

Impacts of glyphosate-based herbicides on soil microbiota

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Master's thesis**Subject:** Biology / MDP Biosciences – Evolutionary Biology**Author(s):** Pawani Liyanage**Title:** Impacts of glyphosate-based herbicides on soil microbiota**Supervisor(s):** Senior Researcher Dr. Suni Anie Mathew, Assistant Professor Dr. Benjamin Fuchs, Adjunct Professor Dr. Marjo Helander**Number of pages:** 62 pages**Date:** 12.06.2025**Abstract**

The present study investigates long term effects of glyphosate-based herbicides (GBHs) on soil microbes. GBHs inhibit the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway of plants, eventually eliminating weeds from agricultural fields. However, several microbes have shikimate pathway and there is very little information on the long-term effects of GBHs on soil bacteria. The study used bacterial culturing techniques, glyphosate-sensitivity prediction bioinformatics tool and *in vitro* glyphosate sensitivity tests to investigate the response of soil bacteria collected from an experimental plot with long-term GBH application. Bacterial colony formation was decreased in soil samples collected from GBH-treated plots compared to the control plot. The abundance of cultured bacteria was lower in the samples from GBH-treated soil compared to that of control soil. Even though, GBH treatment did not significantly change the overall bacterial diversity in terms of number of taxa, bacterial richness and evenness in genus level were notably impacted by GBH exposure. Phyla *Pseudomonadota* was more dominant while *Actinomycetota*, *Bacillota*, and *Bacteroidota* were less dominant in samples from GBH-treated plots compared to those from control plots. Glyphosate sensitivity assays showed variable observations in low and high GBH treatments, when compared to the predictions of EPSPS bioinformatics tool. Most isolates grew in low GBH regardless of predicted sensitivity status or source of sample origin (control or GBH plots). However, except for genus *Variovorax*, *Sphingomonas* and *Pseudarthrobacter* none of the selected isolates grew in high GBH. Remarkably, *Variovorax* and *Pseudarthrobacter* bacterial isolates from GBH-treated plots were resistant to even high GBH treatment whereas those from control plots did not grow. The findings reveal that GBH effect on bacterial growth depends on the applied quantity of the glyphosate, with higher concentrations potentially inhibiting growth while lower concentrations might have stimulated the development of resistant mechanisms. Further studies are essential to deeply understand the response of each bacterial taxa to long-term field application of GBH and how it may affect microbial interactions and soil health.

Key words: GBH, Bacterial diversity, bacterial community composition, shikimate pathway, long term effects, Glyphosate sensitivity

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1 Introduction

The increase in the world population during the 20th century led to a parallel expansion in agricultural production (Tudi et al., 2021). With this agricultural revolution, widespread usage of pesticide was a crucial milestone to improve agricultural productivity. Glyphosate-based herbicides (GBHs) played a major role since their commercialization in the 1970s and are the most widely applied herbicide globally (Mandrioli, 2020). Despite being an effective weed control agent, criticisms have emerged regarding glyphosate's persistence in soil and possible consequences for the ecosystem (Duke, 2020). Glyphosate adsorbs to soil particles, thus contributing to its persistence in soil. This occurs because of its ability to form hydrogen bonds with organic matter and anion exchange processes with clay minerals and oxides, ultimately resulting in reduced potential for degradation. While glyphosate can degrade and manufacturers assure quick degradation, its degradation is often slower when adsorbed compared to when it is in a free state, leading to longer persistence in the environment especially in northern ecosystems (Helander et al., 2012). Furthermore, some studies demonstrated that repeated use of glyphosate led to longer half-lives for total mineralization, increasing from 2.2 to 3.4 months as the number of applications increased, proposing that its degradation can be impacted by the application frequency (Andréa et al., 2003).

1.1 Glyphosate's mode of action,

Glyphosate acts by targeting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an essential enzyme in the shikimate pathway found in plants (Duke & Powles, 2008). By inhibiting EPSPS, glyphosate disrupts the formation of chorismate, which is an essential compound required for the synthesis of the aromatic amino acids tryptophan, phenylalanine, and tyrosine (Maeda & Dudareva, 2012). This subsequently blocks production of phytohormones and other secondary metabolites (Fuchs et al., 2021) vital for plant growth and development. This inhibition leads to biochemical, physiological and structural alterations within the plants, and these indirect effects can also promote plant death indicating that GBHs have an impact on plant health via a variety of pathways other than EPSPS inhibition (Freitas-Silva et al., 2022)

The shikimate pathway is not restricted to plants; it can be found in many other microorganisms such as fungi and bacteria. Consequently, these microorganisms are sensitive to glyphosate. GBHs are applied to the soil approximately two weeks prior to planting crops, to eliminate weeds. During the application process, soil microbes can be directly exposed to glyphosate.

Regular GBH application and persistent glyphosate residues in soil lead to prolonged exposure of soil microbes to glyphosate.

1.2 Long-term effects of glyphosate on nutrient cycling and soil microbiota

Herbicide use has significant impacts on several biological processes in the soil, such as respiration, carbon (C) and phosphorus (P) dynamics and nitrogen (N) mineralization, breakdown of organic material, enzyme activity, and nutrient cycling (Rose et al., 2016). These impacts are shaped by various factors such as rate and time of application of herbicide, soil properties, temperature and moisture, and other agroclimatic conditions, which determine both the extent and direction of the impact (Guijarro et al., 2018). Notably, application of commercial herbicides in soils without prior herbicide exposure indicates more prominent impacts (Lane et al., 2012), suggesting microbial adaptation. Herbicide impacts have been estimated on soil microbes, C-cycling enzymes, and the breakdown and mineralization of organic matter. In general, respiration is relatively unaffected when glyphosate is applied at standard rates of 0.5–5 kg/ha (Araújo et al., 2003; Means et al., 2007), in most instances, respiration is either increased, particularly at elevated application rates (Wardle & Parkinson, 1990a) or remains unchanged (Busse et al., 2001; Houston et al., 1998; Pereira et al., 2008; Wardle & Parkinson, 1991). Some soil bacteria can utilize glyphosate as a source of phosphorus *in vitro*, appearing as a viable strategy for bioremediation of glyphosate-polluted soil (Chávez-Ortiz et al., 2022). Glyphosate applied at standard rates has minimal effect on soil N-cycling, ammonification, nitrification and denitrification processes (Stratton & Stewart, 1991).

Prior research has demonstrated varying effects of glyphosate on soil bacterial populations. Initial studies indicated a temporary increase in bacterial populations and overall microbial activity following glyphosate application (Wardle & Parkinson, 1990b, 1990a). However, with advancements in methodologies to study microbes, later studies observed a slight reduction in bacterial populations (Araújo et al., 2003; Sebiomo et al., 2011). The relative abundance of *Acidobacteria* declined following exposure to glyphosate and long-term glyphosate application affected rhizosphere nutrient status (Newman et al., 2016). Prolonged glyphosate use consistently increase the rate of colonization of crops by *Fusarium* species, which are potentially pathogenic, while reducing the population of *Pseudomonas* bacteria, considered beneficial organisms in the soil. (Kremer & Means, 2009). These results show that prolonged

usage of glyphosate elevates the presence of pathogen while reducing the number of growth-promoting microorganisms in soils. Another study indicated that glyphosate inhibits microbial growth in pure cultures at concentrations of 0.075 g/L (Shehata et al., 2013).

Variations in the composition of the soil microbial population may be due to variations in the dissipation or spread of glyphosate after repeated applications (Lancaster et al., 2010). A single exposure to glyphosate caused only minor changes to soil microbial communities. In pristine soils, microbial respiration increased, likely as a stress response from sensitive species. Microbial biomass in soil was not significantly affected by glyphosate when sprayed at standard application rates (PEC/Predicted Environmental concentration = 3 mg/kg), with stimulation is observed more frequently than inhibition (Rose et al., 2016). Soils with long-term exposure did not exhibit this response. Glyphosate's metabolic breakdown releases nutrients into the soil, lowering nitrogen restriction and favouring species that can make use of these resources (Zabaloy et al., 2012).

1.3 Microbial adaptation to GBH

In contrast to plants, a variety of microbes have been shown to withstand or even overcome the harmful effects of glyphosate (Drouin et al., 2010), by increasing EPSPS production, altering EPSPS structures or rapid metabolism/detoxification of the glyphosate molecule (Hertel et al., 2021a). Sensitivity to glyphosate depends on the class of EPSPS present in the respective organism: Class I EPSPS is glyphosate-sensitive, while Class II EPSPS is glyphosate-tolerant (Funke et al., 2007; Priestman et al., 2005).

Several microorganisms can utilize herbicides as a source of carbon, nitrogen or phosphorus (Mohanty & Das, 2022). Examples include atrazine and glyphosate, which are metabolized by *Pseudomonas* and *Arthrobacter*, acetochlor and dicamba metabolized by *Sphingomonas*, and paraquat metabolized by *Lipomyces* yeast (Huang et al., 2017). Among the bacteria studied, several species utilize glyphosate as a source of phosphorus, which indicating that they possess enzymes breaking the C–P bond. The few exceptions are the mutant *Arthrobacter* sp. GLP 1/Nit strain, which utilizes glyphosate as a nitrogen source (Pipke & Amrhein, 1988), along with *Streptomyces* sp. StC (Obojska et al., 1999) and *Achromobacter* sp. LW9 (McAuliffe et al., 1990), in which glyphosate can serve as a carbon source. Examples of glyphosate-degrading microorganisms with known taxonomic classification are *Achromobacter* sp. LW9,

Agrobacterium radiobacter, *Flavobacterium sp. GDI* and *Pseudomonas sp. LBr* (Sviridov et al., 2015).

There are two main microbial pathways for glyphosate degradation:

1. Glyphosate Oxidoreductase Pathway: Converts glyphosate into amino methyl phosphonic acid (AMPA) and glyoxylate.
2. C-P Lyase Pathway: Cleaves the C-P bond, releasing phosphate and sarcosine, which are further metabolized.

Three important intermediates of glyphosate metabolism, AMPA, sarcosine and acetyl glyphosate, are broken down further through separate metabolic pathways. (Zhan et al., 2018). AMPA is one of the most frequently found intermediates in glyphosate biodegradation. Which is produced by the breaking of the C–N bond by the enzyme glyphosate oxidoreductase. Many bacteria, including laboratory and wild-type strains, can convert glyphosate to AMPA. However, AMPA is often not further mineralized and is released into the environment. Some bacteria can utilize AMPA as a phosphorus source (Zhan et al., 2018).

Another key intermediate, sarcosine, is generated through the direct cleavage of the C–P bond during glyphosate biodegradation (Firdous et al., 2018). It serve as the carbon and the nitrogen source and can be further broken down into formaldehyde and glycine (Sviridov et al., 2012). It has been discovered that bacteria biodegrade via the AMPA pathway in natural settings, while in laboratory setting, they use the sarcosine pathway (Villamar-Ayala et al., 2019). Especially the sarcosine pathway is determined by the level of the inorganic phosphorus present in the environment. These pathways are facilitated by specific enzymes produced by bacteria such as *Pseudomonas*, *Arthrobacter*, and *Achromobacter* (Sviridov et al., 2015).

1.4 Microbes' sensitivity to GBH

It is important to further study the sensitivity of microbial communities to glyphosate since long-term application of GBHs may lead microbes to eventually becoming resistant or tolerant to glyphosate (Chen et al., 2022) and thus change the abundance of certain bacteria (Omorinola et al., 2022). This can disrupt the functions of these bacteria in soil, the composition of soil bacterial communities, their interactions with other microbes, soil functions as well as plant health. Bacteria can develop resistance to glyphosate through mutations in the EPSP synthase gene, resulting the encoded enzyme less sensitive to the herbicide. They can also develop resistance to glyphosate by decreasing glyphosate sensitivity or increasing production of the EPSP synthase, either through gene overexpression or gene amplification. This allows a

subfraction of non-inhibited enzymes to synthesize sufficient EPSP for amino acid biosynthesis, reducing glyphosate's toxicity (Hertel et al., 2021).

Several studies highlight the ability of certain bacteria to adapt and persist in glyphosate-contaminated environments. For example, *Ensifer adhaerens* and *Pseudomonas resinovorans* have demonstrated notable tolerance to glyphosate (Zhumakayev et al., 2021). Mechanisms contributing to glyphosate tolerance include degrading glyphosate, reducing the toxicity of the glyphosate through covalent modification and reducing the uptake or increasing the export of the herbicides. Glyphosate and other similar substances may enter through cells' amino acid transporters. Upon exposure to toxic amino acid analogues, bacteria often inactivate transporter genes to reduce glyphosate sensitivity and ensure survival (Hertel et al., 2021).

Given these concerns, this study investigates the long-term effects of GBH residues on soil bacterial populations and their sensitivity to GBH exposure. By exploring how GBH influences bacterial abundance, diversity, composition, and sensitivity, we aim to provide insights into the broader ecological impacts of GBH use.

Using culturing techniques, bacterial community of GBH -treated soil and control soil were analysed from a long-term field experiment. Further, soil sensitivity test was performed to demonstrate the soil bacterial growth response across a gradient of GBH concentrations. Based on the hypothesis, that GBH treatments in soil alter abundance and diversity of bacterial taxa we asked the following research questions:

- 1) Is the abundance and diversity of isolated bacterial taxa reduced in GBH -treated soils compared to controls?
- 2) How does bacterial sensitivity vary with different concentrations of GBH exposure?

Research objectives were

1. To identify the bacterial abundance and diversity of soil samples collected from GBH treated and control plots.
2. To assess the relative abundance of each bacterial taxa between GBH treated and control samples.
3. To monitor bacterial sensitivity to GBH exposure.

2 Materials and methods

2.1 Study area

The field site for sample collection was located at Ruissalo Botanical Garden (60°26'N, 22°10'E), University of Turku in southwestern Finland. The soil was classified as medium clay, notable for its high organic matter content ($>120 \text{ g kg}^{-1}$) and a pH of 5.9 (Hagner et al., 2019). Since 2013, the field has been treated biannually with the glyphosate-based herbicide (GBH) Roundup Gold, in accordance with typical agricultural practices. The standard application involved 450 g of isopropylamine glyphosate salt (CAS: 38641-94-0) at a rate of 6.4 litres per hectare, diluted in 3 litres of tap water per plot. In contrast, the control plots received only 3 litres of tap water per plot (Helander et al., 2019). The field has been cultivated with agricultural plant species - potatoes, strawberries, fava beans, and oats. The experimental setup has two treatment groups control (K) and GBH (G) with 13 plots for each treatment (23 m x 1.5 m each). The concentration of glyphosate residues in control and GBH treated soil plots were checked by following the protocol in previous studies (Mathew et al., 2024)

2.2 Sample collection

Soil samples at a depth of 5 – 10 cm were collected using soil samplers (Diameter 2.5 cm), from eight replicate plots from both the control (K) and GBH -treatment (G). During sample collection (April 2024), the average temperature was 3.7°C and average precipitation was 46 mm (<https://en.ilmatiteenlaitos.fi/download-observations>). Soil samples were sonicated in 2 ml phosphate buffer, followed by transfer to 1.5 ml Eppendorf tubes. The solution was centrifuged, supernatant was discarded, and 1 ml of Reasoner's 2 broth (R2B) was added. The bacterial solution was serially diluted twice, plated on Reasoner's 2 Agar (R2A) medium and incubated at room temperature for 4-7 days. Individual colonies were subsequently isolated, and pure strains were obtained through repeated streaking onto fresh R2A plates. The morphological characteristics, such as colony size, shape, colour of each isolate was recorded (Appendix 1).

2.3 Sample collection and processing

Five plots were selected to obtain a comparable amount of different bacterial colony numbers. Plot selection was dependent on the diversity of colony morphologies present in each plot to assure a complete taxonomic analysis (Appendix 2). Bacterial isolates were harvested from pure cultures by suspending a 1 μ L loopful of cells in 100 μ L of sterile Milli-Q water. The cell suspensions were stored at -20°C and used as a template for PCR. Pure cultures were preserved at -80°C for long-term storage. Stock cultures were prepared by inoculating a 10 μ L loopful of bacterial cells into 1 mL of R2B broth containing 30% glycerol, (Appendix 3) and stored at -80°C.

2.4 16S rRNA gene targeted PCR for bacterial isolate analysis

The full-length bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the forward primer 27f (5'AGAGTTTGATCCTGGCTCAG3') and the reverse primer 1492R (5'-GGYTACCTTGTTACGACTT-3') Lane (1991). The PCR reaction consisted of DNA (25 ng/ μ l) sample, 1x PCR buffer, 0.2 mM dNTPs, 10 μ M of each primer and 2000 U/ml GoTaq DNA Polymerase (Promega, WI, USA) in a 25 μ L reaction volume. Amplification profile for the PCR reactions was 3 min initial denaturation at 95°C followed by 30 cycles of denaturing at 95°C for 60 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min. Final extension was carried out at 72°C for 5 min. All PCR reactions had negative controls. PCR reaction was run in the Biorad C1000 Touch Thermal Cycler. The amplicons were analysed on 1.0% agarose gel after the PCR amplification to ensure the efficiency of PCR and quality of amplicons. Gel run images were taken from ChemiDoc™ MP Imaging System (BioRad).

The concentration of PCR products was checked on Qubit 4 fluorometer and samples were diluted to contain a concentration of 25 ng/ μ l. The PCR products were then sent to Eurofins for Sanger sequencing. To ensure high-quality sequence data, chromatograms were trimmed to remove low-quality regions including overlapping peaks, low peak intensity, and the initial and terminal 50 base pairs, which often exhibit lower quality. Bacterial taxa were identified using NCBI

(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and SILVA (<https://www.arb-silva.de/aligner/>) databases.

2.5 Assessment of bacterial abundance and diversity

Number of colonies was calculated in each treatment group to identify the total number of colonies per plot sample (Appendix 4). Student's t-test was employed to compare the total bacterial colony count between control and GBH-treated plots.

To assess the diversity of bacterial communities, only morphologically different CFUs were selected from each control and treated replicate plates. The number of genera, family, orders, class and phylum present in each sample was determined (Appendix 5). Species diversity was analysed using richness estimation and Shannon diversity indices. Statistical analysis using Student's t-test was employed to compare the diversity between control and GBH-treated plots.

2.6 Glyphosate sensitivity test

2.6.1 *In silico* prediction of EPSPS sensitivity

The EPSPS enzyme can either be sensitive or resistant to glyphosate, determined by specific conserved amino acids. The EPSPS enzyme classification tool (<https://ppuigbo.me/programs/EPSPSClass/>) predicted the sensitivity of isolated bacterial species. Sensitivity scores were assigned based on the predicted EPSPS class, ranging from 0 to 1 where 0 indicates resistance (no sequences within the taxonomic group were predicted to be sensitive to glyphosate) and 1 indicates full sensitivity (all known sequences in the group were predicted to be sensitive to glyphosate). (Mathew et al., 2022).

2.6.2 Glyphosate sensitivity assay

R2A agar plates were prepared with 0 μL (control), 20 μL (low GBH), and 40 μL (high GBH) of the commercially available GBH, Roundup (glyphosate 480 g/L). Selected bacterial cultures from stocked samples 10 μl loop diluted for 1 in 1,000,000 R2B solution and diluted samples were spread onto these plates and incubated at room temperature. The growth of bacterial colonies was monitored over time to assess their sensitivity to GBH (Appendix 6).

The systematic approach of the study is illustrated in the Figure 1.

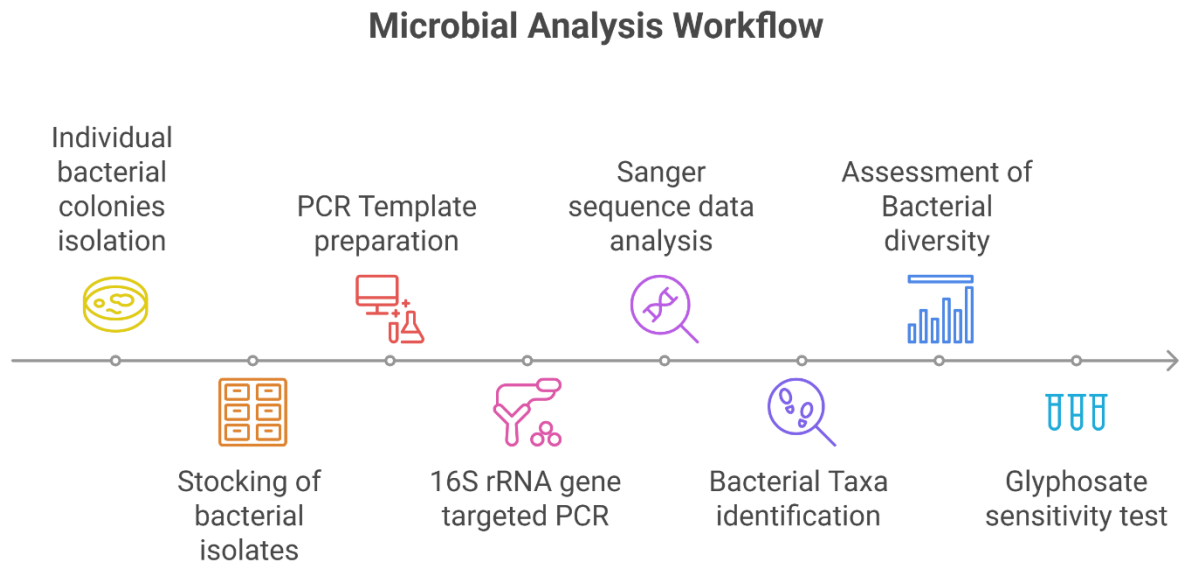


Figure 1. Illustration of the systematic approach of the study. Image created using Napkin AI from the prompt "[<https://www.napkin.ai/>]."

3 Results

3.1 GBH residue concentration in soil were higher in GBH -treated plots

The concentration of GBH residues (Table 1) is significantly higher in the GBH treated plots compared to the control plots according to the T test ($t = -5.1606$, $df = 7.0002$, $p = 0.0013$) (Appendix 7.1). These results demonstrate that GBH concentration in control plots are at minimal levels.

Table 1. Concentration of GBH in soil samples from control and GBH treated field plots

Control plot	GBH concentration in control plots (mg/kg)	GBH treated plot	GBH concentration in treated plots (mg/kg)
K1	0.020	G1	2.2
K2	0.018	G2	4.0
K3	<0.01	G3	3.8
K4	<0.01	G4	0.11
K5	<0.01	G5	1.4
K6	<0.01	G6	2.5
K7	<0.01	G7	2.3
K8	0.020	G8	2.0

3.2 The GBH-treated soil had a lower abundance of bacterial colony forming units (CFUs) compared to the control soil

Bacterial colony forming units (CFUs) count from control soil samples were higher than those from GBH treated soil samples (Figure 2). Representative images of bacterial colony formation in the control and GBH-treated plots are presented in Figure 3. Bacterial colony counts between GBH -treated and control plots were statistically different (T-test: $t = 2.2893$, $df = 10.962$, $p\text{-value} = 0.04291$) (Appendix 7.2).

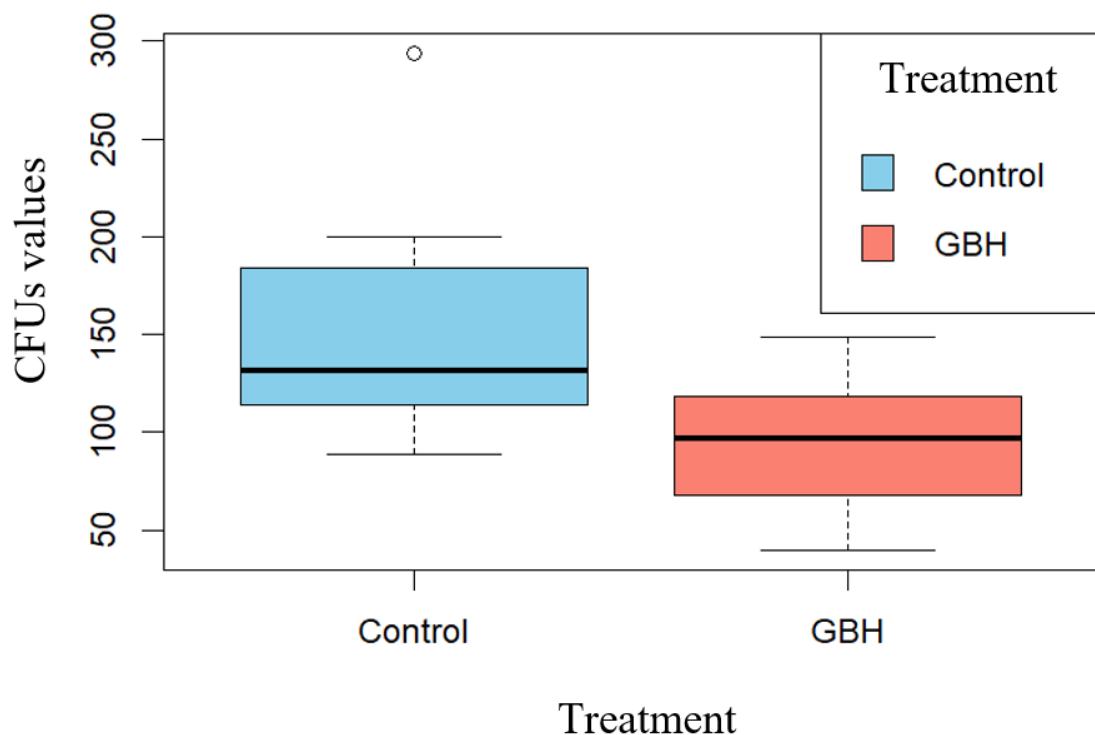


Figure 1. Bacterial CFUs (colony forming units) in control soil and GBH (glyphosate-based herbicide) soil. Box plot displays the distribution of CFU values, and a thick horizontal line indicates the median value.

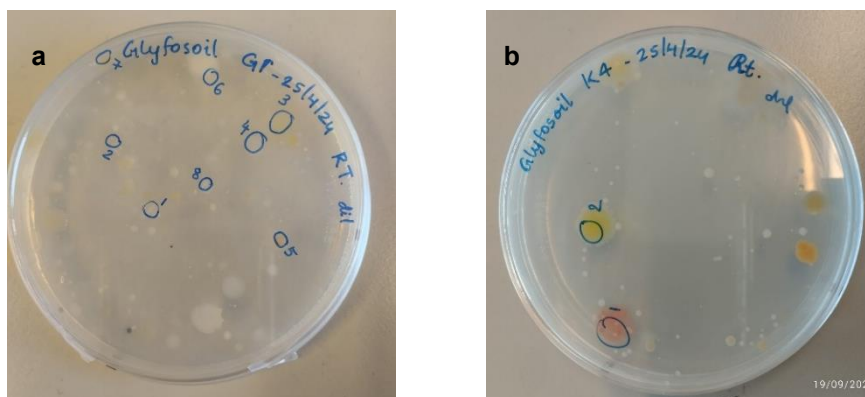


Figure 3. Bacterial colony forming units from (a) GBH (glyphosate based herbicide) soil from plot G1 and (b) control soil from plot K4.

3.3 16s rRNA genes were amplified from all the selected bacterial isolates

Full length 16S rRNA genes were amplified from all the selected bacterial isolates (Figure 4). The size of the amplicons were approximately 1.5kb.

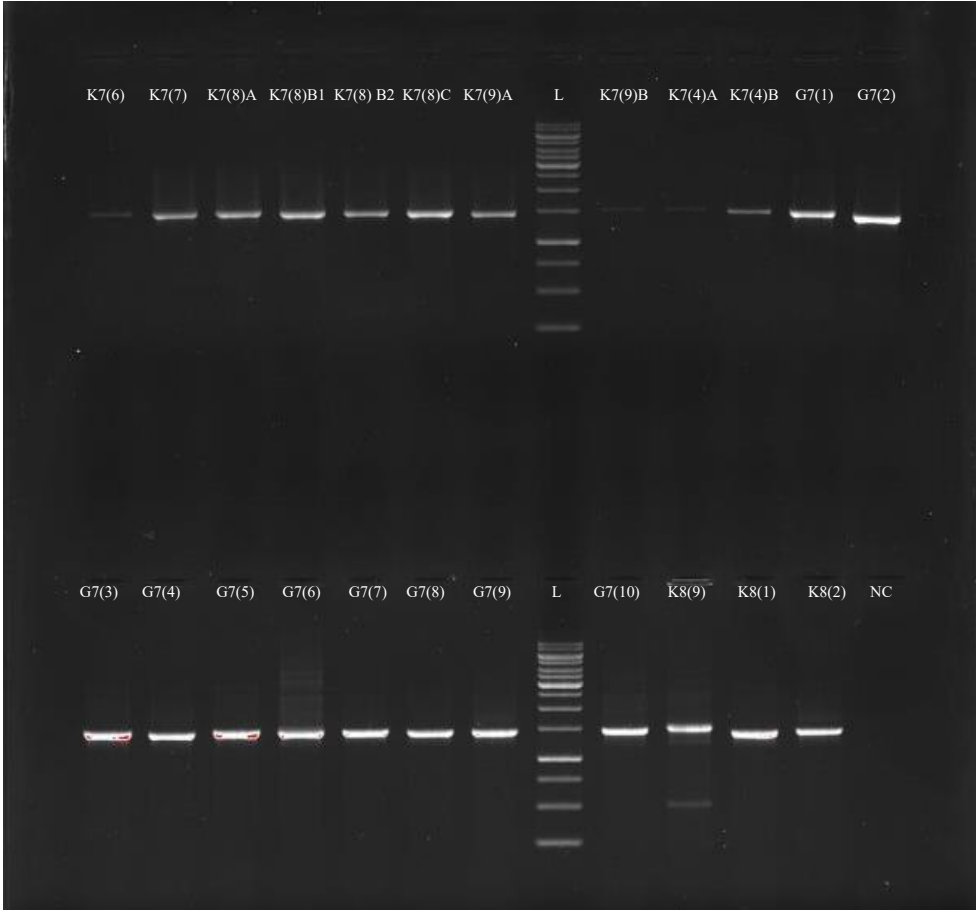


Figure 4. Gel image analysis of 16s rRNA amplification of some selected bacterial isolates from control and GBH (glyphosate based herbicide) treated plot. Each sample lane is labeled with sample codes (G = glyphosate, K = control), L: 1 kb DNA ladder, NC: Negative control

3.4 Diverse bacterial taxa were identified

Diversity of different bacterial taxa at the phylum, class, order, family and genus level are summarized in Table 2. Bacterial isolates identified only in control plots are highlighted in green, those detected only in GBH-treated plots are highlighted in yellow and bacterial isolates identified in control and GBH-treated plots are highlighted in white. Identified culturable bacterial phylum from the soil samples were *Bacillota*, *Actinomycetota*, *Pseudomonadota* and *Bacteroidota*. At the class level, *Bacilli*, *Actinomycetes*, *Betaproteobacteria*, *Alphaproteobacteria*, *Flavobacteriia*, *Sphingobacteriia* were present in both control and GBH treatment plots. Notably, *Gammaproteobacteria* were found only in samples from GBH-treated soil. At the order level *Pseudomonadales* and *Rhodobacterales* were found only in GBH-treated soil. Rest of the taxa were identified from both control and GBH-treated soil and included *Bacillales*, *Micrococcales*, *Mycobacteriales*, *Kitasatosporales*, *Burkholderiales*, *Sphingomonadales*, *Caulobacterales*, *Flavobacteriales* and *Sphingobacteriales*.

At the family level *Sanguibacteraceae* were found only in control soil and *Pseudomonadaceae*, *Paracoccaceae* were only identified in GBH soil. Remaining taxa including *Bacillaceae*, *Micrococcaceae*, *Microbacteriaceae*, *Mycobacteriaceae*, *Nocardiaceae*, *Streptomycetaceae*, *Comamonadaceae*, *Sphingomonadaceae*, *Caulobacteraceae*, *Flavobacteriaceae* and *Sphingobacteriaceae* were found in control and GBH-treated soil. At the genus level, *Peribacillus*, *Agreia*, *Rhodococcoides*, *Pseudomonas*, *Cypionkella* were identified only in GBH-treated soil while *Priestia*, *Bacillus*, *Cryobacterium*, *Leifsonia*, *Salinibacterium*, *Sanguibacter*, *Mycolicibacterium* were identified only in control soil. Other genera, namely *Pseudarthrobacter*, *Arthrobacter*, *Plantibacter*, *Mycobacterium*, *Rhodococcus*, *Streptomyces*, *Variovorax*, *Sphingomonas*, *Brevundimonas*, *Flavobacterium*, *Pedobacter* and *Mucilaginibacter* were identified in both control and GBH-treated soil.

Table 2. Bacterial taxa identified from control and GBH (glyphosate-based herbicide) treated soil.

Phylum	Class	Order	Family	Genus
<i>Bacillota</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Priestia</i>
				<i>Bacillus</i>
				<i>Peribacillus</i>
<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Micrococcaceae</i>	<i>Pseudarthrobacter</i>
				<i>Arthrobacter</i>
			<i>Microbacteriaceae</i>	<i>Cryobacterium</i>

				<i>Leifsonia</i>	
				<i>Plantibacter</i>	
				<i>Salinibacterium</i>	
				<i>Agreia</i>	
			<i>Sanguibacteraceae</i>	<i>Sanguibacter</i>	
			<i>Mycobacteriales</i>	<i>Mycobacteriaceae</i>	<i>Mycolicibacterium</i>
				<i>Nocardiaceae</i>	<i>Rhodococcus</i>
					<i>Rhodococcoides</i>
			<i>Kitasatosporales</i>	<i>Streptomycetaceae</i>	<i>Streptomyces</i>
			<i>Pseudomonadota</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>
<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>		<i>Pseudomonas</i>	
<i>Alphaproteobacteria</i>	<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>		<i>Sphingomonas</i>	
	<i>Caulobacterales</i>	<i>Caulobacteraceae</i>		<i>Brevundimonas</i>	
	<i>Rhodobacterales</i>	<i>Paracoccaceae</i>		<i>Cypionkella</i>	
<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	
	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Pedobacter</i> <i>Mucilaginitibacter</i>	

	Bacterial taxa only found in control plots
	Bacterial taxa only found in GBH-treated plots
	Bacterial taxa found in both control plots and GBH-treated plots

According to the results of the student's t-test, the number of occurrences of selected cultures from different bacterial taxa at the phylum, class, order, family, and genus levels showed no significant differences between control and GBH-treated soil (Appendix 7.3).

3.4.1 Genus and phylum level bacterial diversity

Number of occurrences of different bacterial taxa at the phylum and genus level is summarized in Figure 5.

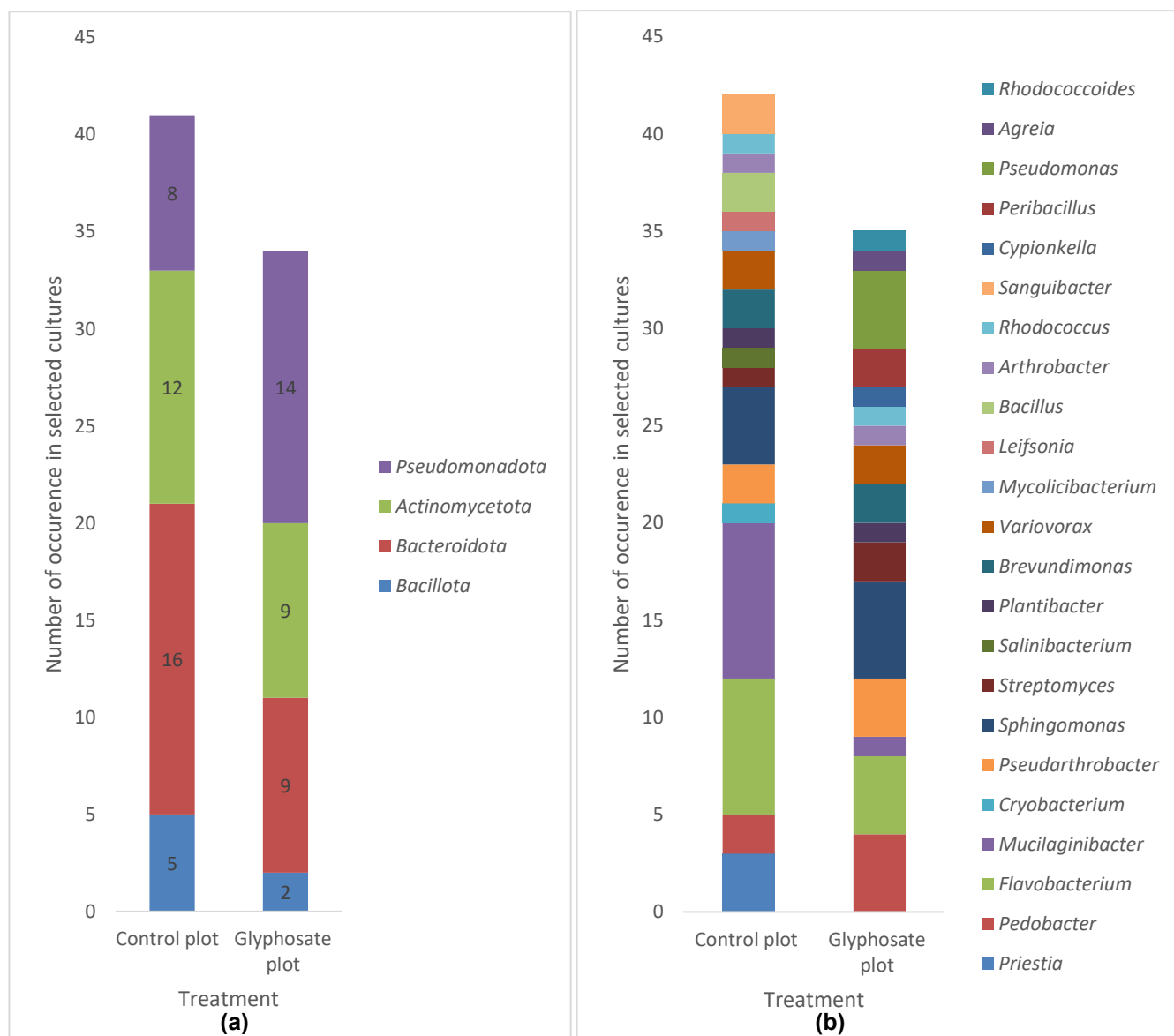


Figure 5. Number of occurrences of different bacterial taxa from selected culture plates at (a) phylum level and (b) genus level in control and GBH-treated soil. A total of 4 phylum were identified in both control and GBH-treated plots. At the genus level 18 different genera were identified in the control soil and 16 different genera were identified in the GBH-treated soil.

The Shannon-Wiener Diversity Index was used to assess genus-level diversity in both the Control plot and the GBH-treated plot, providing insights into species richness and evenness. (Appendix 8). Control Plot exhibits a higher median diversity (~1.75), indicating greater genus richness and a more even distribution of taxa within the field. GBH-Treated Plot displays a lower median diversity (~1.30), suggesting reduced species richness and lower evenness. The Control plot presents a broader diversity range (~1.0 to 2.0). The GBH-treated plot shows a narrower diversity range (~1.25 to 1.5) (Figure 6).

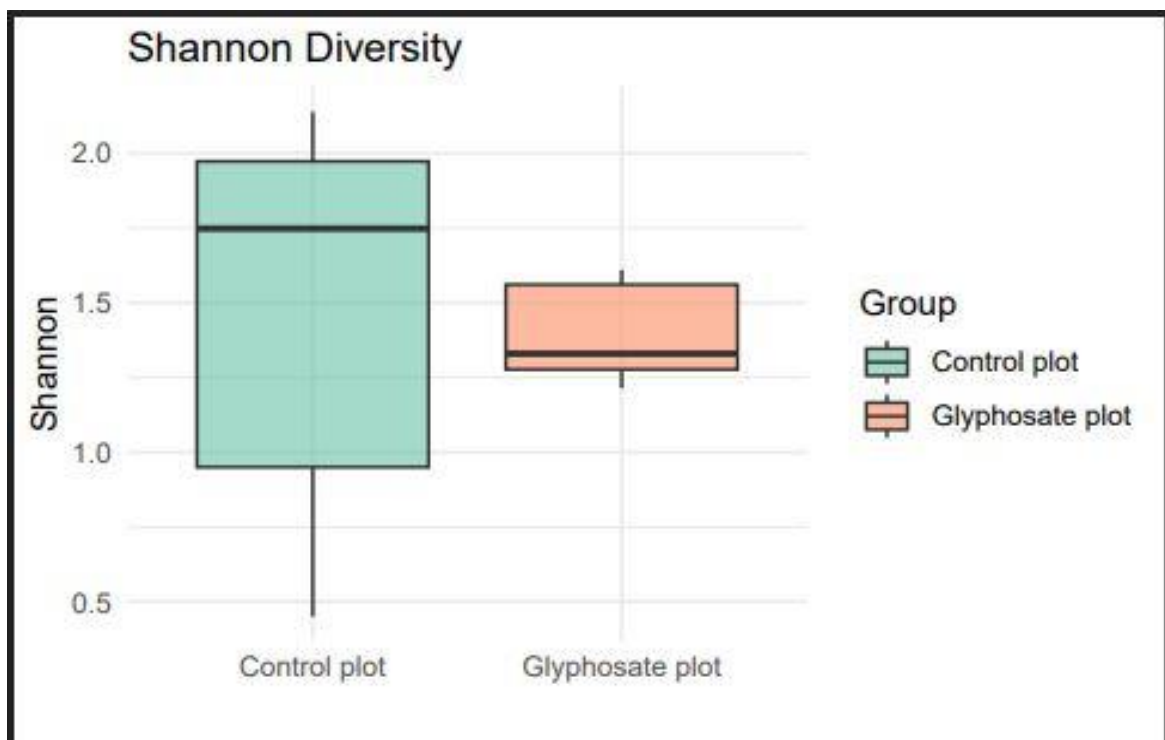


Figure 6 - The box plot compares Shannon Diversity between cultured bacteria from control plot (green) and glyphosate plot (orange). The y-axis represents Shannon Diversity values, while the x-axis labels the two groups.

3.5 Glyphosate sensitivity

3.5.1 GBH sensitivity of bacterial isolates was predicted using EPSPS enzyme classification tool

Bacterial species were classified to glyphosate resistant, sensitive or sensitivity unknown by EPSPS classification tool (Table 3). The analysis identified 11 bacterial species as glyphosate-resistant, 32 species as glyphosate-sensitive and 3 bacterial species as sensitivity unknown.

Table 3. Classification of bacterial species based on glyphosate sensitivity predicted by EPSPS classification tool.

Potential glyphosate-resistant bacterial species	Potential glyphosate-sensitive bacterial species	Bacterial species with unknown sensitivity to glyphosate
<i>Priestia megaterium</i>	<i>Pedobacter alluvionis</i>	<i>Brevundimonas mediterranea</i>
<i>Sphingomonas faeni</i>	<i>Flavobacterium aquidurense</i>	<i>Streptomyces hydrogenans</i>
<i>Brevundimonas intermedia</i>	<i>Mucilaginibacter galii</i>	<i>Streptomyces olivochromogenes</i>
<i>Sphingomonas aurantiaca</i>	<i>Cryobacterium soli</i>	
<i>Bacillus mycoides</i>	<i>Pseudarthrobacter sulfonivorans</i>	
<i>Cypionkella psychrotolerans</i>	<i>Streptomyces camponoticapitis</i>	
<i>Peribacillus simplex</i>	<i>Mucilaginibacter ginsenosidivorax</i>	
<i>Pseudomonas fragi</i>	<i>Salinibacterium xinjiangense</i>	
<i>Pseudomonas silesiensis</i>	<i>Plantibacter flavus</i>	
<i>Pseudomonas chlororaphis</i>	<i>Flavobacterium cupreum</i>	
<i>Pseudomonas fragi</i>	<i>Flavobacterium psychroterrae</i>	
	<i>Flavobacterium piscis</i>	
	<i>Mucilaginibacter rigui</i>	
	<i>Pedobacter frigiditerrae</i>	
	<i>Mucilaginibacter paludis</i>	
	<i>Variovorax ginsengisoli</i>	
	<i>Mycolicibacterium hippocampi</i>	
	<i>Leifsonia flava</i>	
	<i>Arthrobacter nitrophenolicus</i>	
	<i>Rhodococcus maanshanensis</i>	
	<i>Sanguibacter antarcticus</i>	
	<i>Mucilaginibacter boryungensis</i>	
	<i>Variovorax boronicumulans</i>	
	<i>Pseudarthrobacter psychrotolerans</i>	
	<i>Flavobacterium caseinilyticum</i>	
	<i>Flavobacterium hydatis</i>	
	<i>Agreia pratensis</i>	
	<i>Pedobacter insulae</i>	
	<i>Arthrobacter humicola</i>	

	<i>Mucilaginibacter paludis</i>	
	<i>Rhodococcoides fascians</i>	
	<i>Variovorax boronicumulans</i>	

The number of bacterial species categorized on their predicted glyphosate sensitivity using the EPSPS classification tool across different treatment plots illustrated in the Figure 6. From the selected sampling plots 50% of species in GBH-treated soil and 86% of species in control soil were sensitive to glyphosate and 31% of species in GBH-treated soil and 14% of species in control soil were resistant to glyphosate determined by the EPSPS classification tool (Figure 7). Control soil had a higher number of glyphosate sensitive microbial species compared to the resistant species. Bacterial species identified from the GBH-treated soil consisted of glyphosate sensitive and resistant bacterial species.

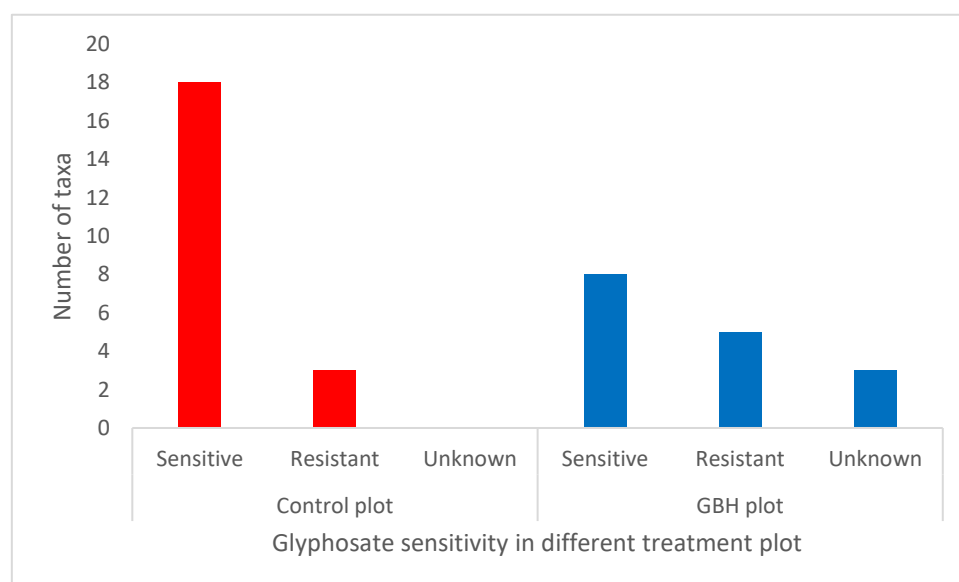
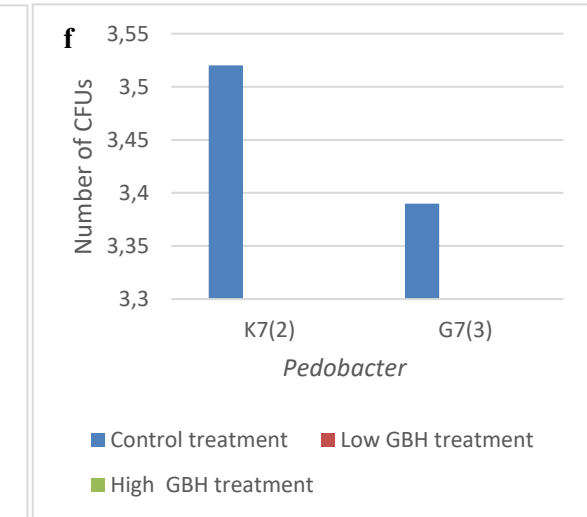
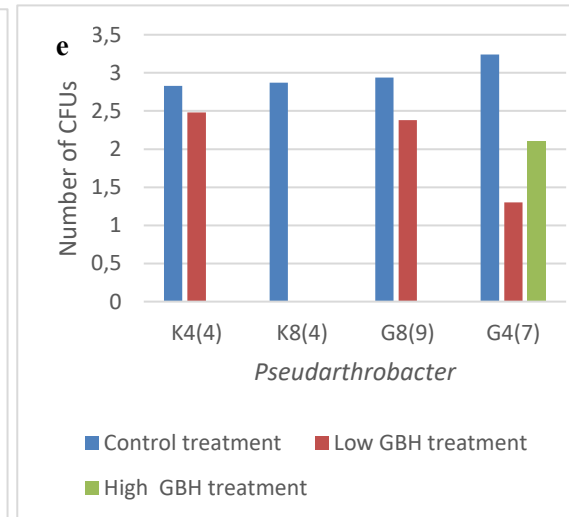
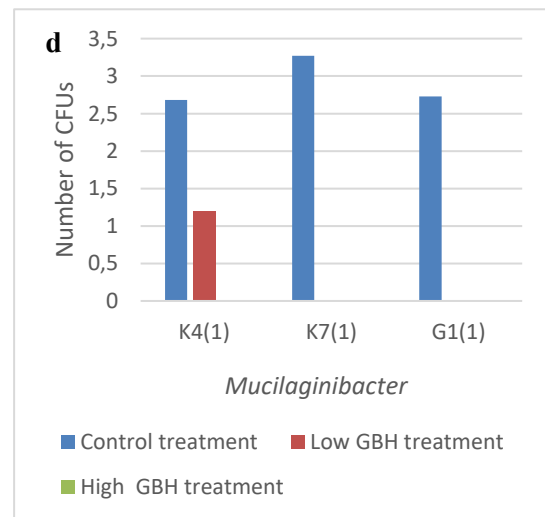
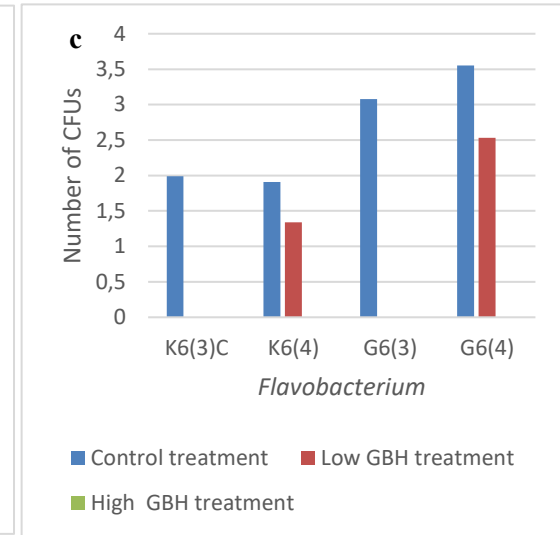
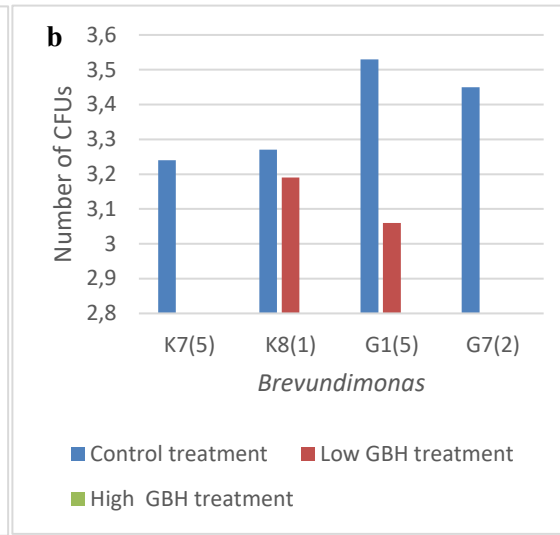
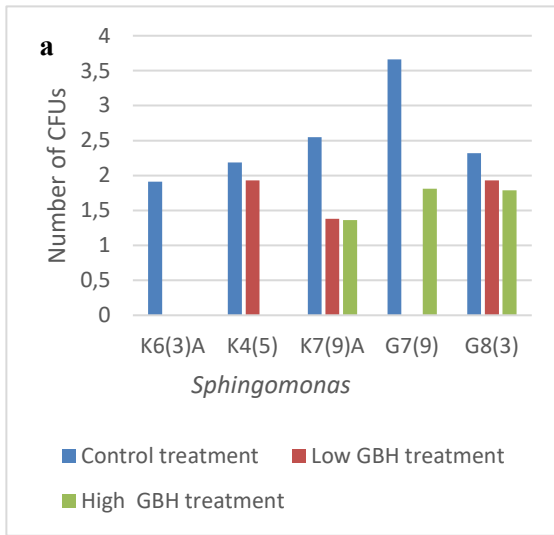


Figure 7. Number of bacterial species grouped by their glyphosate sensitivity predicted by the EPSPS classification tool in control and GBH (glyphosate-based herbicide) soil.

3.5.2 Responses of bacteria to glyphosate varied in *in vitro* conditions

Bacterial genera present in both control and GBH-treated soil were selected for glyphosate sensitivity test, based on the sensitivity predictions. Selected bacteria were *Pedobacter sp*, *Flavobacterium sp*, *Mucilaginibacter sp*, *Pseudarthrobacter sp*, *Sphingomonas sp*, *Brevundimonas sp*, *Variovorax sp*, *Rhodococcus sp*. and *Rhodococcoides sp*. (Figure 8).



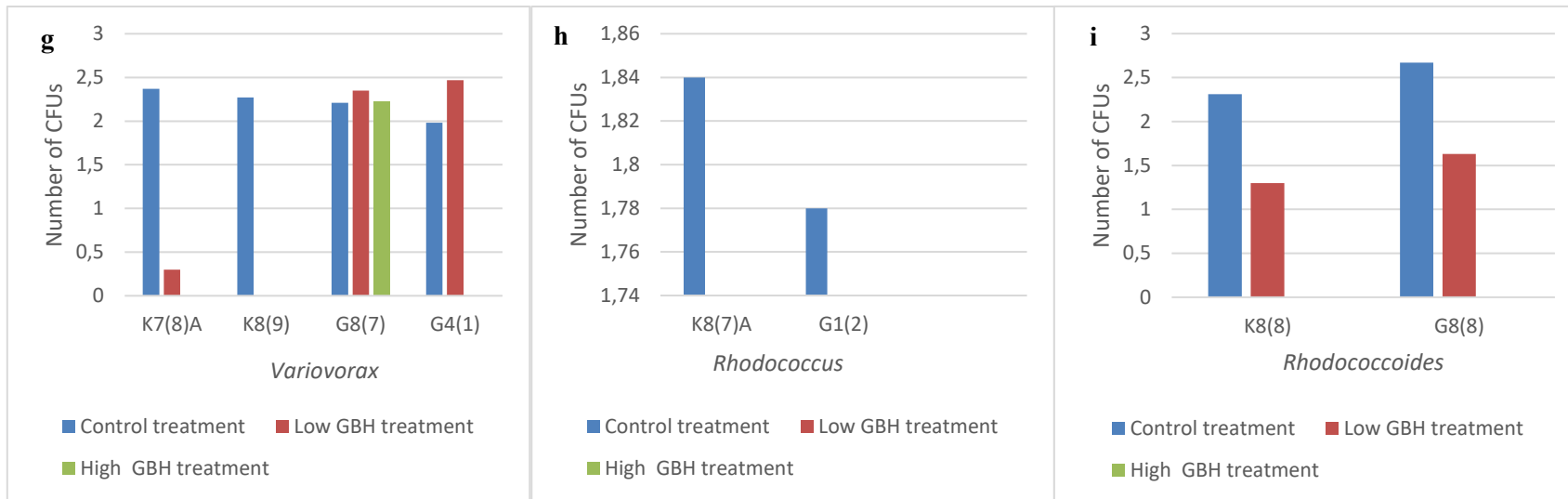


Figure 8-Number of CFUs (Log₁₀ scale) after two weeks of incubation of the selected bacterial isolates in control (0 μ L), low GBH (20 μ L of Roundup with 470 g/l glyphosate), and high GBH (40 μ L of Roundup with 470 g/l glyphosate) R2A plates. Selected bacterial taxa **a** - *Sphingomonas*, **b** - *Brevundimonas*, **c** - *Flavobacterium*, **d** - *Mucilaginibacter*, **e** - *Pseudarthrobacter*, **f** - *Pedobacter*, **g** - *Variovorax*, **h** - *Rhodococcus*, **i** - *Rhodococcoides*. K codes for bacterial isolates identified from control treatment plots and G code for bacterial isolates identified from GBH treated plots. Potential glyphosate-resistant bacterial isolates (**a,b**) and potential glyphosate-sensitive bacteria (**c,d,e,f,g,h,i**)

Bacterial growth on control plates was evident within 2 days. In contrast, bacterial growth was observed on low GBH plates after a 2-week incubation period. High GBH plates did not show any growth except G8(7) belonging to *Variovorax boronicumulans* (Figure 8g) and G7(9), G8(3), K7(9)A belonging to *Sphingomonas faeni* (Figure 8a) and *Pseudarthrobacter psychrotolerans* G4(7) (Figure 8e).

Sphingomonas was predicted to be resistant to glyphosate, and it was confirmed from the growth pattern of bacterial isolates from control and treatment soil (Figure 8a). Colony-forming units (CFUs) were detected across control, low GBH, and high GBH plates. A decreasing trend in CFU counts was observed from the control, low GBH and, high GBH plates, respectively, indicating a concentration based inhibitory effect on overall microbial growth despite the presence of resistant *Sphingomonas* strains. *Brevundimonas* was predicted to be resistant to glyphosate, and it proved in the sensitivity test in some bacterial isolates from both origin G1(5), K8(1) (Figure 8b). High GBH inhibited the growth compared to control plates. Some isolates in lower GBH plates showed comparatively higher growth than other taxa, but lower than control plates growth.

Mucilaginibacter, *Pedobacter*, *Rhodococcus* were predicted to be sensitive to glyphosate, which was supported by sensitivity tests. Growth was inhibited at high GBH and low GBH except one isolate from *Mucilaginibacter* K4(1) (Figure 8d), while isolates from both origins showed higher growth at control plates (Figure 8d,8f,8h). *Flavobacterium* showed no growth in high GBH. Some isolates from GBH-treated soil and control soil exhibited lower growth on low GBH plates and all the isolates showed higher growth in control plates, indicating sensitivity (Figure 8c). *Pseudarthrobacter* was predicted to be sensitive. Growth was inhibited at high GBH for isolates from both origins except for one isolate originating from GBH-treated soil G4(7) *Pseudarthrobacter psychrotolerans* (Figure 8e). Higher growth was detected in control plates compared to low GBH plates by rest of the isolates.

Variovorax was predicted to be sensitive and exhibited exceptional results. Strain G8(7) (*Variovorax boronicumulans*) from GBH-treated soil demonstrated growth at high GBH. Its growth was higher in low GBH plates than control plates (Figure 8g). Same species identified from GBH-treated soil G4(1) also showed higher growth in low GBH plates than control plates. However, *Variovorax* isolates from control plots displayed no growth at high GBH and low GBH proving the sensitivity to glyphosate (Figure 8g). *Rhodococcoides* was predicted to be sensitive to glyphosate. Control plates of *Rhodococcoides* showed higher growth and low GBH plates of *Rhodococcoides* showed low growth in both control and GBH-treated soil. Growth

was inhibited at higher GBH in both control and GBH treated plots bacterial isolates. Bacterial growth after two weeks of incubation of the selected bacterial isolates in control (0 μ L), low GBH (20 μ L), and high GBH (40 μ L) (470g/l) treated R2A plates (Figure 9).

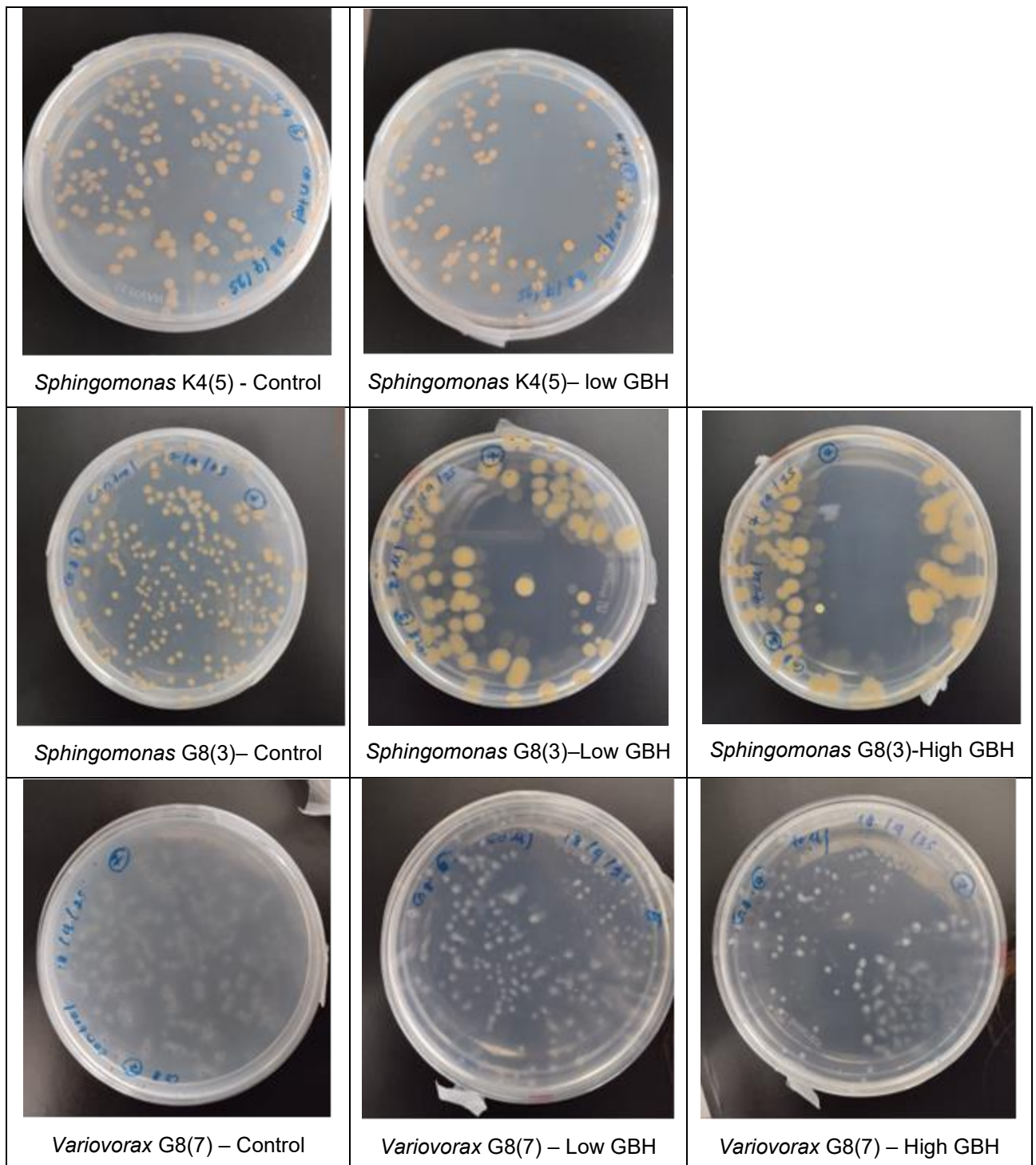


Figure 9. Images of selected bacterial isolates (*Sphingomonas* and *Variovorax*) growth in control, low GBH (20 μ L), and high GBH (40 μ L) (470g/l) treated R2A plates.

4 Discussion

Previous studies on the impact of glyphosate on soil bacteria have yielded contrasting results. Therefore, it is important to investigate this process further to understand the long-term application impact of glyphosate on soil bacterial communities. A comprehensive culture-based taxonomic analysis was conducted to identify representative taxa from control and GBH-treated soil samples, followed by sensitivity assays of cultured isolates to GBH.

The present study indicates a significant difference in bacterial CFUs between samples obtained from control and GBH-treated plots. Samples from control soil demonstrated higher abundance of CFUs compared to samples from GBH-treated soil. The reason may be due to the herbicide's toxic effects on sensitive microbial species. Glyphosate exposure can reduce the abundance of certain microbial populations, as demonstrated by studies showing a decrease in viable bacterial counts in glyphosate contaminated soils (Omorinola et al., 2022). Additionally, studies on microbial abundance in soil following glyphosate application have demonstrated both decrease and increase in microbial population with an initial significant reduction in bacterial populations due to glyphosate treatment, followed by a subsequent increase in abundance over time (Brka et al., 2020). Even though GBH toxic effects act on sensitive microbial species and cause them to initially decrease over time, GBH may act as a nutrient source for some bacteria, or they may develop tolerance to GBH allowing them to survive and proliferate in GBH presence. In addition to changes in abundance, glyphosate application can alter microbial community composition and soil properties, potentially impacting soil health. Glyphosate can shift bacterial community structures, favouring certain taxa while diminishing others, which may disrupt ecological balance (Wang et al., 2023). The Shannon-Wiener Diversity Index was utilized to assess genus-level diversity in both the control plot and the GBH-treated plot, providing insights into species richness (number of genus) and evenness (how evenly individuals are distributed among genus). Control Plot exhibits greater genus richness and a more even distribution of taxa within the field. GBH-Treated plot displays a lower diversity suggesting reduced species richness and lower evenness. The control plot presents a broader diversity range and GBH-treated plot shows a narrower diversity range. As an overall view according to Shannon-Wiener Diversity Index result suggest that glyphosate usage leads to a decline in culturable bacterial diversity of GBH treated plot compared to the control plot. GBH appears to negatively affect genus richness and evenness, reducing the overall bacterial diversity. The lower Shannon Index in the Glyphosate plot suggests that certain genus may be disproportionately affected. Certain taxa were observed to increase in occurrence in GBH plots, while others were more prevalent

in control plots. For example, phyla *Pseudomonadota* seem to be more abundant in the GBH-treated soil. Research has identified various genera within the *Pseudomonadota* phylum that are capable of degrading glyphosate (Aslam et al., 2024). Some studies have shown that the genes responsible for phosphonate degradation are widely distributed among various bacterial taxa. For instance, a significant number of genomes containing phosphonate catabolism genes belong to the *Pseudomonadota* phylum (Ruffolo et al., 2023). On the other hand, phyla like *Bacillota*, *Bacteroidota* and *Actinomycetota* were more abundant in the control soil compared to the GBH-treated soil. These microbial phyla may be more sensitive to glyphosate, leading to reduced growth or inhibited activity in the presence of the herbicide.

From a total of 76 bacterial isolates identified in this study, the class *Gammaproteobacteria* was detected only in GBH-treated soil and absent in control soil. The reason may be that *Gammaproteobacteria* have the ability to utilize glyphosate as a source of energy, which could contribute to their survival in glyphosate containing environments. Supporting this result, Newman et al. (2016) reported a significant increase in the relative abundance of *Gammaproteobacteria* in both corn and soybean rhizospheres following glyphosate treatment. Moreover *Gammaproteobacteria*, has been reported to use glyphosate as a sole P-source (Sviridov et al., 2014). Apart from class *Gammaproteobacteria*, orders *Pseudomonadales* and *Rhodobacterales*, families *Pseudomonadaceae*, *Paracoccaceae*, and genera *Peribacillus*, *Agreia*, *Rhodococcoides*, *Pseudomonas* and *Cypionkella* were only identified in GBH-treated soil. The presence of these taxa in glyphosate-impacted environments, where the fields have been treated with glyphosate for the past 9–10 years, suggests they may have undergone evolutionary adaptations for tolerance or resistance to the toxic effects of glyphosate. These adaptations could include mechanisms such as detoxification, utilization of glyphosate as a nutrient or energy source, or other physiological strategies that allow them to survive and thrive under glyphosate exposure.

Our findings suggest that the impact of GBH on bacterial growth is dependent on the GBH concentration. *Mucilaginibacter*, *Pedobacter*, *Rhodococcus* were predicted to be sensitive, which was supported by sensitivity tests. Growth was inhibited at high GBH and low GBH plates, while isolates from both origins showed higher growth at control plates, demonstrating the toxic effects of glyphosate. Some bacterial isolates predicted to be sensitive to glyphosate e.g., *Rhodococcoides*, *Flavobacterium*, *Variovorax* and *Pseudarthrobacter*, were growing in low GBH plates. High GBH concentration inhibited bacterial growth, while lower

concentration may lead to the development of resistance mechanisms or alternative metabolic pathway. Bacteria exposed to low concentrations of glyphosate may develop mechanisms to utilize glyphosate as a nutrient source, such as phosphorus, or evolve mechanisms to tolerate its toxic effects. This phenomenon may be a result of the hormesis effects, where low doses of glyphosate can promote the growth of certain bacterial species (Hameed et al. 2023).

All tested taxa, except *Variovorax*, *Sphingomonas* and *Pseudarthrobacter* were inhibited by high GBH concentration. *Variovorax*, *Sphingomonas* and *Pseudarthrobacter* demonstrated growth on high GBH, indicating their ability to tolerate higher GBH concentrations. A delay in growth was observed in low GBH plates on all the tested taxa. Bacteria on control plates were growing within two days after inoculation while growth on low GBH plates was detected after two weeks of incubation. *Sphingomonas* was predicted to be resistant to glyphosate and accordingly it was growing in high GBH plates. *Variovorax* was predicted to be sensitive and isolates from control plots displayed no growth at high GBH and low GBH proving the sensitivity to glyphosate. However, isolates from GBH treated plots showed a growth in low and high GBH treated plates. A potential explanation for *Variovorax* could be that *Variovorax* species contain enzymes such as phosphonatease, which is important for breaking the carbon-phosphorus (C-P) bond found in glyphosate. This enzymatic action is essential for breaking down glyphosate into less harmful substances, facilitating its degradation in the environment (Ruffolo et al., 2023). *Variovorax* G8(7) was identified as *Variovorax boronicumulans* (99.89% identity, SILVA database) and it is predicted to be sensitive to glyphosate based on the EPSPS type prediction tool. *Pseudarthrobacter* was predicted to be sensitive to glyphosate. Its growth was inhibited at high GBH for isolates from both origins except for isolate G4(7) that originated from GBH-treated soil and was identified as *Pseudarthrobacter psychrotolerans*. This GBH resistant strains (*Variovorax boronicumulans* and *Pseudarthrobacter psychrotolerans*) were identified from GBH treated soil and this resistance to GBH may have been acquired through evolutionary adaptation resulting from long-term exposure to GBH. In contrast, the *Variovorax* species identified from the control plot was classified as *Variovorax ginsengisoli* (99.80% identity). This strain was similarly predicted to be sensitive to glyphosate based on the EPSPS type prediction tool, and this finding is supported by our study. *Variovorax boronicumulans* and *Variovorax ginsengisoli* are different species and may have different sensitivities or *Variovorax boronicumulans* may have acquired resistance due to long-term exposure to GBH treatment.

The EPSPS bioinformatics tool predicted some taxa such as *Brevundimonas* and *Sphingomonas* to be resistant to glyphosate. Their resistance was further validated *in vitro* as growth was observed in the presence of glyphosate in control and GBH-treated soil bacterial isolates.

The amount of glyphosate applied to the soil is one of the primary determining factors of the soil microbial response. Bacteria have evolved multiple methods to withstand the effects of glyphosate. These approaches include reducing the sensitivity to herbicide by mutating EPSP synthase gene, glyphosate degradation by using glyphosate oxidoreductase (GOX) and phosphonates, detoxification, and changing the glyphosate uptake mechanism (Hertel et al., 2021).

Another possible reason for bacterial growth in glyphosate-containing media is the growth medium as R2A can potentially supply essential nutrients including amino acids. Thus, this potentially allows bacteria to grow even in the presence of glyphosate. The effectiveness of the growth medium may vary depending on glyphosate concentration. Higher concentrations could inhibit bacterial growth regardless of external amino acids.

Our experimental findings regarding glyphosate sensitivity yielded different results for some bacterial isolates compared to predictions generated by the web server (<http://ppuigbo.me/programs/EPSPSClass>) (Leino et al., 2021). Bacteria can resist glyphosate through different methods, including target site and non-target site resistance. These include mutations in the EPSPS gene increased efflux pump activity, modified metabolic pathways and decreased the uptake of the herbicide contributing to glyphosate tolerance. These would not be captured by the web server's prediction that based solely on the EPSPS sequence (Ruuskanen et al., 2023).

This study's results solely rely on culturable bacteria present in the field, limiting the scope of broader microbial diversity analysis. To obtain a more comprehensive understanding of the microbial responses to glyphosate treatment it would be important to expand the number of study plots in future studies. This may increase the diversity and quantity of bacterial isolates. Furthermore, a broader study of the overall microbial abundance and diversity can be obtained through molecular techniques, which provide insights into unculturable microbial populations.

5 Conclusions

In this study, the abundance of cultured bacteria was lower in the samples from GBH-treated soil compared to that of control soil. However, the overall diversity of culturable bacteria remained unaffected when considering the number of taxa present. Despite this, bacterial richness and evenness were notably impacted by GBH exposure. *In vitro* assays showed that bacterial growth depended on the GBH concentration of the growth media. Higher concentrations inhibited bacterial growth, while lower concentrations possibly allowed bacteria to develop resistant mechanisms. Soil microbes exposed to long-term GBH-treatments may have undergone evolutionary adaptations for tolerance or resistance to the toxic effects of glyphosate. This is noted by the results from the genus *Variovorax*, which was predicted to be sensitive to glyphosate but exhibited different responses to GBH. *Variovorax* isolate exposed to long-term application of GBH in the field was resistant, while non-exposed isolates were sensitive. Results of the study emphasize the complexity of glyphosate resistance mechanisms and the need for further in-depth studies on soil microbial communities.

6 Acknowledgement

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ChatGPT was employed to enhance the grammatical accuracy (OpenAI, 2025)

Appendices

Appendix 1 – Morphological feature of bacterial isolates

Table 4. Morphological features of cultured bacterial isolates

Plot	Bacterial colony identifier	Morphological features	Plot	Bacterial tag	Morphological features	Plot	Bacterial tag	Morphological features
1	K4(4)	white, translucent	2	K6(2)	white,translucent	3	K1(4B)	white, opaque
	K4(5)	orange, translucent		K7(1)	pink,translucent		K1(5A)	black dot surrounded by gray circle, bacteria zone has prevented fungus spread
	K3(15)	pale yellow, translucent		K7(2)	yellow,translucent,circular		K1(5B)	white, fungus
	K3(8)	white, translucent		K8(9)	white,translucent		K2(1)	whitish yellow translucent
	K5(5)	white, translucent		K1(1A)	white,opaque		K2(2A)	white,translucent
	K3(9)	yellow,translucent, circular		K1(2A)	white,opaque		K2(2B)	yellow biofilm
	K3(7)	white, translucent		K1(3A)	pink,translucent		K2(3B)	yellow biofilm
	K3(13)	cream,translucent		K1(4A)	yellow biofilm translucent		K2(4)	yellow biofilm
4	K2(5)	yellow,translucent, biofilm	5	K3(4)	yellow,translucent	6	K4(2)	yellow,translucent

	K2(6)	yellow,transluscent, biofilm		K3(5)	cream,transluscent		K4(3)	white,transluscent
	K2(7B)	yellow,transluscent, biofilm		K3(6)	white,transluscent		K4(6)	black dot surrounded by yellow circle, opaque
	K2(8A)	yellow,transluscent, biofilm		K3(10)	white,transluscent,circular		K4(7)	cream,transluscent,circular
	K2(8B)	white,transluscent		K3(11)	white,opaque,circular		K4(8)	white,transluscent
	K3(1)	yellow,transluscent		K3(12)	yellow,transluscent		K4(9)	white,transluscent
	K3(2)	white,opaque		K3(14)	white,transluscent		K4(10)	yellow,transluscent
	K3(3)	white,opaque		K4(1)	pink,transluscent		K4(11)	yellow,transluscent
7	K5(1)	pink,transluscent, circular	8	K5(8)	white,transluscent	9	K7(3)	white,transluscent
	K5(2A)	orange,transluscent		K5(9)	yellow,transluscent		K7(4)	mixed , yellow and orange
	K5(2B)	orange,transluscent		K6(1A)	white,transluscent		K7(5)	pale pink,transluscent
	K5(2C)	white,opaque,circular		K6(1B)	white,transluscent		K7(6)	white,transluscent,circular
	K5(3)	orange,transluscent		K6(3A)	yellow,transluscent		K7(7)	cream, transparent, circular
	K5(4)	white, transluscent		K6(3B)	yellow,transluscent		K7(8A)	white,transluscent
	K5(6)	white,opaque,circular		K6(3C)	yellow,transluscent		K7(8B1)	pink,transluscent
	K5(7)	white, transluscent,circular		K6(4)	yellow,transluscent		K7(8B2)	pink,transluscent
10	K7(8C)	pink,transluscent	11	K8(5)	white,transluscent,circular	12	K7(4)B	yellow,transluscent

	K7(9A)	yellow,transluscent		K8(6A)	white,circular		G1(1)	yellow,transluscent
	K7(9B)	white,transluscent		K8(6B)	white,opaque,circular		G1(2)	white,opaque
	K8(1)	pale pink,transluscent		K8(7A)	white,opaque		G1(3)	yellow,transluscent
	K8(2)	orange,transluscent		K8(7B)	yellow,transluscent		G1(4)	white,transluscent
	K8(3A)	white,opaque,dot surrounded by circle		K8(7C)	yellow,transluscent		G1(5)	orange,transluscent
	K8(3B)	yellow		K8(8)	orange,transluscent		G1(6)	white,transluscent
	K8(4)	white,transluscent,circular		K7(4)A	orange,transluscent		G1(7)	yellow,transluscent
13	G1(8)	white,transluscent	14	G3(2)	white,opaque	15	G4(5)	white,opaque
	G2(1)	white,transluscent		G3(3)	white,transluscent		G4(6)	white,opaque
	G2(2)	orange,transluscent		G3(4)	white,opaque		G4(7)	white,transluscent
	G2(3)	yellow,transluscent		G3(5)	orange,opaque,circular		G4(8)	white,opaque
	G2(4)	cream,transluscent		G4(1)	white,opaque		G5(1)	white,transluscent
	G2(5)	yellow,opaque,circular		G4(2)	brown,opaque		G5(2)	pale pink,opaque,circular
	G2(6)	white,opaque		G4(3)	white,opaque		G5(3)	white,transluscent
	G3(1)	white,transluscent		G4(4)	white,opaque		G5(4)	white,transluscent
16	G5(5)A	white,transluscent	17	G5(11)B	white circular and black margin,transluscent	18	G6(3)	cream,transluscent

	G5(5)B	white,transluscent		G5(12)	white,transluscent		G6(4)	yellow,transluscent
	G5(6)	yellow,transluscent		G5(13)	white,transluscent		G6(5)	white,transluscent
	G5(7)	white,transluscent		G5(14)	yellow,transluscent		G6(6)	white,transluscent
	G5(8)	white,transluscent		G5(15)	yellow,transluscent,circular		G6(7)	white,transluscent
	G5(9)	white,transluscent		G5(16)	white,transluscent		G6(8)	cream,transluscent
	G5(10)	brown,opaque		G6(1)	orange,transluscent		G6(9)	yellow,transluscent
	G5(11)	white,transluscent		G6(2)	white,transluscent		G6(10)	white,transluscent
19	G6(11)	yellow,transluscent	20	G7(8)	orange,transluscent			
	G7(1)	white,transluscent		G7(9)	yellow,transluscent			
	G7(2)	orange,transluscent		G7(10)	yellow,transluscent			
	G7(3)	yellow,transluscent		G8(1)	orange,transluscent			
	G7(4)	yellow,transluscent		G8(2)	dark pink,opaque,circular			
	G7(5)	white,transluscent		G8(3)	orange,transluscent			
	G7(6)	yellow,transluscent		G8(4)	orange,transluscent			
	G7(7)	yellow,transluscent		G8(5)	white,transluscent			

Appendix 2 – Selected plots for analysis

For taxonomic identification, five plots were selected from each of the respective control and glyphosate plot. Plot selection was done by identifying the more similar number of colonies available in both control and glyphosate plot.

Table 5. Number of different colonies identified based on morphological features

Control plot plots		Treatment plot plots	
K1	7	G1	8
K2	10	G2	6
K3	15	G3	5
K4	11	G4	8
K5	9	G5	19
K6	7	G6	11
K7	14	G7	10
K8	12	G8	11

Selected plots for further analysis



Appendix 3 - R2B media preparation for stocking bacterial isolates

Prepared 250 mL of R2B medium by dissolving 0.54 g of R2B powder in 175 mL of MilliQ water. The pH of the solution was adjusted to 6.5. Sterilized 75 ml glycerol and 175ml R2B in separate bottles by autoclaving. After sterilization, glycerol was added to the R2B and mixed well.

Appendix 4 – Bacterial colony forming units (CFUs) count of soil samples

Table 6. Bacterial colony forming units (CFUs) count of soil samples from plot replicates of control and GBH -treated plots

Control plot		GBH plot	
K1	133	G1	149
K2	106	G2	131
K3	130	G3	84
K4	294	G4	102
K5	200	G5	91
K6	89	G6	40
K7	122	G7	51
K8	168	G8	105

Appendix 5 – Taxonomy of bacterial isolates

Table 7. Taxonomy of selected bacterial isolates from control plots

K1	Phylum	Class	Order	Family	Genus	Species
K1(1)A	<i>Bacillota</i>	<i>Bacilli</i>	<i>Caryophanales</i>	<i>Bacillaceae</i>	<i>Priestia</i>	<i>Priestia megaterium</i>
K1(2)A	<i>Bacillota</i>	<i>Bacilli</i>	<i>Caryophanales</i>	<i>Bacillaceae</i>	<i>Priestia</i>	<i>Priestia megaterium</i>
K1(3)A	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Pedobacter</i>	<i>Pedobacter alluvionis</i>
K1(4)A	<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	<i>Flavobacterium aquidurense</i>
K1(4)B	<i>Bacillota</i>	<i>Bacilli</i>	<i>Caryophanales</i>	<i>Bacillaceae</i>	<i>Priestia</i>	<i>Priestia megaterium</i>
K4	Phylum	Class	Order	Family	Genus	Species
K4(1)	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Mucilaginibacter</i>	<i>Mucilaginibacter galii</i>
K4(2)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Microbacteriaceae</i>	<i>Cryobacterium</i>	<i>Cryobacterium soli</i>
K4(4)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Micrococcaceae</i>	<i>Pseudarthrobacter</i>	<i>Pseudarthrobacter sulfonivorans</i>
K4(5)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>	<i>Sphingomonas faeni</i>
K4(6)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Kitasatosporales</i>	<i>Streptomycetaceae</i>	<i>Streptomyces</i>	<i>Streptomyces camponoticapitis</i>
K4(7)	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Mucilaginibacter</i>	<i>Mucilaginibacter ginsenosidivorax</i>

K4(8)	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Mucilaginibacter</i>	<i>Mucilaginibacter paludis</i>
K4(9)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Microbacteriaceae</i>	<i>Salinibacterium</i>	<i>Salinibacterium xinjiangense</i>
K4(10)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Microbacteriaceae</i>	<i>Plantibacter</i>	<i>Plantibacter flavus</i>
K4(11)	<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	<i>Flavobacterium cupreum</i>
K6	Phylum	Class	Order	Family	Genus	Species
K6(2)	<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	<i>Flavobacterium psychroterrae</i>
K6(1)A	<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	<i>Flavobacterium psychroterrae</i>
K6(1)B	<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	<i>Flavobacterium psychroterrae</i>
K6(3)A	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>	<i>Sphingomonas faeni</i>
K6(3)C	<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	<i>Flavobacterium piscis</i>
K6(4)	<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	<i>Flavobacterium psychroterrae</i>
K7	Phylum	Class	Order	Family	Genus	Species
K7(1)	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Mucilaginibacter</i>	<i>Mucilaginibacter rigui</i>

K7(2)	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Pedobacter</i>	<i>Pedobacter frigiditerrae</i>
K7(5)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Caulobacterales</i>	<i>Caulobacteraceae</i>	<i>Brevundimonas</i>	<i>Brevundimonas mediterranea</i>
K7(7)	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Mucilaginibacter</i>	<i>Mucilaginibacter paludis</i>
K7(8)A	<i>Pseudomonadota</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Comamonadaceae</i>	<i>Variovorax</i>	<u><i>Variovorax ginsengisoli</i></u>
K7(8)B1	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Mucilaginibacter</i>	<i>Mucilaginibacter rigui</i>
K7(8)C	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Mucilaginibacter</i>	<i>Mucilaginibacter rigui</i>
K7(9)A	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>	<i>Sphingomonas faeni</i>
K7(4)A	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Mycobacteriales</i>	<i>Mycobacteriaceae</i>	<i>Mycolicibacterium</i>	<i>Mycolicibacterium hippocampi</i>
K7(4)B	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Microbacteriaceae</i>	<i>Leifsonia</i>	<i>Leifsonia flava</i>
K8	Phylum	Class	Order	Family	Genus	Species
K8(9)	<i>Pseudomonadota</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Comamonadaceae</i>	<i>Variovorax</i>	<u><i>Variovorax ginsengisoli</i></u>
K8(1)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Caulobacterales</i>	<i>Caulobacteraceae</i>	<i>Brevundimonas</i>	<i>Brevundimonas intermedia</i>
K8(2)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>	<i>Sphingomonas aurantiaca</i>
K8(3)A	<i>Bacillota</i>	<i>Bacilli</i>	<i>Caryophanales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>	<i>Bacillus mycoides</i>

K8(4)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Micrococcaceae</i>	<i>Pseudarthrobacter</i>	<i>Pseudarthrobacter sulfonivorans</i>
K8(5)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Micrococcaceae</i>	<i>Arthrobacter</i>	<i>Arthrobacter nitrophenolicus</i>
K8(6)A	<i>Bacillota</i>	<i>Bacilli</i>	<i>Caryophanales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>	<i>Bacillus mycoides</i>
K8(7)A	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Mycobacteriales</i>	<i>Nocardiaceae</i>	<i>Rhodococcus</i>	<i>Rhodococcus maanshanensis</i>
K8(7)B	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Sanguibacteraceae</i>	<i>Sanguibacter</i>	<i>Sanguibacter antarcticus</i>
K8(7)C	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Sanguibacteraceae</i>	<i>Sanguibacter</i>	<i>Sanguibacter antarcticus</i>
K8(8)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Mycobacteriales</i>	<i>Nocardiaceae</i>	<i>Rhodococcus</i>	<i>Rhodococcoides fascians</i>

Table 8. Bacterial taxonomy of selected bacterial isolates GBH treated plots

G1(1)	Phylum	Class	Order	Family	Genus	Species
G1(1)	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Mucilaginibacter</i>	<i>Mucilaginibacter boryungensis</i>
G1(2)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Mycobacteriales</i>	<i>Nocardiaceae</i>	<i>Rhodococcus</i>	<i>Rhodococcus maanshanensis</i>
G1(3)	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Pedobacter</i>	<i>Pedobacter frigiditerrae</i>
G1(5)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Caulobacterales</i>	<i>Caulobacteraceae</i>	<i>Brevundimonas</i>	<i>Brevundimonas mediterranea</i>

G1(7)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Microbacteriaceae</i>	<i>Plantibacter</i>	<i>Plantibacter flavus</i>
G1(8)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Rhodobacterales</i>	<i>Paracoccaceae</i>	<i>Cypionkella</i>	<i>Cypionkella psychrotolerans</i>
G4	Phylum	Class	Order	Family	Genus	Species
G4(1)	<i>Pseudomonadota</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Comamonadaceae</i>	<i>Variovorax</i>	<i>Variovorax boronicumulans</i>
G4(2)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Kitasatosporales</i>	<i>Streptomycetaceae</i>	<i>Streptomyces</i>	<i>Streptomyces hydrogenans</i>
G4(4)	<i>Bacillota</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Peribacillus</i>	<i>Peribacillus simplex</i>
G4(5)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Kitasatosporales</i>	<i>Streptomycetaceae</i>	<i>Streptomyces</i>	<i>Streptomyces olivochromogenes</i>
G4(7)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Micrococcaceae</i>	<i>Pseudarthrobacter</i>	<i>Pseudarthrobacter psychrotolerans</i>
G4(8)	<i>Bacillota</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Peribacillus</i>	<i>Peribacillus simplex</i>
G6	Phylum	Class	Order	Family	Genus	Species
G6(1)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>	<i>Sphingomonas faeni</i>
G6(2)	<i>Pseudomonadota</i>	<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i>	<i>Pseudomonas fragi</i>
G6(3)	<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	<i>Flavobacterium caseinilyticum</i>
G6(4)	<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	<i>Flavobacterium cupreum</i>

G6(5)	<i>Pseudomonadota</i>	<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i>	<i>Pseudomonas silesiensis</i>
G6(6)	<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	<i>Flavobacterium hydatis</i>
G6(7)	<i>Pseudomonadota</i>	<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i>	<i>Pseudomonas silesiensis</i>
G6(8)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Microbacteriaceae</i>	<i>Agreia</i>	<i>Agreia pratensis</i>
G6(11)	<i>Bacteroidota</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	<i>Flavobacterium cupreum</i>
G7	Phylum	Class	Order	Family	Genus	Species
G7(1)	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Pedobacter</i>	<i>Pedobacter frigiditerrae</i>
G7(2)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Caulobacterales</i>	<i>Caulobacteraceae</i>	<i>Brevundimonas</i>	<i>Brevundimonas intermedia</i>
G7(3)	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Pedobacter</i>	<i>Pedobacter frigiditerrae</i>
G7(5)	<i>Pseudomonadota</i>	<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i>	<i>Pseudomonas chlororaphis</i>
G7(7)	<i>Bacteroidota</i>	<i>Sphingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Pedobacter</i>	<i>Pedobacter insulae</i>
G7(8)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>	<i>Sphingomonas faeni</i>
G7(9)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>	<i>Sphingomonas faeni</i>

G8	Phylum	Class	Order	Family	Genus	Species
G8(1)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>	<i>Sphingomonas faeni</i>
G8(3)	<i>Pseudomonadota</i>	<i>Alphaproteobacteria</i>	<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>	<i>Sphingomonas faeni</i>
G8(5)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Micrococcaceae</i>	<i>Arthrobacter</i>	<i>Arthrobacter humicola</i>
G8(7)	<i>Pseudomonadota</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Comamonadaceae</i>	<i>Variovorax</i>	<i>Variovorax boronicumulans</i>
G8(8)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Mycobacteriales</i>	<i>Nocardiaceae</i>	<i>Rhodococcoides</i>	<i>Rhodococcoides fascians</i>
G8(9)	<i>Actinomycetota</i>	<i>Actinomycetes</i>	<i>Micrococcales</i>	<i>Micrococcaceae</i>	<i>Pseudarthrobacter</i>	<i>Pseudarthrobacter sulfonivorans</i>

Appendix 6 –Bacterial sensitivity test in vitro bacterial growth observation

Table 9- Number of CFUs in selected bacterial isolates on 0 µL,20 µL,40 µL GBH treated R2A plates (After 2 weeks of incubation)

Genus	Species	Sensitivity prediction		Number of colony forming units (CFUs)		
				Control treatment	20µL GBH	40µL GBH
<i>Sphingomonas</i>	<i>Sphingomonas faeni</i>	Resistant	G7(9)	4620		64
	<i>Sphingomonas faeni</i>		G8(3)	207	85	61
	<i>Sphingomonas faeni</i>		K6(3)A	82	1	
	<i>Sphingomonas faeni</i>		K4(5)	154	85	
	<i>Sphingomonas faeni</i>		K7(9)A	353	24	23
<i>Pedobacter</i>	<i>Pedobacter frigiditerrae</i>	Sensitive	K7(2)	3340		
			G7(3)	2464		
<i>Flavobacterium</i>	<i>Flavobacterium piscis</i>		K6(3)C	97		
	<i>Flavobacterium psychroterrae</i>		K6(4)	82	22	
	<i>Flavobacterium caseinilyticum</i>	Sensitive	G6(3)	1216		
	<i>Flavobacterium cupreum</i>		G6(4)	3524	335	
<i>Mucilaginibacter</i>	<i>Mucilaginibacter boryungensis</i>	Sensitive	G1(1)	540		
	<i>Mucilaginibacter galii</i>		K4(1)	483	16	
	<i>Mucilaginibacter rigui</i>		K7(1)	1844		

Genus	Species	Sensitivity prediction		Number of colony forming units (CFUs)		
				Control treatment	20µL GBH	40µL GBH
<i>Pseudarthrobacter</i>	<i>Pseudarthrobacter sulfonivorans</i>	Sensitive	K4(4)	677	301	
	<i>Pseudarthrobacter psychrotolerans</i>		G4(7)	1736	20	127
	<i>Pseudarthrobacter sulfonivorans</i>		K8(4)	745		
	<i>Pseudarthrobacter sulfonivorans</i>		G8(9)	864	240	
<i>Brevundimonas</i>	<i>Brevundimonas mediterranea</i>	unknown	G1(5)	3380	1140	
	<i>Brevundimonas mediterranea</i>	unknown	K7(5)	1720		
	<i>Brevundimonas intermedia</i>	Resistant	G7(2)	2800		
	<i>Brevundimonas intermedia</i>	Resistant	K8(1)	1872	1536	
<i>Variovorax</i>	<i>Variovorax boronicumulans</i>	Sensitive	G4(1)	95	295	
	<i>Variovorax ginsengisoli</i>		K7(8)A	237	2	
	<i>Variovorax ginsengisoli</i>		K8(9)	187		
	<i>Variovorax boronicumulans</i>		G8(7)	163	224	166
<i>Rhodococcus</i>	<i>Rhodococcus maanshanensis</i>	Sensitive	G1(2)	60		
	<i>Rhodococcus maanshanensis</i>		K8(7)A	69		
<i>Rhodococcoides</i>	<i>Rhodococcoides fascians</i>	sensitive	K8(8)	203	20	
	<i>Rhodococcoides fascians</i>		G8(8)	465	43	

Appendix 7 – Student T-test analysis

Appendix 7.1- Comparison of glyphosate residues concentration in treatment plots

Two Sample t-test

data: Control_plot and Glyphosate_plot

$t = -5.1606$, $df = 7.0002$, $p\text{-value} = 0.001308$

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-3.317771 -1.232729

sample estimates:

mean of x mean of y

0.01350 2.28875

Appendix 7.2 – Bacterial colony counts of samples from control and glyphosate treated plots

Two Sample t-test

data: Control_plot and Glyphosate_plot

$t = 2.2893$, $df = 10.962$, $p\text{-value} = 0.04291$

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

2.333455 119.916545

sample estimates:

mean of x mean of y

155.250 94.125

Appendix 7.3 – Bacterial diversity analysis of treatment plots

Two Sample t-test _Genus

data: Control_plot and Glyphosate_plot

$t = 0.56846$, $df = 40.661$, $p\text{-value} = 0.5729$

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-0.7771771 1.3858728

sample estimates:

mean of x mean of y

1.826087 1.521739

Two Sample t-test _Family

data: Control plot and Glyphosate plot

$t = 0.67761$, $df = 21.075$, $p\text{-value} = 0.5054$

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-1.181921 2.324778

sample estimates:

mean of x mean of y

3.000000 2.428571

Two Sample t_test_Order

data: Control_plot and Glyphosate_plot

$t = 0.53559$, $df = 16.24$, $p\text{-value} = 0.5995$

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-1.722778 2.889445

sample estimates:

mean of x mean of y

3.416667 2.833333

Two Sample t-test_Class

data: Control_plot and Glyphosate_plot

$t = 0.5406$, $df = 10.515$, $p\text{-value} = 0.6$

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-3.094397 5.094397

sample estimates:

mean of x mean of y

5.857143 4.857143

Two Sample t-test_Phylum

data: Control_plot and Glyphosate_plot

$t = 0.50918$, $df = 5.9946$, $p\text{-value} = 0.6288$

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-6.661702 10.161702

sample estimates:

mean of x mean of y

10.25 8.50

Appendix 8 – Shannon diversity Index assement

```
# Calculate diversity indices
shannon <- diversity(abundance_data, index = "shannon")
simpson <- diversity(abundance_data, index = "simpson")
richness <- specnumber(abundance_data)
# Combine into one data frame
alpha_div_Paw <- data.frame(
  Sample = sample_names,
  Group = group_info,
  Shannon = shannon,
  Simpson = simpson,
  Richness = richness
)
# Plotting function
plot_diversity <- function(metric_name) {
  ggplot(alpha_div_Paw, aes(x = Group, y = .data[[metric_name]], fill = Group)) +
    geom_boxplot(alpha = 0.6) +
    #geom_jitter(width = 0.2, size = 2, shape = 21, color = "black") +
    theme_minimal() +
    labs(title = paste(metric_name, "Diversity"), y = metric_name, x = "") +
    scale_fill_brewer(palette = "Set2")
}
# Generate plots
plot_diversity("Shannon")
plot_diversity("Simpson")
plot_diversity("Richness")

wilcox.test(Shannon ~ Group, data = alpha_div_Paw)
```

Result:

Wilcoxon rank sum exact test

data: Shannon by Group

W = 15, p-value = 0.6905

alternative hypothesis: true location shift is not equal to 0.