacteriota in all treatments (16S rRNA analysis **Figure 4**). These two phyla are widely-known to dominate in the soil bacterial community (Deng et al., 2018; Montecchia et al., 2015). Both phyla play a key role in biogeochemical cycling of elements and have a capability to degrade monomeric and polymeric substrates (Kalam et al., 2020; Mhete et al., 2020). The abundance of four phyla Proteobacteria, Acidobacteriota, Gemmatimonadota and Bacteroidota was highest in glyphosate and glycine treatments on day 2/4. These findings are consistent with results of previous studies. An increased abundance of Proteobacteria, Acidobacteria (Newman et al., 2016), Gemmatimonadota (Arango et al., 2014) and Bacteroidota (Guijarro et al., 2018) was also found in soils exposed to glyphosate as compared to controls. Proteobacteria and Acidobacteria were shown to increase their abundances in soils contaminated with metals (Kim et al., 2021). Therefore, we suppose that both phyla might have been glyphosate-tolerant and possibly utilized it as a C-, N- or P-substrate. The Gemmatimonadota phylum has been poorly investigated and its abundance was positively correlated with high C and nutrient contents in soils (Liu et al., 2021; Mujakić et al., 2022).



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Both glyphosate and glycine as an additional C-substrate thus might have enhanced the abundance of Gemmatimonadota in this study. Furthermore, another phylum - Bacteroidota were reported to be copiotrophs growing on nutrient- and C-rich substrates as well as to degrade complex organic C-substrates (Fierer et al., 2012). We have already remarked the highest microbial activity (based on $PLFAs_{tot}$) of soil spiked with glyphosate or glycine in the previous **section 3.1**. Therefore, the noticeable abundance of Bacteroidota in these two treatments could have been induced by a C-substrate added, especially the readily biodegradable glycine. Contrastingly, we also noticed an increase in the abundance of Bacteroidota on last day 75 in soils spiked with sarcosine and AMPA as compared to their counterparts on day 2/4 sampling. The increased abundance of Bacteroidota in the sarcosine treatment indicated that bacteria of this phylum could use decaying biomass of primary degraders of sarcosine as a potential C-substrate, whereas that in the AMPA treatment the slowly biodegradable AMPA. Interestingly, we also observed that abundance of Acidobacteriota at the end of incubation (day 75) was lowest in soil spiked with AMPA compared to all treatments including control (**Figure 4**). This indicates AMPA might have had toxic impact on microorganisms belonging to this phylum.

Second most abundant group as indicated by $PLFAs_{tot}$ was Gram-positive bacteria, whereas both actinobacteria and fungi were least abundant in all treatments (**Figure 3**). The abundance of Gram-positive bacteria, actinobacteria and fungi were also greatest in soil spiked with glyphosate or glycine and especially after day 4 sampling. The Gram-positive bacteria including actinobacteria and fungi are known to act as decomposers of decaying microbial biomass (necromass) and when easily available C-substrates are depleted (Kramer and Gleixner, 2006; Moore-Kucera and Dick, 2008; Rinnan and Baath, 2009). This thus explains a higher abundance of these three groups of bacteria a later period of soil incubation in this study. Contrastingly, Actinobacteriota phylum (16S rRNA analysis **in Figure 4**) was least



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abundant in soil treatments spiked with glyphosate and glycine compared to control or sarcosine treatments, whereas that of AMPA was highest. This result is difficult to explain. Nevertheless, the trend in time-dependent abundances of actinobacteria in the $PLFAs_{tot}$ analysis seems to be more consistent than the one when looking at the 16S rRNA data.

Noteworthy here is that the time-dependent abundances of Gram-negative and positive bacteria, actinobacteria and fungi as well as of four phyla Proteobacteria, Acidobacteriota, Gemmatimonadota and Bacteroidota of soil spiked with glyphosate shared similarity with that of glycine treatment (**Figure 3 and Figure 4**). This similarity might be attributed to the fact that high amounts of glycine could have been formed during glyphosate biodegradation (Sun et al., 2019). Therefore, we also assume that glyphosate might have been biodegraded mainly to glycine rather than to sarcosine or AMPA.

We observed a higher abundance of fungi during the first 4 days of incubation in glycine and glyphosate treatments (**Figure 3**). An addition of glyphosate or AMPA to soil also affected species richness and abundance of fungi. Fungal species richness (ITS rRNA; **Figure S3**) and abundance (**Figure S4**) in both glyphosate (richness: 167, abundance: 3.74) and AMPA (richness: 153, abundance: 3.57) treatments were lower than that of in control (richness: 177, abundance: 3.9). We also noticed shifts in the fungi at the phylum level in glyphosate and AMPA samples as compared to control (**Figure S5**). Ascomycota were more abundant in glyphosate and AMPA treatment as compared to control soil. The Ascomycota prevailed in heavy metal (Narendrula-Kotha and Nkongolo, 2017) and microplastic (Temporiti et al., 2022) contaminated soils than in the uncontaminated ones. Therefore, an addition of glyphosate or AMPA to soil might have induced the shift of fungi towards the Ascomycota due to either enhanced tolerance to the compound or capability to degrade the compound. It is noteworthy that the abundance of Basidiomycota in the AMPA treatment was greatest; this fungal phylum might have a capability to degrade AMPA in this study. A white-rot



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Basidiomycota fungi were reported to degrade a great variety of organic contaminants like pharmaceuticals, pesticides, heavy metals and polymeric compounds (Bosco and Mollea, 2019; Henn et al., 2020; Nguyen et al., 2022; Šnajdr et al., 2011; Vaksmaa et al., 2023).

### 3.3. Mineralization of glyphosate and its three degradation products

Daily mineralization rates of $^{13}C$ labeled compounds ($^{13}CO_2$ $day^{-1}$) were significantly different from each other especially during early days of incubation experiment (**Figure 5**). At day 2 sampling, highest mineralization rate was observed for glycine treatment (18±0.4% of initial $^{13}C_2$-glycine equivalents; $p<0.05$). Second fastest mineralization occurred for sarcosine treatment (8.5±0.3% of initial $^{13}C_3$-sarcosine equivalents) followed by glyphosate (2.2±0.01% of initial 2-$^{13}C$-glyphosate equivalents) and slowest in AMPA (0.3±0.02% of initial $^{13}C$-AMPA equivalents). Mineralization of sarcosine and glycine was fastest in the first 2 days of incubation and after 32 days was negligible (glycine: 0.03 – 0.1%; sarcosine: 0.02 – 0.07%). Glycine and sarcosine are readily biodegradable compounds; therefore, both compounds are quickly mineralized by microorganisms (Aslam et al., 2023; McFarland et al., 2010; Sun et al., 2019).

Glyphosate mineralization also decreased over time, but at slower rates than glycine and sarcosine and after 32 days ranged between 0.1% and 0.4% of initial 2-$^{13}C$-glyphosate equivalents. Mineralization rate of glyphosate in our study was comparable (0.3±0.004% of 2-$^{13}C$-glyphosate equivalents on day 45) with that (0.2% of $^{13}C_3$-glyphosate equivalents on day 40 and at 20°C) reported by Muskus et al. (2022) who incubated the same soil as we did.

The lowest mineralization rates of AMPA (0.1 – 0.4% of initial $^{13}C$-AMPA equivalents) are associated with the persistent nature of this compound in soil (Aslam et al., 2023; Battaglin et al., 2014). Noteworthy here is that the mineralization rate of AMPA was highest among all



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treatments on day 75 (0.1±0.02%) suggesting that microorganisms degraded this compound, albeit slowly.

### 3.4. Compound specific $^{13}C$ assimilation into microbial biomass and microbial degraders based on $^{13}C$-PLFAs and and 16S/ITS rRNA

Highest contents of $^{13}C$-PLFAs during the entire incubation period and among all treatments were found for glycine (0.2 – 0.4% of initial $^{13}C_2$-glycine equivalents, see **Figure 6**) and glyphosate (0.1 – 0.3% of initial 2-$^{13}C$-glyphosate equivalents) treatments. We observed the lowest amounts of $^{13}C$-PLFAs for AMPA treatment (in range of 0.005 – 0.007% of initial $^{13}C$-AMPA equivalents).

The dynamics of $^{13}C$ incorporation into PLFAs differed among four treatments. The maximum amounts of $^{13}C$-PLFAs for glycine (0.4±0.03% of initial $^{13}C_2$-glycine equivalents) and sarcosine (0.2±0.02% of initial $^{13}C_3$-sarcosine equivalents) treatments were measured on day 4. Thereafter, the $^{13}C$-PLFAs decreased in both sarcosine and glycine treatments, but for the $^{13}C_3$-sarcosine (73% of the maximum) it was reduced greater than for $^{13}C_2$-glycine (22% of the maximum). The rapid incorporation of $^{13}C$-label from 2-$^{13}C$-glycine into PLFAs in different soils was also noticed by Xue et al. (2022). In contrast, the $^{13}C$ assimilation into PLFAs from 2-$^{13}C$-glyphosate was slower and reached its maximum during 16-32 days (0.3% of initial 2-$^{13}C$-glyphosate equivalents) which remained nearly constant at the end. The measured $^{13}C$-PLFAs content (0.3% of 2-$^{13}C$-glyphosate equivalents) on day 32 in the glyphosate treatment was higher than that in the study by Muskus et al. (2022) who reported only 0.1% of $^{13}C_3$-glyphosate equivalents in $^{13}C$-PLFA in the same soil incubated at 20°C. Lower assimilation of $^{13}C$ of $^{13}C_3$-glyphosate into PLFAs in the study by Muskus et al. (2022) compared to this study might be due to the two following reasons. First reason might be a lower microbial activity of soil caused by 1-year long storage at 4°C prior to incubation



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406 resulting in a slower biodegradation of $^{13}C_3$-glyphosate and thereby lower $^{13}$C-PLFAs in the
407 study by Muskus et al. (2022). The second explanation could be a different labeling of
408 glyphosate with $^{13}$C, i.e. all three C were labeled ($^{13}C_3$-glyphosate; Muskus et al., 2022) from
409 which a big portion and especially the $^{13}$C-COOH-group could have been directly released as
410 $^{13}CO_2$.

411 The incorporation of $^{13}$C into PLFAs from $^{13}$C-AMPA was low and it was nearly constant
412 throughout the entire incubation (0.006±0.001% of initial $^{13}$C-AMPA equivalents). The
413 AMPA is the most resistant compound to microbial degradation among all tested compounds
414 (Aparicio et al., 2013); hence, the lowest $^{13}$C-PLFAs were as expected.

415 Gram-negative bacteria were the most abundant in $^{13}$C during biodegradation of all
416 compounds and throughout the entire incubation period (**Figure 7**). Gram-negative bacteria
417 were suggested in previous studies to be the major group of degraders of organic pollutants
418 like glyphosate (Muskus et al., 2022), 2,4-D (Nowak et al., 2011) or ibuprofen (Nowak et al.,
419 2013) in soils. Increased $^{13}$C abundance of the Gram-negative bacteria starvation marker after
420 4 days suggests that Gram-negative bacteria were presumably involved in glyphosate
421 degradation in an early biodegradation of glyphosate in soils. We did not find any of well
422 described Gram-negative bacterial degraders of glyphosate at the genus level as follows;
423 *Achromobacter* (Ermakova et al., 2017; Sviridov et al., 2012)*, Agrobacterium* (Wacket et al.,
424 1987), *Comamonas* (Firdous et al., 2017), *Flavobacterium* (Balthazor and Hallas, 1986),
425 *Ochrobactrum* (Hadi et al., 2013; Sviridov et al., 2012) and *Pseudomonas* (Dick and Quinn,
426 1995; Peñaloza-Vazquez et al., 1995). However, we found increased abundances of other
427 genera of Gram-negative bacteria that might have degraded glyphosate as compared to control
428 on day 4 (**Figure S6**). These are as follows: *RB 41* and *Subgroup_7* (Acidobacteriota
429 phylum), *Gemmatimonas* (Gemmantimonadota phylum) and *Arenimonas* (Proteobacteria
430 phylum). Noteworthy is here that the abundances of these four genera were nearly similar in



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both glyphosate and glycine treatments at early degradation (day 2 and 4) compared to control. This finding suggests again our assumption that glyphosate was biodegraded mainly to glycine in **section 3.2** (time-dependent shifts between the four classes of $PLFAs_{tot}$ and four phyla). The two genera *RB41* and *Gemmatimonas* were also highly abundant in sarcosine treatment, but the abundance of both *Subgroup_7* and *Arenimonas* was rather similar to these in control soil. Therefore, it is less likely that high amounts of sarcosine were formed from glyphosate. Interestingly, the abundance of genus *Massilia* (Pseudomonadota phylum) on day 75 was highest in AMPA treatment as compared to other treatments indicating a possible contribution of this bacterial group in AMPA biodegradation. The novel genus *Massilia* was reported to utilize phenanthrene as the sole carbon source and energy (Du et al., 2021; H. Wang et al., 2016).

In addition to the Gram-negative bacteria, both sarcosine and especially glycine treatments exhibited increased $^{13}C$ abundance in the Gram-positive positive bacterial marker throughout the entire incubation period as compared to AMPA and glyphosate (**Figure 7**). This finding is in good agreement with Xue et al. (2022) who showed that Gram-positive bacteria were most important degraders of glycine in a forest soil. However, increased $^{13}C$ abundance in the starvation marker of Gram-negative bacteria was also found in the glycine treatment after 4 days. This suggests that the Gram-negative bacteria were rather the primary degraders of glycine which mineralized quickly (see **section 3.3**).

Actinobacteria were most abundant in $^{13}C$ in the glyphosate treatment on day 75; however, this microbial group was comparably highly abundant in $^{13}C$ in the glycine treatment on day 75 (**Figure** 7). Time-dependent shifts in $^{13}C$ abundance of fungal degraders were not straightforward in all treatments. However, the $^{13}C$-PLFA fungal marker was most abundant in glyphosate treatment at the end of soil incubation, i.e. day 32 and 75. Gram-positive bacteria, actinobacteria and fungi were suggested as decomposers of the necromass of



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degraders that had consumed C-substrates (Kramer and Gleixner, 2006; Moore-Kucera and Dick, 2008; Rinnan and Baath, 2009). Therefore, actinobacteria and fungi prevailed at later phases of glyphosate degradation and Gram-positive bacteria and actinobacteria in glycine treatment.

We found genus of fungi *Sordariomycetes* (OTU 8; Ascomycota phylum) that was highly abundant in glyphosate treatment (**Figure S7**) on day 75 compared to very low abundance in control and AMPA treatments. In contrast, two *Ascomycota* genera were highly abundant in AMPA treatment as compared to glyphosate treatment and control. Fungi from Ascomycota phylum were widely reported to decompose complex organic substrates like plant litter, organic matter or microplastics in soils with the help of laccase enzymes (Ekanayaka et al., 2022; Koechli et al., 2019; Xiao et al., 2022). Therefore, Ascomycota fungi in both glyphosate and AMPA treatments presumably decomposed the necromass of primary glyphosate or AMPA degraders. Interestingly, we found a genus *Sistotrema* (Basidiomycota phylum) only in the AMPA treatment. This finding implies that this genus of fungi could be either AMPA tolerant or AMPA degrader. The study by Op De Beeck et al. (2015) showed that the abundance of *Sistotrema* increased in soils in response to heavy metals pollution suggesting the adaptation of this genus to the pollution.

## 4. Conclusions

This is the first report evaluating the effect of glyphosate and its degradation products on microbial activity and community composition, as well as identifying potential microbial degraders of each compound. Rates of soil respiration and compound mineralization were much greater in soil which was spiked with glycine or glyphosate followed by sarcosine and was least in soil spiked with AMPA. Gram-negative bacteria were presumably major degraders of all compounds, whereas Gram-positive bacteria, actinobacteria and fungi



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(especially Ascomycota) acted as decomposers of primary degraders. The *Sistotrema* fungi might have been the AMPA degrader. Similar time-dependent shifts between the four $PLFAs_{tot}$ classes and abundances of four genera (*RB41, Subgroup_7*, *Gemmatimonas* and *Arenimonas*) in glyphosate and glycine treatments suggest that glyphosate was biodegraded mainly to glycine. However, further studies investigating the capability of specific bacterial and fungal genera to degrade glyphosate to either glycine or to AMPA product are necessary. This could be e.g., metagenomics or proteomics combined with the stable isotope labeling approach.

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# Figures

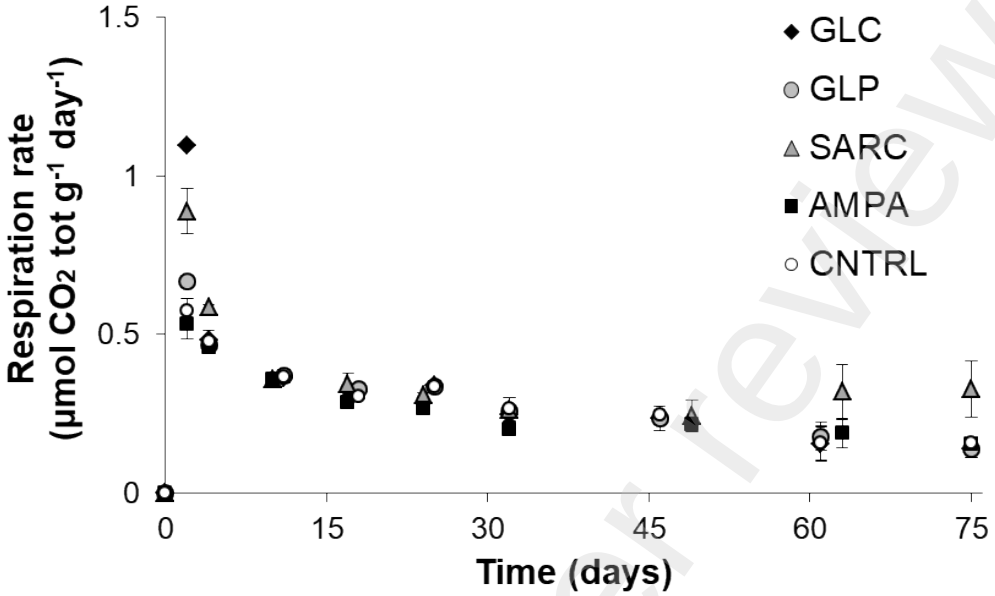


**Figure 1:** Respiration rate (µmol $CO_2$ $g^{-1}day^{-1}$) of control soil (CNTRL) and of soil spiked with glycine (GLC), glyphosate (GLP), sarcosine (SARC) or AMPA during 75-day incubation experiment. Error bars represent standard deviations of means (n = 3).

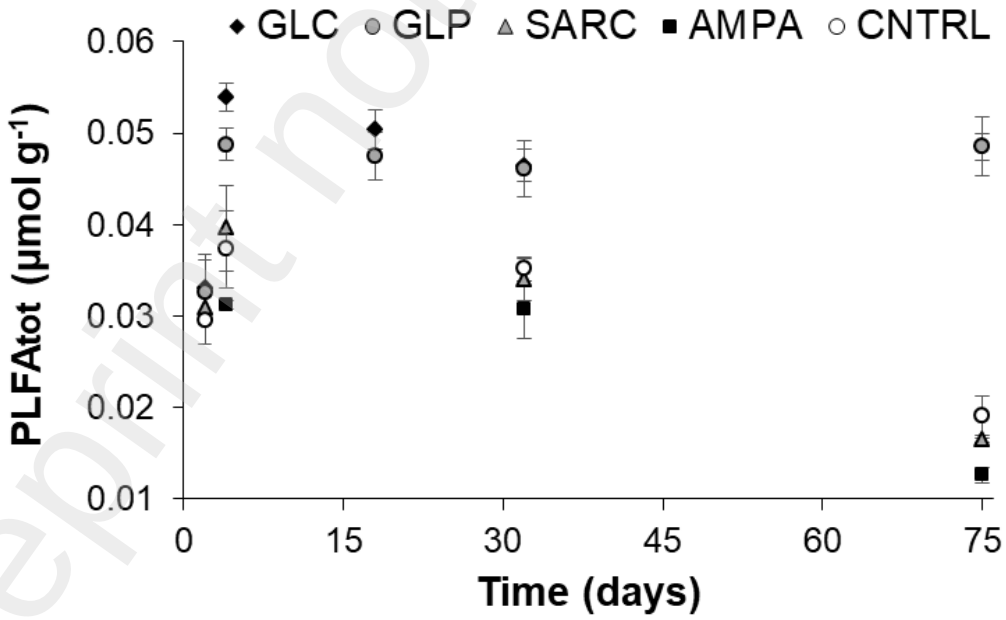


**Figure 2:** $PLFA_{stotal}$ (µmol $g^{-1}$) in control soil (CNTRL) and spiked with glycine (GLC), glyphosate (GLP), sarcosine (SARC) and AMPA during 75-day incubation experiment. Error bars represent standard deviations of means (n = 3).



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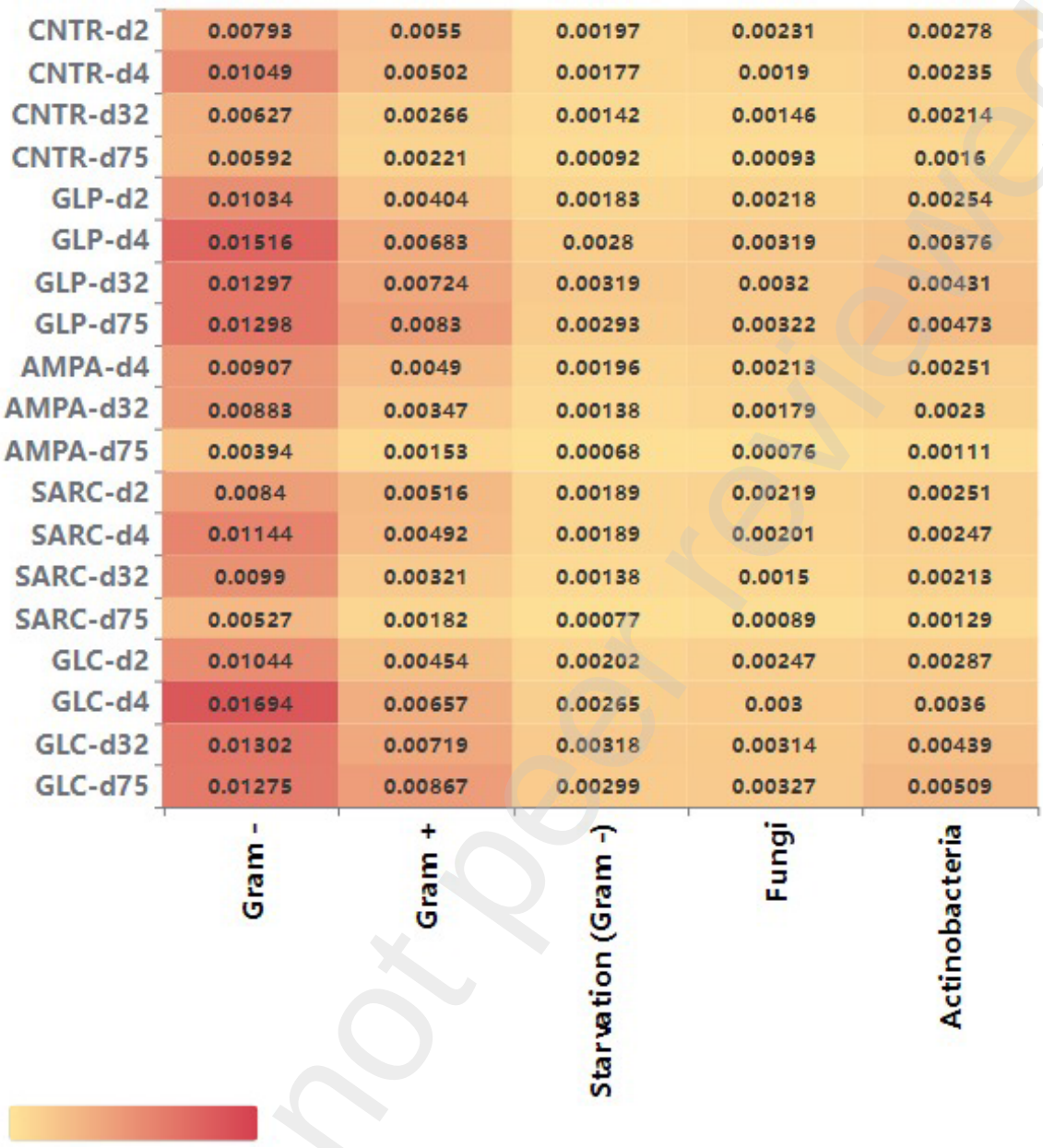


**Figure 3:** Heat map representing abundance of four major groups (Gram-positive & Gram-negative bacteria, actinobacteria and fungi) of soil microbial community and starvation biomarker for Gram-negative bacteria based on $PLFA_{stotal}$ (µmol $g^{-1}$) in control soil (CNTRL) and spiked with glycine (GLC), glyphosate (GLP), sarcosine (SARC) and AMPA during 75-day incubation experiment. Scale in the heat maps shows minimum (light brown color) to maximum (reddish color) contribution of microbial groups.



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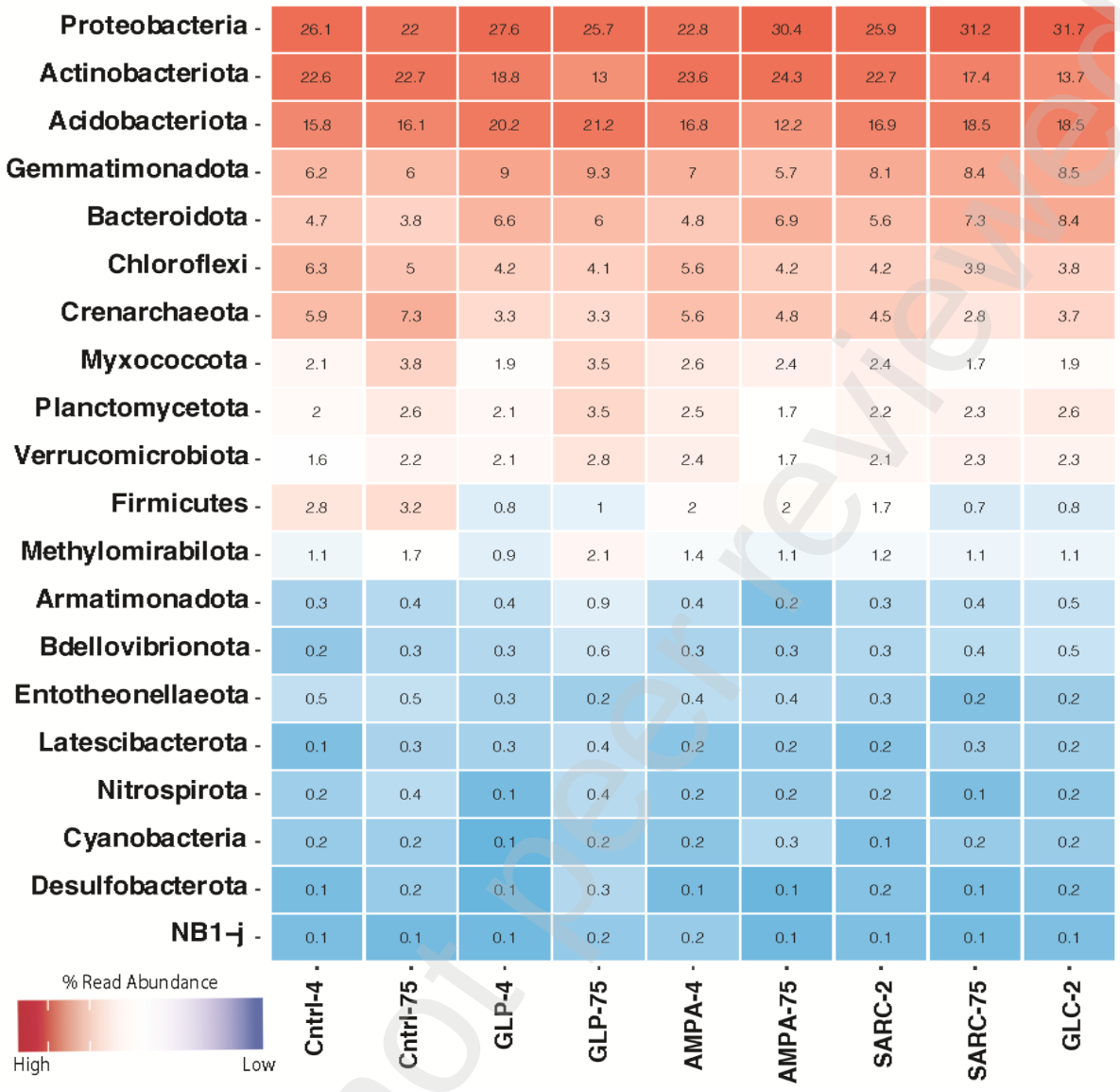


**Figure 4.** Heat map of relative abundance of bacterial phyla based on 16S rRNA analysis in control soil (CNTRL) and spiked with glycine (GLC), glyphosate (GLP), sarcosine (SARC) and AMPA on day 2/4 versus day 75. **Gram-negative bacteria:** Proteobacteria, Acidobacteriota, Gemmatimonadota, *Bacteroidota*, Chloroflexi, Crenarchaeota, Myxococcota, Verrucomicrobia, Methylomirabilota, Armatimonadota, Bdellovibrionota, Latescibacterota, Nitospirota, Cyanobacteria, Desulfobacterota. **Gram-positive bacteria:** Firmicutes. **Actinobacteriota** (Gram-positive bacteria phylum). **Unclassified:** Planctomycetota, Entotheonellaeota. Scale in the heat maps shows minimum (blue color) to maximum (reddish color) contribution of microbial groups.

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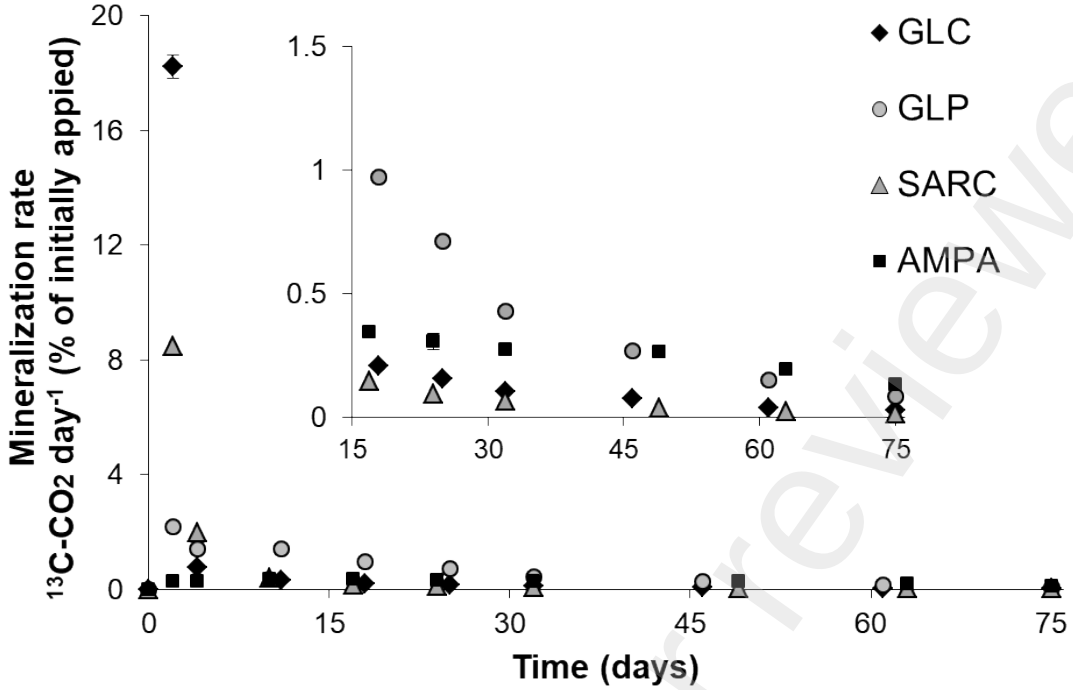


800 **Figure 5:** Net mineralization rates of glyphosate (GLP) and its major transformation products
801 sarcosine (SARC), glycine (GLC) and AMPA in soil during 75-day incubation experiment
802 expressed in % of the initially applied $^{13}C$ label. Data from day15 to day75 are also shown
803 with smaller scale. Error bars represent standard deviations of means (n = 3).
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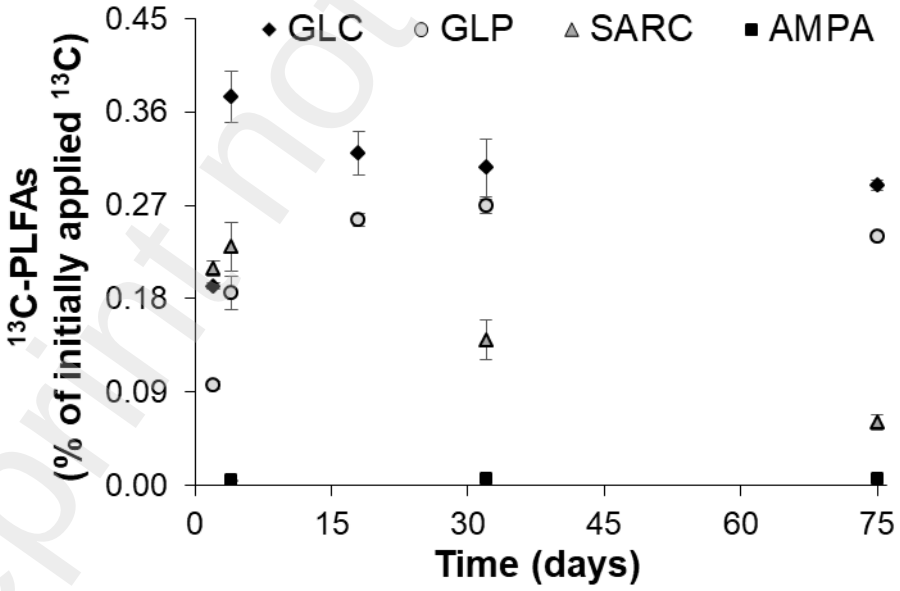


805
806 **Figure 6:** Incorporation of $^{13}C$ from $^{13}C$-glyphosate (GLP) or its major transformation
807 products $^{13}C$-glycine (GLC), $^{13}C$-sarcosine (SARC) and $^{13}C$-AMPA into PLFAs in soils
808 during 75-day incubation experiment expressed in % of the initially applied $^{13}C$ label. Error
809 bars represent standard deviations of means (n = 3).



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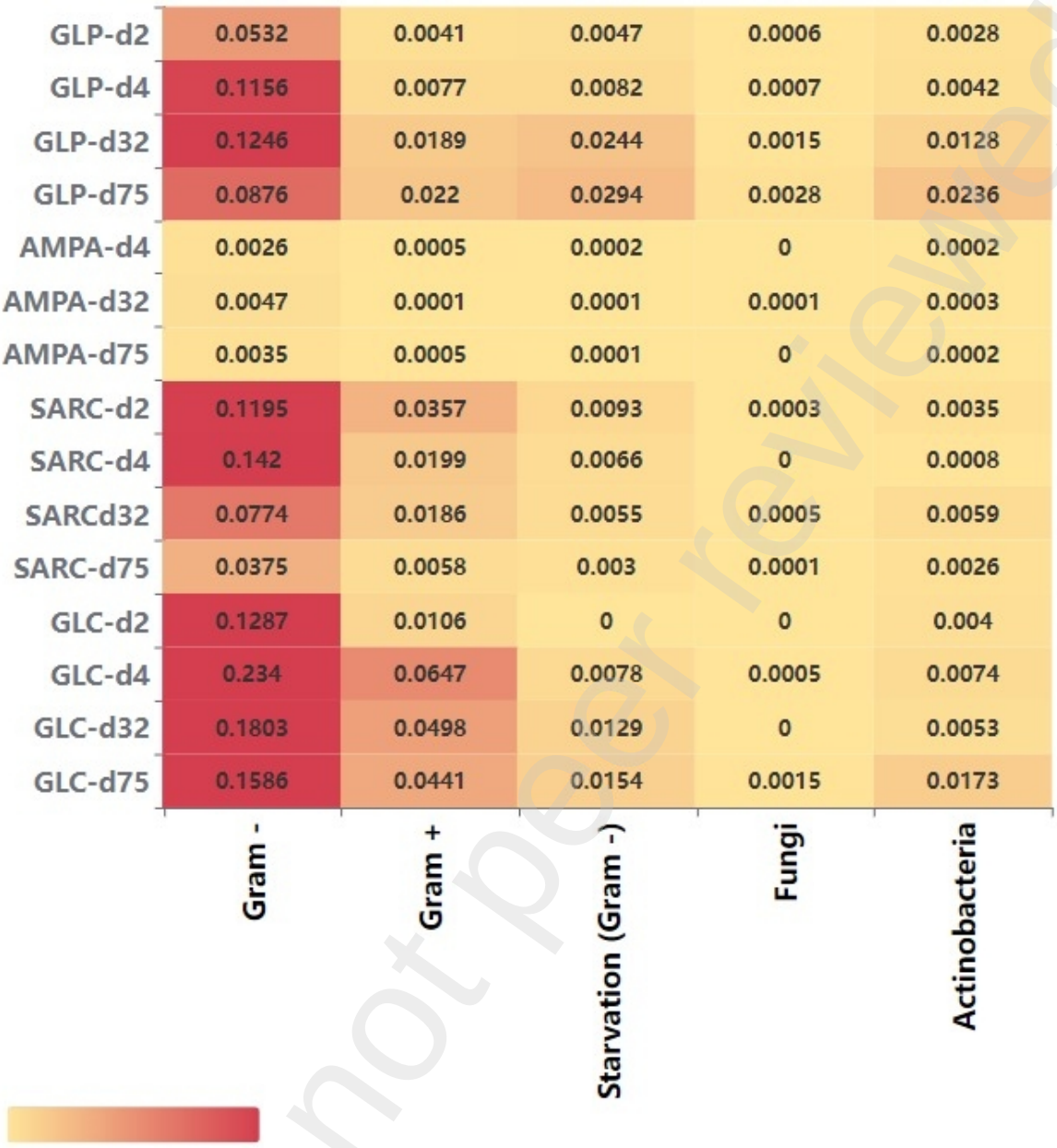


**Figure 7.** Heat map representing abundance of major four groups of microbial degraders (Gram-positive & Gram-negative bacteria, actinobacteria and fungi) and starvation biomarker for Gram-negative bacteria based on $^{13}$C-PLFA (PLFA-SIP analysis) in soil spiked with glycine (GLC), glyphosate (GLP), sarcosine (SARC) and AMPA during 75-day incubation experiment. Scale in the heat maps shows minimum (light brown color) to maximum reddish color) contribution of microbial groups.



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