



Quantifying glyphosate and its AMPA metabolite from environmental samples in South Africa

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Thesis accepted in fulfilment of the requirements for the degree
Doctor of Philosophy in Science with Environmental Sciences at
the North-West University

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Graduation: November 2025

ACKNOWLEDGEMENTS

All glory and praise go to the Heavenly Father YHWH (יהוה) my Elohim, for giving me the opportunity, talents and blessings to pursue my Ph.D. degree and everything it encompasses. May this thesis only glorify and magnify your Holy Name, YHWH (יהוה).

"The quest for knowledge, like everything else of value, is not to be obtained easily. It must be worked for, studied for, thought for, and more than all, must be prayed for", Thomas Arnold.

I would like to sincerely thank my supervisors for their invaluable advice and support throughout my doctoral journey.

First and foremost, I am deeply grateful to my promoters, Prof. Rialet Pieters, Prof. Suranie Horn, and Prof. Charlotte Mienie. All your contributions, thoughts, and participation in this thesis have helped to shape it into something that I am proud of. Prof. Rialet, your ability to explain complex concepts in simple terms has been a gift and a skill that I have come to admire and make my own. You have taught me that there is always a way and that any obstacle can be overcome. You truly are someone I admire both in academia and in personal matters. I will forever be grateful for the time and energy you invested in me. Thank you, Prof. Suranie, for keeping me focused when things got overwhelming and for being there all the time to give me your insightful advice. I could not have asked for a better mentor: Your astute observations, unwavering support, and work ethic inspired me to think more deeply and set higher goals for my research and in my personal life. Prof. Mienie, your patience, wisdom, and attention to detail have helped a lot. I greatly appreciate your thoughtful discussions and support during the most challenging phases of my research.

A special thanks to Prof. Jaco Bezuidenhout for all his help with data analysis and motivational words during the study. Also, thank you to Ms. Prudent Mokgokong for her assistance in the 16S sequencing.

To my colleagues, Dr. Hannes Erasmus and Dr. Anja Erasmus, your willingness to help and mentorship have made this journey easier. Both of you are excellent role models, and I am grateful for any role you played. My colleagues at ENVITROX, thank you for all the shared experiences.

To my friends Ahmed Nosir, Chané de Bruyn, Ilzé Horak (Engelbrecht), Vickey-Luanne Harris, Raeesa Bhikhoo, Hussain Kaka, Ian Luyt, Velesia Lesch, Mariclélle Botes and Francois Bothma, you have shared this rollercoaster with me. Your support, encouragement, and all the good times made the days easier. I am truly blessed with all of you.

My parents, Hennie and Riana, thank you for everything. Your infinite love, patience, and faith in me have been my anchor. I could not have done this without you, and I am forever grateful for the sacrifices you made to support me in all possible ways. I promise I am done studying now.

The NWU Faculty of Natural and Agricultural Sciences Research Ethics Committee (FNASREC) is acknowledged for providing and examining the necessary ethical approvals for this project (Ethics No: NWU-00525-21-A9).

This work is based on research and is supported by the South African Water Research Commission (WRC) (project number C2020/202100195). Student financial support provided by the WRC with the Water RDI Roadmap Student Support Fund - C2020/2021-00195 (2021), EWSETA (2022) is also recognised. Finally, support for students from the Faculty of Natural and Agricultural Sciences of North-West University (FNAS-NWU) and the Environment Sciences and Management Unit of North-West University (UESM-NWU) is also recognised and appreciated. The South African Weather Service (SAWS) is also acknowledged for providing data on ultraviolet and temperature measurements from 2017–2022. The opinions, findings, conclusions, and recommendations expressed in this thesis are those of the author and are not necessarily attributed to NWU, the WRC, EWSETA, or SAWS.

ABSTRACT

Glyphosate-based herbicides (GBHs) are integral to modern agriculture, but concerns about their persistence in the environment and potential impacts on non-target organisms have prompted investigations into their environmental concentrations. It is globally considered to be the most used herbicide. The study aimed to quantify the levels of glyphosate (GLY) and its metabolite, aminomethylphosphonic acid (AMPA), in soil, sediment and water samples from selected agricultural and non-agricultural regions in South Africa and to assess the impact of these compounds on microbial communities and public health risks. To contextualise herbicide application, GBH-use in South Africa was geospatially mapped to understand the distribution of GLY use in key South African crops: maize, soybeans, wheat, and sunflower. Water, sediment, and soil samples were collected from the Vaalharts irrigation scheme in the Northern Cape, which uses canal irrigation from the Vaal and Harts rivers and from rain-fed crop fields in the catchments of the Klein Olifants and Vaal rivers in Mpumalanga. Samples were also collected from non-agricultural areas in the North West and Gauteng provinces. Water, soil, and sediment samples were collected during four events. Analytical methods involved the use of liquid chromatography-tandem mass spectrometry for the quantification of GLY and AMPA in environmental matrices. The microbial diversity was evaluated by sequencing the 16S rRNA gene to evaluate whether the presence of GLY affected the structure of the microbial community. Lastly, a Quantitative Microbial Risk Assessment (QMRA) was conducted to evaluate the probable risk of infection posed by GLY-altered microbial populations selecting for environmental pathogens. Unfortunately, the GLY and AMPA concentrations were below the limit of detection in all the samples. This caused the focus to shift to assessing microbial diversity in response to agricultural chemicals in general at sites expected to present microbial diversity. The study pivoted to attempting to explain the absence of GLY and AMPA by investigating the role of UV-radiation, cation-exchange capacity (CEC), pH and GLY's adsorption coefficient. Throughout the study sites, the UV-B indices ranged from high to extremely high (6.7–8.7), which may have contributed to the deterioration of GLY. The CEC mean for the soils ranged from 1.6–2.2 meq/100 g, with some sites having values >6 meq/100 g. The calculated GLY sorption coefficients (mean K_d = 70 L/kg) using the CEC, clay, and pH indicated that GLY was mobile, except for 5/46 sites. Beta diversity communities were classified by matrix (soil vs. water) rather than by location, and microbial sequencing revealed that alpha diversity was higher in Mpumalanga. The QMRA showed that inhalation was the primary exposure route with *Legionella* and *Mycobacterium*, with probable infection rates of 1.0 (100%) at 10/20 and 19/20 sites, respectively. Together, these findings create a paradox: heavy reported GBH use with no quantifiable levels of GLY or AMPA. At the same time, the diverse microbial communities and probable pathogen infection risks suggest rapid GLY breakdown conditions, thereby helping to explain the non-detection of GLY and AMPA.

Key terms: Pesticide use, non-target organisms, microbial diversity, microbial risk assessment

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ACRONYMS & ABBREVIATIONS

Designation	Declaration
A	
AChE	Acetylcholinesterase
ACN	Acetonitrile
a.i.	Active ingredient
Al ³⁺	Aluminium ion
AMPA	Aminomethylphosphonic acid
ANOSIM	Analysis of similarities (non-parametric statistical test in ecology)
ANOVA	Analysis of variance
ASE	Accelerated solvent extractor
ASTER	Advanced Spaceborne Thermal Emission and Reflection Radiometer
ASV	Amplicon sequence variants
AT	Average lifetime
ATSDR	Agency for Toxic Substances and Disease Registry
B	
BaCl ₂ ·2H ₂ O	Barium chloride dihydrate
BFAP	Bureau for Food and Agricultural Policy
C	
Ca ²⁺	Calcium ion
CE	Collision energy
CEC	Cation exchange capacity
CE-MS	Capillary electrophoresis-mass spectrometry
Cl ⁻	Chloride ion
CP4	Agrobacterium tumefaciens strain CP4
CR	Cancer risk
CV	Cone voltage
Cw	Concentration of herbicide residue
D	
DADA2	Divisive Amplicon Denoising Algorithm 2
DEA	Department of Environmental Affairs
DNA	Deoxyribonucleic acid
DR	Dose-response
DWS	The Department of Water and Sanitation
E	
EC	Electrical conductivity
EC50	Effects concentration producing a 50% effect
ED	Exposure duration
eDNA	Environmental deoxyribonucleic acid
EF	Exposure frequency
EFSA	European Food Safety Authority

Designation	Declaration
EPA	Environmental Protection Agency
EPSPS	5-enolpyruvyl-shikimate-3-phosphate
ER	Oestrogen receptor
F	
FAO	Food and Agriculture Organisation
FI	Fluorescence immunoassay
G	
GAT	Glyphosate N-acetyltransferase
GBH	Glyphosate-based herbicide
GC-MS	Gas chromatography-mass spectrometry
GDP	Gross domestic product
GIS	Geographic Information Systems
GLY	Glyphosate
GM	Genetically modified
GOX	Glyphosate oxidoreductase
GT	Glyphosate tolerant
H	
H ⁺	Hydrogen ion
HDPE	High-density polyethylene
HEK293	Human embryonic kidney
HepG2	Human liver cells
HI	Health index
HPLC	High-performance liquid chromatography
I	
IARC	International Agency for Research on Cancer
IC-MS	Ion chromatography-mass spectrometry
IR	Ingestion rate
IS	Internal standard
J	
JEG3	Human placental cells
K	
K ⁺	Potassium ion
K _d	Linear pedotransfer isotherm
K _f	Freundlich pedotransfer isotherm
L	
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography with tandem mass spectrometry

Designation	Declaration
LDPE	Low-density polyethylene
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
M	
m/v	Mass to volume
Mg ²⁺	Magnesium ion
MgSO ₄	Magnesium sulphate
MPA	Mpumalanga
MPA-ORC	Mpumalanga's Olifant River catchment
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometer
N	
NAMC	National Agricultural Marketing Council
NAS	Non-agricultural sites
NASA	National Aeronautics and Space Administration
NAS-UVC	Non-agricultural sites-Upper Vaal River Catchment
NFEPA	National Freshwater Ecosystem Priority Areas
NH ₄ ⁺	Ammonia ion
NMDS	Non-metric multidimensional scaling
NMISA	National Metrology Institute of South Africa
NO ₂ ⁻	Nitrite ion
NO ₃ ⁻	Nitrate ion
NWU	North-West University
P	
PCA	Principal component analysis
PCR	Polymerase chain reaction
pKa	Acid dissociation constant (describes the acidity of a particular molecule)
PO ₄ ³⁻	Ortho-phosphate ion
POEA	Polyethoxylated tallow amine
POST-MPA	After-herbicide application-Mpumalanga
POST-NAS	After-herbicide application-Non-agricultural sites
PRE-MPA	Before-herbicide application-Mpumalanga
PRE-NAS	Before-herbicide application-Non-agricultural sites
PTFE	Polytetrafluoroethylene
Q	
QC	Quality control
QIIME2	Quantitative Insights Into Microbial Ecology
QMRA	Quantitative Microbial Risk Assessment
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe

Designation	Declaration
QuPPE	Quick Polar Pesticides Extraction
R	
rcf	Relative centrifugal force
RR	Roundup Ready®
RSD	Relative standard deviation
RWQO	Resource water quality objectives
S	
SA	South Africa
SANBI	South African National Biodiversity Institute
SO ₄ ²⁻	Sulphate ion
SPE	Solid phase extraction
spm	Suspended particulate matter
SS	Suspended solids
sw	Surface water
T	
TEMP	Temperature
TH	Total hardness
TOC	Total organic carbon
TSS	Total suspended solids
TURB	Turbidity
U	
UHPLC	Ultra-high performance liquid chromatography
US\$	United States Dollar
USA	United States of America
USEPA	United States Environmental Protection Agency
UV	Ultra-violet
V	
VIS	Vaalharts Irrigation Scheme
VIS-LVC	Vaalharts Irrigation Scheme - Lower Vaal River catchment
W	
WfW	Working for Water
WHO	World Health Organisation
WWTP	Wastewater treatment plant
Z	
ZAR	South African Rand

CHAPTER 1: BACKGROUND

1.1 General background

One of the necessary practices in agricultural activities to reduce crop losses is the use of pesticides, specifically glyphosate-based herbicides (GBH), a subcategory of pesticides, which are widely used in the agricultural sector. In contrast, agricultural practices without the use of pesticides show a 20–25% decrease in yield (crop-specific) (Azarbad, 2022). Agricultural activities, vital to sustain food production, inadvertently introduce various chemicals into the environment. Examples of chemicals include medications given to animals, artificial fertilisers applied to crop fields (Zhang *et al.*, 2018) and herbicides like GBHs, broad-spectrum, non-selective, post-emergent herbicides used against weeds and for vegetation control. Glyphosate ([N-(phosphonomethyl)glycine]) (GLY) is the active ingredient in GBHs and is a polar, non-volatile compound that is also highly water-soluble (more than 100 g/L at 20°C) (Lewis *et al.*, 2016). Glyphosate is approved in 130 countries (Kaszkowiak *et al.*, 2021), including South Africa, and is the most used herbicide in the world (Dai *et al.*, 2016). Glyphosate has a specific mechanism of action that makes it safe for both humans and animals (Jarrell *et al.*, 2020). Even though GLY is designed to be degradable, its frequent use continues to pollute the environment. Glyphosate is claimed to be environmentally benign because it bonds tightly to soil, making it immobile; it has no soil activity and has limited persistence (Reddy, 2001). Glyphosate has been found in water sources in studies conducted worldwide, raising concerns about human and animal health.

Concentrations of GLY have been documented worldwide, and to name a few, Skeff *et al.* (2015) reported concentrations of 0.028–1.7 µg/L in the water of German Baltic estuaries. Glyphosate was also found in groundwater in Catalonia, Spain, at a maximum concentration of 2.5 µg/L and a mean concentration of 0.2 µg/L (Sanchís *et al.*, 2012). Van Stempvoort *et al.* (2016) reported GLY concentrations of 0.7 µg/L in rural groundwater within the Nottawasaga River watershed, Canada. Smith *et al.* (1996) found 45 µg/L GLY in well water seven weeks after spraying. This well was located at the Massey Drive substation, Newfoundland, Canada, where vegetation around the electric substation was sprayed with Roundup®. This station is built on a limestone bed that is highly permeable and could have allowed contaminants sprayed on the weeds to move from the surface to the groundwater. Börjesson and Torstensson, (2000) reported levels of GLY in groundwater ranging from 0.12–1.42 µg/L due to its application to railway weeds. In the USA, GLY has been detected in a stream and wastewater treatment plant (WWTP) effluent at a maximum concentration of 2.2 µg/L (Kolpin *et al.*, 2006). A very extensive study by Battaglin *et al.* (2014), in the USA,

reported the following maximum levels in environmental matrices: streams: 73 µg/L; groundwater: 2.03 µg/L; ditches and drains: 427 µg/L; large rivers: 3.08 µg/L; soil water: 1 µg/L; lakes, ponds and wetlands: 301 µg/L; precipitation: 2.5 µg/L; soil and sediment: 476 µg/L, and WWTP outfall: 0.3 µg/L. It is evident that GLY ends up in water sources.

While GBHs is primarily applied as a herbicide, emerging evidence suggests GLY can affect non-target organisms such as bacteria; these biological effects may provide early signals relevant for both ecological and human risk assessment, and are addressed in more detail in Section 2.5. Given that South Africa is the largest user of pesticides in sub-Saharan Africa (Dabrowski, 2015a), GLY pollution is becoming a growing concern for South Africa. By evaluating the concentrations of GLY and its primary metabolite, AMPA, in soil, sediment, and water sources, we can have a holistic understanding of the current state of our environment.

1.2 Problem statement

Agricultural activities are a potential source of several chemicals that end up in the environment. Current-use pesticides, in contrast to the historically used persistent organochlorine pesticides traditionally used, are biodegradable, but the continuous use of high volumes causes contamination of the environment (Carvalho, 2017). The development of Roundup-Ready® crops has contributed to the high usage of Roundup® because farmers can spray larger amounts of GLY-containing herbicides, increase application rates, and spray them on the plants during the growing season while leaving the crops unharmed (Benbrook, 2012). Evidently, GLY ends up in water sources. However, no studies have ever been conducted to investigate the concentrations of GLY and its main metabolite AMPA (aminomethylphosphonic acid) in South African water sources, except for a single study by Horn *et al.* (2019), which reported traces of GLY in water samples from the dam (B3) on Farm B, with levels ranging from LOD (0.2 µg/L) to LOQ (0.4 µg/L) following the spraying event. At Farm B's (B2) in-flow dam, GLY levels of 0.42 µg/L were found following the spraying event. When the season ended, these levels dropped to <LOD.

South Africa was estimated to use approximately 23 253 tonnes of GLY in 2012 only (Gouse, 2014). In a water-scarce country such as South Africa, water contaminated with chemicals is of great concern as concentrations of chemicals increase as water decreases. Another issue to be highlighted is the fact that many residents depend on untreated surface and groundwater resources due to the lack of supply of treated drinking water (Dabrowski, 2015a). So far there is only one South African study with publically available data that screened water sources in the North West Province for Cry1Ab (crystalline protein), GLY and 2,4-D (2,4-dichlorophenoxyacetic acid) using enzyme-linked immunosorbent assays (ELISAs) (Horn *et al.*, 2019).

This study aims to measure GLY and AMPA concentrations in selected water sources, soils, and sediments from agricultural and non-agricultural areas of South Africa. The persistence of GLY and AMPA in the environment and their toxicity are still under scientific discussion worldwide. In addition, the aim was to use an analytical method to measure GLY/AMPA concentrations in South Africa, with samples selected based on areas where GBH use was known or suspected.

1.3 Hypothesis

There are quantifiable levels of GLY and AMPA in South African environmental water, soil, and sediment in areas of expected application of the herbicide.

1.4 Aims and objectives

Aim 1: Visualise the GBH-use data for maize, soybean, wheat, and sunflower crops by creating a geospatial map using geographic information systems.

- 1) Buy data from a commercial data mining company, Kynetechn[®].

Aim 2: Determine the concentrations of GLY and AMPA in water, soil and sediment samples collected, before, during, and after pesticides are applied on crops in two agricultural regions in different provinces and different water sources: Vaalharts irrigation scheme (Northern Cape), and the upper catchment of the Vaal River (Mpumalanga), as well as in non-agricultural sites (North West).

- 1) Collecting samples for analysis.
- 2) Quantification of GLY and AMPA using a liquid chromatography-mass spectrometer (triple quadrupole) after establishing the appropriate methods.

***Aim 3:** Compare the microbial diversity in surface water areas where GLY was detected to those where GLY was not detected to determine the effect of GLY on the microbial community by sequencing the 16S rRNA gene (V3–V4).

- 1) To analyse microbial sequences using the QIIME2 pipeline to determine the composition and diversity of the bacterial community.
- 2) To conduct sequence data refinement, visualisation, and comparative analysis using R software.

***Aim 4:** Perform a quantitative microbial risk assessment based on the sequences from Aim 3.

- 1) Search for published studies identifying pathogenic and pollutant-degrading bacteria reported to be influenced by glyphosate exposure.
- 2) Determine the hazard identification, dose-response assessment, exposure assessment, and risk characterisation specifically via inhalation and ingestion exposure.

*Aims 3 and 4 had to be reframed during the study. This is explained in Section 3.6.

1.5 Structure of thesis

This thesis is an assembly of six chapters that were written in the NWU format to fulfil the requirements of the university for the Doctor of Philosophy in Science with Environmental Sciences degree.

Chapter 1 is the current chapter and provides a synopsis of the research. The problem statement, hypothesis, aims, and objectives of the study are also included in this chapter.

Chapter 2 comprises an extensive review of the current literature, including the importance of agriculture in South Africa, GLY's mechanism of action, South African cultivation practices, effects on target and non-target organisms, environmental fate of GLY, methods used to analyse GLY and AMPA and finally the health risk posed by GLY.

Chapter 3 presents the materials and methods used in this study clearly and concisely.

Chapter 4 describes the results obtained about water nutrients, soil properties, geospatial analysis maps, chemical analysis, microbial diversity, and risk assessment.

Chapter 5 presents an interpretation and in-depth discussion of the results reported in Chapter 4 and how they relate to the current literature.

Chapter 6 provides a general conclusion of the study based on the results obtained. The main findings of the study are discussed. Limitations identified during the study and recommendations for future research avenues are also included.

References are given at the end of the thesis to avoid repetition of certain references. Harvard's reference style (parenthetical referencing) was used.

Appendix A contains supplementary information for Chapter 3.

Appendix B contains supplementary information for Chapter 4's water, sediment and soil data.

Appendix C contains supplementary information for Chapter 4's chemical analysis data.

Appendix D contains supplementary information for Chapter 4's QMRA data.

CHAPTER 2: LITERATURE REVIEW

2.1 General overview

Glyphosate is a widely used herbicide central to modern agricultural practices due to its effectiveness in controlling a broad spectrum of weeds. Its role in enhancing crop yields has made it vital to global food security, particularly in genetically modified cropping systems where it targets weeds without damaging the crop. GLY also supports conservation and no-till farming practices, contributing to sustainable agriculture. However, long-term dependence has led to challenges, including the rise of GLY-resistant weed species that complicate weed management. Many GBHs include surfactants to enhance plant absorption and increase efficacy. Also, GBH manufacturers' labels require adding ammonium sulphate (an inorganic salt used to increase the herbicide's effectiveness) (Mueller and Steckel, 2019). Glyphosate [N-(phosphomethyl)glycine] is a weak acid, highly hydrophilic and water-soluble (Table 2.1). Despite its agricultural advantages, concerns persist about its environmental persistence and potential long-term health effects (See Section 2.5), fuelling ongoing debate about its safe use.

Table 2.1: General overview of glyphosate

Aspect	Details	Reference
Chemical name	N-(phosphonomethyl)glycine	*Lewis <i>et al.</i> (2016); Maria <i>et al.</i> (2020)
Molecular formula	C ₃ H ₈ NO ₅ P	*Lewis <i>et al.</i> (2016)
CAS number	1071-83-6	*Lewis <i>et al.</i> (2016)
Molar mass (g/mol)	169.1	*Lewis <i>et al.</i> (2016)
Aqueous solubility at 20°C (mg/L)	100	*Lewis <i>et al.</i> (2016)
Vapour pressure, mPa at 25°C	0.0131, low volatility	*Lewis <i>et al.</i> (2016); Rawat <i>et al.</i> (2023)
pH (in aqueous solution 1% m/v) at 25°C	4.68	*Lewis <i>et al.</i> (2016)

Aspect	Details	Reference
Soil adsorption and mobility (mL/g)	Linear K_d : 209.4 Freundlich K_f : 54.2	*Lewis <i>et al.</i> (2016)
Acidity (pKa)	<2.0, 2.32, 5.86, 10.86	Maria <i>et al.</i> (2020)
Surfactants	Polyethoxylated tallow amine (POEA); propoxylated quaternary ammonium surfactant	Klátyik <i>et al.</i> (2023)
Mechanism of action	Inhibits the shikimate pathway in plants & microbes, preventing the production of essential amino acids	*Lewis <i>et al.</i> (2016)
Type of herbicide	Broad-spectrum, non-selective, post-emergent	Karasali <i>et al.</i> (2019)
Common use	Weed control in agriculture, forestry, and non-agricultural settings.	*Lewis <i>et al.</i> (2016)
Manufacturers and suppliers (current and historic)	Bayer CropScience, Dow AgroSciences, Headland, Makhteshim-Agan, Monsanto, Nufarm UK, Syngenta, Cheminova	*Lewis <i>et al.</i> (2016)
Common GBH brands	Asteroid, Envision, Touchdown, Glyphos, Daker, Glyphosate 360, Glyphogan, Kernel, Manifest	*Lewis <i>et al.</i> (2016)
Environmental concerns	Persistence in soil; Non-target effects	Karasali <i>et al.</i> (2019); Lewis <i>et al.</i> (2016)
Health considerations	Debates about carcinogenicity and long-term exposure risks	*Lewis <i>et al.</i> (2016)
Contribution to food security	Helps improve crop yields by controlling weed competition	Daramola <i>et al.</i> 2022

*The Lewis *et al.* (2016) source refers to both the paper and the database (PPDB: Pesticide Properties Database: <http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/373.htm#1>). The paper explains how and why the database was created, and the PPDB database provides the information in the table above.

2.2 Importance of agriculture for the South African economy and food security

Agriculture provides food, income, and employment to approximately 70% of the population in southern Africa (SADC, 2012). South Africa has a poverty rate of 22%, with a significant number of people living in extreme poverty (World Data Lab, 2025). Therefore, agriculture is crucial to create employment and alleviate poverty (Olujimi *et al.*, 2010). Agriculture contributed US\$349.6 billion (ZAR4.7 trillion) in 2017 to the GDP and increased by 2.5% in the second quarter of that year. The years 2017 and 2019 are important for this study, as

these were the years glyphosate-use data were obtained to illustrate its extensive use in the country. The 15 million ha of South Africa's agricultural land accounts for 12–13% of the country's total surface area of 122 million ha (Van der Laan *et al.*, 2017).

In 2017, there were 40 122 farms involved in the commercial agricultural industry (Stats SA, 2017b), with cereals and other crops accounting for 21% of the total. Maize, soybeans, wheat, and sunflower are among the most cultivated crops in the country. Maize contributed ~0.66% in 2017, wheat ~0.13%, soybeans ~0.11%, and sunflower ~0.10% (BFAP, 2018). Table 2.2 shows the total annual yields of maize, soybean, sunflower, and wheat crops in South Africa for three periods. Maize contributed the most to total grain production: 17 million tonnes. The consumption of all four crops increased from 2017 to 2022 (Table 2.2), except for soybeans. South Africa's GDP was US\$381.4 billion (ZAR5 trillion) in 2017 and US\$388.5 billion (ZAR5.6 trillion) in 2019. The biggest sector contributors in 2017 were agriculture, fishing, and forestry, with agriculture representing 33.6%, resulting in an annual growth of 0.7% in GDP (Stats SA, 2017a). More recently, the GDP for 2022 was US\$807 billion (ZAR13.2 trillion). GDP decreased by 0.7% in the second quarter of 2022, with the agriculture sector contributing 7.7%, resulting in a 0.2% annual decrease in GDP (Stats SA, 2022). Approximately 21% of the commercial agricultural industry is responsible for cereals and other crops (Stats SA, 2017b). Table 2.2 displays the total annual yields of these crops. Maize contributed the most to overall grain production at 17 million tonnes from the 2017–2020 growing seasons.

Table 2.2: Average crop yields and consumption in tonnes of the August to September marketing season in South Africa (NAMC. National Agricultural Marketing Council, 2017, 2019, 2022).

Crop	2017/18 annual yield	Consumption	2019/20 annual yield	Consumption	2021/22 annual yield	Consumption
Maize	17 million	Human: 4.9 million Animal: 2.5 million ⁱ Industry: 2.5 million	17 million	Human: 5.2 million Animal: 5.6 million ⁱ Industry: 5.6 million	14.9 million	Human: 5.3 million Animal: 5.9 million ⁱ Industry: 5.9 million
Soybean	4 million	Human: 26 000 Animal: 125 000 ⁱⁱ Industry: 1 million	2.3 million	Human: 25 500 Animal: 230 000 ⁱⁱ Industry: 1.1 million	2 million	Human: 23 000 Animal: 195 000 ⁱⁱ Industry: 1.6 million
Sunflower	1 million	Human: 1 800 Animal: 7 500 ⁱⁱⁱ Industry: 830 000	892 000	Human: 1 500 Animal: 6 000 ⁱⁱⁱ Industry: 740 000	832 050	Human: 1 550 Animal: 6 000 ⁱⁱⁱ Industry: 820 000
Wheat	1 million	Human: 3.1 million Animal: 2 300 ^{iv} Industry: not reported	4.3 million	Human: 3.2 million Animal: 3 000 ^{iv} Industry: not reported	3.6 million	Human: 3.3 million Animal: 18 500 ^{iv} Industry: not reported

i) shoe polish & cosmetics; ii) soaps & disinfectants; iii) soaps, detergents, surfactants, adhesives, fabric softeners; iv) synthetic rubber, explosives

Zdráhal *et al.* (2020) reported that South Africa's agricultural exports have consistently exceeded imports, highlighting the sector's strong contribution to the national economy. Over a 13-year period, imports and exports doubled, underscoring agriculture's growing global relevance. To maintain the quality of export crops, chemical pest and weed control remains a common practice, one example being the use of glyphosate-based herbicides (GBHs). GBHs are widely applied to manage weeds and improve crop productivity. Given that agriculture supports national food security, employment, and export earnings, understanding the scale and role of staple crop production further emphasises the importance of GBH use. This context reinforces the rationale for investigating GBH application in maize, soybean, wheat, and sunflower cultivation, as it provides critical insights into agricultural practices and their broader economic and environmental impacts.

2.3 Glyphosate-based herbicides (GBH): Mechanism and application

Glyphosate-based herbicides, such as Roundup[®], are widely used for weed control in agriculture and horticulture. The active ingredient glyphosate (GLY) inhibits the shikimate pathway enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Figure 2.1). This metabolic pathway is found in plants, bacteria, fungi, archaea, protozoans and algae (Van Bruggen *et al.*, 2021). Glyphosate competitively binds EPSPS, blocking the conversion of shikimate-3-phosphate and phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate. Such inhibition stops the shikimate pathway, depleting plants of the aromatic amino acids' phenylalanine, tyrosine, and tryptophan required for protein synthesis and growth (Duke *et al.*, 2012; Leino *et al.*, 2021). Aromatic amino acid deficiency leads to chlorosis, necrosis, stunting, and death. Accumulated shikimate in vacuoles serves as a biomarker of GLY exposure (Lozano *et al.*, 2018; Okumu *et al.*, 2019; Leino *et al.*, 2021).

Glyphosate-based herbicides control a broad spectrum of weeds with minimal impact on animals and humans (Singh, 2024) because they lack the shikimate pathway, a selectivity that underpins GBH popularity. In bacteria, the compound is metabolised via two routes: an oxidase cleaves the carboxymethylene-nitrogen bond to yield AMPA and glyoxylate, and C–P lyase severs the carbon-phosphorus bond to produce sarcosine; the same lyase also splits AMPA's C–P bond during further breakdown (Klátyik *et al.*, 2023).

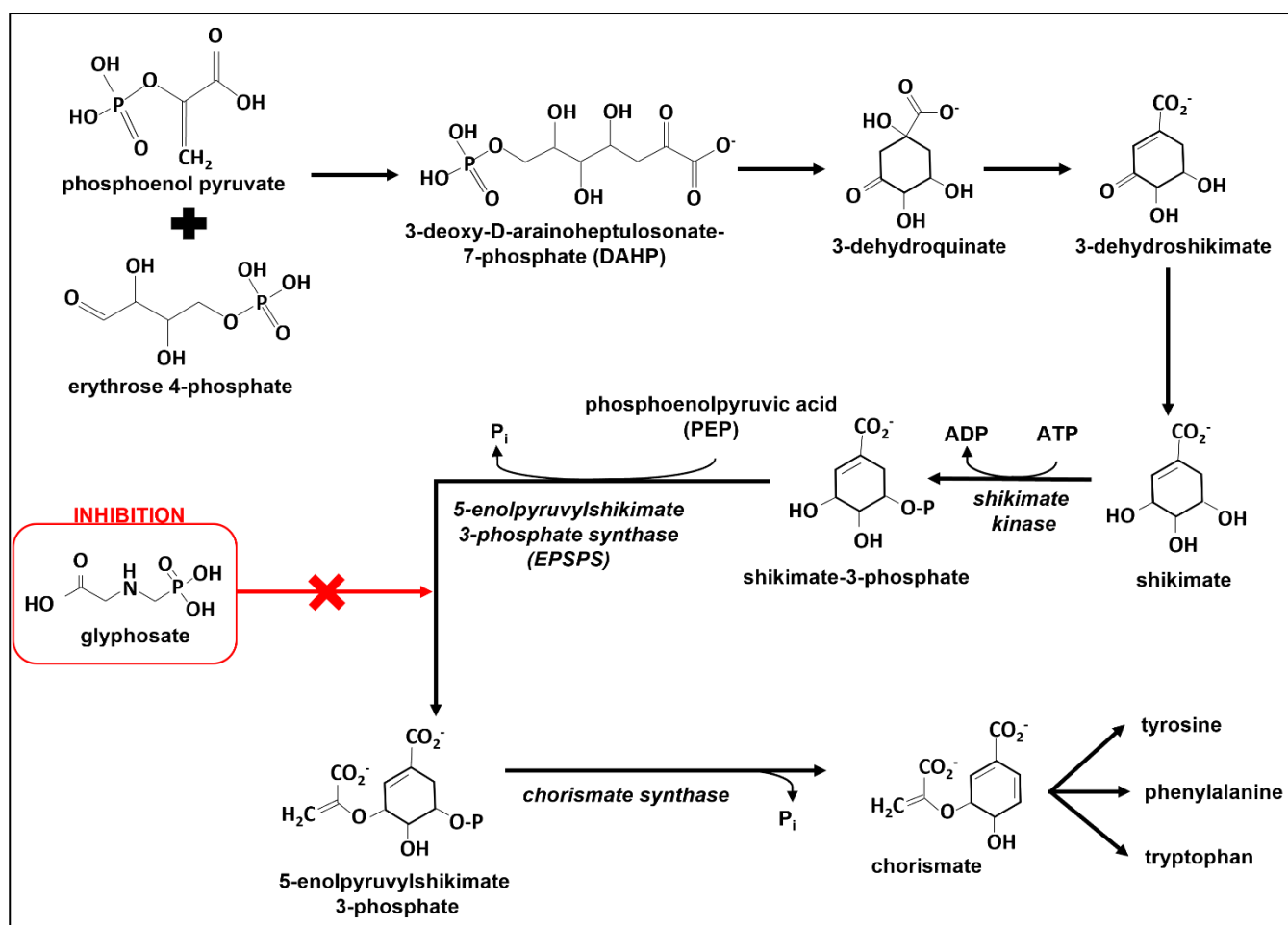


Figure 2.1: The mode of action of glyphosate inhibition in the shikimate pathway. Adapted from Rueda-Ruzafa *et al.* (2019) and Costas-Ferreira *et al.* (2022).

2.4 Cash crop cultivation practices in South Africa

Cash-crop practices shape fertiliser and pesticide use, driving both economic returns and environmental impacts. South Africa's four main cash crops, maize, wheat, sunflower and soybeans, illustrate how cultivation methods influence yields as well as ecological outcomes.

2.4.1 Conservational and conventional cultivation practices

Conservation agriculture (CA) combines crop rotation, minimal soil disturbance (e.g., no-till) and permanent soil cover to sustain yields while enhancing biodiversity, water retention, and nutrient efficiency (FAO, 2025). Conventional (industrial) farming relies on high inputs, intensive tillage, irrigation, synthetic fertilisers, fungicides, insecticides, herbicides, monocultures, and genetically modified high-yield cultivars, implemented with heavy machinery (Le Campion *et al.*, 2020). Some synthetic fertilisers used in South Africa use

nitrogen: phosphorus: potassium, and are typically applied to maize, soybean and sunflower crops (Haarhoff and Swanepoel, 2024).

Commercial grain production in South Africa already strains soil and water, freshwater depletion, salinisation, degradation and eutrophication are documented (Van der Laan *et al.*, 2017). About 80 % of grain farmers follow conventional systems (Blignaut *et al.*, 2015), relying on repeated ploughing and harrowing plus pre-and post-emergence herbicides for weed control (Fess and Benedito, 2018; Romero-Salas *et al.*, 2021; Haarhoff *et al.*, 2020). Glyphosate-based products dominate the market, but at least six other actives, atrazine, terbutylazine, S-metolachlor, mesotrione, and 2,4-D, are routinely sprayed on white-maize fields (Ala-Kokko *et al.*, 2021). However, tillage mixes treated and untreated soil, spreading residues, and cover-crop rotations receive additional sprays, creating a continuous, cumulative herbicide load that favours long-term glyphosate build-up (Bonny, 2016).

Monocropping, planting the same crop every season without rotation or intercropping (Wang and Ortiz-Bobea, 2019), leaves fields bare between harvests, exposing the soil to wind and rain and heightening erosion risk (Carlson and Stockwell, 2013). Paired with conventional practices, monocropping intensifies reliance on herbicides; diverse modes of action are applied to curb weed resistance (Haarhoff *et al.*, 2020).

Conservation agriculture represents only about 20% of South African grain farming (Blignaut *et al.*, 2015), yet it typically lowers herbicide demand relative to conventional systems. Adopted for long-term resource protection and yield stability (Shrestha *et al.*, 2020), CA maintains soil integrity through minimal disturbance, permanent cover and crop rotations (Katiski *et al.*, 2018; Shrestha *et al.*, 2020; Romero-Salas *et al.*, 2021). Zero-till or direct seeding preserves surface residues, boosting organic matter, nutrient retention and water-holding capacity while cutting synthetic inputs (Malobane *et al.*, 2020). Farmers shifting to CA often face weed surges and still apply herbicides for control (Thierfelder *et al.*, 2024); consequently, CA reduces but does not eliminate GBH-use. Reduced herbicide efficacy has prompted heavier short-term GLY use, lowered biodiversity and sped up weed shifts (Firbank and Forcella, 2000; Watkinson *et al.*, 2000). Conservation farming is spreading slowly across South Africa. Farmers value its higher yields, lower chemical costs and improved water efficiency (Kuschke and Jordaan, 2017). Adoption rates vary about 30% of operations in Mpumalanga, ~40% in parts of the Free State, >70% in the Western Cape, and ~1% in the Vaalharts Irrigation Scheme (Kuschke and Jordaan, 2017).

Farmers now pair diverse herbicides with crop rotation to ease selection pressure and curb total chemical inputs (Liebman and Dyck, 1993; Weisberger *et al.*, 2019). Conventional systems still account for the highest glyphosate-based herbicide use in South Africa.

2.4.2 Genetically modified glyphosate-tolerant (GT) crops

Crops were genetically modified (GM) to tolerate glyphosate so fields could be sprayed without harming yields. Since GLY's global introduction in 1974 (Nandula *et al.*, 2005), GM varieties have simplified weed control and reduced crop losses (Brookes and Barfoot, 2018). Nations cultivating >50 000 ha of GM crops are termed "biotech mega-countries" (James, 2013); South Africa ranks eighth, planting 2.7 million ha in 2019, about 0.015 % of the 175 million ha global total (Duke, 2015). Advocates cite GM crops as a route to higher-yield food security, yet evidence of this for Southern Africa remains limited and contested (Muzhinji and Ntuli, 2020).

Glyphosate-tolerant crops, marketed as Roundup Ready® (RR), carry the CP4 gene from *Agrobacterium tumefaciens*, a strain first isolated from wastewater at a GLY production plant (Vicini *et al.*, 2021). The gene encodes a CP4 EPSP synthase that resists GLY, enabling crops to withstand herbicide applications (Van Bruggen *et al.*, 2018). Adoption of these GLY-tolerant (GT) varieties has increased GBH use and raised environmental GLY levels (Funke *et al.*, 2006). Roundup Ready® soybean was the first (GT) crop to be introduced globally in 1996, and it was quickly followed by RR cotton, RR maize, RR canola, RR alfalfa and RR sugar beets (Szekacs and Darvas, 2012). Roundup Ready® soybeans were first commercialised in South Africa in 1999 (Steenkamp, 2004), and the South African government later approved the release of RR cotton and maize in 2002 and 2003, respectively (SeedQuest, 2005).

Plant genetic engineering uses two main approaches to create GT crops: (i) insertion of CP4 EPSPS, MEPSPS or 2MEPSPS alleles (different forms of the same gene) that are less sensitive to GLY, and (ii) introduction of genes for GLY-metabolising enzymes such as N-acetyltransferase (GAT) and oxidoreductase (GOX). Both strategies equip plants to tolerate or degrade the herbicide (Pollegioni *et al.*, 2011; Szekacs and Darvas, 2012; Fartyal *et al.*, 2018).

Mounting evidence links the additional GBH applications on GM crops to adverse human, animal and microbial health effects (De Roos *et al.*, 2003; Eriksson *et al.*, 2008; Krüger *et al.*, 2014; McDuffie *et al.*, 2001; Paganelli *et al.*, 2010; Shehata *et al.*, 2013). Farmers and labourers face the greatest risk, and concerns will grow as GM cultivars tolerant to other herbicides, such as 2,4-D, already detected in South African streams (Horn *et al.*, 2019), enter the market.

2.5 Effects of glyphosate (GLY) on target and non-target organisms

Since animals lack the shikimate pathway, GLY was long deemed harmless to them (Reddy 2001; Simonsen *et al.*, 2008). However, accumulating studies report adverse human and animal effects that challenge this assumption (Moneke *et al.*, 2010).

2.5.1 Development of glyphosate-resistant weeds

Glyphosate-based herbicides now face widespread weed resistance, complicating GT-crop management. Resistant individuals pre-exist in weed populations, and selection has produced 375 GLY-tolerant species worldwide (Gaines *et al.*, 2020; Heap, 2025). Farmers counter with higher doses and tank mixes such as GLY plus metachlor (Benbrook, 2016; Zemolin *et al.*, 2014). Resistance arises through reduced uptake or translocation, altered binding sites and amplified EPSPS expression (Nandula *et al.*, 2005; Salas *et al.*, 2012). South Africa already reported five resistant weeds: *Lolium rigidum*, *Conyza bonariensis*, *Amaranthus palmeri*, *Amaranthus hybridus* and *Plantago lanceolata* (Heap, 2025; Pieterse, 2010). Integrated weed management now pairs herbicides with differing modes of action; mixes such as GLY plus 2,4-D outperform 2,4-D alone (Osipe *et al.*, 2017). Repeated spraying, however, continues to select resistant weed mutants.

2.5.2 Effect of glyphosate (GLY) on invertebrates and vertebrates

2.5.2.1 Effects on invertebrates

Numerous studies report adverse effects of GLY on invertebrates. Glyphosate exposure reduced juvenile size in *Daphnia magna* at just 0.05 mg a.i./L (Cuhra *et al.*, 2013). At 0.45 mg a.i./L Roundup®, growth, fertility and abortion rate were negatively affected, decreasing fecundity, while 1.35 mg/L caused 100% abortion of eggs and embryos. *D. magna* also exhibited feeding inhibition and oxidative stress responses, including elevated lipid peroxidation and antioxidant activity (Puértolas *et al.*, 2010). Faena®, a GLY formulation, was slightly more toxic to *D. magna* and 11 times more toxic to *Lecane quadridentata* than technical GLY (Domínguez-Cortinas *et al.*, 2008). A four-day exposure to Roundup® at concentrations between 0.05 and 5 mg/L significantly increased antioxidant enzyme activity in *Lumbriculus variegatus*, notably superoxide dismutase and membrane-bound glutathione S-transferase (Contardo-Jara *et al.*, 2009). *Pseudosuccinea columella*, an intermediate host of the fluke *Fasciola hepatica*, was exposed to 0.1–10 mg/mL GLY while already infected. Glyphosate exposure enhanced parasite reproduction and development, potentially increasing infection rates in animals and humans (Tate *et al.*, 1997).

2.5.2.2 Effects on vertebrates

Roundup Ready® was tested on *Piaractus mesopotamicus*, the small-scale pacu (fish), at 0.0–4.5 mg/L GLY over 48 hours. No gill or liver damage appeared up to 3.0 mg/L. At 3.5 and 4.0 mg/L, however, histological changes emerged: gill hyperplasia and hypertrophy, along with disorganised hepatic cords, nuclear hypertrophy and atrophy, potentially impairing detoxification and tissue repair (Shiogiri *et al.*, 2012). In *Oreochromis niloticus* (Nile tilapia), GLY exposure at 2–310 mg/L over 4 days caused liver vacuolation, necrosis and leukocyte infiltration; kidney congestion, pyknosis and hyaline droplets; gill necrosis, oedema and epithelial irregularities; and brain congestion and spongiosis. Behavioural effects included erratic swimming, respiratory stress and sudden death at just 1.05 mg/L (Ayoola, 2008). The genotoxic effects of Roundup® were assessed in *Caiman latirostris* embryos using comet and micronucleus assays. Exposure to sub-lethal doses (50–1750 µg/egg) during early development caused significant DNA damage at concentrations above 500 µg/egg compared to controls (Poletta *et al.*, 2009). Roundup® also suppressed immune function in this species, reducing complement system activity and increasing susceptibility to illness (Siroski *et al.*, 2016).

The eggs of *Trachemys scripta elegans* (red-eared slider terrapin) were dipped for 30 seconds in seven separate solutions: a water control; a surfactant control (3% LI700 in reconstituted water); and geometrically spaced Glypro concentrations of 1.2, 3.5, 10.6, 31.7, and 95% (each also containing 3% LI700). Only the highest herbicide dose (95% Glypro) greatly reduced hatching success. The combination of GLY and LI700 posed a greater embryonic risk than GLY alone (Sparling *et al.*, 2006). Agpro® GLY 360 did not affect heat-seeking behaviour in skinks, unlike Yates Roundup®, which triggered a fever response, raising body temperature to help manage physiological stress (Carpenter *et al.*, 2016). Glyphosate reduced gastrointestinal motility in rats, with contractions observed at 0.003 g/L and significant biphasic reactions at 0.014 g/L after 20 minutes of jejunum exposure (Chłopecka *et al.*, 2014). In pregnant rats, 21-day exposure to a 1% GLY tap water solution increased lipid peroxidation, overwhelming both maternal and foetal antioxidant defences (Beuret *et al.*, 2005). Zebra finches (*Poephila guttata*) died after consuming seeds treated with 5 000 µg/g GLY over 23 days (Evans and Batty, 1986).

A South African study by Babalola and Van Wyk (2017) investigated the early life stage toxicity of the African clawed frog (*Xenopus laevis*) when exposed to three commercial glyphosate-based herbicide formulations (Roundup, Kilo Max, and Enviro Glyphosate). Embryos (NF-stage 8–11), premetamorphic larvae (NF-stage 48), and prometamorphic larvae (NF-stage 60) were exposed for 96 hours across measured concentration ranges. Mortality was recorded

as the endpoint, and LC50 values were calculated, showing that the premetamorphic NF-stage 48 larvae were consistently the most sensitive, with LC50 values of 0.89 mg/L (Roundup), 58.1 mg/L (Kilo Max), and 134.6 mg/L (Enviro Glyphosate).

A broader review of pesticide-induced endocrine disruptions confirms that thyroid hormone signaling is commonly affected across vertebrate taxa and highlights the regulatory oversight gaps concerning GBH impacts on thyroid-related development and neuroendocrine health (Leemans *et al.*, 2019).

2.5.2.3 *In vitro* studies

Roundup® may act as an endocrine disruptor. In human placental cells (JEG3), exposure to low concentrations (0.05–2.0%) for 18 hours, where 2.0% reflects typical agricultural use, disrupted aromatase gene expression and reduced mRNA levels, impairing enzyme function (Richard *et al.*, 2005). Aromatase converts androgens into oestrogens.

Growing evidence indicates that GLY and GBHs may disrupt thyroid axis function, contributing to broader endocrine effects. Romano *et al.* (2021) provide a targeted review of how GLY's chemical properties could interfere with key components of the hypothalamus–pituitary–thyroid axis, including iodide oxidation and thyroid hormone synthesis, potentially linking exposure to increased incidences of thyroid disorders (Romano *et al.*, 2021). Supporting this mechanism, an *in vitro* study by Coperchini *et al.* (2023) found that rat thyroid FRTL-5 cells (2-D and 3-D cultures) exposed to GLY concentrations ranging from 16.9 to 1690.7 mg/L for up to 72 hours. The effect reduced viability and proliferation in the cultured thyroid cells and increased oxidative stress, further implicating thyroid-specific toxicity. Importantly, South African work by Babalola & Van Wyk (2018) as mentioned in Section 2.5.2.2, showed the ecological relevance of this mechanism in South African contexts.

Glyphosate has been associated with harmful effects on both adult and foetal cells. An *in vitro* toxicity assessment tested four GLY formulations, Roundup Express® (R7.2), Bioforce® (R360), Grands Travaux® (R400), and Grands Travaux Plus® (R450) (Gasnier *et al.*, 2009). At 2 mg/L, all GBHs inhibited the transcriptional activity of oestrogen receptors ER α and ER β in HepG2 cells, possibly through receptor interference or direct DNA interaction. R400 also showed anti-androgenic effects at 0.5 mg/L by suppressing androgen receptor transcription in MDA-MB453-kb2 cells. Despite not having the highest GLY concentration, R400 was the most cytotoxic. R400 and R450 were 20–200 times more toxic than R7.2 and R360. R400 damaged HepG2 cells at 10 mg/L and caused DNA damage in MDA-MB453-kb2 cells at 5 mg/L. Another study found DNA strand breaks in human peripheral blood mononuclear cells

exposed to GLY at concentrations between 0.08–1.69 g/L (Kwiatkowska *et al.*, 2017). The buccal epithelial cell line TR146 exhibited cytotoxic effects, including membrane damage and reduced mitochondrial activity, after exposure to GLY and Roundup® at concentrations below 40 mg/L. At concentrations above 40 mg/L, Roundup® caused significant membrane and DNA damage, marked by increased extracellular lactate dehydrogenase release (Koller *et al.*, 2012).

Polyethoxylated tallow amine (POEA), an adjuvant used to enhance GLY solubility and plant uptake (Mesnage and Antoniou, 2018), was tested on HepG2 (hepatic), HEK293 (embryonic kidney), and JEG3 (placental) cell lines. Toxic effects occurred at 1–3 mg/L, including compromised cell integrity, necrosis during micellization, and potential disruption of endocrine and respiratory systems. JEG3 cells were twice as sensitive as HEK293 and HepG2 (Mesnage *et al.*, 2013).

2.5.2.4 Effects on humans

Glyphosate-based herbicides poorly penetrate human skin but can enter the body through dietary exposure. Glyphosate residues are commonly ingested via treated fruits, vegetables and animal products. Some formulations also contain formaldehyde, classified as a human carcinogen, as an impurity (Soares *et al.*, 2021). Glyphosate exposure during pregnancy has been linked to an increased risk of preterm birth, defined as delivery before 37 weeks (Varde *et al.*, 2023). Chronic GBH exposure in infants and children has been associated with a range of health issues, including cancer, kidney damage, and neurodevelopmental and neurodegenerative conditions such as Attention-deficit/hyperactivity disorder, autism, Parkinson's and Alzheimer's diseases (Rawat *et al.*, 2023).

Glyphosate-based herbicides have been linked to long-term effects including hepatorenal damage, teratogenicity, tumorigenicity, oxidative stress and endocrine disruption (Mesnage *et al.*, 2015). However, the USEPA considers GLY safe when used according to manufacturer guidelines. A joint evaluation by the European Food Safety Authority (EFSA) and the Food and Agriculture Organisation (FAO) of the United Nations's joint meeting on pesticide residues also concluded that GLY is not carcinogenic (Brueck, 2019; Centner *et al.*, 2019). In contrast, the International Agency for Research on Cancer (IARC) found that GLY is "probably carcinogenic to humans" (Centner *et al.*, 2019).

The GLY controversy remains unresolved. In August 2018, Dewayne "Lee" Johnson, a school groundskeeper, won a lawsuit against Monsanto after developing non-Hodgkin lymphoma from regular use of Roundup® and Ranger Pro®, both GBHs produced by the company. Bayer acquired Monsanto in June 2018 and has since paid nearly \$11 billion to settle similar claims

(Glenna and Bruce, 2021; Voorhees, 2021). Supporting this concern, Weisenburger and others have reviewed evidence linking GLY and GBHs to non-Hodgkin lymphoma in humans (Weisenburger, 2021; Kaur *et al.*, 2024). Recently, a former French landscaper said GLY exposure caused his rare cancer, intravascular B-cell lymphoma (Gauriat, 2025).

2.5.3 Effect on microorganisms as primary glyphosate (GLY) degraders

Glyphosate-based herbicides can alter microbial communities by selecting tolerant species, thereby shifting species composition and interactions (Dorigo *et al.*, 2007). The gut microbiota is vital to human health, supporting digestion, nutrient synthesis, detoxification of xenobiotics, immune regulation and gastrointestinal integrity through a complex symbiotic relationship (Samsel and Seneff, 2013; Littman and Pamer, 2011). The increasing prevalence of inflammatory bowel diseases, including Crohn's disease, in Western Europe and the United States has raised concerns about a possible link between GLY exposure and gut microbial dysbiosis (Samsel and Seneff, 2013).

Microbial sensitivity to GLY depends on the class of EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) enzyme they carry (Table 2.3). Initially, two classes were known: GLY-sensitive class I and GLY-resistant class II (Funke *et al.*, 2007). More recently, four distinct EPSPS groups have been identified based on genetic variation (Leino *et al.*, 2021).

Table 2.3: Bacterial glyphosate sensitivity/resistance classification criteria.

Class	Glyphosate status	Key feature	Examples	References
Class I	Sensitive	Alpha and beta variants, common in plants	Plants, <i>E. coli</i>	Light <i>et al.</i> (2016); Firdous <i>et al.</i> (2018)
Class II	Resistant	Specific amino acid markers (use one amino acid)	<i>Agrobacterium</i> spp.	Priestman <i>et al.</i> (2005)
Class III	Varies	Presence of specific motifs (uses multiple amino acids)	Experiments	Carozzi <i>et al.</i> (2006)
Class IV	Resistant	Newly identified amino acid markers	New emerging bacterial strains	Lira <i>et al.</i> (2016)

Long-term, intensive GLY-use selected resistant bacterial and fungal strains through mechanisms such as altered EPSPS binding sites, active efflux, and reduced cell wall permeability (Van Bruggen *et al.*, 2021). Glyphosate toxicity varies by species: beneficial bacteria like *Bacteroides fragilis*, *Bifidobacterium breve* and *Escherichia coli* are highly sensitive, while pathogens such as *Clostridium bartlettii*, *Enterococcus casseliflavus* and

Salmonella, *Ruminococcus obeum* show resistance (Leino *et al.*, 2021; Shehata *et al.*, 2014). As a result, GLY exposure may suppress beneficial microbes and promote GLY-resistant pathogens (Shehata *et al.*, 2014). It may also contribute to the loss of microbial species that normally suppress *Clostridium botulinum* (Shehata *et al.*, 2014; Rueda-Ruzafa *et al.*, 2019). Roundup PowerFlex® altered soil microbial and fungal communities, reducing colony-forming units at 0.8 kg GLY a.i./ha (Zaller *et al.*, 2021). In bacteria, herbicide exposure has been linked to changes in antibiotic tolerance: *Escherichia coli* and *Salmonella enterica* exposed to GLY and other herbicides showed increased tolerance responses to ampicillin, chloramphenicol and tetracycline, suggesting a potential link to antibiotic resistance (Kurenbach *et al.*, 2015). Glyphosate's impact on microbial communities also extends to wildlife exposed via contaminated water or food, with altered gut microbiota observed in green turtles (*Chelonia mydas*), rainbow trout (*Oncorhynchus mykiss*) and bumblebees (*Bombus* spp.) (Kittle *et al.*, 2018; Bellec *et al.*, 2022; Helander *et al.*, 2023). These reductions in microbial diversity highlight the broad ecological implications of GLY exposure.

Fei *et al.* (2013) examined regulatory genes in *Enterobacter* responsible for resistance to GLY (10–60 mg/L). Their findings showed that GLY influences not only the shikimate pathway but also multiple metabolic pathways, either directly or indirectly. Resistance may result from the combined action of EPSPS and stress-induced genes responding to osmotic, acidic and oxidative stress. This underscores the complexity of gene regulation in GLY resistance.

Glyphosate's antimicrobial properties were formally recognised when it was patented as an antibiotic (US Patent No. 7771736 B2) by the inventor William Abraham, after decades of herbicidal use (Abraham, 2010; Walsh *et al.*, 2023). Monsanto, the company that the inventor assigned the rights to, filed the patent citing GLY's activity against bacteria, fungi and parasites containing the EPSPS enzyme. Its antibiotic effect is comparable to fosfomycin, another phosphonic acid antibiotic (Abraham, 2010; Raoult *et al.*, 2021). According to Van Bruggen *et al.* (2018), local bacterial susceptibility may determine whether environments favour GLY- or antibiotic-resistant strains, resistance that could eventually spread to clinical pathogens. Even bacteria lacking EPSPS can face selective pressure from GLY.

While these studies highlight GLY's potential to alter microbial communities and ecological interactions, it is also important to consider the broader debate around GLY's safety, its use in invasive alien plant management programmes, and findings from South African experimental work. This wider context is reviewed below

2.5.4 Glyphosate (GLY) use, debates, and South African context

The regulatory status and safety profile of GLY, particularly in its commercial formulation Roundup®, remains a subject of international debate. Some authorities reaffirm its safety under prescribed use, while others highlight potential carcinogenic and endocrine-disrupting risks (Van Bruggen *et al.*, 2018; Van Bruggen *et al.*, 2021; Rawat *et al.*, 2023).

In South Africa, GLY formulations are not only used in agriculture but also in invasive alien plant eradication through the national Working for Water (WfW) programme. The programme includes management of aquatic weeds such as water hyacinth in catchment areas (Andrade-Rivas & Rother, 2015; Babalola & Van Wyk, 2018; Dabrowski, 2015a).

Local experimental research has also highlighted ecological risks. For example, Babalola & Van Wyk (2018) (see Section 2.5.2.2) study with formulations used in WfW contexts. At a national scale, the Water Research Commission has published reports assessing pesticide use, environmental mobility, and aquatic risk hotspots across South Africa (Dabrowski, 2015a; Dabrowski, 2022).

2.6 The environmental fate of glyphosate (GLY) predicted by its characteristics

Glyphosate and AMPA undergo mineralisation, immobilisation, or leaching in the environment (Bai and Ogbourne, 2016). Mineralisation, driven by microbial activity, is considered the primary degradation pathway, producing AMPA, methylphosphonic acid, glycine and sarcosine. AMPA is further broken down into methylamine and phosphate, eventually decomposing into CO₂ and NH₃. Soil properties such as pH, iron and copper content, and organic carbon influence microbial mineralisation. Due to its strong adsorption to clay and organic debris, GLY often becomes immobile, reducing microbial access and increasing the risk of long-term soil accumulation (Meftaul *et al.*, 2020). When not mineralised or immobilised, GLY and AMPA can leach through the soil matrix. Additional degradation occurs via photodegradation and chemical disintegration (Degenhardt *et al.*, 2012; Zhan *et al.*, 2018; Feng *et al.*, 2020; Nagy *et al.*, 2020). In aquatic systems, GLY dissipates rapidly from pond surfaces with high suspended sediments, showing hydrolytic half-lives between 1.5 and 11 days. In soil, GLY binds to carbon particles, with half-lives ranging from a few hours to over 110 days depending on soil type (Bai and Ogbourne, 2016), and in some cases extending to 300–428 days (Mertens *et al.*, 2018). Sediment acts as the primary sink for GLY residues in aquatic environments (Rawat *et al.*, 2023).

2.6.1 Formation of glyphosate (GLY) metabolites

Glyphosate degrades through several pathways into the intermediate metabolite AMPA (Figure 2.2) (De Brito Rodrigues *et al.*, 2019; El Agrebi *et al.*, 2020; Kwiatkowski and Harasim, 2020). The two primary degradation routes are cleavage by GLY oxidoreductase and carbon–phosphorus (C–P) lyase–mediated biodegradation (Klátyik *et al.*, 2023; Singh *et al.*, 2024; Zhao *et al.*, 2015). *Pseudomonas* spp. is among the most frequently isolated GLY-degrading bacteria (Feng *et al.*, 2020; Zhao *et al.*, 2015). These pathways occur naturally in bacteria and plants found in both soil and water environments (Szekacs and Darvas, 2012). Although the plants and bacteria can break down GLY, it will ultimately die as a result of the Shikimate inhibition.

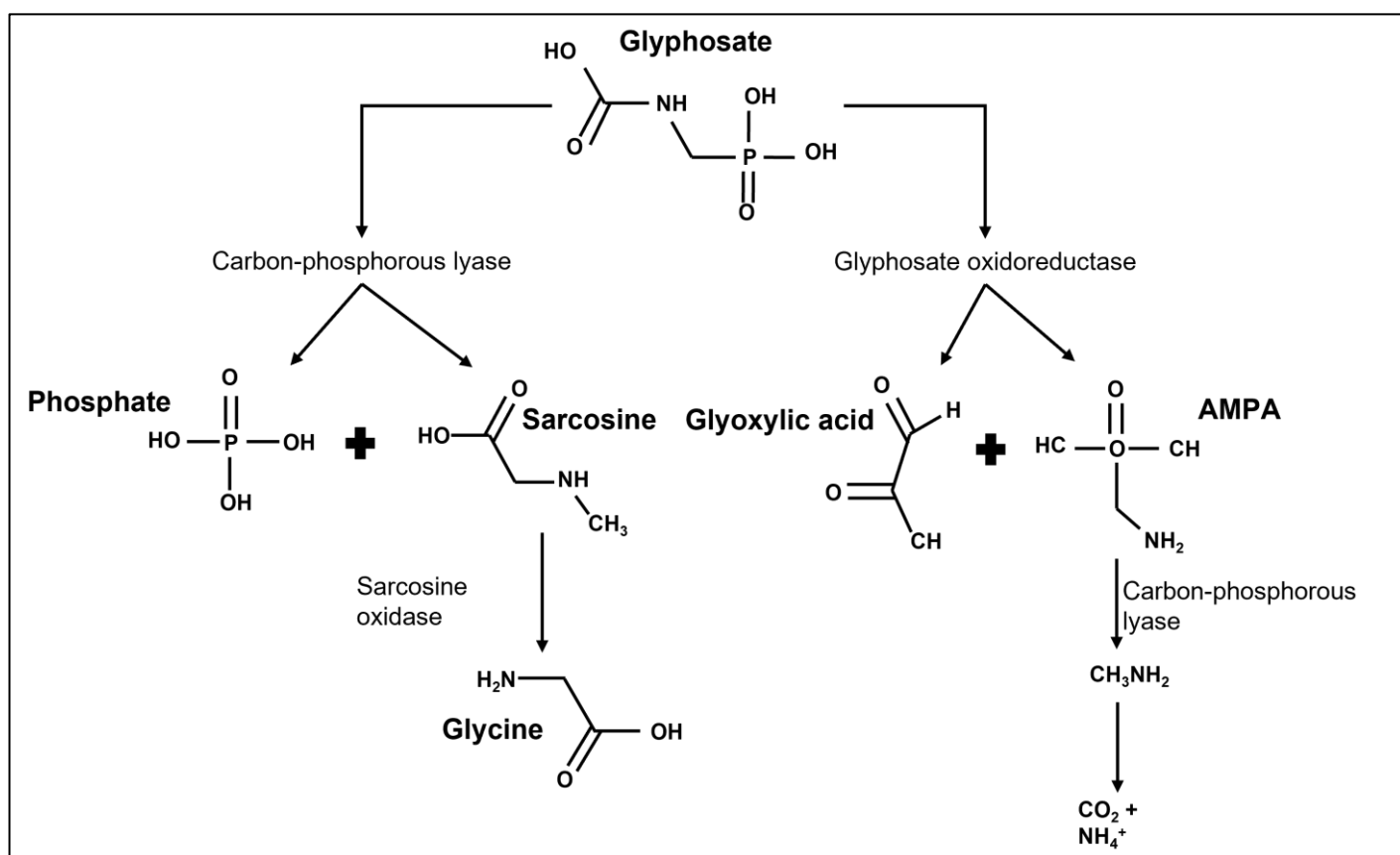


Figure 2.2: Glyphosate microbial degradation pathways in water, soil and plants. Adapted from Vicini *et al.* (2021).

Although AMPA can also result from the breakdown of detergents (Battaglin *et al.*, 2014) and artificial sweeteners like acesulfame (Van Stempvoort *et al.*, 2016), its primary environmental source is GLY degradation (Van Stempvoort *et al.*, 2016). AMPA is highly water-soluble and degrades more slowly than GLY. Its further breakdown yields inorganic phosphate, methylamine, ammonium and carbon dioxide (Gimsing *et al.*, 2007; Duke *et al.*, 2012). Compared to GLY, AMPA is more readily detectable in streams and rivers.

2.6.2 Sorption-desorption, and mobility of glyphosate (GLY)

Once a pesticide enters the soil, its chemical properties influence its sorption, solubility and persistence, and the soil's physicochemical characteristics, including organic matter, moisture, biomass, pore structure, and pH, also affect the pesticide's destiny (Ortiz *et al.*, 2017). For GLY, leaching depends on dose, preferential flow, sorption and degradation rates (Norgaard *et al.*, 2014). Rainfall and soil structure are key drivers of GLY transport, which can occur in dissolved or particulate forms, enabling entry into surface waters (Aparicio *et al.*, 2013). Glyphosate binds readily to clay and organic matter in soil, limiting its mobility and making microbial degradation more difficult, which can lead to soil accumulation over time (Cassigneul *et al.*, 2016). Although GLY is water-soluble, it strongly adsorbs to clay upon contact (Kovács *et al.*, 2024) and therefore GLY and AMPA persist longer in clay-rich soils than in sandy soils, where higher porosity allows easier washout. In South Africa, one of the dominant clay types is kaolinite (Kgabi and Ambushe, 2023). Glyphosate competes with phosphate compounds for binding sites, as both share similar functional groups that attach to soil hydroxides (Wang *et al.*, 2023a).

Soil pH also influences GLY sorption. Alkaline conditions increase the negative charge of GLY molecules through deprotonation, which may enhance electrostatic repulsion and reduce sorption efficiency (Dollinger *et al.*, 2015). Amorphous iron and aluminium oxides in soil are key sorption sites (Figure 2.3) for both negatively charged phosphate and GLY ions (Munira *et al.*, 2018; Karasali *et al.*, 2019). However, cations compete for binding sites to GLY and PO_4^{2-} based on cation exchange capacity (CEC), with the order of competition as follows: $\text{Al}^{3+} > \text{Fe}^{3+} > \text{Mn}^{2+} > \text{Zn}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+} > \text{Na}^+$ (Dollinger *et al.*, 2015).

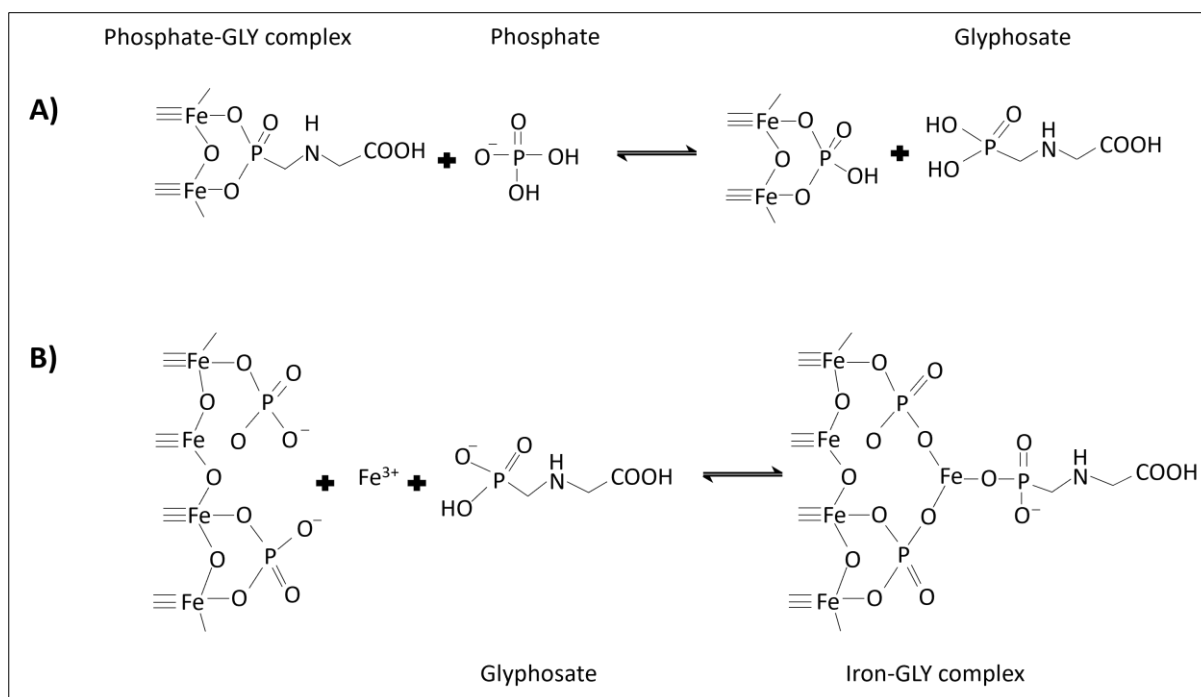


Figure 2.3: A simulated reaction of the sorption of glyphosate and phosphate. A) competitive sorption; B) sorption in two planes (glyphosate on phosphate). Since glyphosate has a zwitterionic structure of phosphate, carboxyl and amino groups, the phosphate group or volatile phosphor will compete to bind to the surface complex. Adapted from Borggaard and Gimsing (2008).

2.6.3 Glyphosate (GLY)/AMPA (aminomethylphosphonic acid) metal complexes

The role of the CEC of soil in GLY adsorption remains unclear. Some studies suggest a direct link between GLY adsorption, clay content and soil CEC (Gunarathna *et al.*, 2018). High concentrations of metal cations in water can reduce herbicide effectiveness, likely due to GLY forming precipitates or chelates that plants cannot absorb (Duke *et al.*, 2012). In soil, GLY may increase the solubility and bioavailability of heavy metals, forming metal-GLY complexes that contribute to toxicity and potentially harm crops (Mertens *et al.*, 2018). These complexes can either adsorb to soil or leach out during rainfall, depending on factors such as soil type, porosity, temperature and pH.

Jayasumana *et al.* (2014) have identified three possible sources where GLY/AMPA-metal complexes may occur:

- [GLY/AMPA + Ca/Mg/Fe/Sr] complex in drinking water
- [GLY/AMPA + Cd/Cr/Ni/Co/Pb/V or As] complex in food
- [GLY/AMPA coming from dermal/respiratory route] + low number of [metals/As] from water and foods, here the complex is formed within the circulation of water and food.

When the pH of moderately buffered soils and water exceeds 6.5, GLY exists as a di-anion, increasing its affinity for metal ions (Jayasumana *et al.*, 2014). Metal ion presence affects all GLY-related processes, including adsorption, photodegradation and biodegradation. Thelen *et al.* (1995) used nuclear magnetic resonance to show that hard water cations like Ca^{2+} and Mg^{2+} bind to the phosphate and carboxyl groups of GLY. Figure 2.4 illustrates the formation of these GLY-metal complexes. Glyphosate-metal complexes reduce the bioavailability of metal micronutrients, potentially causing deficiencies that lower plant resistance to pathogens. These complexes also disrupt plant-microorganism interactions, such as nitrogen fixation (Klátyik *et al.*, 2023).

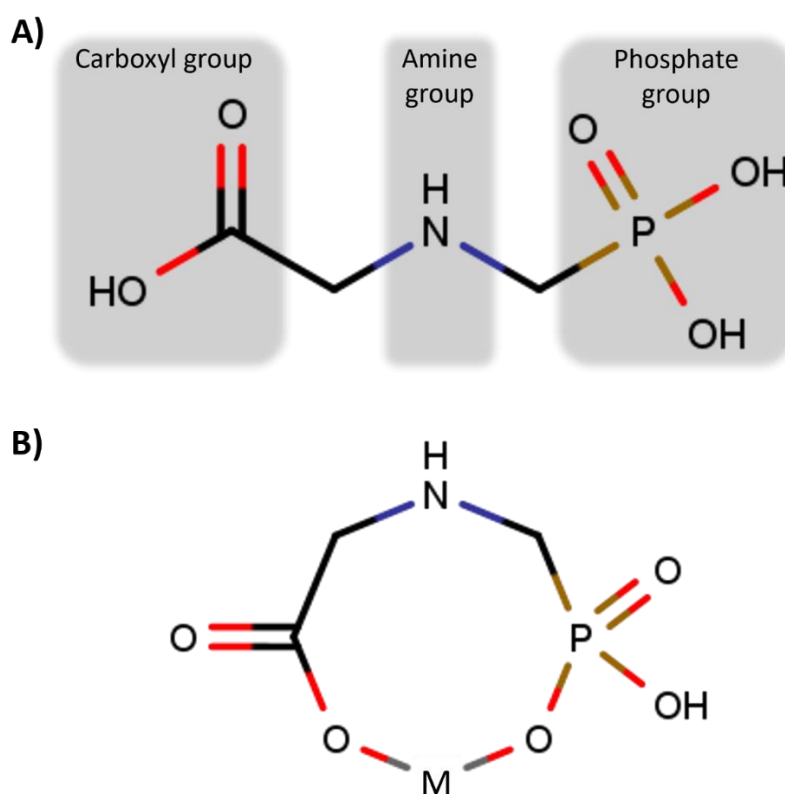


Figure 2.4: A) The chemical structure of glyphosate with its three functional groups and B) the chemical structure of glyphosate that underwent metal ion chelation to create a glyphosate-metal complex. The ion 'M' serves as a substitute for the possible metal ions that might undergo chelation. Chemical structures were created and stoichiometrically corrected using MarvinSketch v23.7 (ChemAxon Ltd).

2.6.4 UV degradation of glyphosate (GLY) and AMPA (aminomethylphosphonic acid)

Heat and photodegradation by solar radiation play a major role in the breakdown of pesticide molecules (Rad *et al.*, 2022). Solar ultraviolet (UV) radiation is a key driver of photodegradation in environmental systems, especially in aquatic and terrestrial ecosystems where direct sunlight exposure is prevalent. Although studies specifically investigating GLY photodegradation under natural UV exposure are few, it is well known that sunlight can initiate degradation processes for other organic compounds.

Studies investigating the transformation of GLY with UV irradiation alone, without added oxidising agents or catalysts, are limited. For example, one study reported that GLY concentration in a deionised water solution (1 mg/L) decreased by 50% after 4 days of exposure to UV-C at 20°C with a UV intensity of 30 W/cm² (Lund-Høie and Friestad, 1986). Balah *et al.* (2017) found that an aqueous solution of 30 µg/L GLY decreased by 99% after 120 minutes of UV irradiation at 254–336 nm (UV dose not stated). Assalin *et al.* (2010) observed degradation of GLY and AMPA formation after UV-B irradiation ($\lambda > 290$ nm) of 42 mg/L of GLY in water, while Sandy *et al.* (2013) reported the generation of orthophosphate after UV-mediated photo-oxidative degradation of GLY within the euphotic zone of natural aquatic environments. These results suggest that UV-mediated photolysis can decrease GLY concentrations by over 90% in aqueous solutions and generate different metabolites as by-products.

A study from Argentina has shown that UV irradiation (in tandem with peroxide) can reduce the toxicity of GLY (Vidal *et al.*, 2015). The authors also mentioned that in commercially formulated GLY (GLY-monoisopropylamine salt), the salt cation competes with the phosphonate moiety of GLY when scavenging for hydroxyl radicals, slowing down the GLY oxidation degradation. This observation is also seen in a study by Assalin *et al.* (2010), who concluded that the process of forming hydroxyl radicals by photocatalytic degradation (UV-TiO₂) (another form of oxidation degradation) and photolysis (UV) plays a role in breaking down GLY and AMPA after a short treatment time.

A study by Papagiannaki *et al.* (2020) exposed GLY to UV-A, UV-B, and UV-C to investigate which UV decreased GLY toxicity to exposed organisms, *Daphnia magna*, *Raphidocelis subcapitata* and *B. subtilis*. The effective concentration at 50% producing an effect (EC₅₀) of GLY for the *D. magna*, *R. subcapitata*, and *Bacillus subtilis* were 0.99, 1.13 and 3.67 mg/L, respectively (Papagiannaki *et al.*, 2020). The EC₅₀ of GLY increased after UV-C irradiation two-fold, five-fold and 23-fold for *D. magna*, *R. subcapitata* and *B. subtilis*. Exposure to UV-B also showed a significant decrease in toxicity, but not as much as UV-C. Finally, the authors

suggested that UV-C and UV-B exposure can decrease the ecotoxicity of GLY if the UV dose is high enough. These findings indicate that UV irradiation may help reduce GLY concentration and decrease overall toxicity in environmental settings.

2.7 Various analytical methods for quantifying glyphosate (GLY) and AMPA (aminomethylphosphonic acid)

Glyphosate and AMPA can be detected and quantified through various analytical methods applied to different environmental and biological matrices. Table 2.4 presents seven techniques used in publications from 2008 to 2022 for analysing GLY and AMPA in matrices such as plant tissues (seeds and fruit), water (fresh and marine), soil, sediment, suspended matter and animal tissues (invertebrates and chordates). These methods include, but are not limited to, capillary electrophoresis–mass spectrometry (CE-MS), gas chromatography–mass spectrometry (GC-MS), immunoassays, ion chromatography–mass spectrometry (IC-MS), liquid chromatography–mass spectrometry (LC-MS) and spectrophotometry.

Detection limits for GLY and AMPA vary by matrix. In water, the limit of detection (LOD) for GLY ranges from 0.03 to 5 µg/L, while in solids like soil, it can be as high as 25.7 mg/kg. AMPA LODs in water range from 0 to 0.25 µg/L, and in serum, up to 0.25 µg/mL. Limits of quantification (LOQ) for GLY span 0.01–3 mg/kg in solids and 0.1–0.5 µg/L in water; AMPA LOQs range up to 3 mg/kg in solids and 0.1–0.5 µg/L in water. Recovery rates range from 70–127.2% for GLY and 65–106.5% for AMPA. These varied analytical approaches reflect GLY's complex chemistry: a zwitterion with four dissociation constants, GLY shifts ionic form with a change in pH and acts as both an acid and a base (ATSDR, 2020).

Among the 30 studies listed in Table 2.4, LC-MS was the most frequently used analytical method, accounting for 43% of the total. This preference likely stems from LC-MS being better suited for analysing highly polar compounds like GLY and its metabolite AMPA, in contrast to GC-MS (Stachniuk and Fornal, 2016).

Table 2.4: The various analytical methods used to quantify glyphosate and AMPA (aminomethylphosphonic acid) from various matrices.

Analytical method	Matrix	Extraction	LOD	LOQ	Recovery	Country	Reference
CE-MS	Soil	LLE	25.7 mg/kg.	77.1 mg/kg.	G: 92.0–98.0%	Mexico	Muñoz <i>et al.</i> (2019)
CE-MS	<i>Daphnia</i> , Beer.	<i>Daphnia</i> : LLE. Beer: SPE	G _{water} : 4.2 µg/L. G _{Daph} : 4.1 µg/L. G _{beer} : 5.3 µg/L. A _{water} : 3.3 µg/L. A _{Daph} : 6.2 µg/L. A _{beer} : 30.6 µg/L.	-	-	Germany	Wimmer <i>et al.</i> (2020)
GC-MS	Urine (human, cows, rabbit); cow organs	LLE	-	-	-	Germany	Krüger <i>et al.</i> (2014)
GC-MS	Human serum	SPE	G: 0.25 µg/mL. A: 0.25 µg/mL.	-	87.5–96.6%	Japan	Motojyuku <i>et al.</i> (2008)
GC-MS	Plants, water, benthic macroinvertebrate organs	LLE	-	-	-	Poland	Rzymiski <i>et al.</i> (2016)
GC-MS	Water	LLE	G: 0.011 µg/kg.	-	-	Iran	Samarghandi <i>et al.</i> (2017)
FI	Plant tissues, river water, tea, soil	SPE	G: 8 ng/mL.	-	G: 87.4%–103.7%	China	Wang <i>et al.</i> (2016)
FI	Water	None	G: 5 µg/L.	-	G _{water} : 80–115.8%.	China	Yang <i>et al.</i> (2022)
Immunogenic	Water	None	G: 0.05 ng/mL.	-	G: 28.2–127.2%	Hungary	Mörtl <i>et al.</i> (2013)
Immunogenic	Spiked meat	LLE	-	-	G: 91%	Germany	Krüger <i>et al.</i> (2014)
Immunogenic	Urine (Human, Cows, Rabbit); Cow organs	LLE	-	-	-	Germany	Krüger <i>et al.</i> (2014)

Analytical method	Matrix	Extraction	LOD	LOQ	Recovery	Country	Reference
Immunogenic	Tap water	SPE	G: 0.1 µg/L.	-	-	Poland	Kaszkowiak <i>et al.</i> (2021)
IC-MS	Human urine	Samples were spiked and diluted	G: 0.2–0.8 µg/L.	-	G: 85–115%	United States of America	Schütze <i>et al.</i> (2021)
IC-MS	Grapes, honey, wheat	SPE	-	G: 0.01 mg/kg. A: 0.05 mg/kg.	G _{grapes} : 107–109%. G _{honey} : 93–97%. G _{wheat} : 105%. A _{grapes} : 89–99%. A _{honey} : 85–89%. A _{wheat} : 89–103%	Italy	Gasparini, <i>et al.</i> (2020)
IC-MS	Water	None	G: 0.015 mg/L. A: 0.08 mg/L.	G: 0.05 mg/L. A: 0.25 mg/L.	G _{tap} : 86.2–96.9%. G _{spring} : 92.2–99%. A _{tap} : 81.2–83%. A _{spring} : 80.8–102.6%.	Brazil	Dovidauskas, <i>et al.</i> (2020)
IC-MS	Honey, fish (bass), bovine muscle	SPE	-	G _{honey} : 4.30 ng/g. G _{fish} : 5.08 ng/g. G _{bovine} : 6.47 ng/g. A _{honey} : 9.26 ng/g. A _{fish} : 5.38 ng/g. A _{bovine} : 6.44 ng/g.	G _{honey} : 99–100%. G _{fish} : 80–93%. G _{bovine} : 75–80%. A _{honey} : 91–95%. A _{fish} : 95–96%. A _{bovine} : 75–79%.	Germany	Chiesa <i>et al.</i> (2019)
LC-MS	Soybean, maize, carrot, apple, spicy cabbage (SC)	SPE	G _{soybean} : 0.005 mg/kg. G _{maize} : 0.006 mg/kg. G _{carrot} : 0.007 mg/kg. G _{apple} : 0.008 mg/kg. G _{SC} : 0.007 mg/kg.	G _{soybean} : 0.016 mg/kg. G _{maize} : 0.02 mg/kg. G _{carrot} : 0.025 mg/kg. G _{apple} : 0.026 mg/kg. G _{spicy cabbage} : 0.023 mg/kg.	G: 83.1–100.8%	China	Ding <i>et al.</i> (2016)
LC-MS	Soil, water, sediments	SPE	G _{soil} : 0.5 µg/kg. G _{sed} : 0.5 µg/kg. G _{water} : 0.1 µg/kg. A _{soil} : 0.5 µg/kg.	G _{soil} : 3 µg/kg. G _{sediment} : 3 µg/kg. G _{water} : 0.5 µg/kg. A _{soil} : 3 µg/kg.	Water: >90%. Soil: 75–110%	Argentina	Vialle <i>et al.</i> (2013)

Analytical method	Matrix	Extraction	LOD	LOQ	Recovery	Country	Reference
			A _{sed} : 0.5 µg/kg. A _{water} : 0.1 µg/kg.	A _{sediment} : 3 µg/kg. A _{water} : 0.5 µg/kg.			
LC-MS	Water	LLE	-	G: 0.1 µg/L; A: 0.1 µg/L	-	France	Vialle <i>et al.</i> (2013)
LC-MS	Water	SPE	G: 0.1 µg/L.	-	-	Poland	Kaszkowiak <i>et al.</i> (2021)
LC-MS	Surface water, suspended particulate matter (SPM), sediments	Surface water: LLE. SPM: SPE. Sediments: QuEChERS	G _{sw} : 0.03 µg/L. G _{spm} : 0.2 µg/L. G _{sediment} : 0.3 µg/kg _{dw} . A _{sw} : 0.04 µg/L. A _{spm} : 0.3 µg/L. A _{sediment} : 0.6 µg/kg _{dw} .	G _{sw} : 0.1 µg/L. G _{spm} : 0.5 µg/L. G _{sed} : 1 µg/kg _{dw} . A _{sw} : 0.1 µg/L. A _{spm} : 0.9 µg/L. A _{sed} : 2 µg/kg _{dw} .	G+A: 60%–140%	Argentina	Mac Loughlin <i>et al.</i> (2022)
LC-MS	Water	SPE	G: 1 µg/L. A: 1 µg/L	-	-	New Zealand	Close <i>et al.</i> (2021)
LC-MS	Florida manatee (<i>Trichechus manatus latirostris</i>) plasma, water	None	G: 0.05 µg/L.	G: 0.05 µg/L.	-	United States of America	De María <i>et al.</i> (2021)
LC-MS	Soil, earthworms (<i>Allolobophora chlorotica</i>)	Soil: LLE+SPE. Earthworm: SPE	G _{soil} : 0.009 µg/g. A _{soil} : 0.007 µg/g. G _{worm} : 0.07 µg/g. A _{worm} : 0.065 µg/g.	G _{soil} : 0.03 µg/g. A _{soil} : 0.025 µg/g. G _{worm} : 0.23 µg/g. A _{worm} : 0.2 µg/g.	G: 70%. A: 65%	France	Delhomme <i>et al.</i> (2021)
LC-MS	Milk, cucumber, honey, porridge formula, bovine kidney & liver	Dansyl chloride: SPE. QuPPE: SPE.	-	G: 10–25 µg/kg. A: 10 µg/kg.	G: 80%–120%	Latvia	Jansons <i>et al.</i> (2021)
LC-MS	Mealworm (<i>Tenebrio molitor</i>) larva	SPE	-	G: 0.5 µg/L. A: 0.5 µg/L.	G: 89.9–91.7%. A: 90–105.6%	Republic of Korea	Kim <i>et al.</i> (2021)

Analytical method	Matrix	Extraction	LOD	LOQ	Recovery	Country	Reference
LC-MS	Water, suspended particulate matter.	Filtered water: SPE SPM: SPE	G _{sw} : 0.03 µg/L. A _{sw} : 0.04 µg/L. G _{spm} : 0.06 µg/L. A _{spm} : 0.08 µg/L.	G _{sw} : 0.1 µg/L. A _{sw} : 0.14 µg/L. G _{spm} : 0.2 µg/L. A _{spm} : 0.3 µg/L.	G: >90% A: >90%	Argentina	Mac Loughlin <i>et al.</i> (2020)
LC-MS	Soybean, maize, cotton seeds	SPE	-	G: 0.05 mg/kg. A: 0.05 mg/kg.	-	Brazil	Rodrigues <i>et al.</i> (2021)
LC-MS	Water	None	-	G: 0.025 µg/L A: 0.025 µg/L.	G _{water} : 85–92%. G _{soil} : 80–87%. G _{wheat} : 95–102%. G _{water} : 85–92%. G _{soil} : 80–87%. G _{wheat} : 95–102%.	Italy	Campanale <i>et al.</i> (2022)
Spectrophotometric	Water, soil, wheat	All SPE.	G: 1.1 µg/mL.	G: 3.7 µg/mL.	G _{water} : 85–92%. G _{soil} : 80–87%. G _{wheat} : 95–102%	Pakistan	Jan <i>et al.</i> (2009)

CE-MS = Capillary electrophoresis - mass spectrometry; GC-MS = Gas chromatography-mass spectrometry; FI = Fluorescence immunoassay; IC-MS = Ion chromatography-mass spectrometry; LC-MS = Liquid chromatography-mass spectrometry; SPE = Solid phase extraction; LLE = Liquid-liquid extraction; G = Glyphosate; A = aminomethylphosphonic acid; LOD = Limit of detection; LOQ = Limit of quantification; QuEChERS = quick, easy, cheap, effective, rugged, and safe; - (dash) = not mentioned; sw = surface water; spm = suspended particulate matter.

2.8 Bacterial ecological risk assessment

A quantitative microbial risk assessment (QMRA) was employed in this study to model the potential health risks to humans consuming water from the study sites (Haas *et al.*, 2014; Ahmed *et al.*, 2020). QMRA estimates the probability of infection or disease following exposure to pathogenic microorganisms. It involves a structured process that quantifies both exposure and the resulting health effects across environments such as water, soil and air. The framework is adaptable to various pathogens and scenarios, making it applicable to water safety, food contamination and airborne disease risk (Haas *et al.*, 2014).

The QMRA framework consists of four key components: (1) hazard identification, (2) dose-response assessment, (3) exposure assessment and (4) risk characterisation. Hazard identification determines which microorganisms are relevant and what health effects they may cause. The dose-response step describes the relationship between exposure levels and health outcomes, often using animal data extrapolated to humans, supplemented by epidemiological insights. Exposure assessment considers the quantity, route (e.g., inhalation, ingestion) and duration of exposure, as well as the characteristics of the exposed population. Risk characterisation integrates the previous steps to estimate the likelihood of adverse outcomes, yielding information on risk magnitude, variability and uncertainty.

Ahmed *et al.* (2020) used QMRA to estimate infection risks in schoolchildren consuming contaminated school water. Detected pathogens included *Escherichia coli*, *Shigella* spp., *Salmonella* spp. and *Vibrio cholerae*. The QMRA tool predicted high probabilities of infection and illness: *E. coli* (99.6%, 34.8%), *Shigella* spp. (94.4%, 33%), *Salmonella* spp. (100%, 45%) and *V. cholerae* (98.8%, 19.8%). In agricultural settings, especially those exposed to faecal contamination, such as wastewater or settlements near irrigation rivers, *E. coli* is commonly used as an indicator of potential pathogen presence (Panagiotou *et al.*, 2023).

A systematic review by Owens *et al.* (2020) examined QMRA applications in public drinking water and identified thirteen major reference pathogens across 86 studies. Most assessments focused on the parasites *Cryptosporidium* and *Giardia*. Bacterial pathogens included *E. coli*, *Campylobacter*, faecal coliforms, *Legionella*, *Pseudomonas* and *Salmonella*, while viral targets included Rotavirus, Norovirus, Enterovirus, Adenovirus and non-specific viruses.

Prominent waterborne pathogens such as *Enterococcus* spp. and *Pseudomonas* spp. are frequently considered in QMRA studies due to their known association with water sources. There is growing concern that GLY may exert indirect selection pressure, favouring opportunistic pathogens with antibiotic resistance. The presence of potentially resistant strains like *Staphylococcus* and *Mycobacterium* further elevates the risk of infection. Species such as *Bacillus*

and *Clostridium*, known for their spore-forming abilities and resilience, along with *Legionella*, commonly associated with waterborne disease outbreaks, were also evaluated. These bacteria pose risks due to their resistance to environmental stressors like temperature fluctuations and adverse conditions, increasing their potential to impact human health when transmitted through water.

2.9 Need for monitoring glyphosate (GLY) and AMPA (aminomethylphosphonic acid) in the South African environment

Glyphosate-based herbicides are extensively used in South Africa, mirroring global patterns. Since the mid-1990s, usage has surged, largely due to the widespread adoption of GLY-tolerant crops (Duke, 2015; Rodrigues *et al.*, 2021; Viljoen *et al.*, 2021). The continued expansion of Roundup Ready® crops such as maize, soybeans, cotton, canola, alfalfa and sugar beet is expected to further increase GBH application, even in regions where these cultivars were previously uncommon. As a result, elevated GBH use raises the risk of contaminating rainwater, surface water and groundwater, with potential implications for drinking water and other common exposure pathways (Borggaard and Gimsing, 2008; Rendón-von Osten and Dzul-Caamal, 2017). As noted in Section 2.6, the half-life of GLY ranges from a few hours to as long as 428 days. Field studies across various soil types have reported half-lives spanning from several days to months, and in some cases up to a year (Mertens *et al.*, 2018). This variability creates a high risk of long-term, incremental GLY contamination in soil, surface water and groundwater. Site-specific factors complicate prediction and contribute to the difficulty and cost of effective monitoring. To sustain high crop yields and manage GLY-resistant weeds, as outlined in Section 2.5.1, farmers increasingly rely on additional herbicides, including older chemicals with established environmental and health risks, as well as newer alternatives. This approach is especially concerning in grain and row-crop systems where Roundup Ready® GM crops have been cultivated continuously over multiple seasons (Myers *et al.*, 2016).

Given that GLY and AMPA have been detected in both water sources and food supplies (Viljoen *et al.*, 2021), ongoing monitoring of these compounds is essential. Particular attention should also be given to surfactants such as POEA, which are known to be more toxic than GLY or AMPA (Gandhi *et al.*, 2021). Such contamination presents risks to human and animal health and raises broader concerns about environmental sustainability and future management strategies (Viljoen *et al.*, 2021). Emphasising GLY concentrations and their environmental impact will be critical for developing effective mitigation approaches in South Africa.

2.10 Conclusion

There is a concerning lack of data on GLY and AMPA levels in the South African environment, despite the widespread and growing use of GBHs to support agriculture, a sector vital to both food security and the national economy. This gap is troubling given evidence of GLY presence in food and soil, with unclear health implications. The South African Cancer Association aligns with the WHO's IARC classification of GLY as a Group 2A carcinogen. Currently, no known laboratory in South Africa routinely analyses these compounds in environmental samples, nor is there a designated agency tasked with monitoring them, likely due to the absence of official regulatory guidelines for acceptable levels in soil or water.

What is known so far: (1) GBHs are the most extensively used herbicides globally, with use continuing to rise; (2) GLY regularly contaminates drinking water, precipitation and air, especially in agricultural regions; (3) GLY has a longer environmental half-life in soil and water than previously thought; (4) GLY and its metabolites are prevalent in the global food supply; (5) Human exposure to GBHs is increasing; (6) GLY is classified as a probable human carcinogen; (7) South Africa's weak enforcement of pesticide regulations complicates the control of pesticide usage; and (8) Current assumptions about GLY contamination in South Africa rely on outdated data and inadequate analytical and monitoring systems.

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

The sequence of the present chapter was based on the completion of methods during the execution of the study. Water, soil, and sediment samples were collected from agricultural sites where primarily maize crops are grown over the entire growing season and from non-agricultural sites. These were analysed for GLY and AMPA. Furthermore, nutrients and total suspended solids were also analysed in all water samples. Soil and sediment description, soil particle size distribution and cation exchange capacity were done. Maps were created to show GBH use in South Africa for four crops during 2017 and 2019. Microbial diversity was determined using a subset of water and soil samples, from which a bacterial ecological risk assessment was generated.

3.2 Mapping glyphosate-use in South Africa

For the current study, the data mining company, Kynetech® (<https://www.kynetec.com>), provided information on the volumes (tonnes) of GBHs applied to maize, soybean, wheat, and sunflower crops in 2017 and 2019. They gathered this information through interviews with farmers, who were asked about the pesticides used, the target crops sprayed, the crop variety, the sowing rate and the application rate. Glyphosate-based herbicide-use patterns were mapped for South Africa to locate regions where soil, water, and sediment sampling would be likely contaminated with GLY and/or AMPA.

The following data sources were used to create the maps: The National Aeronautics and Space Administration (NASA) Advanced Spaceborne Thermal Emission and Reflection Radiometer's (ASTER) 90 m digital elevation dataset; the Department of Environmental Affairs' (DEA) 2013–2014 South African National Land Cover Dataset; the Department of Water and Sanitation's Hydrology–Dam's dataset; and the South African National Biodiversity Institute's (SANBI) National Freshwater Ecosystem Priority Areas (NFEPA) river network. The Environmental Systems Research Institute's software, ArcGIS Desktop 10.5, was used to make the maps. ArcGIS was used to import the Kynetech® Excel® file. The tonnage of GBH applied to the various municipal boundaries was added to the geo-referenced municipal boundary information. The WGS 1984 geographical coordinate system was used to project the various vector dataset coordinates (in decimal degrees) to show where the GBH applications are located. The 2017 and 2019 GBH data volumes were divided into six intervals in ArcGIS. The first interval shows when no correspondence from the interviewed farmers was received. This was labelled as 'No data.' The Jenks natural breaks classification was used to create the remaining 5 intervals, which optimise the arrangement where the dataset values would naturally break. The Jenks natural

break is one of ArcGIS' data classification method tools. This classification was utilised to reduce the average deviation from the class mean while increasing the divergence from the other groups' averages (Weber *et al.*, 2020)

When geospatial analysis tools like ArcGIS are combined with diverse datasets such as land cover and hydrology, the GLY-use patterns identify high-risk areas that should be prioritised for more sampling and analysis.

3.3 Sampling

3.3.1 Site selection and site description

Soil, water, and sediment samples were collected from sites based on the areas of GBH use in South Africa from the study by Dabrowski (2015b). They used pesticide-use data from 2009, also obtained from Kynetech®. Non-agricultural sites were also included to reflect GBH-use in urban and industrial settings, such as landscaping, maintenance of golf courses, and clearing railway tracks. The agricultural sites were chosen to represent areas with different water sources: rain-fed lands in Mpumalanga and canal-irrigated fields in Vaalharts. These two regions also differ in annual rainfall: Mpumalanga has higher rainfall than the Vaalharts area. The selection strategy ensures the collection of samples from environments with expected high and low levels of GLY and AMPA, providing a way to compare contamination levels and their potential effects.

The descriptions of the sites in Mpumalanga, Vaalharts irrigation scheme, and non-agricultural areas are laid out in Table 3.1. All descriptions were made on site, noting the geographical location and landmarks, the topography (the slope), and any natural features like hills, valleys, or water bodies. Notes on other information included vegetation types like crops or natural vegetation, land-use such as grazing cattle, and anthropogenic constructions such as roads, buildings, and bridges. Finally, the hydrology of the rivers and dams was also noted in terms of water level and flow rate (as high or low flow). This was based on observation only and not measuring the velocity with instrumentation. Ease of access and safety considerations were also noted where applicable. Sites were selected to capture a range of environmental contexts, from heavily impacted agricultural fields to less impacted non-agricultural areas. The site diversity allows for a comprehensive evaluation of GLY distribution and its possible correlation with land-use and anthropogenic activities.

Table 3.1: Individual site descriptions of all three sampling areas

Site name	Abbreviation	Site type	Coordinates	Description
Mpumalanga sites				
Water and sediment				
Downstream Middelburg Dam	DSD	Dam	25°46'10.3"S 29°31'44.7"E	Rocky topography, personal products and waste dumping, the riverbank was at a 20–30° angle.
Middelburg Dam	MD	Dam	25°46'20.7"S 29°33'12.6"E	The topography is flat with grass fields.
Mpumalanga 4	MP4	River	25°52'36.5"S 29°37'47.7"E	Flat topography, maize croplands with pivot points visible but not close to the site, surrounded by grass vegetation.
Mpumalanga 6	MP6	River	25°56'45.9"S 29°39'04.8"E	Surrounded by grass fields.
PAN1	PAN1	Pan	25°56'43.3"S 29°39'34.7"E	Croplands surrounded the site with cattle grazing in the distance (approximately 1 km), but not at the immediate site, close to the Arnot power station.
Mpumalanga 10	MP10	River	26°08'49.7"S 29°45'08.5"E	Flat topography, grazing cattle, and croplands were visible.
Life Water River 1	X1	River	26°26'54.5"S 29°47'50.4"E	Grazing cattle, maize croplands, flat topography.
Life Water River 2	X2	River	26°33'03.9"S 29°49'05.2"E	Maize croplands and grazing cattle, flat topography.
Life Water River 3	X3	River	26°46'51.0"S 29°48'23.5"E	Multiple maize croplands around the site, a water monitoring station was observed, and topography consisted of bedrock and flat topography.
Vaal River 9	V9	River	26°49'55.0"S 29°45'01.8"E	Hilly topography, steep riverbank (30°–45° inclination), cattle grazing.
Vaal River 11	V11	River	26°51'22.4"S 29°41'50.3"E	Steep topography next to the main road at approximately 30°. Under a bridge. The riverbank was flat.
Vaal River 12	V12	River	26°52'44.5"S 29°38'55.2"E	The riverbank was steep (estimated at 60–90°) while above the bank it is flat, maize croplands around the river, pivot points visible.
Vaal River 13	V13	River	26°51'17.2"S 29°28'52.1"E	Topography has small to medium hills. Consists mainly of grass vegetation.
Grootdraai Dam	GD	Dam	26°53'49.6"S 29°22'27.1"E	Rocky topography, mainly grass fields. Lots of algae in the water.

Site name	Abbreviation	Site type	Coordinates	Description
Vaal River 15	V15	River	26°56'09.7"S 29°15'54.7"E	Hilly topography at river site, pivot points, and crops. The area consists mainly of grass vegetation. Waterworks were observed.
Soil				
Maize 1	M1	Maize field	25°46'41.3"S 29°38'31.2"E	The site has a flat topography close to a main road (R104) with maize fields and a small dam with urban activity.
Maize 2	M2	Maize field	25°50'37.3"S 29°39'48.3"E	Flat topography with coal mining activity close to the site.
Maize 3	M3	Maize field	25°53'17.8"S 29°35'02.0"E	Flat topography with a small dam to the southwest of the site. The site is also situated next to the N11 highway.
Maize 4	M4	Maize field	26°28'24.8"S 29°49'37.4"E	The site is situated on a busy dirt road and surrounded by maize fields.
Maize 5	M5	Maize field	26°28'55.5"S 29°44'51.0"E	The topography is flat. The site is close to a bridge with a train track to the west.
Maize 6	M6	Maize field	26°41'24.2"S 29°47'21.6"E	The site is situated on a busy dirt road and surrounded by maize fields, but also has a river to the northwest.
Maize 7	M7	Maize field	26°49'22.7"S 29°46'21.9"E	The site is situated on a busy dirt road and surrounded by maize fields, but also has some uncultivated lands to the northwest.
Maize 8	M8	Maize field	26°49'43.8"S 29°43'50.2"E	The site is situated on a busy dirt road and surrounded by maize fields.
Maize 9	M9	Maize field	26°53'05.6"S 29°39'00.7"E	Topography varies from steep to flat. The site is surrounded by maize fields and some urban activity close by to the north-east.
Maize 10	M10	Maize field	26°53'19.3"S 29°23'54.1"E	Flat topography, surrounded by maize fields.
Maize 11	M11	Maize field	26°34'15.6"S 29°52'30"E	Topography is slightly angled towards the river (left), adjacent to the field.
Vaalharts irrigation scheme sites				
Water and sediment				
Taung Outflow	TO	River	27°32'5.3"S 24°50'19.7"E	A low bridge contributes to sediment movement. Near an informal settlement (estimate of 5–10 km). Grazing cattle and donkeys. No observable crops.

Site name	Abbreviation	Site type	Coordinates	Description
Harts River 1	HR1	River	27°32'27.2"S 24°49'29.2"E	Directly under a bridge. Next to an informal settlement (1 km). Grazing cattle, pigs. Smells like urine. Filamentous algal growth.
Canal 1	C1	Man-made canal	27°41'40.1"S 24°40'55"E	Opposite of a maize field.
Harts River 2	HR2	River	27°41'46.4"S 24°40'36.5"E	Grazing cattle. Small informal settlement. Maize croplands with pivot points. Wetlands were also present.
Canal 2	C2	Man-made canal	27°50'34"S 24°46'15.3"E	Surrounded by a pecan orchard.
Main Inflow Canal	MIC	Man-made canal	27°50'59.7"S 24°51'7"E	Surrounded by grass fields.
Harts River 3	HR3	River	27°47'13.7"S 24°42'18.1"E	Algal growths. Grass fields. No visible agricultural activities.
Harts River 4	HR4	River	27°54'12.7"S 24°36'56.6"E	Surrounded by grass fields and tree vegetation.
Spitskop Dam	SD	Dam	28°44'1.3"S 24°31'41.6"E	No observed crop activities. Presence of grazing wild animals. Filamentous algal growth.
Downstream Spitskop Dam	DSD	Dam	28°7'51.6"S 24°30'3.6"E	Under a bridge. Surrounded by grass fields.
Soil				
Maize 12	M12	Maize field	27°39'40.5"S 24°42'30.7"E	The topography is flat. The maize field is next to a pecan nut orchard.
Maize 13	M13	Maize field	27°40'41.8"S 24°42'22.2"E	Flat topography with maize fields and a thicket of trees surrounding the crop field, in addition to wheat fields
Maize 14	M14	Maize field	27°41'50.3"S 24°42'16.8"E	Flat Topography with maize fields and a thicket of trees surrounding the crop field
Maize 15	M15	Maize field	27°41'44.6"S 24°42'30.3"E	Flat Topography with maize fields and a thicket of trees surrounding the crop field
Pecan 1	P1	Pecan orchard	27°42'9.9"S 24°43'53.8"E	Flat topography with total coverage of vegetative grassy soil. Juvenal pecan nut trees mostly.
Pecan 2	P2	Pecan orchard	27°43'16.7"S 24°44'1.8"E	Flat topography with total weed vegetative soil coverage. Mature pecan nut trees mostly.

Site name	Abbreviation	Site type	Coordinates	Description
Pecan 3	P3	Pecan orchard	27°43'14"S 24°45'51.4"E	Flat topography with no vegetative soil coverage. Mature pecan nut trees mostly.
Pecan 4	P4	Pecan orchard	27°39'40.5"S 24°42'30.7"E	Flat topography with no vegetative soil coverage. Mature pecan nut trees mostly.
Non-agricultural sampling sites				
Water and sediment				
Vaal River Barrage	VB	River	26°44'51.5"S 27°50'14.8"E	The site was at a golf course and a private residence. Landscaping activities. Algal growth. Flat topography.
Downstream Potchefstroom Country Club	DGP	River	26°43'30.7"S 27°06'27.3"E	At a bridge. The golf course is upstream and downstream and is surrounded by various vegetation.
Mooi River Mall	MM	River	26°42'47.7"S 27°06'19.5"E	The site is next to a mall. Various landscaping activities were observed. Flat riverbank.
Tuscany Ridge Estate	TRE	Man-made dam/pond	26°39'54.9"S 27°06'27.8"E	Pond in a residential estate. Landscaping activities observed. The site has filamentous algal growth and weeds.
Gimmie Gat	GG	River	26°41'09.8"S 27°06'03.6"E	Surrounded by residential properties on one riverbank and various vegetation on the other. Rocky topography.
Water				
NWU Botanical Gardens	BT	Man-made dam/pond	26°40'54.1"S 27°05'42.8"E	Landscaping activities observed. Still standing pond with floating lotus leaves.

3.3.1.1 Upper Vaal and Olifants rivers catchments (Mpumalanga Province) (rainfed region)

The Mpumalanga area (MPA) spans the province of Mpumalanga, from Middelburg in the north to Standerton in the south, along the Klein Olifants-, Life Water-, and Vaal rivers (Figure 3.1). The Klein Olifants River drains into the north-eastern flowing Olifants River, and the Life Water River drains into the Vaal River, which forms part of the Orange River catchment. The Orange River flows south-west. Water, sediment, and soil samples were collected from 15 sites (Table 3.1). Coal mines surround the sites upstream of the Middelburg Dam (OMD) to PAN1 along the Klein Olifants River (Figure 3.1). Other sites represented dams and wetlands (MP4 and MP6). All the soil samples were located at maize crop fields Maize 1–10: M1–M10, as shown in Figure 3.2.

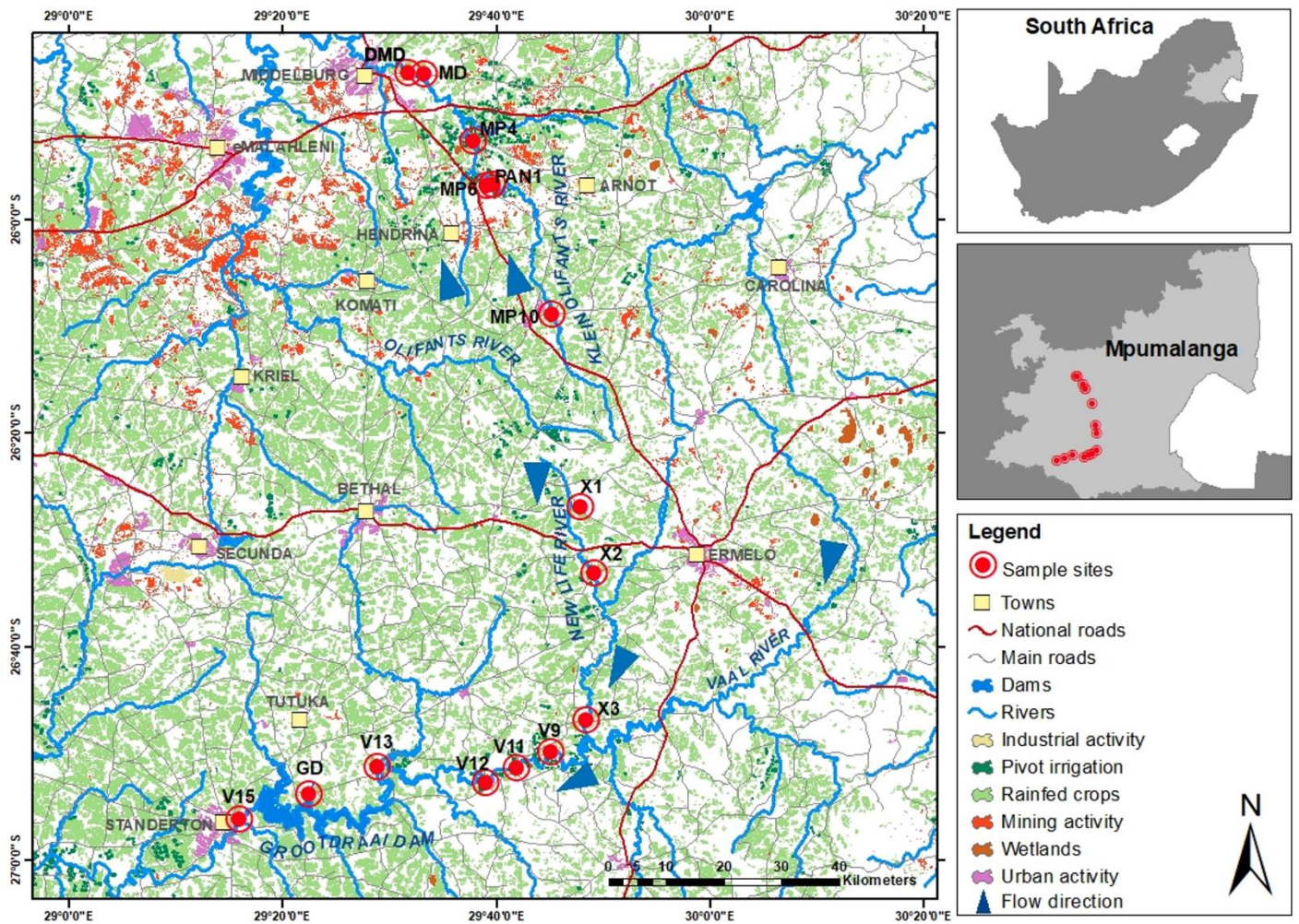


Figure 3.1: Map of the Mpumalanga (MPA) sites along the Klein Olifants River, New Life River and Vaal River in South Africa, where water and sediment samples were collected.

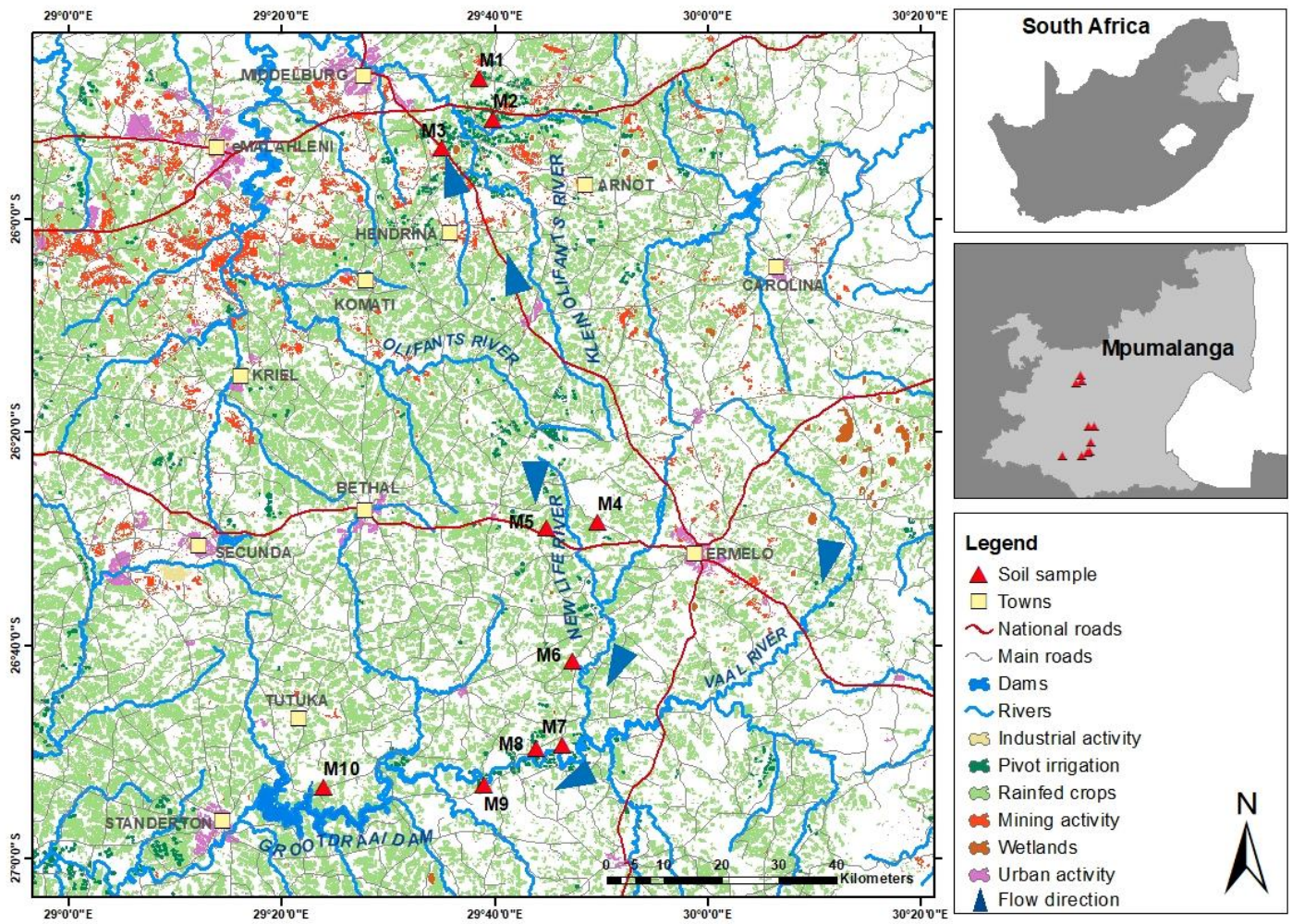


Figure 3.2: Map of the Mpumalanga (MPA) sites where the soil was collected.

3.3.1.2 Vaalharts Irrigation scheme, Northern Cape Province (irrigated region)

The Vaalharts irrigation scheme (VIS) is on the border between the North West and Northern Cape provinces. There were eleven sites in the VIS (Figure 3.3; Table 3.1). Man-made canals in this area deliver water from the Harts River to help with irrigation. Canals, a wetland and dams were among the features of the VIS. Soil samples were collected from pecan orchards, soybean, maize and wheat fields, which were the most common crops grown in the area (Figure 3.4).

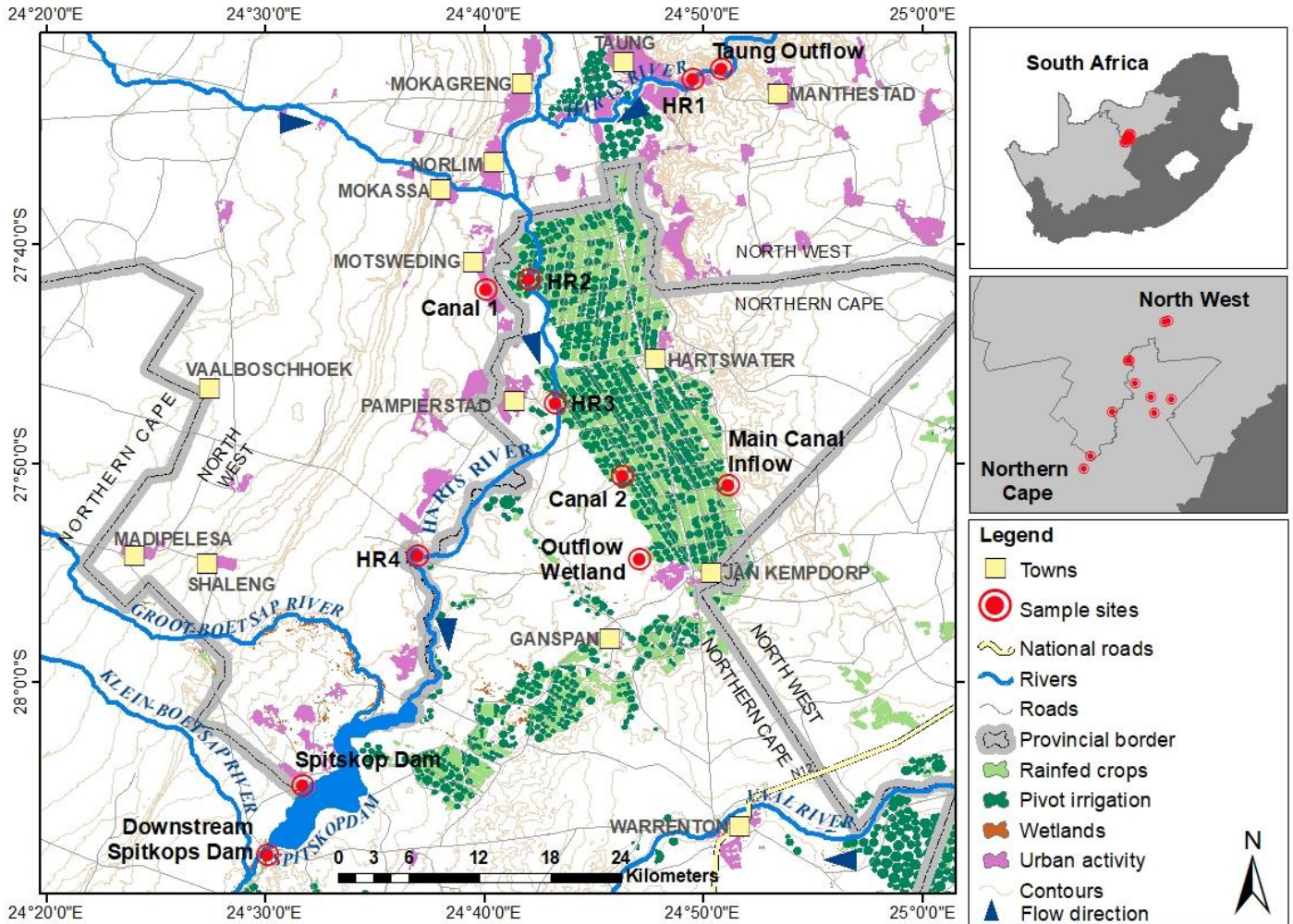


Figure 3.3: Map of the Vaalharts irrigation scheme (VIS) in South Africa where water and sediment samples were collected.

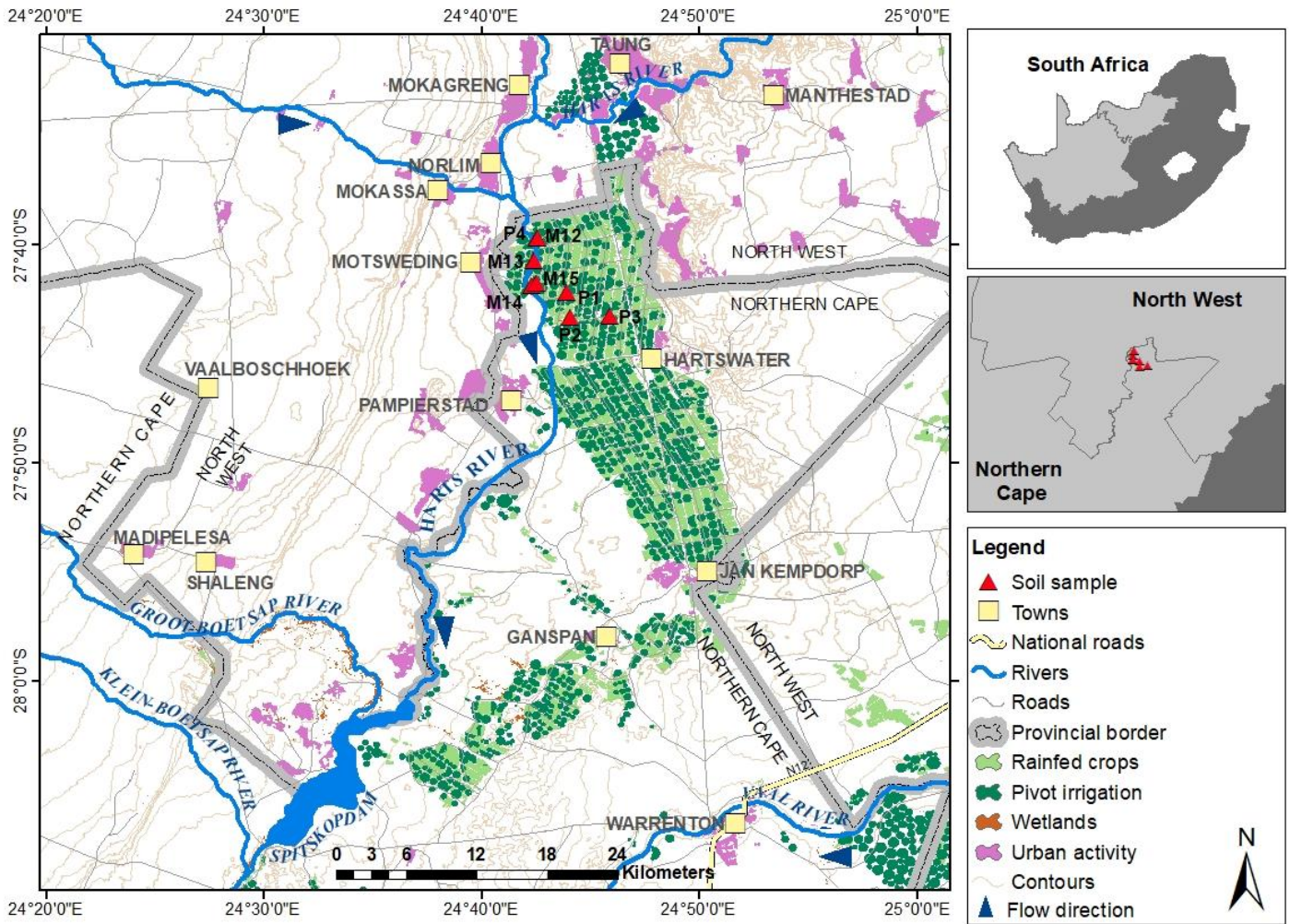


Figure 3.4: Map of the Vaalharts irrigation scheme (VIS) indicating the sites where soil was sampled.

3.3.1.3 Non-agricultural sites (North West and Gauteng provinces)

The sites selected to represent non-agricultural sites (NAS) (Figure 3.5) are in the Upper Vaal catchment: the Mooi River, a tributary of the Vaal River, that runs through the town of Potchefstroom was sampled at Potchefstroom Country Club, MooiRiver Mall, and Gimmie Gat (a slow flowing pool in the Mooi River that flows through the suburbs of Potchefstroom). Two ponds, not directly associated with the river, were also sampled: the Tuscany Ridge Estate and North-West University (NWU) Botanical Gardens. These sites were representative of landscaping areas, residential areas and a golf course. The Vaal Barrage, a dam within the Vaal River, was chosen because of its location in an industrialised area of Vanderbijlpark. There were six sites in the Upper Vaal catchment: Vaal Barrage, Tuscany Ridge Estate, Botanical Gardens, Gimmie Gat, MooiRiver Mall and Downstream Potchefstroom golf course.

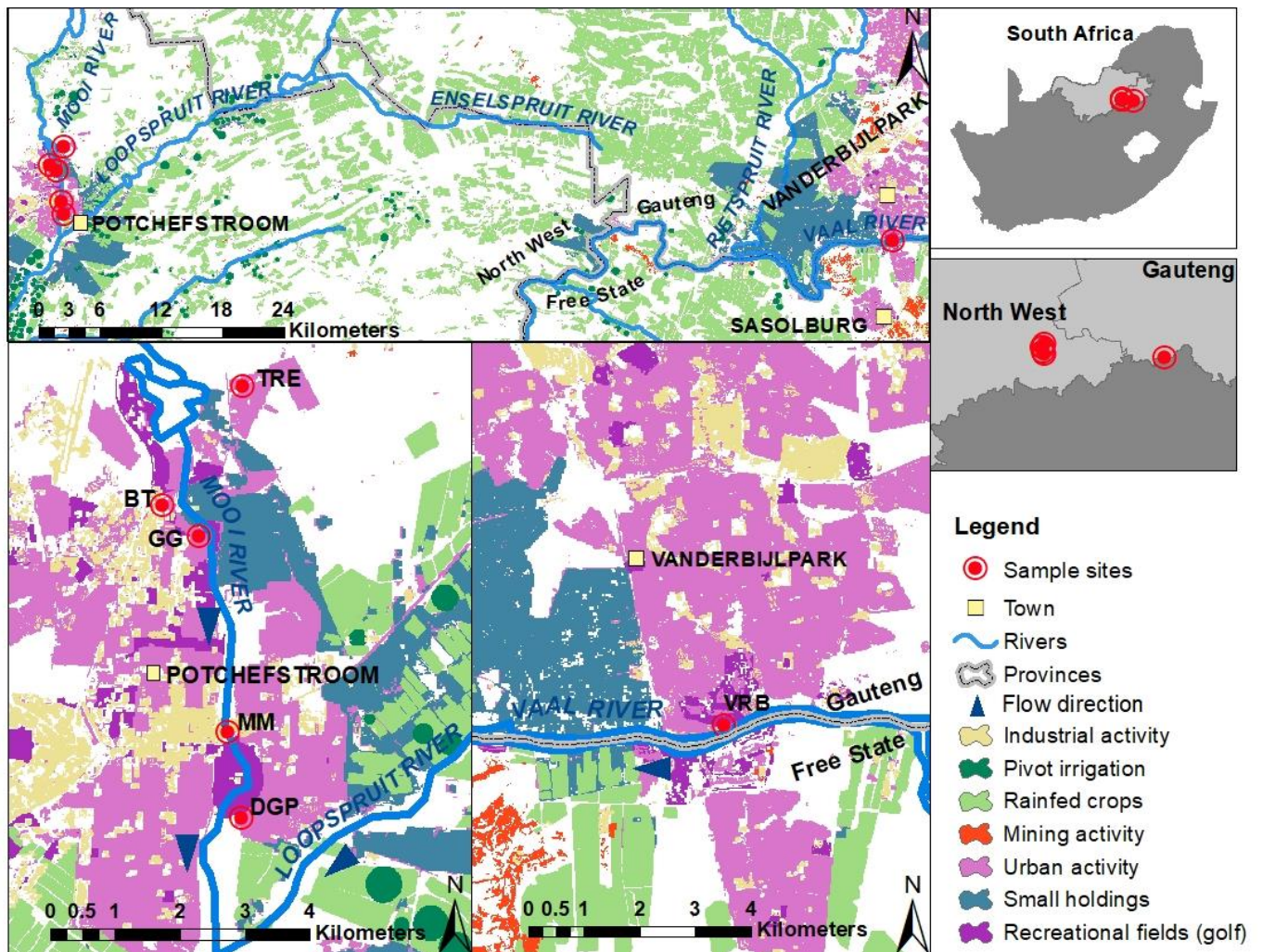


Figure 3.5: Non-agricultural sites (NAS) where water and sediment samples were collected in North West and Gauteng provinces.

3.3.2 Sampling times

The sampling timing strategy was designed to capture both the highest and lowest GLY and AMPA concentrations expected in the environment. Water, soil and sediment samples were collected during four distinct events from October 2020 to June 2021: before spraying (21–30 September 2020; 22 October 2020), after herbicide application (16 November to 1 December 2020), after a rain event (10–24 March 2021), and post-harvest (25 May to 8 June 2021) end of the season. These events aimed to understand the temporal variations in GLY and AMPA concentrations.

3.3.3 Water sampling

Water samples were collected in triplicate in 250 mL high-density polyethylene (HDPE) bottles (Thermo Scientific™ Nalgene®, AEC-Amersham SOC Ltd), which were ethanol-rinsed (70% ethanol) to remove bacteria and polar contaminants. One of these was filtered at the laboratory to determine the concentration of total suspended solids (TSS). The other two were stored at -80°C until processed for chemical analysis of GLY and AMPA. An additional water sample (500 mL) was taken in a low-density polyethylene (LDPE) bottle (shop-bought with no specific brand) for the water nutrient analysis, which was done upon return to the laboratory. All water samples were kept at 4°C during the sampling trip and temporarily stored at that temperature unless otherwise indicated above. The physico-chemical parameters pH, electrical conductivity (EC) and temperature were determined using an ExStik II EC500 (Extech Instruments) at each site where water samples were collected. The probes were inserted into the sampled water bottle to measure the physico-chemical parameters. The probes were rinsed with deionised water between the samples.

3.3.3.1 Total suspended solids in water

The concentration of TSS in water samples (mg/L) was determined by filtering the water samples. In short, the mass of a cellulose nitrate filter (0.45 µm, Sartorius Stedim Biotech) was recorded with an analytical balance (Radwag AS 220/C/2, RADWAG®). Water samples were inverted a few times to homogenise the sample, and 100 mL of water was filtered. The membrane filter was oven-dried in an empty Petri dish at 37°C overnight (Mohamad-Zainal *et al.*, 2021). The dried filter mass was determined again, and the difference between the filter before and after filtering was reported as the total suspended solids. The g/100 mL was converted to mg/L (Equation 3.1).

$$TSS = \text{measured TSS in g} \times \left(\frac{1000 \text{ mg}}{1 \text{ g}} \div 0.1 \text{ L} \right) \quad \text{Equation 3.1}$$

3.3.3.2 Water nutrient analysis

The unfiltered water samples were analysed for nutrients and other chemical water quality variables with a spectrophotometer (Spectroquant® Pharo 300, Merck) using the appropriate test kits (purchased from Merck) by following the manufacturer's instructions. The list of nutrients determined includes the test name followed by its pre-programmed test code: ammonium (NH_4^+ -N, 114752), chloride (Cl^- , 114897), nitrate (NO_3^- -N, 109713), nitrite (NO_2^- -N, 114776), sulphate (SO_4^{2-} , 114791), ortho-phosphate (PO_4^{3-} -P, 114848) and total hardness (TH, 100961).

3.3.4 Soil and sediment sampling

Sediment and soil samples were dug up using a metal spade at 2–3 close-by areas and transferred into a metal bowl to mix and create a composite sample. Subsamples were transferred into HDPE bottles (Thermo Scientific™ Nalgene®, AEC-Amersham SOC Ltd). The HDPE and other plastic bottles were pre-cleaned by rinsing with 70% ethanol. The soil samples were transported to the laboratory at 4°C and stored at -20°C until soil property determination and extraction for chemical composition. All sampling equipment was rinsed with deionised water between sites to prevent cross-contamination.

3.3.4.1 Total organic carbon content

The total organic carbon (TOC) content in soil indicates the total amount of organic material that can be oxidised. This includes organic matter that is dissolved, particulate, and suspended (USEPA, 2001). One characteristic of GLY is that it tightly binds to organic content. To determine the %TOC in soil samples, the semi-quantitative loss-on-ignition method was used. This method involves heating the soil to destroy all organic matter. The soil sample (~5 g) was placed in an acid-washed ceramic crucible that was pre-weighed. The crucible's content was incinerated at 600°C for six hours in a muffle furnace (Nabertherm GmbH, Lilienthal, Germany). After cooling to room temperature, the crucible was weighed again to determine the %TOC loss with Equation 3.2 (Schumacher, 2002; Gerber *et al.*, 2015)

$$\%TOC = \left(\frac{M_b - M_a}{M_b} \right) \times 100 \quad \text{Equation 3.2}$$

Where:

%TOC = Total organic carbon content (%).

M_b = Mass of crucible and its contents before incineration (g)

M_a = Mass of crucible and its contents after incineration (g).

The %TOC for each sample was classified into one of four categories as explained by Cyrus *et al.* (2000): very low (<0.05%); low (0.05–1%); moderately low (1–2%); medium (2–4%); and high (>4%).

3.3.4.2 Soil particle size distribution and characterisation

Glyphosate strongly binds to soils containing clay (Wang *et al.*, 2023a). The Endecotts dry sieving method was used to determine the soil particle size distribution and describe the soil type at different sampling locations. The method involved stacking Endecotts sieves with varying mesh sizes (53, 212, 500, 2 000 and 4 000 μm) on top of each other, starting from the largest to the smallest mesh size, with a collection pan at the bottom. The soil was dried overnight at 35°C. Twenty grams of this soil (total mass) was manually sieved for 10 minutes. The following soil particle fractions were collected: clay (<53 μm); fine sand (53–212 μm); medium sand (212–500 μm); coarse sand (500–2 000 μm); very coarse sand (2 000–4 000 μm); and gravel (>4 000 μm). The mass of the soil fraction retained in each sieve was recorded, and the soil particle size distribution (%Composition) was determined as a percentage of the total mass of the sample (Equation 3.3).

$$\%Composition = \left(\frac{\text{Mass of fraction (g)}}{\text{Total mass (g)}} \right) \times 100 \quad \text{Equation 3.3}$$

3.3.4.3 Soil cation exchange capacity

Glyphosate is known to be adsorbed to soil mineral fractions such as clay, composed of, for example, amorphous iron and aluminium oxides and competes for these binding sites (De Gerónimo *et al.*, 2018). The measure of a soil's ability to retain positively charged ions (cations) like calcium (Ca^{2+}), magnesium (Mg^{2+}), and potassium (K^{+}) is called the cation exchange capacity (CEC). This capacity is determined by the number of negatively charged sites on the soil's surface that can electrostatically attract and hold cations. Soils with higher CEC values can maintain sufficient levels of these nutrients because the retained cations can be easily exchanged with other cations in the soil solution. However, it is worth noting that a higher CEC does not necessarily make the soil more fertile since acidic cations like hydrogen (H^{+}) and aluminium (Al^{3+}) can also occupy the soil's CEC sites. The Gillman and Sumpter method was used to determine CEC (Gillman and Sumpter, 1986). This method determines the CEC at the pH and ionic strength of the soil. Knowing the soil's CEC helps interpret the bioavailability of the GLY and AMPA and its mobility in the soil.

First, the mass of an empty 50 mL centrifuge tube was measured to the nearest milligram (Shimadzu AY220). Next, 2 g of soil sample was added to the tube, along with 20 mL of 0.1 M $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ extraction solution. The tube was shaken for two hours on a Labcon mechanical shaker. Following the shaking step, the pH was measured with a CRISON Basic20 pH meter and recorded. Afterwards, the tube was centrifuged at a relative centrifugal force (rcf) of 10 000 rcf for 10 minutes, and the supernatant was carefully removed. Subsequently, 20 mL of 2 mM $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ equilibrating solution was added to the tube and capped. The tube was briefly vortexed to disperse the soil pellet and shaken on the mechanical shaker for one hour, followed by the centrifugation step and careful removal of the supernatant. This step was repeated another two times so that a total of 60 mL of equilibrating solution was used. Before the third centrifugation, the pH of the slurry was measured and recorded. After the final decantation of 2 mM $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mL of 5 mM MgSO_4 was added, and the mixture was shaken gently for 1 hour.

The conductivity of a separate 1.5 mM MgSO_4 solution was determined (it should be 300 μS), which served as the reference solution to compare to the conductivity of the sample solution. If the conductivity of the sample solution is not 1.5 times that of the reference solution value, 100 μL increments of 0.1 M MgSO_4 (an adjusting solution) were added until it was. The number of 0.1 M MgSO_4 increments added was recorded when needed. After the adjustments, the sample solution's pH was re-evaluated, and if it varied by more than 0.1 units from the previous reading, it was adjusted by lowering the pH by using 50 mM H_2SO_4 . The sample solution was then diluted with distilled water until its conductivity approached that of a 1.5 mM MgSO_4 standard solution (300 μS). At the end, the sample centrifuge tube was allowed to dry, and the mass of the tube and its contents were recorded.

The CEC was finally calculated as milli-equivalents/100 g (meq/100 g). The data points collected included the mass of the total solution (mL) (assuming 1 mL weighs 1 g) by Equation 3.4, the amount of magnesium in the solution (Equation 3.5), the total magnesium added (Equation 3.6) and finally the CEC (Equation 3.7). The monitoring and capturing of the pH and EC were done to ensure the stability for reactions to occur.

$$\text{TS (mL)} = \text{TM}_f \text{ (g)} - \text{TM}_{\text{tare}} \text{ (g)} - 2 \text{ g (mass soil used)} \quad \text{Equation 3.4}$$

Where:

TS = total solution (assumes 1 mL weighs 1 g)

TM_f = total mass final

TM_{tare} = total mass tared

$$\mathbf{Mg_{sol}} \text{ (meq)} = \mathbf{TS(mL)} \times \mathbf{0.003} \text{ (meq/mL)} \quad \text{Equation 3.5}$$

Where:

Mg_{sol} = Mg in the solution

TS = total solution

0.003 = constant of the reference solution [1.5 mM MgSO₄]

$$\mathbf{T_{Mg}} \text{ (meq)} = \mathbf{0.1} \text{ meq} + (\mathbf{n} \text{ (mL)} \times \mathbf{0.2} \text{ meq/mL}) \quad \text{Equation 3.6}$$

Where:

T_{Mg} = total Mg added

0.1 = constant in 10 mL of [5 mM MgSO₄]

n = increments added of adjusting solution [0.1 M MgSO₄]

0.2 = constant of adjusting solution [0.1 M MgSO₄]

$$\mathbf{CEC} \text{ (meq/100 g)} = (\mathbf{T_{Mg}} \text{ (meq)} - \mathbf{Mg_{sol}} \text{ (meq)}) \times \mathbf{50} \quad \text{Equation 3.7}$$

Where:

CEC = cation exchange capacity

T_{Mg} = total Mg added

Mg_{sol} = Mg in the solution

50 = converts 2 g soil to 100 g soil

3.4 Predicted pedotransfer of glyphosate (GLY)

The rate at which GLY is transferred from soil to water is dependent on GLY's sorption to soils and sediment. Sorption coefficients are the most sensitive parameters in models of risk assessment of GLY. However, different soils have varying sorption abilities for GLY. Dollinger *et al.* (2015) reviewed the sorption parameters of GLY and concluded that the best predicting sorption parameter to determine GLY sorption is the linear pedotransfer function (K_d). The K_d function is based on the CEC and clay of a particular soil and is expressed in Equation 3.8.

$$\mathbf{K_d} \text{ (L/kg)} = 7.20 \times \mathbf{CEC} \text{ (meq/100)} - 1.31 \times \mathbf{clay}(\%) + 24.82 \quad \text{Equation 3.8}$$

Where:

K_d = linear pedotransfer

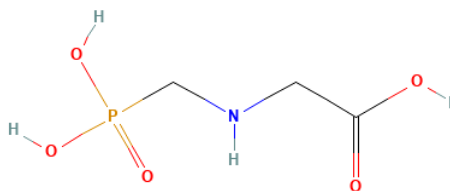
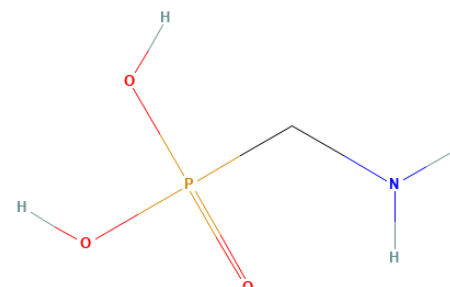
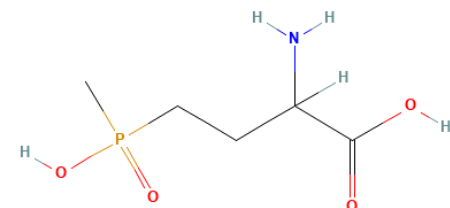
CEC = cation exchange capacity

clay = clay percentage of soil/sediment

3.5 Chemical analysis

The objective of this section is to describe the methods used to determine the concentration of GLY and AMPA in the samples. Given that GLY and AMPA are found in low concentrations in the environment, it is essential to concentrate the target compounds in environmental matrices to enable effective detection using analytical techniques. In this study, water samples were enriched and evaporated, soil and sediment were extracted and evaporated, and both types of extracts were analysed using an ultra-high pressure liquid chromatograph coupled mass spectrometer UHPLC-MS/MS. These analyses were done by the National Metrology Institute of South Africa (NMISA). The targeted compounds, GLY and AMPA, belong to the class of highly polar pesticides, and their chemical properties are listed in Table 3.2. Analysing the concentrations of GLY and AMPA in collected samples can determine the extent of environmental contamination. The concentrations are essential for assessing potential ecological risks and understanding the degradation patterns of GLY in different environmental matrices.

Table 3.2: Chemical properties of the target compounds and internal standard (Source: Pubchem, <https://pubchem.ncbi.nlm.nih.gov/>)

Compound	CAS#	Molecular formula	Molecular mass (g mol ⁻¹)	Structure	Dissociation constants	Water solubility
Glyphosate	1071-83-6	C ₃ H ₈ NO ₅ P	169.07		pKa1 = 2.0; pKa2 = 2.6; pKa3 = 5.6; pKa4 = 10.6	10 mg/mL
Aminomethyl-phosphonic acid (AMPA)	1066-51-9	CH ₆ NO ₃ P	111.037		pK1: 2.35; pK2: 5.9; pK3: 10.8	50 mg/mL
Glufosinate (Internal standard)	51276-47-2	C ₅ H ₁₂ NO ₄ P	181.128		pKa1 = <2; pKa2 = 2.9; pKa3 = 9.8	1 370 mg/mL

3.5.1 Chemicals and reagents

The analytical reference standards GLY, $\geq 98\%$ purity (CAS# 1071-83-6) and AMPA, $\geq 99\%$ purity (CAS# 1066-51-9) were both acquired from Sigma-Aldrich, Germany. The internal standard (IS) was glufosinate (CAS# 51276-47-2) and was purchased from Dr. Ehrenstorfer GmbH, LGC Ltd. Spectrometry-grade Romil methanol was acquired from Microsep in South Africa, while using Fluka 98% formic acid (Sigma, Germany) solutions for mass spectrometry. Acetonitrile (ACN) (Honeywell Burdick & JacksonTM brand) was purchased from Anatech, South Africa. Double-distilled, nanopure water ($18.2 \text{ M}\Omega\cdot\text{cm}$) was obtained from an in-house ELGA PURELAB Ultra water purification system.

A gravimetric method was used to prepare individual stock solutions for GLY, AMPA and glufosinate. Weighing 10 mg of the compound and dissolving it in 10 g (9.27 mL) of 10% acetonitrile (analytical grade) yielded 1 mg/g. The stock solutions were placed in plastic 15 mL tubes and covered with foil to prevent UV degradation. Also, an additional stock of glufosinate (2.1 mg/g) was made only for water samples. These stock solutions were used to prepare standard mix solutions to be used to prepare the calibration curve, and also to spike the samples for recovery determination. The glufosinate stock solution was used as the internal standard (IS) for water and soil samples and for calculating the percentage recovery.

3.5.2 Water sample enrichment

The method to enrich the water samples with GLY and AMPA was adapted from the EURL-SRM QuPPe technique designed for highly polar pesticides in fruits and vegetables (Anastassiades *et al.*, 2021). The water samples were thawed in the dark at room temperature and then inverted 200 times to ensure thorough mixing. Next, 15 mL of the sample was transferred to a 50 mL centrifuge tube (Falcon[®], Lasec SA (Pty) Ltd, South Africa), which was properly labelled and covered with foil, and 250 μL glufosinate IS (2.1 $\mu\text{g/g}$) was added to the sample. The sample was then vortexed to ensure proper mixing, after which 1 mL of methanol was added, vortexed again for an additional 2 minutes. The water was evaporated until 5 mL remained, and 1 mL was filtered using a 0.22 μm polytetrafluoroethylene (PTFE) syringe filter (Pall Acrodisc[®], Promolab (Pty) Ltd T/A Separations). Finally, the extract was analysed using UHPLC-MS/MS (for instrument details, see Section 3.5.3).

3.5.3 Glyphosate (GLY) and AMPA (aminomethylphosphonic acid) extraction from soil/sediment

Multiple extraction methods were evaluated by NMISA to achieve optimal recovery of GLY and AMPA from soil and sediment samples. These are documented in the method development report (Appendix A). For the final method, soil samples were air-dried in the dark at room temperature and ground to a fine powder using a mortar and pestle to create a homogeneous sample matrix.

A subsample of 10 g of sediment/soil was then placed into a labelled 50 mL centrifuge tube wrapped in foil to prevent exposure to light, and the mass was recorded. The sample was mixed with an equal amount (1:1) of diatomaceous earth (Anatech Instruments (Pty) Ltd, South Africa), and the resulting mixture was placed in a pre-cleaned and labelled accelerated solvent extractor (ASE) extraction cell with two cellulose filters at the bottom. Then, 126 μ L IS (glufosinate, 1 μ g/g) was added, and the remainder of the cell was filled with diatomaceous earth, with the top end cap hand-tightened onto the cell body. No cellulose filter was required on top. To ensure repeatable solvent volumes with extraction on the ASE, the cell must be packed consistently with a similar density. The amount of diatomaceous earth to mix with the sample depended on the size of the cell used. Methanol with 0.1% formic acid and 2% water was used as the extraction solvent. The following ASE parameters were used (Schäfer *et al.*, 2008): temperature at 110°C, static time of 6 min, 3 cycles, 80% purge volume, and 120 s purge time. The resulting extract was evaporated with nitrogen gas, resuspended with 5% methanol to 1 mL and filtered using a 0.22 μ m PTFE syringe filter (Pall Acrodisc®, Promolab (Pty) Ltd T/A Separations) before being subjected to UHPLC-MS/MS for analysis.

3.5.4 Instrumentation

The GLY and AMPA analysis were carried out using a Waters UHPLC-MS/MS. The LC-MS/MS conditions were optimised for GLY and AMPA analysis, including chromatographic separation, identification of precursor and product ions for specific multiple reaction monitoring (MRM) transitions, and associated cone voltage (CV) and collision energy (CE) for GLY, AMPA, and glufosinate. Negative ion mode was used to analyse the compounds. A summary of the instrument parameters is presented in Table 3.3 to Table 3.7. Data were acquired using MassLynx version 4.1 (Waters, San Jose, CA, USA).

Table 3.3: Liquid chromatography (LC) parameters

Parameter	Condition
Sample temperature	4°C
Column	Hypercarb analytical column (2.1 x 100 mm; 5 μ m) (Anatech)
Column temperature	40°C
Injection volume	10 μ L
Flow rate	0.2–0.4 mL/min (refer to Table 3.4 for gradient)
Mobile phase A	1% acetic acid, 5% methanol in Milli-Q water
Mobile phase B	1% acetic acid in methanol
Run time	30 minutes

Table 3.4: Liquid chromatography (LC) mobile phase solvent gradient

Time (minutes)	Flow (mL/min)	% Mobile phase A	% Mobile phase B
Initial	0.2	100	0
10.00	0.2	70	30
11.00	0.4	70	30
18.00	0.4	70	30
19.00	0.4	10	90
22.00	0.4	10	90
22.10	0.2	100	0
30.00	0.2	100	0

Table 3.5: Liquid chromatography (LC) wash solvent composition

Wash step	Wash solvent
Seal wash	90% Milli-Q water:10% methanol
Purge solvent	90% Milli-Q water:10% methanol
Strong needle wash	5% Milli-Q water:5% isopropanol:90% methanol

Table 3.6: Source conditions of the mass spectrometer (MS).

Parameter	Condition
Source temperature	150°C
Desolvation temperature	600°C
Desolvation gas flow	1 000 L/h
Source offset	70 V
Collision gas flow	0.15 mL/min

Table 3.7: Multiple reaction monitoring (MRM) transitions and mass spectrometer (MS) parameters

Analyte	RT (min)	Dwell time (ms)	Q1 (m/z)	Q3 (m/z)	Cone voltage (V)	Collision voltage (V)
Glyphosate	2.98	Auto	168	150, 124, 81, 63	25	18
AMPA	1.8	Auto	110	79, 63	35	13
Glufosinate	2.1	Auto	180	95, 85	30	16

RT: retention time

3.5.5 Method validation

Quality control samples were prepared to determine the percentage of target compounds recovered from each type of matrix. These control samples were analysed along with each batch of samples. This allowed for the evaluation of degradation and correction of this deviation in the final data. Three samples per matrix were spiked with a known concentration before extraction, and three blank samples were extracted and spiked after extraction (Table 3.8). These are known as spike before (SB) and spike after (SA) quality control samples. Moreover, a matrix blank was added to evaluate the presence of any analytes of interest in the matrix used for the spike samples.

Table 3.8: Preparation of quality control samples.

Matrix			End concentration (ng/g)
Spike before (SB)			
Water	18.2 MΩ·cm water	Three samples spiked, extracted, analysed	1 000
Soil	Blank soil	Three samples spiked, extracted, analysed	1 000
Spike after (SA)			
Water	18.2 MΩ·cm water	Three samples were extracted, spiked, analysed	1 000
Soil	Blank soil	Three samples were extracted, spiked, analysed	1 000
Blank			
Water	18.2 MΩ·cm water	No spike, extracted, analysed	0
Soil	Blank soil	No spike, extracted, analysed	0

3.5.5.1 Calibration curve and matrix effect

Potential suppression and enhancement effects of the matrix should be addressed to ensure accurate quantification within a specific concentration range of 0–1 µg/g, with 11 intervals, by preparing an external matrix-matched calibration curve spiked post-extraction. Concentrations (0, 0.002, 0.005, 0.01, 0.02, 0.06, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 µg/g) were determined based on the expected levels of GLY and AMPA in the environment, as well as the performance of the instrument. The calibration standards were not serially diluted but instead originated from separate stocks and were analysed in triplicate to determine the reportable range. To prevent carry-over, the standards were injected in ascending order of concentration with blank injections in between.

3.5.5.2 Limit of detection (LOD)/Limit of quantification (LOQ)

The sensitivity of an analytical method refers to its increased responsiveness to changes in analyte concentration, as described by Whitmire *et al.* (2011). This sensitivity is represented by the slope of the calibration curve, which can also be used to determine the method's limit of detection (LOD) and limit of quantification (LOQ) using linear regression statistics from an external matrix-matched calibration curve. The LOD is calculated using Equation 3.9, derived from ($y=mx+c$), with Sa representing the standard deviation of the intercept (abundance) and b representing the slope of the calibration curve. Similarly, the LOQ is calculated using Equation 3.10 (Schoeman and Mashiane, 2015). Linearity is evaluated using the R-squared value of the calibration curve, which for all compounds analysed in this study was ≥ 0.9 , indicating excellent linearity. The LOD and LOQ were evaluated with gravimetric preparation of the calibration curve.

$$LOD = 3 \times \frac{Sa}{b} \quad \text{Equation 3.9}$$

$$LOQ = 10 \times \frac{Sa}{b} \quad \text{Equation 3.10}$$

3.5.5.3 Data analysis

The concentration of GLY and AMPA was determined by using Equation 3.11:

$$X_{compound} = \frac{\left(\frac{\text{native}}{\text{internal standard}} \right) - c}{m} \times IS_{conc} \quad \text{Equation 3.11}$$

Where:

$X_{compound}$ = calculated analysed concentration.

Native = native abundance.

Internal standard = internal standard abundance.

c = calibration curve y-intercept.

m = slope of the calibration curve.

IS concentration = internal standard concentration.

3.5.5.4 Precision

The precision is a measure of the consistency and repeatability and was determined by analysing quality control (QC) samples (Table 3.8). Equation 3.12 was used to calculate precision. The % relative standard deviation (RSD) should be less than 15% for excellent repeatability (Schoeman and Mashiane, 2015). Intra-day precision replicates of six injections of the QC samples from the

same day and inter-day precision based on six injections of a QC sample on another day were done to determine the precision.

$$\% RSD = \left(\frac{\text{mean SDEV}_{QC}}{\text{mean}_{QC}} \right) \times 100 \quad \text{Equation 3.12}$$

Where:

%RSD = percent relative standard deviation
mean SDEV_{QC} = the mean standard deviation of the QC samples
mean_{QC} = the mean of the QC samples

3.5.5.5 Accuracy

Accuracy is a measure of the closeness of the results to the true value of the known concentration. The accuracy was determined by the mean recovery of the spiked compounds in the water and sediment/soil matrices that were analysed. The formula used was in Equation 3.13:

$$\% \text{ Accuracy} = \left(\frac{\text{mean recovery obtained}}{\text{true concentration}} \right) \times 100 \quad \text{Equation 3.13}$$

Where:

mean recovery obtained = average recovery of the spiked compounds
true concentration = the known concentration of the spiked compounds

3.6 Microbial diversity

The analysis of microbial diversity provides insights into the ecological impacts of GLY on soil and water microbial communities, which are crucial for assessing ecosystem health and help guide regulatory decision-making. However, as is evident in the Results chapter, no quantifiable levels of GLY or AMPA were found. Since the initial planning was to compare microbial diversity between sites with varying levels of GLY and AMPA, we had to adjust the selection of sites based on other information.

3.6.1 Bacterial environmental DNA extraction

The samples for the analysis of the selection of microbial diversity were designed to explore potential relationships between GLY exposure and the composition of the microbial community. This part of the research was to follow after the levels of GLY and AMPA in the samples had been determined. Since no quantifiable levels were detected in any of the samples, the water samples for this part of the research were selected according to a different set of criteria. The research

question changed to whether there were different microbial communities distinguishable between sites up- and downstream from crop fields, expecting that the run-off from the crop would have introduced a higher level of agricultural chemicals to downstream sites compared with upstream sites. Sample selection was aided by information from the doctoral research conducted by Horak (2023), which screened soil samples for known agrochemical pesticides and where GLY was detected in the after-spraying GBH event. The sample selection also considered the GBH applications (based on the 2019 Kynotech® data use maps) and the site descriptions of the sites. Furthermore, water samples were chosen to reflect diverse land uses such as agriculture, mining activities and urban development. Soil sampling sites were determined based on GLY screening and the historic data from Kynotech® of the application volumes of GBH use from the use maps. The samples from these selected sites are shown in Table 3.9.

Table 3.9: List of sample sites chosen for environmental DNA extraction and sequencing

Site	Matrix	Description	2019 GBH application (tonnes)
Vaalharts			
TO	Water	Upstream site to all agricultural activity	No data
HR1	Water	Start to agricultural activities	No data
HR2	Water	Harts River flows through the sample site	No data
HR3	Water	Harts River flows through the sample site	No data
HR4	Water	Downstream of most agricultural sites	No data
M12	Soil	Soil collected at the agricultural site M12	No data
M13	Soil	GLY detected in 2023 study	No data
M15	Soil	Low pesticide application site M15	No data
Mpumalanga			
DMD	Water	Downstream to agriculture sites of M1, M2 and M3	248–404
MP10	Water	Upstream site to agriculture sites of M1, M2 and M3	248–404
MP4	Water	Close to the agriculture site M2	248–404
M1	Soil	Medium GBH application (Figure 4.1)	126–247
M2	Soil	GLY detected in 2023 study + high GBH application	248–404
M3	Soil	High GBH application (Figure 4.1)	248–404
M4	Soil	Soil collected from agricultural site M4	248–404
M6	Soil	Low GBH application (Figure 4.1)	53–125
M7	Soil	Soil collected from agricultural site M7	53–125
M8	Soil	Upstream of agriculture sites Vaal River	53–125
M11	Soil	Medium GBH application (Figure 4.1)	126–247
X3	Water	Downstream agriculture sites M4, M5 and M6	126–247

Microbial genomic environmental DNA (eDNA) was extracted from water samples filtered through cellulose nitrate membrane filters (0.45 µm, Sartorius Stedim Biotech) and from dried soil samples (thawed and oven-dried at 20°C) using a NucleoSpin soil kit (Macherey-Nagel, Germany). The protocol provided by the manufacturers was followed. For soil samples, 200–500 mg was added to the NucleoSpin bead tube, marked as sample A and sample B to create duplicates of the same sample. Similarly, the nitrate membrane filters were halved (creating a pseudo-sample), and each

half was labelled sample A and sample B before being inserted into respective NucleoSpin bead tubes. The same extraction protocol was followed for both the water filter and the soil sample tubes according to the manufacturer's instructions. The procedure was followed by adding 700 μ L lysis buffer SL1 and 150 μ L enhancer to the same tube, vortexing for 5 minutes at room temperature, and centrifuging for 2 minutes at 11 000 rcf. Afterwards, 150 μ L lysis buffer was added and incubated for 5 minutes at 4°C, followed by centrifugation for 1 minute. During centrifugation, a NucleoSpin inhibitor removal column was prepared by placing the column in a collection tube with a lid. The clear supernatant (700 μ L) from the centrifuged tube was applied to the inhibitor removal column filter, centrifuged for 1 minute, and the column was discarded. The binding conditions were adjusted by the addition of 250 μ L binding buffer to the collection tube, briefly vortexed and 550 μ L sample was applied to a newly prepared NucleoSpin soil column filter in a collection tube without a lid. This was followed by centrifugation for 1 minute to discard the flow-through. This process was repeated to maximise extraction.

The silica membrane was washed by adding 500 μ L binding buffer SB, centrifuging for 30 s at 11 000 rcf and discarding the flow through. The wash steps were repeated with 550 μ L buffer SW1. This was followed by 2 washes with 650 μ L of wash buffer SW2. The filter was centrifuged for 2 minutes to dry and placed in a clean 1.5 mL collection tube, where 50 μ L elution buffer SE was added, and the filter was incubated at room temperature for 1 minute. Finally, the filter was centrifuged for 30 s at 11 000 rcf, and the flow-through collected was the extracted eDNA.

3.6.2 Amplicon PCR

The 16S rRNA gene (V3–V4) region (460 bp) of each sample was analysed by using locus-specific primers with Illumina-specific adapter overhang nucleotide sequences: forward overhang adapters with 16S amplicon PCR forward primer: Illumina_16S_341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse overhang adapters with 16S amplicon PCR reverse primer: Illumina_16S_805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Herlemann *et al.*, 2011). The polymerase chain reaction (PCR) was set by adding the following to a PCR tube: 2.5 μ L of 20 ng/ μ L extracted microbial eDNA, 12.5 μ L 2x KAPA HiFi HotStart ReadyMix, 5 μ L 341F forward primer (1 μ M) and 805R reverse primer (1 μ M), making a total volume of 25 μ L reaction mixture. The reaction was carried out using a C1000 Touch™ Thermal cycler (BioRad Hercules, California, USA). The thermal cycling conditions were as follows: initial denaturation at 95°C for three minutes, 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The final extension step was executed at 72°C for five minutes and held at 4°C until further processing.

3.6.3 First cleanup of PCR products

AMPure XP beads (Beckman Coulter, South Africa) were used to eliminate any residual nucleotides, enzymes and unwanted products that might have developed during the PCR process. The PCR products were added to a 96-well PCR plate along with 20 μ L of AMPure XP beads, mixed for 2 minutes, and incubated for 5 minutes at room temperature. After incubation, the mixture was set aside on a magnetic stand for two minutes, or until the supernatant had cleared. The supernatant was carefully removed, and each sample was washed twice with 200 μ L freshly prepared 80% ethanol. The plate was incubated on the magnetic stand for 30 s, and the supernatant was removed and discarded. The beads were left to air-dry for ten minutes. The amplicon PCR plate was removed from the magnetic stand, and each well received 52.5 μ L of Tris (pH 8.5). The product in each well was pipetted up and down to ensure that the beads were properly resuspended. The plate was then incubated at room temperature for two minutes. The plate was placed on a magnetic stand for two minutes until the supernatant was clear. The supernatant from the Amplicon PCR plate was transferred to a clean PCR tube for further testing and determining the DNA concentration and quality. A NanoDrop™ 1000 Spectrophotometer v3.5.2 (Thermo Scientific, US) was used to determine the product's concentration (ng/ μ L) and quality A260:280 (nm). An A260:280 ratio of 1.7–2.1 was considered acceptable. The quality of the PCR fragments was also assessed by agarose gel electrophoresis (Bio-Rad, UK).

3.6.4 16S Library preparation and dual index PCR

During library preparation, Illumina dual-index barcodes were added to the amplicon target. The Nextera XT Index Kit v2, Set D (previously sold as IDT index, set D) from Illumina Inc. (CA, USA) was used for this purpose. The index PCR was set up with the following components: 5 μ L of PCR product, 5 μ L index 1 primer (N7XX), 5 μ L index 2 primer (S5XX), 25 μ L of 2x KAPA HiFi HotStart ReadyMix, and 10 μ L of PCR-grade water, resulting in a final volume of 50 μ L. The mixture was gently pipetted up and down to ensure thorough mixing, and the PCR 96-well plate was covered with a microseal film. The index PCR was performed using a C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The thermal cycling conditions included an initial denaturation at 95°C for five minutes, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s. A final extension step was carried out at 72°C for ten minutes, after which the samples were held at 4°C until further processing.

3.6.5 Second clean-up of indexed PCR products

The indexed PCR products were cleaned using AMPure XP beads (Beckman Coulter, South Africa). The protocol is the same as that used for the first clean-up of the PCR products, with a few adjustments to the volumes of the reagents. The quantities were as follows: 56 μ L of AMPure XP beads, 27.5 μ L of 10 mM Tris pH 8.5, and 400 μ L of 80% ethanol per sample.

3.6.6 Library quantification, normalisation, and pooling

The Qubit™Flex Fluorometer (Thermo Scientific, US) was used to determine the library concentration following the manufacturer's instructions. The required standards and reagents (Qubit reagent and Qubit buffer) were supplied by Thermo Scientific, US. After calibration, a working solution of the Qubit reagent and Qubit buffer was added to a solution reservoir, and by using a multi-channel pipette, 190 µL working solution was added to a tube strip consisting of 8 tubes followed by 10 µL of standard solution. The tube strip was briefly vortexed and centrifuged. The tube strip was inserted into the Qubit™Flex Fluorometer to standardise the machine. Thereafter, 198 µL working solution and 2 µL of PCR product were added to a tube strip. The strip tube was briefly vortexed and centrifuged before determining the DNA concentration.

The concentrations of the respective indexed libraries were calculated with the following formula Equation 3.14:

$$\frac{\text{DNA concentration (ng/}\mu\text{L)}}{(660 \text{ g/mol} \times \text{average library size})} \times 10^6 = \text{concentration (nM)} \quad \text{Equation 3.14}$$

The libraries were normalised using Tris 10 mM (pH 8.5) to 2 nM. To pool libraries with unique indices, 5 µL of diluted DNA from each was aliquoted and mixed.

3.6.7 Illumina NextSeq sequencing

The pooled libraries were combined with 40% (v/v) PhiX control spike-in and loaded onto a flow cell at a final concentration of 1 000 pM. Sequencing was done on an Illumina® NextSeq™ 2000 using a NextSeq 2000 P1 reagent 600-cycle kit. The machine's reporter software then generated FASTQ files for downstream analysis.

3.6.8 Microbial data analysis

Reads obtained from the sequencing platform, indexes and primers removed, were processed using the DADA2 software package (Callahan *et al.*, 2016a; <https://benjjneb.github.io/dada2/tutorial.html>). The filtering and trimming step was applied with the following settings: truncLen = c(250,250), minLen = c(220,220), maxEE = c(10,10), maxN = 0, rm.phix=TRUE. The step was applied to both the forward and reverse reads. This was followed by the learnErrors and dada functions, applied separately to the forward and reverse reads, and the reads were merged using mergePairs. The resulting sequence table was processed to remove chimeric reads using the removeBimeraDenovo function (method = "consensus") (Flegontova *et al.*, 2023). The function, assignTaxonomy, was used to assign taxonomy using the Silva 138.1 database (McLaren and Callahan, 2021). At key points, the "rds" data structures were saved,

including the sequence and taxonomy tables. The processed data were visualised using phyloseq (McMurdie and Holmes, 2013; Callahan *et al.*, 2016b).

3.7 Risk assessment

A bacterial ecological risk assessment was conducted to predict the probable risks of bacterial infection, which GLY potentially influenced the bacterial pathogenicity. The specific quantitative microbial risk assessment (QMRA) explained by Haas *et al.* (2014) and adapted by Ahmed *et al.* (2020) was used. The risk assessment process includes four steps: 1) hazard identification; 2) dose-response assessment; 3) risk characterisation; and 4) hazard characterisation (WHO, 2021). A literature search aided in identifying which bacterial pathogens are more likely GLY-resistant or GLY-tolerant. The specific bacteria from the sequenced bacterial eDNA samples were used for the QMRA tool by filtering for pathogens identified in the literature and the amplicon sequence variant (ASV) counts at which they were detected. The hazard identification step identifies the potential pathogens that can cause various health effects in humans. The dose-response assessment summarises the amount of pathogenic bacteria exposure by calculating the probability of infection per day. The exposure assessment evaluates the duration of pathogenic bacteria in soil and water and how they reach a target population. The final step is risk characterisation, which includes a calculation of the probability of annual infection and the probability of illness.

3.7.1 Identified environmental pathogens

Environmental pathogens, those not typically associated with urban or clinical environments, are commonly found in water, soil and air. These organisms pose significant health risks due to their release via natural processes, industrial discharges and urban or agricultural runoff (Fong and Lipp, 2005). Transmission can occur through ingestion, inhalation or direct contact with contaminated sources such as groundwater, estuarine water, seawater, rivers, aerosols from sewage treatment facilities, untreated or poorly treated water, and private wells (Fong and Lipp, 2005). A list of known human pathogens found in the environment was summarised from the literature (Table 3.10). While not all bacterial genera selected for the QMRA are inherently GLY-resistant or pathogenic, for this study, all were considered potential environmental pathogens with the capacity for developing GLY resistance or tolerance.

Pathogens such as *Escherichia coli*, *Salmonella* spp., *Klebsiella* spp. and *Pseudomonas* spp. are commonly found in GLY-impacted environments like agricultural fields and runoff-receiving water bodies (Mohy-Ud-Din *et al.*, 2023; Pöppe *et al.*, 2020). *Staphylococcus* spp. and *Mycobacterium* spp. were included due to their potential for antibiotic resistance development (Raoult *et al.*, 2021; Chekan *et al.*, 2016), a concern intensified by widespread antibiotic exposure. GLY may select antibiotic-resistant strains, raising the risk of hard-to-treat infections. Spore-forming bacteria such

as *Bacillus* spp. (Hertel *et al.*, 2021) and *Clostridium* spp. (Van Bruggen *et al.*, 2021) were included in this study because of their resilience in GLY-exposed environments, where their protective spore coats enhance persistence. *Enterococcus* spp. and *Legionella* spp. were also considered due to their well-documented links to waterborne transmission (Van Bruggen *et al.*, 2021).

Table 3.10: Pathogenic bacteria and their transmission and exposure routes from literature sources.

Pathogen	Transmission	Exposure	Reference
<i>Pseudomonas</i> spp.	Ingestion	Ingesting contaminated soil/water	Balfour-Lynn, (2021)
<i>Enterococcus</i> spp.	Ingestion	Faecal contamination in aquatic and terrestrial vegetation, beach sand, soil, and sediments.	Byappanahalli <i>et al.</i> (2012)
<i>Klebsiella</i> spp.	Inhalation & ingestion	Contaminated river sediment, river water, and wastewater.	Chi <i>et al.</i> (2019)
<i>Salmonella</i> spp.	Ingestion	Eating/drinking contaminated food/water.	Christidis <i>et al.</i> (2020)
<i>Staphylococcus</i> spp.	Inhalation & ingestion	Inhalation of aerosols and contact ingestion.	Davis <i>et al.</i> (2018)
<i>Mycobacterium</i> spp.	Inhalation	Aerosolised water, farm animals, soil, dust particles.	Mtetwa <i>et al.</i> (2022)
<i>Escherichia coli</i>	Ingestion	Faecal-oral, undercooked food, swimming, and drinking contaminated water.	Muniesa <i>et al.</i> (2006)
<i>Campylobacter</i> spp.	Ingestion	Eating/drinking contaminated food/water	Reichelt <i>et al.</i> (2022)
<i>Bacillus</i> spp.	Inhalation & Ingesting	Inhalation or ingestion of spores by humans or animals	Turner <i>et al.</i> (2016)
<i>Legionella</i> spp.	Inhalation	Breathing in an aerosol	Vermeulen <i>et al.</i> (2021)
<i>Clostridium</i> spp.	Ingestion	Eating/drinking spore-contaminated food/water.	Warriner <i>et al.</i> (2017)

All bacterial pathogens mentioned above, except *Legionella*, were reported to be susceptible to the GLY-related antibiotic fosfomycin (Abraham, 2010). It is important to note that the pathogens used in this study's QMRA were identified only to the genus level, while the risk assessment parameters are species-specific. Although 16S rRNA sequences can confirm genus identity, pathogenicity and antibiotic resistance can vary widely between species within the same genus. For instance, not all species in a genus are harmful or fosfomycin-resistant, but exceptions like

Legionella (Chatziprodromidou *et al.*, 2022) illustrate that genus-level classification does not always reflect risk accurately. As a result, the QMRA in this study was conducted at the genus level.

3.7.2 Hazard identification measures

There are pathogenic bacteria in water and sediment: *E. coli*, *Salmonella* spp., *Vibrio cholerae*, *Shigella* spp., and *Campylobacter* spp., to name a few (Abia *et al.*, 2016; Ahmed *et al.*, 2020). And some bacterial species are resistant to GLY, such as *Salmonella enterica*, *E. coli*, *Salmonella* serovar Typhimurium, and *Clostridium* (Bote *et al.*, 2019a; Pöppe *et al.*, 2020; Van Bruggen *et al.*, 2021; Mohy-Ud-Din *et al.*, 2023), and *Campylobacter* is GLY-sensitive (Puigbò *et al.*, 2022). Pathogens within water and soil samples, as well as those that were described as GLY-resistant or -sensitive, were selected for further analysis in the QMRA tool. Additionally, *Klebsiella* spp., *Bacillus* spp., *Enterococcus* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Legionella* spp., and *Mycobacterium* spp. were included in the assessment. All selected pathogens were identified in the water and soil samples of this study.

3.7.3 Dose-response assessment.

The dose-response (DR) assessment considers factors such as the infectious dose required to cause illness and the variability in individual susceptibility. A dose-response model is selected based on how well a pathogen's behaviour is described by the model. This behaviour is based on variations in infection mechanisms and host-pathogen interactions. The models used were the Exponential model (Equation 3.15) and the β -Poisson dose-response model (Equation 3.16). The models used for each chosen pathogen are listed in Table 3.11. The optimised parameters, such as the k , N_{50} and α , were determined for each organism based on assumptions from literature and the QMRAwiki database (<https://www.qmrawiki.org/pathogens>); see Table 3.11. Given that bacteria were only identified at the genus level, and the proposed variables in Table 3.11 are species-specific, the risk assessment was performed based on the assumption that all chosen genera and all species within those genera were pathogenic. The parameters are also from clinical settings only and not environmental settings. Only literature on clinical settings was available.

$$\frac{P_{inf}}{day} = 1 - \exp(-kd) \quad \text{Equation 3.15}$$

$$\frac{P_{inf}}{day} = 1 - \left[1 + \left(\frac{d}{N_{50}} \right) \left(2^{\frac{1}{\alpha}} - 1 \right) \right]^{-\alpha} \quad \text{Equation 3.16}$$

Where:

- $P_{\text{inf/day}}$** = the probability of infection,
 k = infection probability per dose
 d = dose: the ASV counts/250 mL
 N_{50} = the dose to the median response
 α = the shape of dose-response variability.

Table 3.11: Dose-response parameters of pathogenic bacteria using the β -Poisson or exponential model

Organisms	Parameters	Type of model	Reference
<i>Escherichia coli</i>	$\alpha = 1.55\text{E-}01$ $N_{50} = 2.11\text{E+}06$	β -Poisson	QMRAwiki (2024)
<i>Klebsiella</i> spp.	$k = 1.62\text{E-}06$	Exponential	Domenico <i>et al.</i> (1982)
<i>Salmonella</i> spp.*	$\alpha = 1.09\text{E+}00$ $N_{50} = 1.34\text{E+}06$	β -Poisson	QMRAwiki (2024)
<i>Bacillus</i> spp.	$k = 1.65\text{E-}05$	Exponential	QMRAwiki (2024)
<i>Enterococcus</i> spp.	$\alpha = 1.60\text{E-}01$ $N_{50} = 5.99\text{E+}04$	β -Poisson	Niknejad <i>et al.</i> (2024)
<i>Pseudomonas</i> spp.	$k = 1.05\text{E-}04$	Exponential	QMRAwiki (2024)
<i>Staphylococcus</i> spp.	$k = 7.64\text{E-}08$	Exponential	QMRAwiki (2024)
<i>Campylobacter</i> spp.	$\alpha = 1.44\text{E-}01$ $N_{50} = 8.90\text{E+}02$	β -Poisson	QMRAwiki (2024)
<i>Clostridium</i> spp.	$k = 1.82\text{E-}11$	Exponential	Seo <i>et al.</i> (2021)
<i>Legionella</i> spp.	$k = 5.99\text{E-}02$	Exponential	QMRAwiki (2024)
<i>Mycobacterium</i> spp.	$k = 6.93\text{E-}04$	Exponential	QMRAwiki (2024)

*Used the average of all the listed pathogenic *Salmonella*.

3.7.4 Exposure assessment

The exposure assessment evaluates the population exposed to the bacteria, considering factors such as the volume consumed and the duration of exposure. By assessing the pathways of exposure, such as drinking water, recreational water use, agricultural water, or agricultural soil, the exposure to the bacterial pathogens can be calculated. The variables in Table 3.12 were obtained from Haas *et al.* (2014).

Table 3.12: The suggested point estimates for the respective exposure routes with the frequency and contact rate.

Exposure route	Contact rate	Exposure frequency	Used (L/day)*
Ingestion of drinking water	1.4 L/day	365 days/year	511.0024
Ingestion of surface water while swimming	50 mL/hour	2.5 hours/swim, 7 swims/year	
Soil ingestion by child (<6 years old)	200 mg/day	Depends on context	0.0003
Soil ingestion by child (>6 years old)	100 mg/day	Depends on context	
Inhalation	20 m ³ /day (adult), 15 m ³ /day (child)	365 days/year	6 413 050
Inhalation during showering (using surface water, due to lack of piped water in rural areas)	0.07 m ³ /shower	365 showers/year	

*The used point estimates were reworked to L/day and grouped as ingestion by water, ingestion by soil, and inhalation.

3.7.5 Risk characterisation

For optimal risk characterisation, the integration of hazard identification, dose-response assessment, and exposure assessment is needed to quantify the risk of pathogenic infections in the community. To do this, calculations on the probability of illness need to be made using Equation 3.17.

$$P_{inf_annual} = 1 - \left[1 - \frac{P_{inf}}{day} \right]^n \quad \text{Equation 3.17}$$

Where:

P_{inf_annual} = probability of annual infection

P_{inf/day} = probability of infection obtained from Equation 3.16 or Equation 3.17

n = the number of exposure days in a year.

3.8 Statistical analysis

GraphPad Prism v7 was used to test the data's normality and homogeneity of variance using the D'Agostino and Pearson Omnibus (a combined test that analyses both skewness and kurtosis of a data set to assess normality, whereas D'Agostino and Pearson refer to the method used to measure skewness and kurtosis) and the Shapiro-Wilk normality test, respectively. When significant differences in the sampling events coupled to geographic locations were investigated,

a one-way ANOVA with Tukey's multiple comparison test was used when the data were parametric and for non-parametric data, Kruskal-Wallis tests with Dunn's multiple comparison tests were used (Pyrzrak, 2016). A p-value of less than 0.05 was set to determine the significance of the spatial and temporal variation of the water and sediment quality variables. A principal component analysis (PCA) (Canoco v5.12) was also used to evaluate the temporal and spatial patterns associated with water nutrients and sediment quality. All data used in PCA analysis were log-transformed $x=\log(y+1)$, centred, and standardised (Šmilauer and Lepš, 2014).

In statistical analyses of data from the bacterial community, the range of diversity indices was calculated using the vegan library. Differences in alpha diversity indexes between conditions were tested with a Kruskal-Wallis test followed by a pairwise Wilcoxon test. To determine abundance and richness, respectively, the Shannon index and the Chao1 index were used as alpha-diversity indices. The Bray-Curtis distance measure was used to cluster the samples for the beta-diversity analysis. The ggplot2 tool was used to represent the resulting distance matrices, which resulted in an NMDS plot.

Alpha diversity indicates diversity within individual sites, but not whether they share the same species. Beta diversity indicates the difference between communities or sites (Kers and Saccenti, 2022).

CHAPTER 4: RESULTS

In this study, GIS was used to visually represent the application of GBH on maize, wheat, soybean, and sunflower crops in the geographical context of South Africa. Water, soil, and sediment samples were collected from carefully selected locations: agricultural areas (rain-fed fields and canal-irrigated fields) as well as non-agricultural areas such as landscaped areas. The water samples were thoroughly tested for physico-chemical and nutrient parameters, while soil and sediment samples were analysed using the Endecotts dry sieving system to determine composition. Additionally, the CEC of the soil and sediment was assessed. Glyphosate and its metabolite AMPA were extracted using the EURL-SRM QuPPe method designed for soil and sediment, specifically to extract highly polar pesticides. The solid samples were extracted with ASE. Analysis was conducted via UHPLC MS/MS to quantify the GLY and AMPA compounds. The bacterial communities within selected samples at the time of collection were identified by next-generation sequencing of the V3-V4 region of the 16S rRNA gene up to genus level, followed by a bacterial risk assessment focusing on organisms known for resistance to GLY and degradation of GLY. This chapter presents the results of map visualisation, and water and soil analysis, including nutrient content and physico-chemical properties of the water samples, soil composition, soil CEC, instrumental chemical analysis, bacterial diversity sequencing, and bacterial risk assessment.

4.1 Mapping the GBH (glyphosate-based herbicide) application

Kynetech®, a market research company for animal health and agriculture, provided data on the volumes of GBHs applied to maize, soybean, wheat, and sunflower crops in 2017 and 2019. These four crops are the most widely cultivated crops in the country, representing approximately 4.2 million ha per annum (SAGL, 2021), and account for approximately 80% of the total GBH market in South Africa when determined at an a.i. level. Kynetech® obtained this information by interviewing farmers whose farms use GBH. The information collected included the target crop, its variety, seeding rate, and pesticide application rate, not just herbicide data that were used in the current study.

The map shows the distribution of GBH applications across different regions, with higher concentrations observed in certain areas. Figure 4.1 shows the tonnes of GBH applied to specific magisterial locations in South Africa. In 2017, the Free State Province bordering the Vaal River had the highest GBH applications (1 753 tonnes) (see red in Figure 4.1). Furthermore, the highest GBH applications in 2019 were again in the Free State (see red in Figure 4.2) but were slightly lower (405–1 065 tonnes) than in 2017 and were spread across three magisterial districts rather than one in 2017.

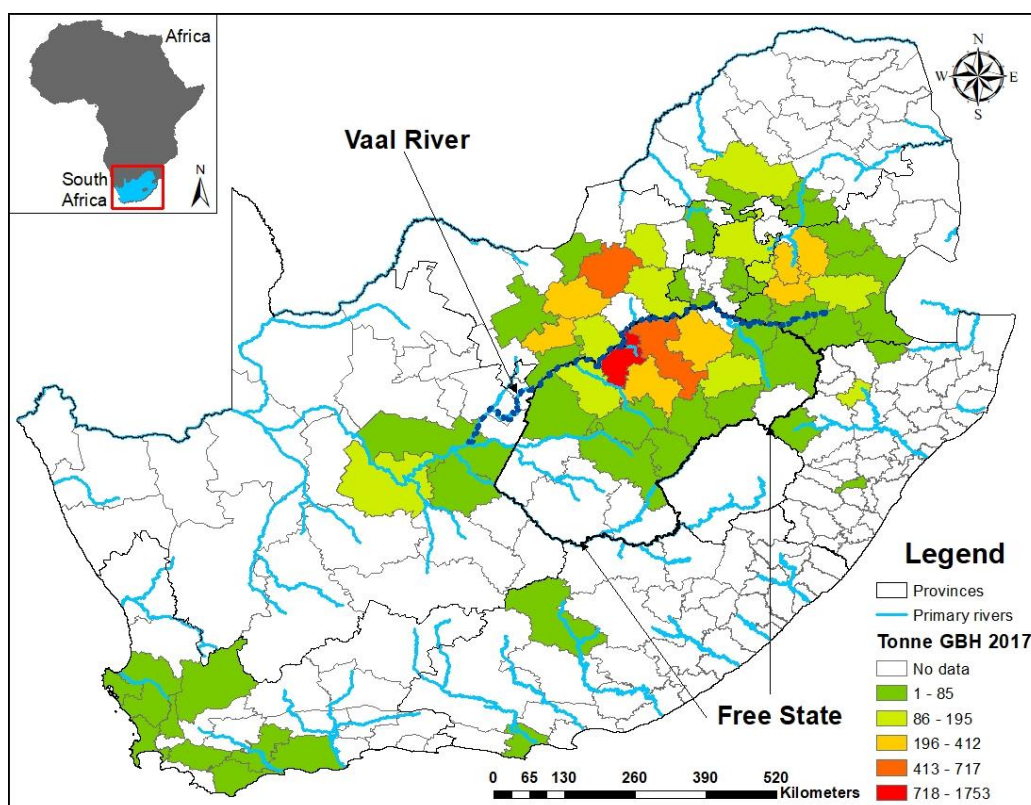


Figure 4.1: The application of glyphosate-based herbicides (GBHs) on maize, soybean, wheat, and sunflower crops in South African municipal districts for the 2017 season (in tonnes a.i./ha). The vector data set coordinates (in decimal degrees) were projected using the World Geodetic System (WGS) 1984 geographical coordinate system to identify where the chemicals were used.

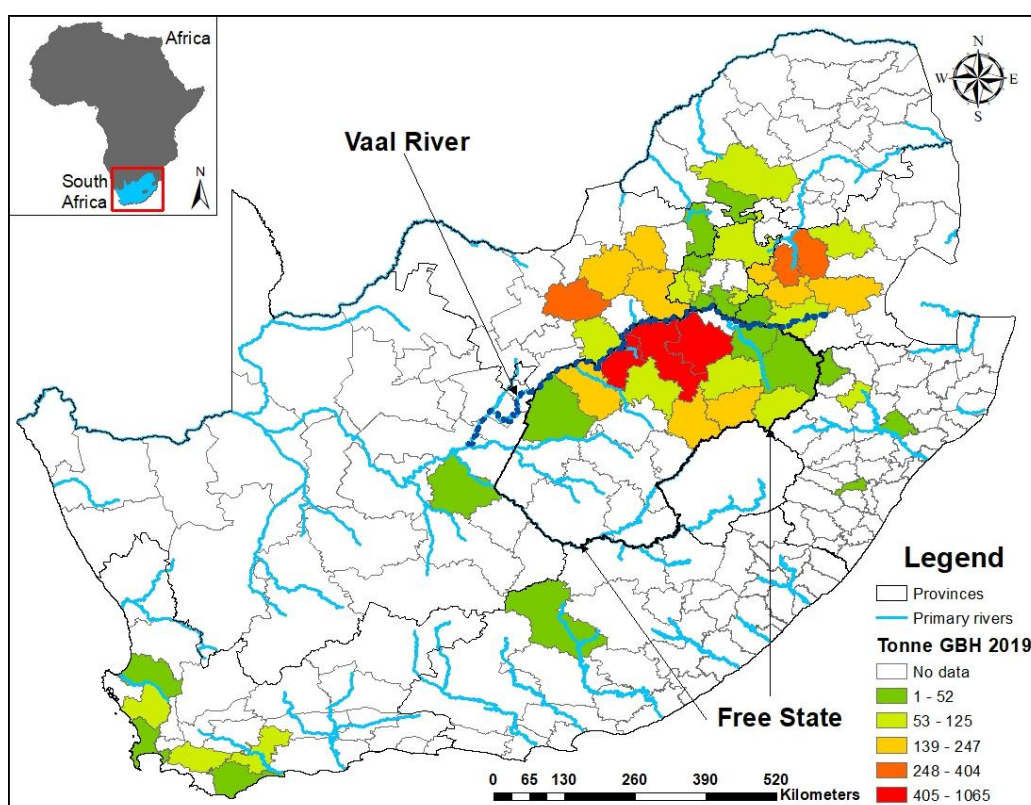


Figure 4.2: The application of glyphosate-based herbicides (GBHs) on maize, soybean, wheat, and sunflower crops in South African municipal districts for the 2019 season (in tonnes a.i./ha). The vector data set coordinates (in decimal degrees) were projected using the World Geodetic System (WGS) 1984 geographical coordinate system to identify where the chemicals were used.

4.2 Water quality and soil composition

Anthropogenic impacts affect the water quality of water, soil, and sediment. South Africa's Department of Water and Sanitation (DWS), under the National Water Act (Act No. 36 of 1998), set the standards for managing and monitoring the quality of surface water in the country, known as resource water quality objectives (RWQOs). These RWQOs are set to meet water quality for irrigation use. These RWQOs were developed using data on the quantity, quality, habitat, and biota of resource water. The RWQOs vary depending on the water catchment in which the sample sites are located. These differ due to the different lithologies and geologies of the areas, as well as anthropogenic activities that may influence these different water quality parameters. The Harts River in the Vaalharts irrigation scheme in the Northern Cape Province falls in the Lower Vaal water catchment (DWS, 2016a), the Klein Olifants River in the Mpumalanga Province belongs to the Olifants water catchment (DWS, 2016b), the Life Water- and Vaal rivers in the Mpumalanga province, and non-agricultural sites in the North West Province are in the Upper Vaal water catchment (DWS, 2016c). The water quality parameters of the current study were compared to the RWQOs of the respective catchments.

4.2.1 Water quality parameters

The water quality parameters (physical parameters) include pH, temperature, electrical conductivity (EC), turbidity, total hardness, and total suspended solids. The chemical parameters include nitrates (NO_3^-), nitrites (NO_2^-), phosphates (PO_4^{3-}), sulphates (SO_4^{2-}), ammonia (NH_4^+) and chloride (Cl^-). The evaluation of water quality at different sites and catchment areas revealed variability in both physical and chemical parameters, possibly influenced by activities such as irrigation, rainfall, and harvest periods.

The pH values in Figure 4.3 generally fell within the RWQO range of 6.5–8.5 across most sites. However, there were deviations where the pH dropped below 6.5, notably in the MPA-ORC and NAS-UVC areas, and exceeded 8.5, especially after spraying and after the harvest periods. The water temperature ranged from around 10–35°C, showing consistent patterns across the different sampling periods, with slight increases after harvest and after spraying, possibly attributed to a specific time in the growing season and the climate. Electrical conductivity showed high variability, particularly in the MPA-ORC region. The before-spraying event showed that the conductivity had sharp spikes that occurred at the before-spraying event (orange) and after-spraying event (green), with values ranging from 1 600–2 000 mS/m at site PAN1, far above the RWQO threshold of 111 mS/m. The turbidity levels increased sharply after spraying, especially in the MPA-ORC and MPA-UVC areas. After rainfall, turbidity further increased, indicating a strong response to both spraying and precipitation events. The total hardness fluctuated between sites, with higher values observed in the MPA-ORC area. After spraying, there was a noticeable increase in hardness, particularly in MPA-ORC and VIS-LVC, with values remaining elevated after

harvest. The TSS levels in Figure 4.3 showed variability at some sites, with elevated TSS for most sampling events, compared to the before-spraying event. Total suspended solids dramatically increased in the MPA-ORC area at some sites after spraying, after rainfall and post-harvest. Before spraying, the TSS was generally low, but at PAN1, the value exceeded 9 000 mg/L.

The nitrate levels had considerable variation between sites, with the MPA-ORC and MPA-UVC areas showing the highest concentrations after spraying (Figure 4.4). Before spraying (orange line), nitrate levels were relatively low in most sites but increased sharply after spraying (green line), particularly in MPA-ORC, where values exceeded 20 mg/L, five times above the RWQO of 4 mg/L. After harvest (purple line) and after rainfall (blue line), nitrate levels remained elevated at several sites, especially in the MPA-ORC region. Nitrite levels were generally low, with most sites sampled before spraying (orange line) showing values within the RWQO. However, after spraying (green line) and specifically after rainfall (blue line), there were noticeable spikes in nitrite levels at specific sites in MPA-ORC, MPA-UVC, and NAS-UVC, where the values exceeded the RWQO of 0.858 mg/L for MPA-UVC. The post-harvest period (purple line) showed some reduction in nitrite levels, although certain sites in VIS-LVC and NAS-UVC still exhibited elevated concentrations.

The phosphate levels were generally above the RWQO of 0.025 mg/L for most sites before spraying (orange line), but several sites in MPA-ORC were below the threshold for after spraying, after rainfall and after harvest (Figure 4.4). The blue line (after rainfall) indicates further increases in phosphate levels, particularly in MPA-UVC, where the concentrations were the highest. After harvest (purple line), phosphate levels decreased slightly but remained elevated at some sites, particularly in the MPA-ORC and NAS-UVC regions. The sulphate levels varied across the sites, with all the sites showing values within the RWQO limit of 500 mg/L. However, the RWQO for VIS-LVC was less than 200 mg/mL, and a few sites exceeded this lower RWQO in samples from the 'before-spraying' event (orange line) and after harvest (purple line). Interestingly, the sulphate levels in MPA-UVC were all roughly similar. Ammonium levels generally exceeded the RWQO at most sites. Additionally, sites in MPA-ORC and MPA-UVC showed a sharp increase in both the before-spraying (orange line) and after-spraying (green line) events, where values exceeded the RWQO. Ammonium levels were high at VB in NAS-UVC for all sampling events. There is no RWQO for chloride levels (Figure 4.4). The chloride levels were relatively low after rainfall (blue line). However, the other events, i.e. before spraying, after spraying and after harvest, all share a similar trend with increases and decreases in concentration. The chloride concentrations increased at several sites over time, particularly in MPA-ORC (PAN1) and VIS-LVC (C2, HR3 and HR4). The chloride concentrations were higher at MPA-ORC and specifically VIS-LVC, where both share ranges from 23.5–252 mg/L.

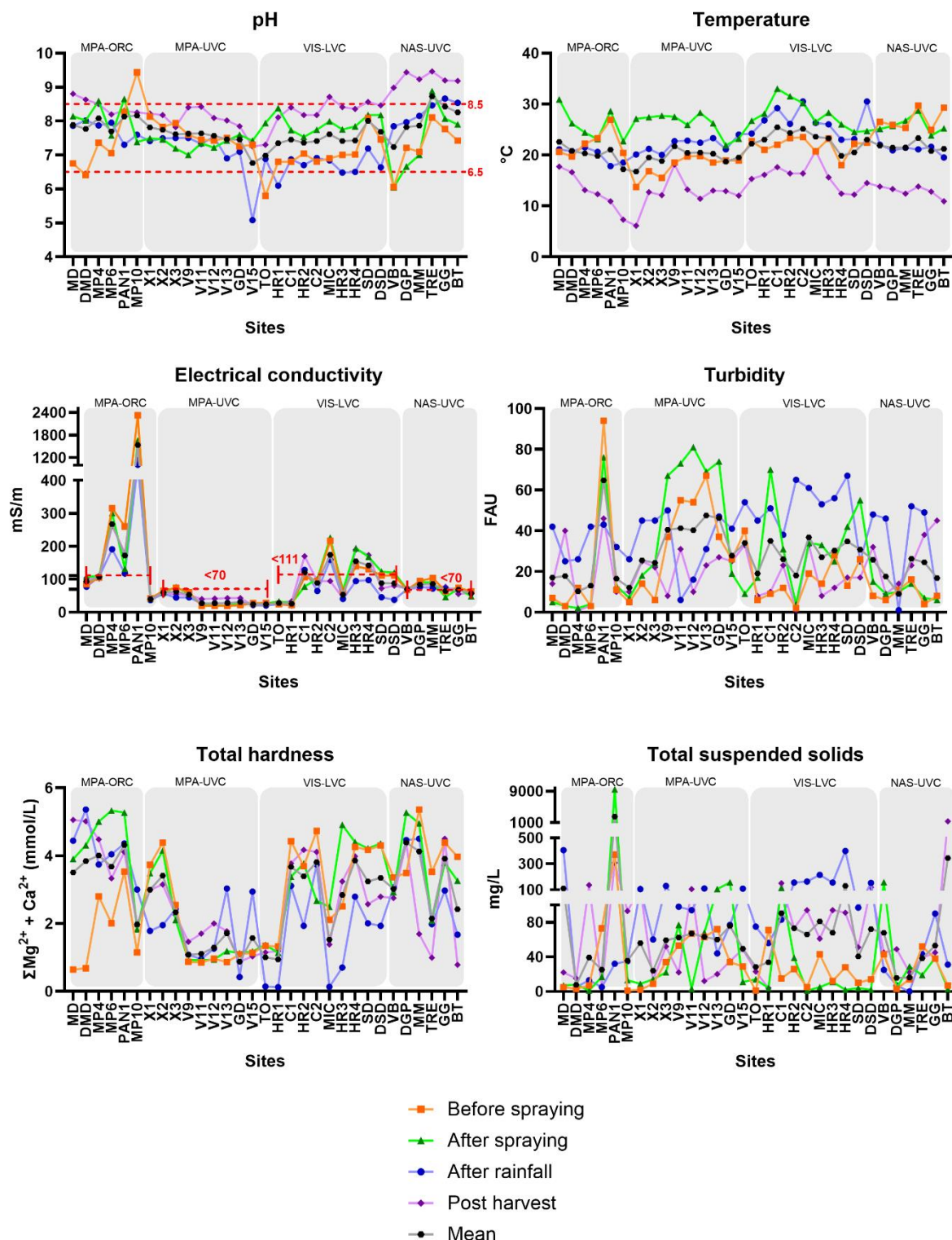


Figure 4.3: The physical parameters of the physico-chemical analysis of the water samples collected during the growing season before spraying pesticide, after spraying pesticide, after rainfall and after harvest. The resource water quality objectives (RWQO) levels are indicated in red dotted lines in the respective areas, Mpumalanga's Olifants River catchment (MPA-ORC), and upper Vaal River catchment (MPA-UVC), the Northern Cape's Vaalharts irrigation scheme's lower Vaal River catchment (VIS-LVC) and the non-agricultural areas' upper Vaal River catchment (NAS-UVC).

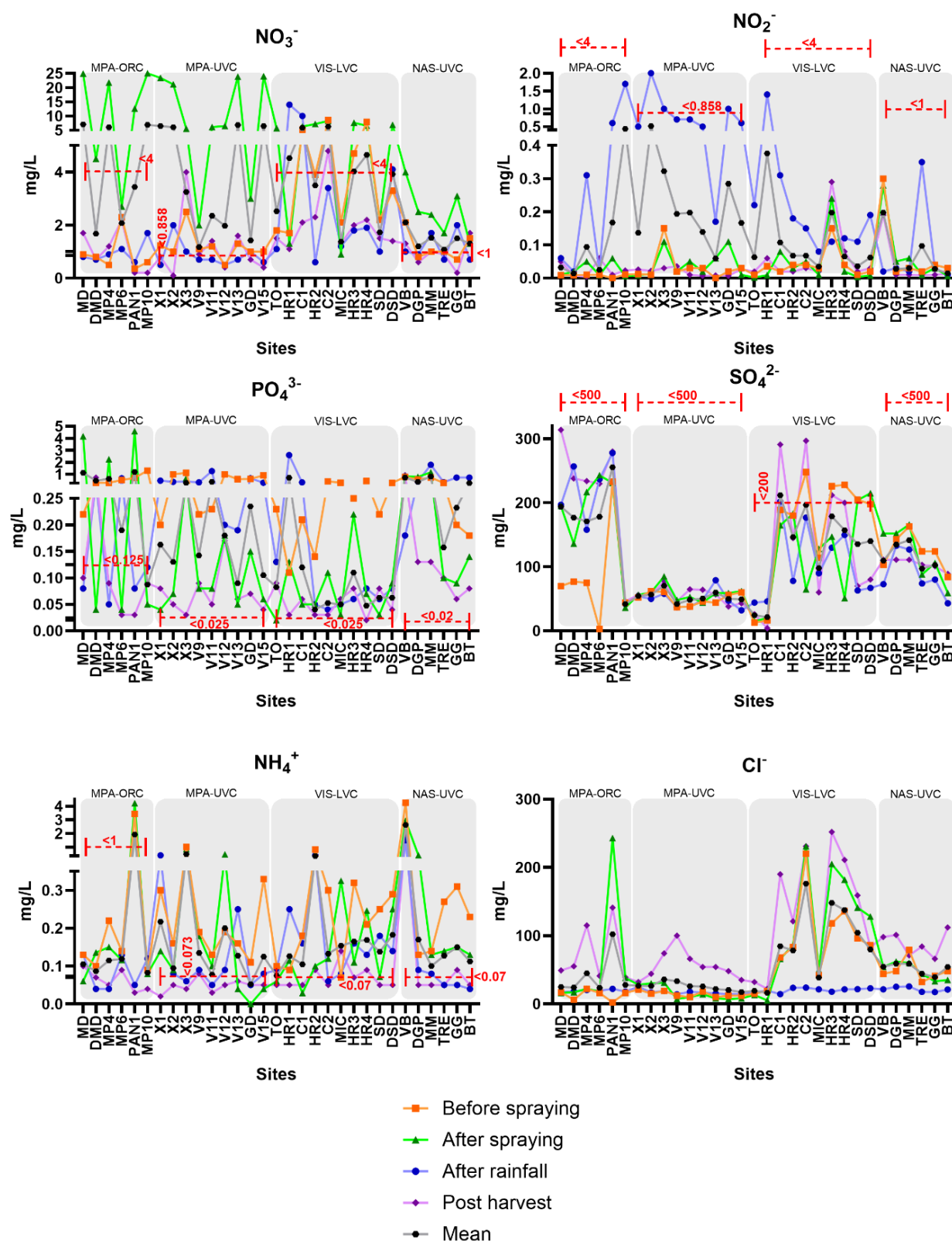


Figure 4.4: The chemical parameters of the physico-chemical analysis of the water samples collected during the growing season before spraying pesticide, after spraying pesticide, after rainfall and after harvest. The resource water quality objectives (RWQO) levels are indicated in red dotted lines in the respective areas, Mpumalanga's Olifants River catchment (MPA-ORC), and upper Vaal River catchment (MPA-UVC), the Northern Cape's Vaalharts irrigation scheme's lower Vaal River catchment (VIS-LVC) and the non-agricultural areas' upper Vaal River catchment (NAS-UVC).

The analysis of water quality across the MPA, VIS, and NAS sites across all four sampling events (before spraying, after spraying, after rainfall and post-harvest) revealed several key statistical differences ($p < 0.05$) (Table 4.1). Before spraying (PRE) in MPA (PRE-MPA), it had the highest electrical conductivity (EC) at 231.0 $\mu\text{S}/\text{cm}$, indicating more salts, while PRE-NAS had a much lower EC at 78.1 $\mu\text{S}/\text{cm}$. Sulphate levels were significantly higher in PRE-VIS (161.7 mg/L) compared to PRE-MPA (63.85 mg/L). Nitrate and phosphate concentrations were significantly elevated in PRE-VIS relative to PRE-MPA and PRE-NAS. Chloride levels were also higher in PRE-VIS compared to the other sites.

After spraying (POST), POST-MPA exhibited notably higher nitrate levels than POST-NAS, while phosphate and chloride concentrations were higher in POST-VIS compared to other areas (Table 4.1). As for the POST-RAIN event, POST-VIS had significantly lower pH and higher temperature compared to POST-MPA and POST-NAS, with increased levels of turbidity, nitrate, and chloride in POST-VIS. In the after-rainfall event (RAIN), significant differences in nitrite concentrations were observed between RAIN-MPA and RAIN-NAS, and TSS levels were higher in RAIN-VIS compared to RAIN-NAS.

Post-harvest (HARVEST), the pH in HARVEST-NAS was significantly higher than that of HARVEST-VIS and HARVEST-MPA. Phosphate levels showed a significant increase in HARVEST-VIS compared to HARVEST-NAS, and chloride concentrations were notably higher in HARVEST-VIS than in HARVEST-MPA. These findings highlight the variability in water quality parameters influenced by geographical location and agricultural practices.

Table 4.1: Means and standard deviation of all water quality variables for the sampling event before spraying (PRE), after spraying (POST), after rainfall event (RAIN) and after harvest (HARVEST) per sampling area: Mpumalanga (MPA) (n=15), Vaalharts irrigation scheme (VIS) (n=10), and non-agricultural sites (NAS) (n=6).

	pH	Temp	EC	Turbidity	TH	NO ₃ ⁻	NO ₂ ⁻	PO ₄ ³⁻	SO ₄ ²⁻	NH ₄ ⁺	Cl ⁻	TSS
PRE-MPA	7.6±0.7 ^a	19.6±3.3 ^a	231.0±584.7	29.7±27.3	1.8±1.3 ^{ab}	1.1±0.6 ^b	0.02±0.0	0.6±0.4 ^a	63.9±50.3 ^a	0.4±0.9	13.9±5.5 ^{ab}	0.005±0.0
PRE-VIS	7.0±0.6 ^a	21.9±1.8	102.2±57.5	16.9±11.5	3.3±1.3 ^a	4.1±2.5 ^{ab}	0.04±0.0	0.3±0.1 ^a	161.7±85.5 ^a	0.3±0.2	88.3±60.9 ^b	0.002±0.0
PRE-NAS	7.3±0.7	26.9±2.1 ^a	78.1±17.3	8.8±4.2	4.0±0.8 ^b	1.2±0.5 ^a	0.07±0.1	0.5±0.3	123.5±28.0	0.9±1.6	48.7±16.0 ^a	0.003±0.0
POST-MPA	7.6±0.5	26.1±2.5	179.0±415.0	35.7±32.6	2.8±1.8	13.8±9.7 ^a	0.04±0.0	0.8±1.5	105.6±76.5	0.4±1.1	33.1±58.9 ^a	0.066±0.2
POST-VIS	7.9±0.3	28.0±2.9	113.8±66.5	32.0±20.2	3.3±1.3	5.3±2.8	0.05±0.1	0.1±0.1 ^a	119.3±75.8	0.1±0.1	110.7±79.4 ^a	0.002±0.0
POST-NAS	7.4±1.0	25.9±1.7	69.5±19.4	10.2±3.7	3.7±1.2	2.5±1.0 ^a	0.07±0.1	0.5±0.5 ^a	120.5±42.7	0.7±1.1	48.0±12.5	0.004±0.0
RAIN-MPA	7.4±0.7 ^b	21.2±1.7 ^{ab}	122.7±250.0	34.5±12.7 ^a	2.7±1.5	0.9±0.4 ^a	0.66±0.6 ^a	0.4±0.3	107.5±90.7	0.1±0.1	18.0±3.0 ^a	0.009±0.0
RAIN-VIS	6.7±0.3 ^{ab}	26.6±2.8 ^b	71.4±46.9	51.5±12.8 ^a	1.7±1.3	3.9±4.5 ^a	0.29±0.4	0.3±0.8 ^a	104.7±56.4	0.2±0.1	20.3±3.1 ^a	0.016±0.0 ^a
RAIN-NAS	8.3±0.3 ^a	21.1±0.9 ^a	70.3±5.9	34.0±23.0	3.1±1.2	1.2±0.6	0.08±0.1 ^a	0.7±0.6 ^a	88.3±34.8	0.3±0.6	21.4±3.5	0.003±0.0 ^a
HARVEST-MPA	8.2±0.4 ^b	12.6±3.3	147.3±284.0	19.7±13.2	2.7±1.4	1.1±1.0 ^a	0.02±0.0	0.1±0.2	122.9±102.1	0.1±0.0	63.1±31.7	0.007±0.0
HARVEST-VIS	8.3±0.4 ^a	15.7±2.5	98.2±59.8	16.2±10.7	2.8±1.2	2.0±1.1 ^a	0.06±0.1	0.1±0.0 ^a	138.5±106.8	0.1±0.0	134.2±86.5	0.008±0.0
HARVEST-NAS	9.2±0.2 ^{ab}	12.8±1.1	69.1±9.8	26.8±14.0	2.5±1.6	1.0±0.5	0.04±0.1	0.2±0.3 ^a	104.3±8.7	0.3±0.7	88.7±18.1	0.026±0.1
OVERALL MEAN	7.7±0.8	21.3±5.7	127.2±280.2	27.7±21.4	2.8±1.5	3.6±5.5	0.14±0.3	0.4±0.7	111.8±76.6	0.3±0.7	54.0±57.7	0.014±0.1

Superscript letters **a**, **b** indicate statistically significant differences ($p < 0.05$) (Kruskal-Wallis + Dunn) among MPA, VIS, and NAS within each sampling event (PRE, POST, RAIN, HARVEST). Statistically significant difference between the groups is indicated with the same alphabet letter. Letters do not extend across different events. Each event "resets" the lettering, so the same letters in different events do not imply any statistical comparison between those events. Temp = temperature; EC = electrical conductivity; TH = total hardness; TSS = total suspended solids.

4.2.2 Soil and sediment composition of the three areas

The 'soil samples' referred to here are the samples taken from an agricultural field, whereas sediment refers to soil collected from the bottom of a dam or river. Both the soil and the sediment were dried before analysis (see Section 3.3.4).

The contents of sand, silt and clay in soil and sediment compositions varied throughout the study period, as did the total organic content (TOC) (Figure 4.5). Before spraying, the sand content of the VIS sites was higher than the mean of 26.2% (DSD sediment: 91.8%, HR1 sediment: 82.8%, VB soil: 95.5%). Except for locations such as PAN1 and DGP, where levels approached 3% and 2.5%, TOC levels were generally low. Although the proportions of sand, silt, and clay remained mostly constant during the study period, TOC increased slightly in a few sites, especially in P1 soil, reaching approximately 6% after spraying.

Following the rainfall event, TOC levels in the M9 soil and DPG sediment increased, with RAIN-VIS having a higher clay content than RAIN-MPA and RAIN-NAS having a significantly different sand content. Following RAIN, there were variations in TOC levels between RAIN-NAS and RAIN-VIS.

In the HARVEST event, the proportions of sand, silt, and clay stayed constant, but the TOC levels varied more, reaching over 10% in places like PAN1. The clay content was higher than HARVEST-MPA, which was the only noteworthy difference. The physical makeup of the soil and sediment remained relatively constant overall (Table 4.2), despite some variability in the TOC, suggesting that agricultural practices and environmental factors had little effect.

Most of the sites were classified by their soil textures into the silt section of the tri-plot in Figure 4.6. Some outlier sites, such as VB, DGP, and M9, were sandy loam, X2 and X3 were purely silt, and HR3 and C2 were more silty clay.

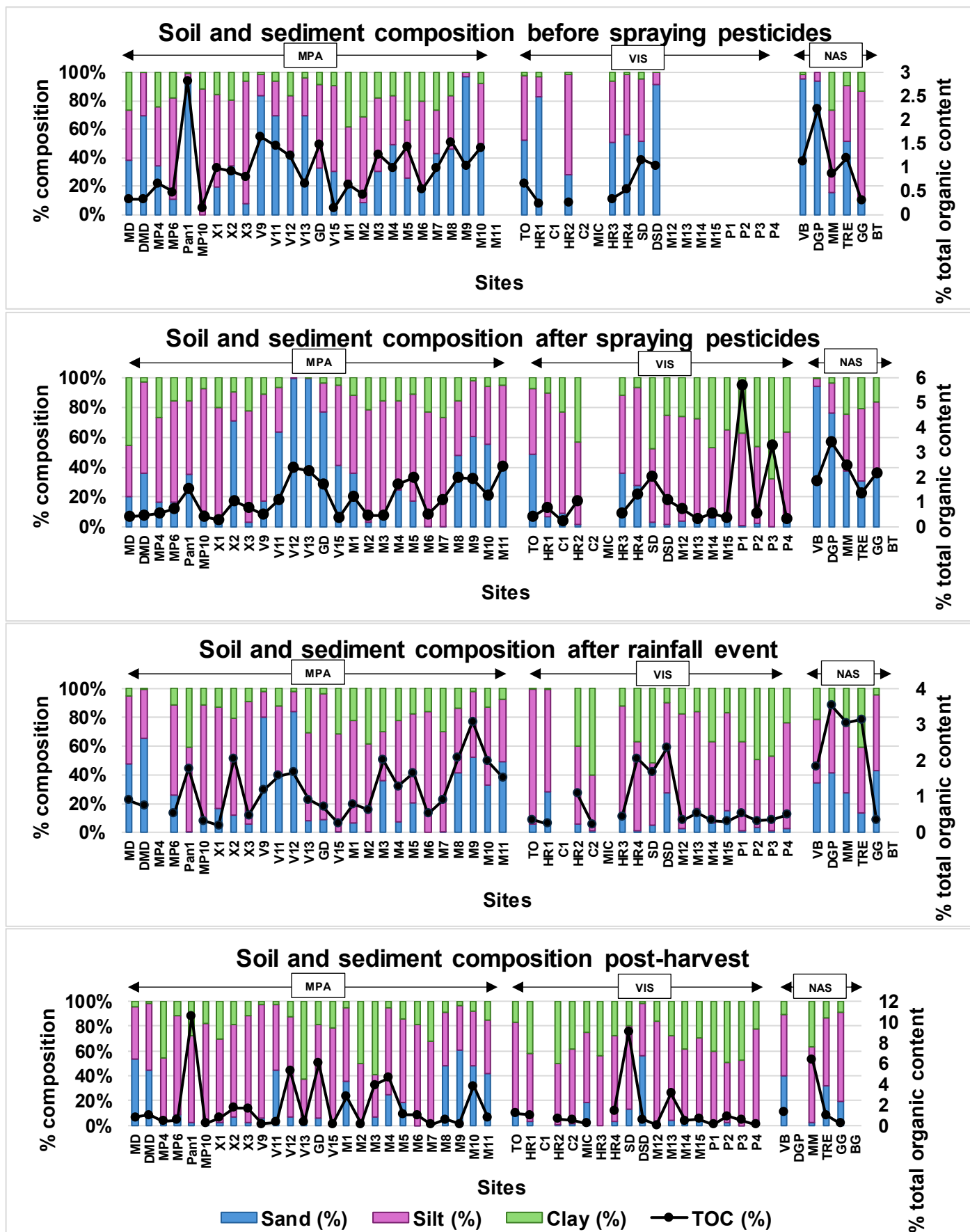


Figure 4.5: Soil and sediment composition of the sites during the growing season, before & after spraying with pesticides, after rainfall and after harvest. The sites were located in Mpumalanga (MPA), Vaalharts irrigation scheme (VIS), and non-agricultural sites (NAS). Sites without plots were inaccessible at the time of sampling.

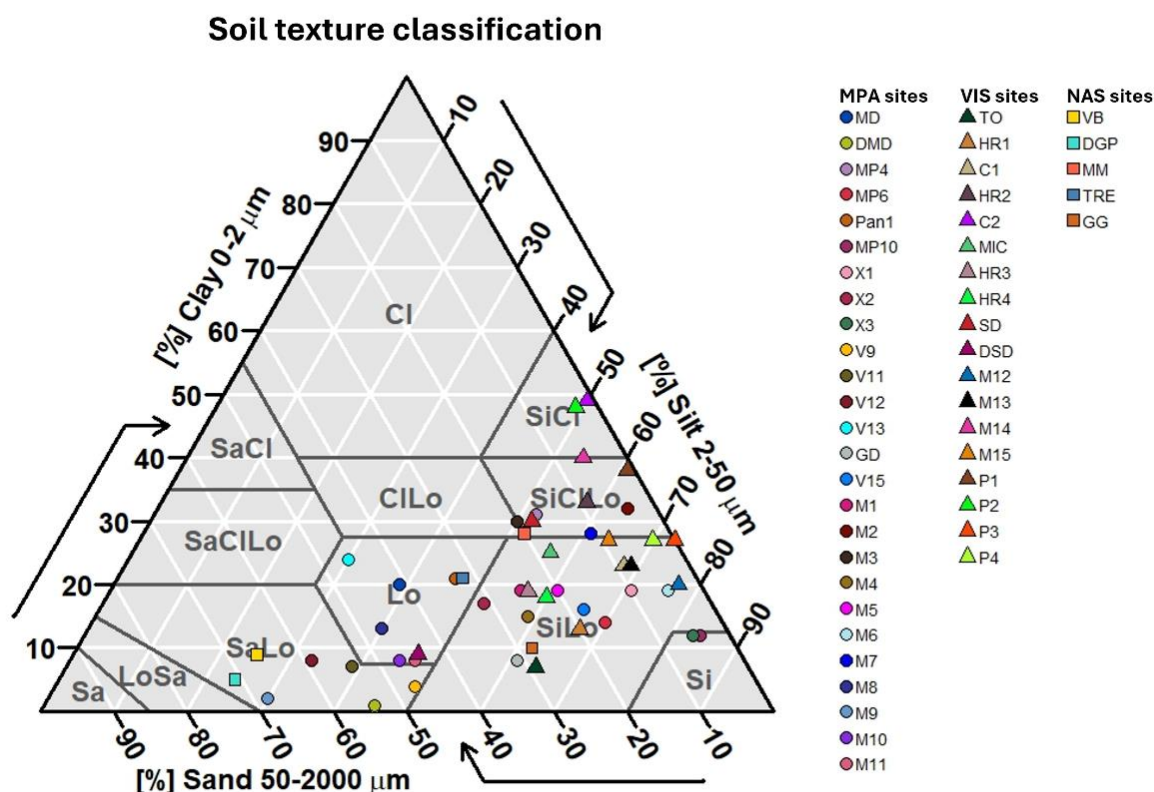


Figure 4.6: A soil texture classification tri-plot for sand, silt and clay based on the sample areas Mpumalanga (MPA), Vaalharts irrigation scheme (VIS), and non-agricultural sites (NAS). CI = clay, SiCl = silty clay, SaCl = sandy clay, ClLo = clay loam, SiClLo = silty clay loam, SaClLo = sandy clay loam, Lo = loam, SiLo = silty loam, SaLo = sandy loam, Si = silt, LoSa = loamy sand, Sa = sand.

Table 4.2: Means of all soil and sediment variables for the sampling event before spraying (PRE), after spraying (POST), after rainfall event (RAIN) and after harvest (HARVEST) per sampling area: Mpumalanga (MPA) (n=26), Vaalharts irrigation scheme (VIS) (n=17), and non-agricultural sites (NAS) (n=6).

	Sand (%)	Silt (%)	Clay (%)	TOC (%)
PRE-MPA	41.0±26.8	42.7±21.3	14.7±10.6 ^a	1.0±0.6
PRE-VIS	52.5±19.5	36.3±22.1	2.4±2.1 ^a	0.6±0.4
PRE-NAS	53.4±40.7	35.8±31.4	9.9±10.4	1.2±0.7
POST-MPA	34.7±29.6 ^b	51.5±23.7	13.0±10.2 ^a	1.1±0.7
POST-VIS	10.0±14.2 ^{ab}	58.1±12.7	30.7±17.1 ^a	1.2±1.4 ^a
POST-NAS	54.8±28.5 ^a	31.2±18.7	13.1±10.3	2.3±0.8 ^a

	Sand (%)	Silt (%)	Clay (%)	TOC (%)
RAIN-MPA	25.7±25.6	56.5±19.3	15.6±11.1	1.2±0.7
RAIN-VIS	8.2±9.0 ^a	62.6±14.6	27.8±18.5	0.7±0.7 ^a
RAIN-NAS	31.2±11.8 ^a	45.8±3.7	20.3±13.3	2.4±1.3 ^a
HARVEST-MPS	18.4±20.5	61.4±17.7	19.2±17.0 ^a	1.9±2.5
HARVEST-VIS	8.0±13.5	60.1±10.1	31.2±13.6 ^a	1.3±2.2
HARVEST-NAS	23.5±16.4	58.7±9.5	17.3±13.0	2.2±2.8
OVERALL MEAN	26.2±26.4	53.2±20.1	19.0±15.1	1.3±1.5

Superscript letters a, b indicate statistically significant differences ($p < 0.05$) (Kruskal-Wallis + Dunn) among MPA, VIS, and NAS within each sampling event (PRE, POST, RAIN, HARVEST). Statistically significant difference between the groups are indicated with the same alphabet letter. Letters do not extend across different events. Each event “resets” the lettering, so the same letters in different events do not imply any statistical comparison between those events. Grey-shaded indicates which of the sand, silt, or clay was the highest in each of the three regions.

4.2.3 Multivariate analysis of water quality and sediment composition

Multivariate analysis of water quality and sediment composition involves the simultaneous examination of multiple variables to identify patterns, relationships, and key factors that influence aquatic ecosystems. The approach enables a broad understanding of the interactions between chemical and physical characteristics of water, soil, and sediment.

The first two factors in the principal component analysis (PCA) of the water quality and sediment composition of the samples collected before spraying started explained 45.7% of the variation observed in the data (Figure 4.7). The Mpumalanga sites (MD, DMD, MP4, MP6, GD, X1, X2, X3, and V12) are grouped, showing a stronger association with the silt and clay variables. In contrast, sites DG, V11, V12, V13, and V9 were associated with PO_4^{3-} , pH, and turbidity. However, PAN1 is associated with variables TSS, TOC, and NH_4^+ . The Harts River sites (HR2, HR3 and HR4) along with non-agricultural sites (MM and GG) formed a cluster, indicating the corresponding variables and higher concentrations of Cl^- and NO_3^- . The DSD site is associated with temperature, TH, NO_3^- , and SO_4^{2-} . The HR1 and TO sites showed a lower association with water quality variables and a stronger link with clay and silt sediment. Among the non-agricultural sites, VB showed a strong association with temperature and SO_4^{2-} , while TRE exhibited a stronger association with EC and NH_4^+ .

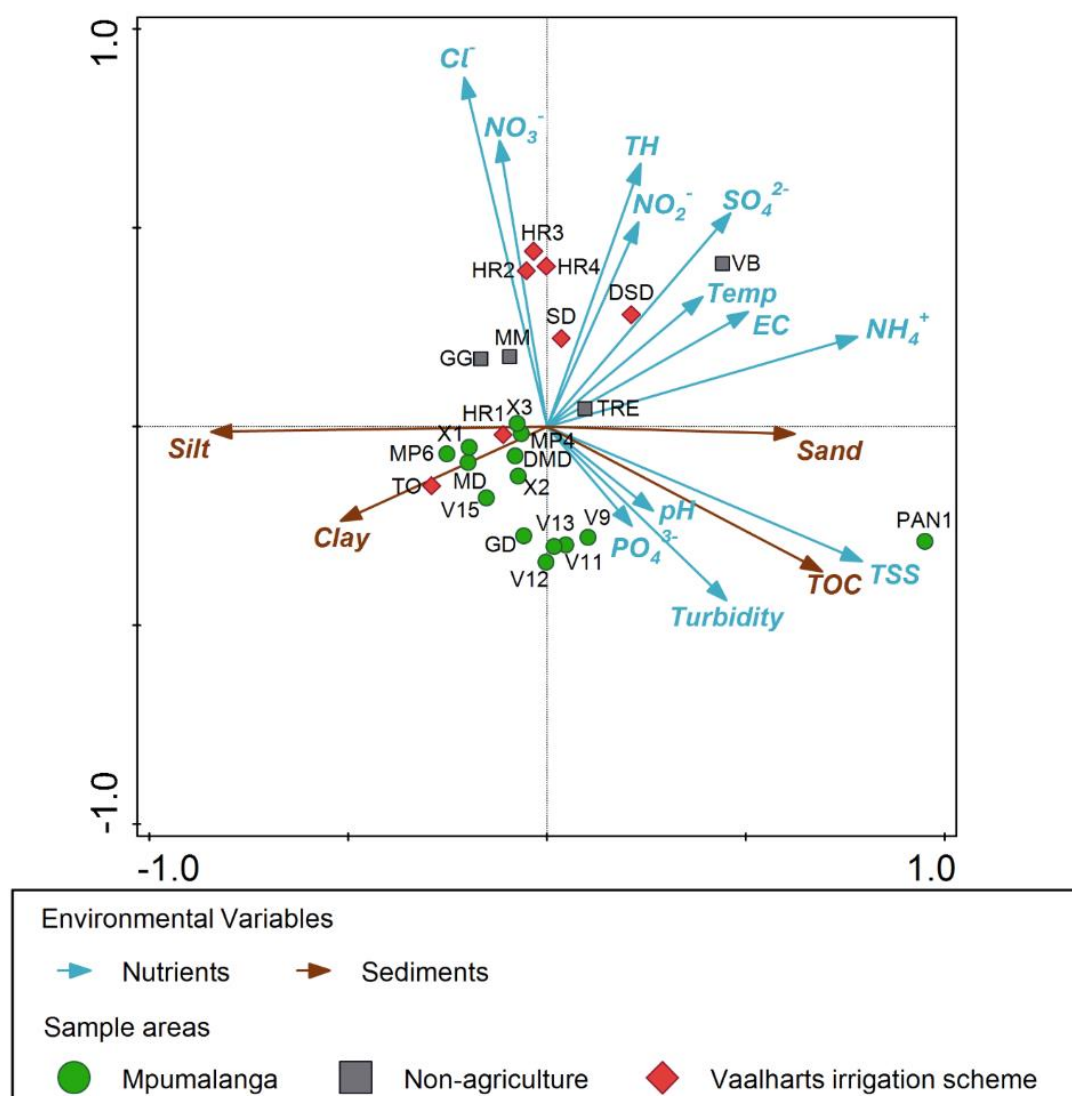


Figure 4.7: Principal component analysis (PCA) biplot of the before-spraying sampling event's water and sediment quality variable measurements. The biplot accounts for 45.7% of the variation, with 26.2% on the first axis (Factor 1) and 19.5% on the second axis (Factor 2). Nutrients (NH_4^+ , NO_3^- , NO_2^- , SO_4^{2-} , PO_4^{3-} , Cl^-) and physico-chemical (pH, electrical conductivity (EC), temperature, turbidity, total hardness (TH), total suspended solids (TSS)) are examples of water quality variables. The percentage composition of sand, silt, and clay, as well as the percentage of total organic content, are all variables of sediment quality. The missing sites are due to the lack of sampling data.

A PCA of the water and sediment variables after spraying the herbicides had Factors 1 and 2 explaining 49.5% of the variation in the data (Figure 4.8). Most sites (including MP4, MP6, X1, X3, HR2, DSD, SD, MD, DMD, C1, MP4, MP6, and TRE) were associated with temp, silt, clay, pH, and NO_3^- variables. In contrast, sites V15, V9, TO, MP10, and HR1 showed a negative association with water quality, nutrients, and physico-chemical variables. Mpumalanga sites, such as X2, V11, GD, and V13, associated with the sand variable, PAN1, were strongly

associated with the variables TSS and PO_4^{3-} , and MM and HR3 to a lesser extent, while VB showed a strong association with NO_2^- , sand, and TOC.

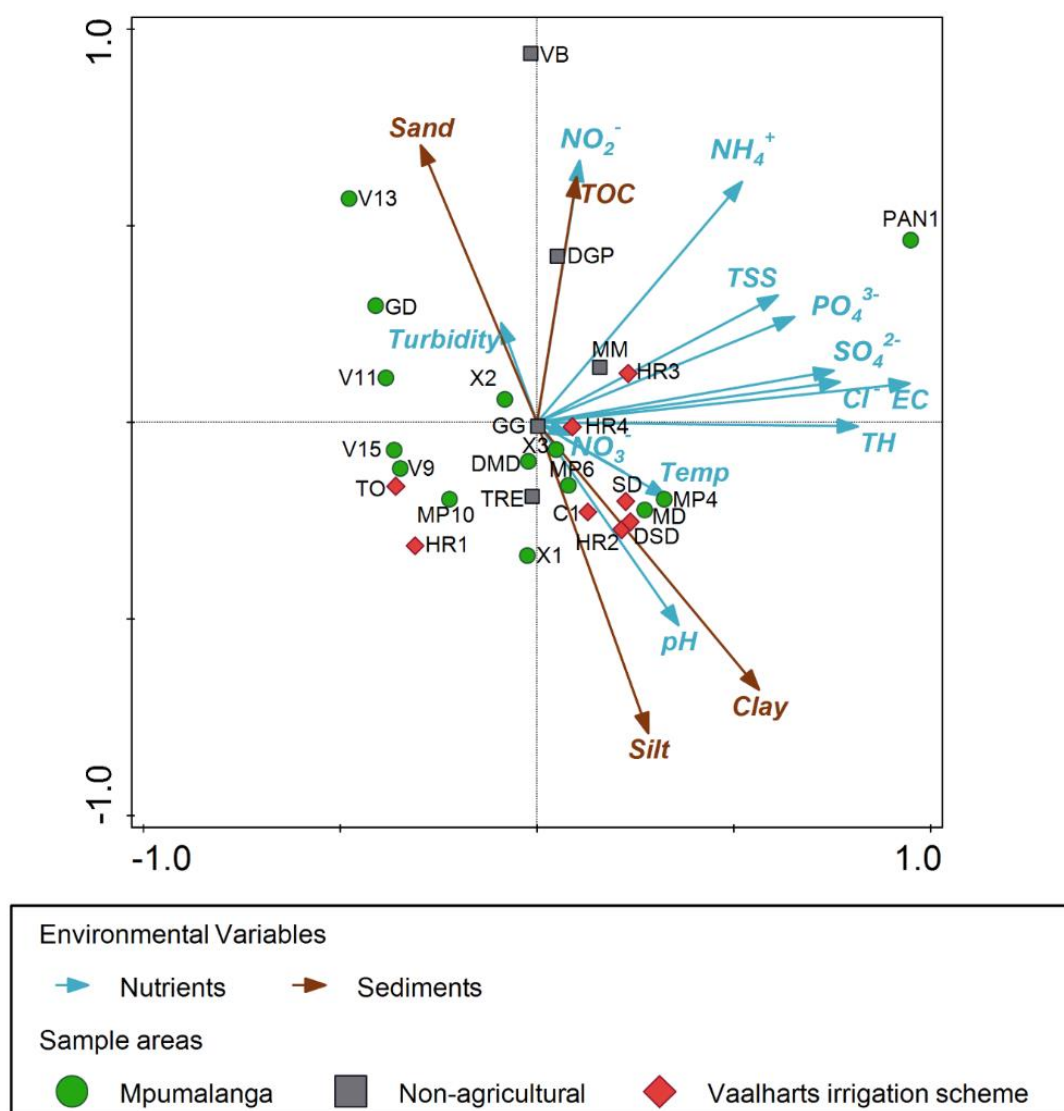


Figure 4.8: Principal component analysis (PCA) biplot of the water and sediment quality variable measurements of the post-herbicide application sampling event. The biplot accounts for 49.5% of the variation, with 28.5% on the first axis (Factor 1) and 21.0% on the second axis (Factor 2). Nutrients (NH_4^+ , NO_3^- , NO_2^- , SO_4^{2-} , PO_4^{3-} , Cl^-) and physico-chemical (pH, electrical conductivity (EC), temperature, turbidity, total hardness (TH), total suspended solids (TSS)) are examples of water quality variables. The percentage composition of sand, silt, and clay, as well as the percentage of total organic content, are all variables of sediment quality. The missing sites are due to the lack of sampling data.

Despite a greater spread among the sites depicted in Figure 4.9, after rainfall, with a variance of 42.5%, the Mpumalanga sites appear to be scattered across all vectors (arrows). Furthermore, within the Vaalharts irrigation scheme, the sites were spread to a lesser extent, showing a negative association with SO_4^{2-} , TH, pH, PO_4^{3-} and sand. Non-agricultural sites clustered together, showing a strong association with TOC and pH. In particular, PAN1 showed strong associations with SO_4^{2-} and EC.

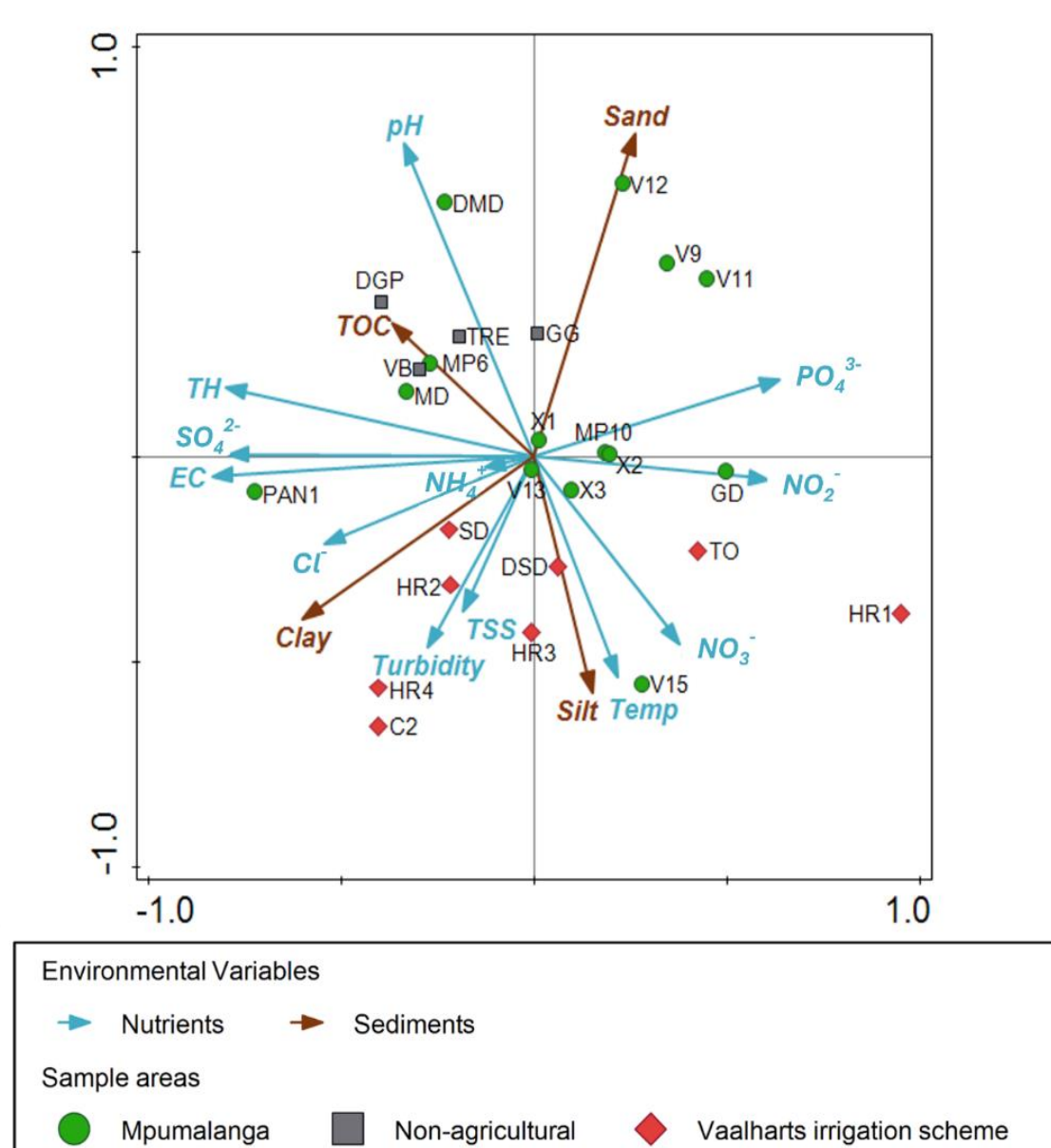


Figure 4.9: Principal component analysis (PCA) biplot after the rainfall sampling event's water and sediment quality variable measurements. The biplot accounts for 42.5% of the variation, with 25.3% on the first axis (Factor 1) and 17.2% on the second axis (Factor 2). Nutrients (NH_4^+ , NO_3^- , NO_2^- , SO_4^{2-} , PO_4^{3-} , Cl^-) and physico-chemical (pH, electrical conductivity (EC), temperature, turbidity, total hardness (TH), total suspended solids (TSS)) are examples of water quality variables. The percentage composition of sand, silt, and clay, as well as the percentage of total organic content, are all variables of sediment quality. The missing sites are due to lack of data.

The PCA explained 41.8% of the variance across all three geographical locations for the post-harvest event (Figure 4.10). In particular, the TO, HR1, GD, V12, V13, V15, MP10, X1, X2 and X3 were negatively associated with all nutrients and physico-chemical variables and were associated with silt. The MP6 clustered with NO_3^- , SO_4^{2-} and TH, while V9 and V11 displayed strong associations with turbidity. Outliers such as PAN1 exhibited a significant correlation with TSS, while sites VB, DSD, MD and DMD showed an association with NH_4^+ . The sites MP6, HR2, MM, SD, HR4, C2, and MP4 are all grouped in the statistical space around NO_3^- , SO_4^{2-} , TH, Cl^- , EC, and TSS.

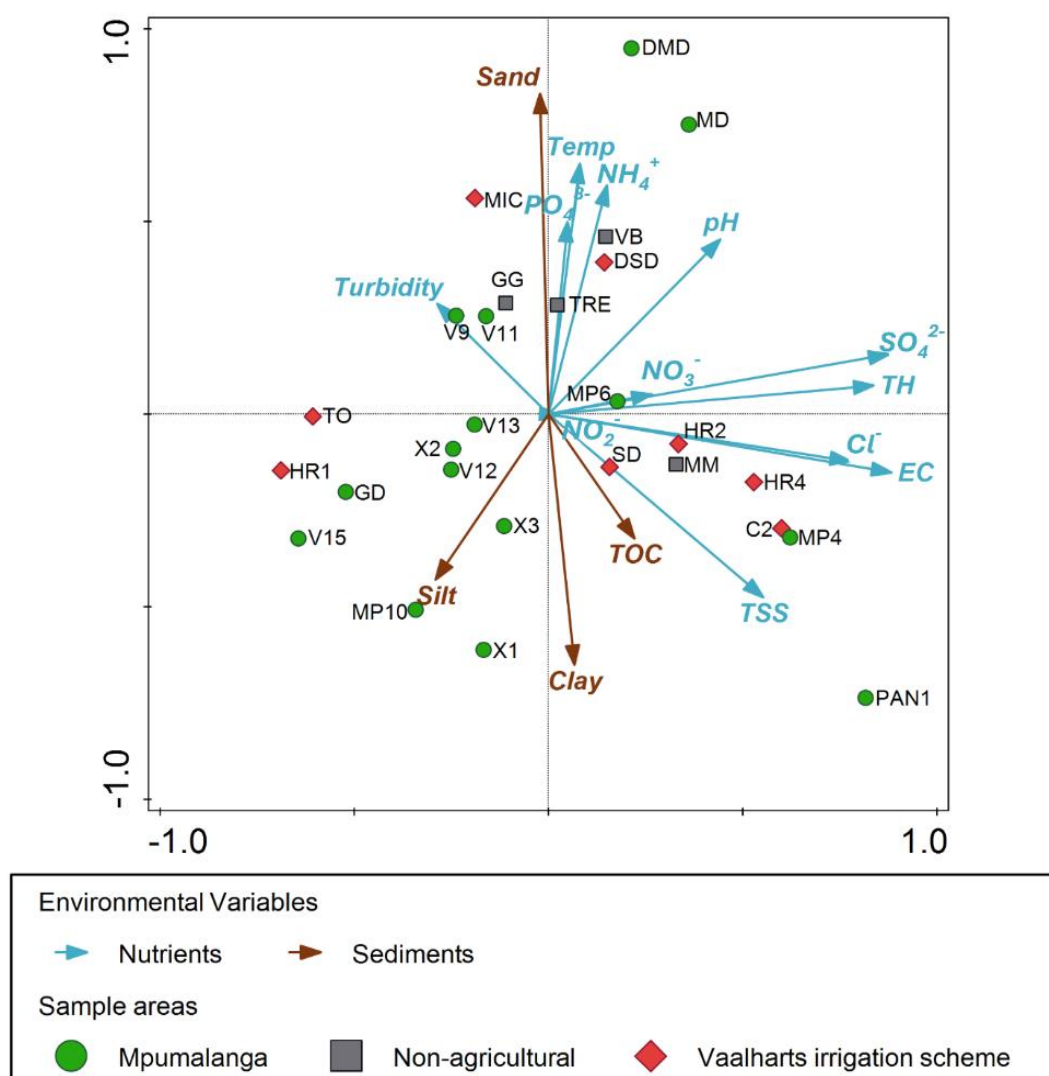


Figure 4.10: Principal component analysis (PCA) biplot after the water and sediment quality variable measurements of the harvest sampling event. The biplot accounts for 41.8% of the variation, with 23.0% on the first axis (Factor 1) and 18.8% on the second axis (Factor 2). Nutrients (NH_4^+ , NO_3^- , NO_2^- , SO_4^{2-} , PO_4^{3-} , Cl^-) and physico-chemical (pH, electrical conductivity (EC), temperature, turbidity, total hardness (TH), total suspended solids (TSS)) are examples of water quality variables. The percentage composition of sand, silt, and clay, as well as the percentage of total organic content, are all variables of the sediment quality. The missing sites are due to a lack of data.

4.3 Cation exchange capacity

The cation exchange capacity (CEC) of soil refers to the proportion of exchangeable cations capable of neutralising its charge. The CEC indicates the ability to retain nutrients or contaminants. By evaluating the CEC of the soil and sediment after the rainfall event (due to more turbulent river flows and creating mixing in sediment and infiltration in soils), we might be able to explain the behaviour of the highly polar GLY and AMPA. A high CEC might explain the lack of GLY and AMPA concentrations in an extract because it is likely that the mentioned compounds sorbed to the soil, causing difficulty in the extraction of GLY. The CEC values were highest for M10>SD>M5>DSD (Figure 4.11).

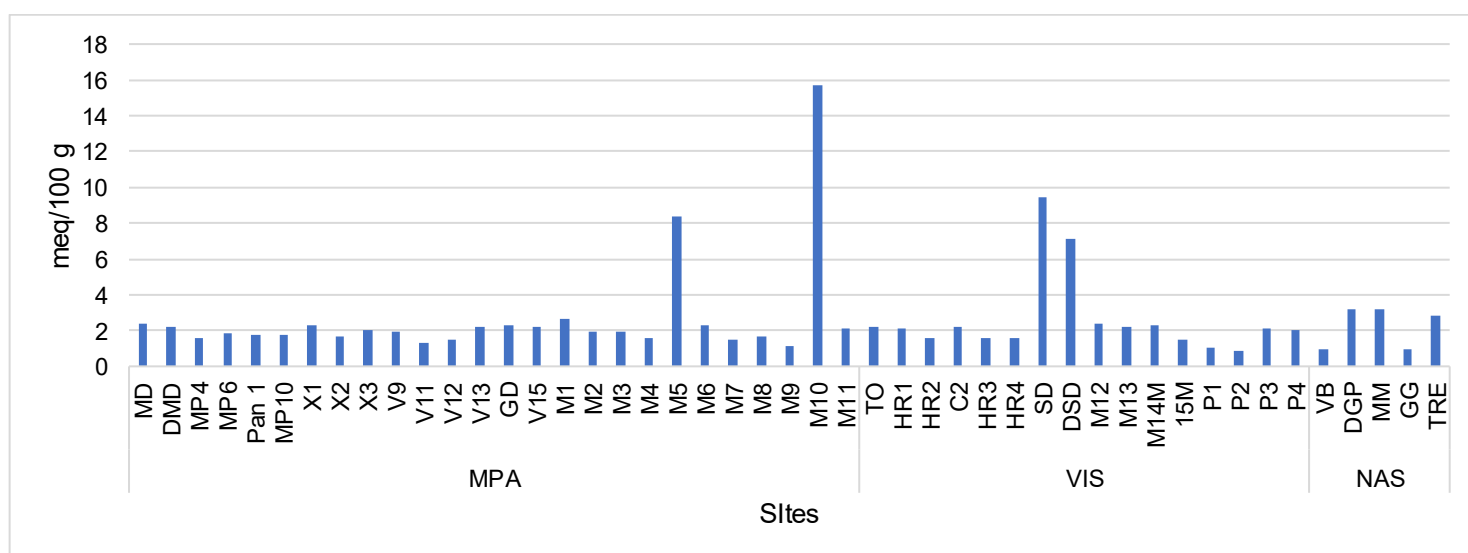


Figure 4.11: The cation exchange capacity of soil and sediment samples from the ‘after rainfall event’.

In the PCA (Figure 4.12), the CEC, pH, and TOC vectors show a strong association, indicating that these variables share some variance within the multivariate space. However, a direct Spearman correlation analysis (Figure 4.13) between CEC and TOC had a correlation of $r_s = 0.26$ ($p = 0.0909$), suggesting a weak and borderline non-significant linear association when assessed one-on-one. These findings indicate that, despite their strong association within the PCA, the CEC and TOC exhibit only limited correlation in a bivariate context.

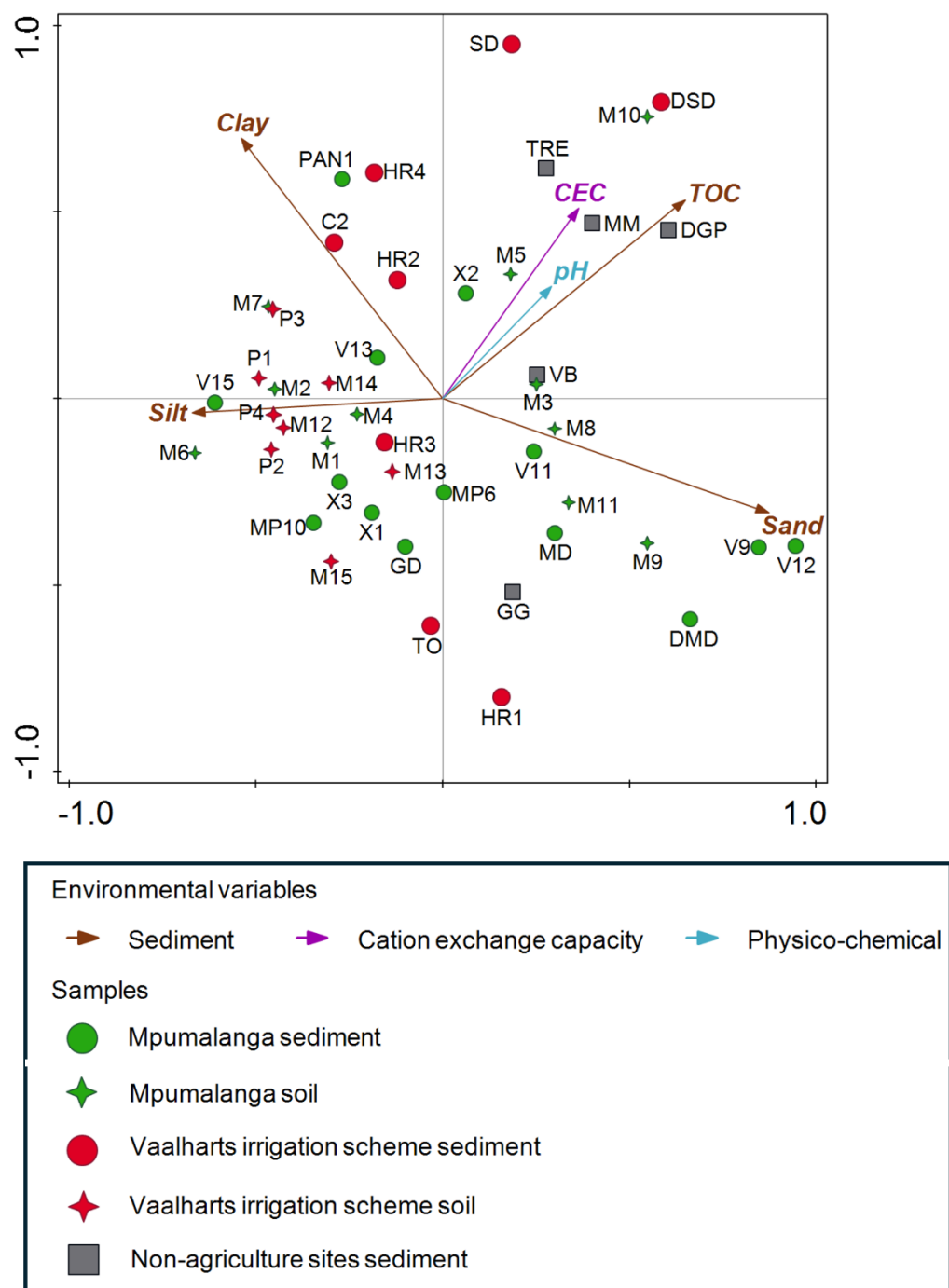


Figure 4.12: Principal component analysis (PCA) biplot after the rainfall sampling event's soil and sediment quality variable and cation exchange capacity measurements. The biplot accounts for 65.64% of the variation, with 42.51% on the first axis (Factor 1) and 24.13% on the second axis (Factor 2). The percentage composition of sand, silt, and clay, as well as the percentage of total organic content, are all variables of sediment quality. The number of exchangeable cations is a variable of the sediment's exchange capacity. The missing sites are due to lack of data.

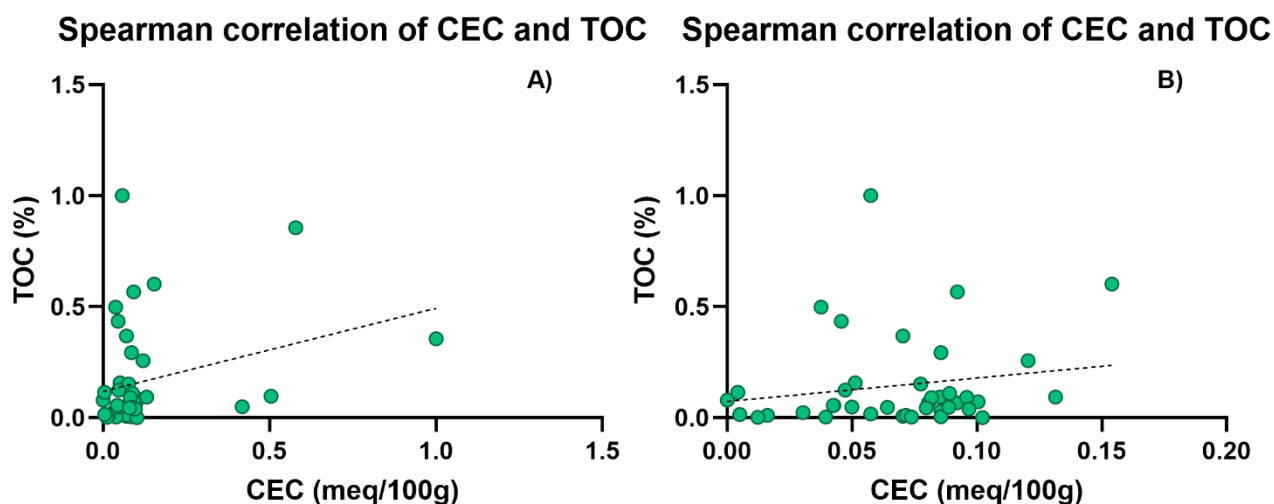


Figure 4.13: Spearman correlation between total organic content (TOC) and cation exchange capacity (CEC). Left (A) is the correlation that is not statistically significant ($p = 0.0909$; 2-tailed) with a correlation of $r_s = 0.26$ of all the data points. Right (B) is the correlation between TOC and CEC with the four outliers removed. It is not statistically significant ($p = 0.28$; 2-tailed) with a correlation of $r_s = 0.18$.

4.4 Predicting glyphosate (GLY) sorption based on CEC (cation exchange capacity) and clay

Dollinger *et al.* (2015) tested various sorption models for GLY and found that $K_d = 7.20 \times \text{CEC} - 1.31 \times \text{clay} + 24.82$ is the best predictor of sorption of GLY, as listed in Section 3.4, Equation 3.8. According to ECETOC (2025), if a K_d value is greater than 100 L/kg, the pesticide is likely to adsorb onto the soil and not leach out into solution, but it does not indicate how strong the sorption is. Knowing the K_d value for the sediment and soil of this study will help understand why no GLY was quantified and whether it was indeed the sorption that could have explained this.

The calculated K_d values of this study are shown in Figure 4.14. In the MPA region (green bars), K_d values show variability, with all but two sites, N5 and M10, below the 100 L/kg threshold, indicating higher leaching potential. The VIS region (blue bars) also shows that most sites are below the 100 L/kg threshold, suggesting that GLY leached out. Three sites exceeded the 100 L/kg threshold, C2, SD and P4, and they are indicative of GLY retention in the soil. The NAS region (orange bars) presents K_d values lower than the 100 L/kg threshold, suggesting leaching, but TRE is just shy of the 100 L/kg K_d threshold, possibly indicating limited adsorption. The VIS sites had the highest overall K_d mean of 80 L/kg, followed by NAS with a K_d mean of 67 L/kg and MPA with a K_d mean of 65 L/kg. For all the study sites, the K_d

mean was 70 L/kg. The VIS sites show a greater number of sites closer to the 100 L/kg threshold, where 9 of the 16 VIS sites are higher than the group mean of 80 L/kg.

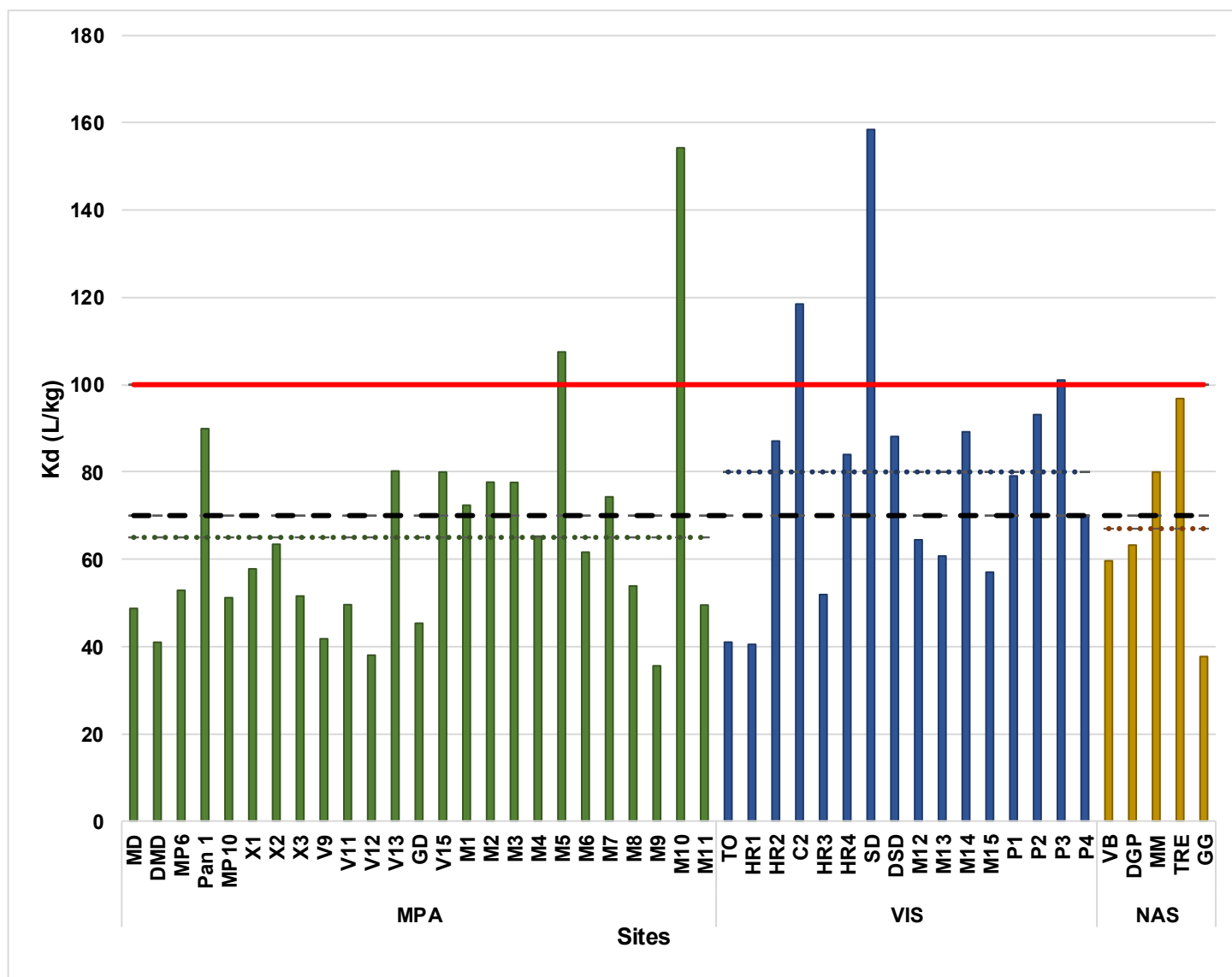


Figure 4.14: Sorption coefficient (K_d) values of glyphosate (GLY) across different sampling sites in Mpumalanga (MPA), Vaalharts irrigation scheme (VIS), and non-agricultural sites (NAS) regions of the after-rainfall event. The red line represents the K_d threshold. $K_d > 100$ means low GLY mobility, and $K_d < 100$ means high GLY mobility. The black dashed line represents the K_d mean of 70 over all the sites. The green, blue and orange dashed lines represent the mean over their corresponding-coloured sites as K_d mean = 65, 80, 67, respectively. There are no statistically significant differences between the groups (Kruskal-Wallis: $p > 0.05$).

4.5 Chemical analysis

4.5.1 Method validation

All the samples and controls were analysed at NMISA using the method described in Section 3.5. The analysis report can be seen in Appendix A. The use of UHPLC with MS/MS quantifying compounds in environmental matrices may be affected by matrix interference caused by ion suppression or enhancement (Matuszewski *et al.*, 2003). In this study, the chosen extraction process was applied to blank water and soil/sediment. The extracts containing the matrix (water, sediment, or soil) were spiked with GLY and AMPA and separated by liquid chromatography. This post-extraction method considers potential matrix interference and evaluates its effect on the chromatographic separation of GLY and AMPA (Figure 4.15 to Figure 4.18). As observed in other studies, the GLY and AMPA peaks in the water and sediment matrices had a slight tailing effect (Ulrich and Ferguson, 2021). Given the difficulty of analysing GLY and AMPA in environmental matrices, these peaks were deemed satisfactory. When several injections were performed, the peaks eluted at the same retention time and peak shape. This confirmed that the chromatography of this method was repeatable. There are some tailing and fracturing in Figure 4.18 that could be explained by the chelating effect of GLY on the metal apparatus during the analysis.



Figure 4.15: Multiple reaction monitoring (MRM) chromatogram for AMPA (aminomethylphosphonic acid) in extracted spiked water. Optimal recovery and adequate peak were observed. The two runs indicated by the repeatability of the peaks validate the analytical method.

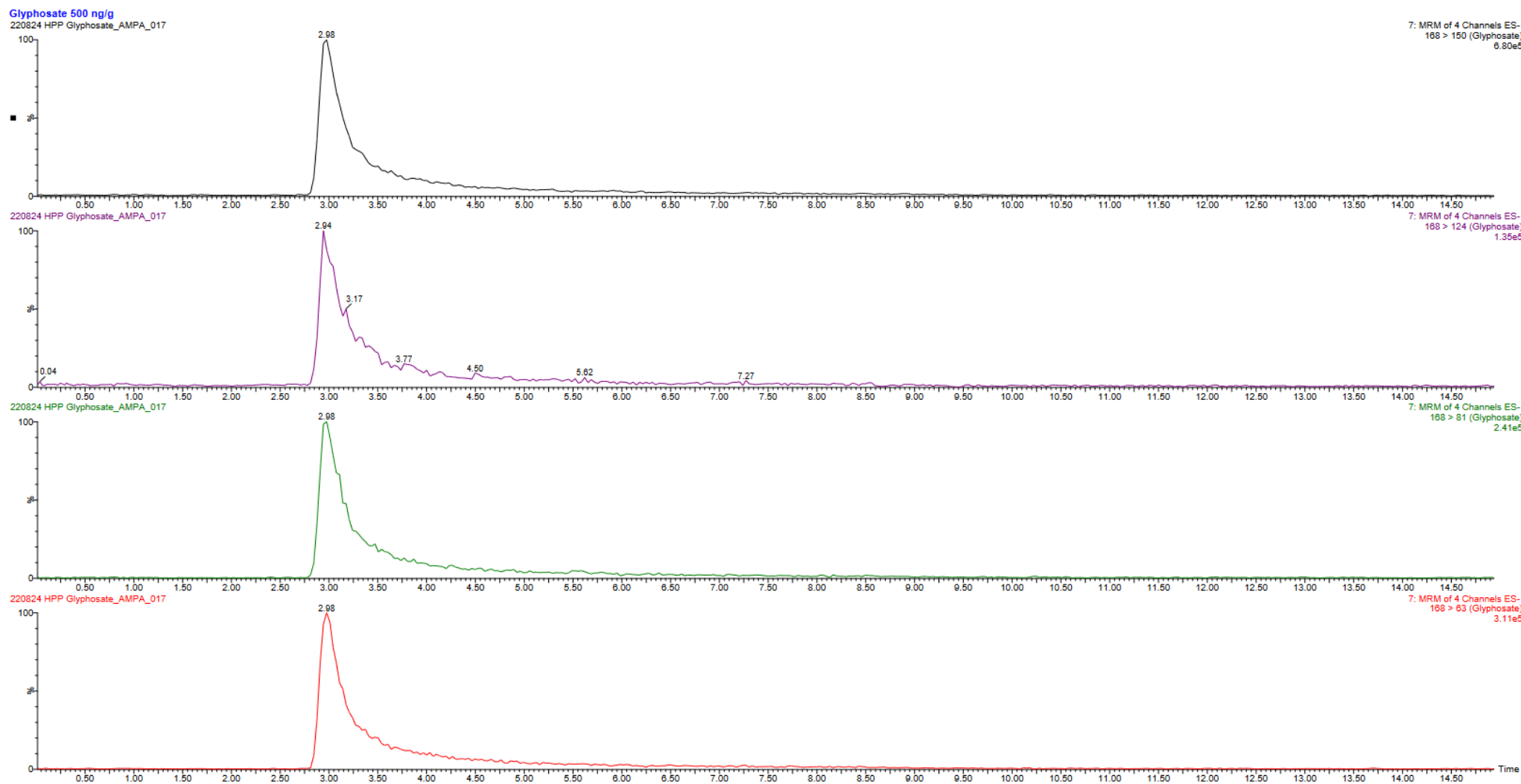


Figure 4.16: Multiple reaction monitoring (MRM) chromatograms for glyphosate in the extracted spiked water. Four runs indicated by the peaks validate the analytical method repeatability and ensure no matrix interference, but there is slight tailing.

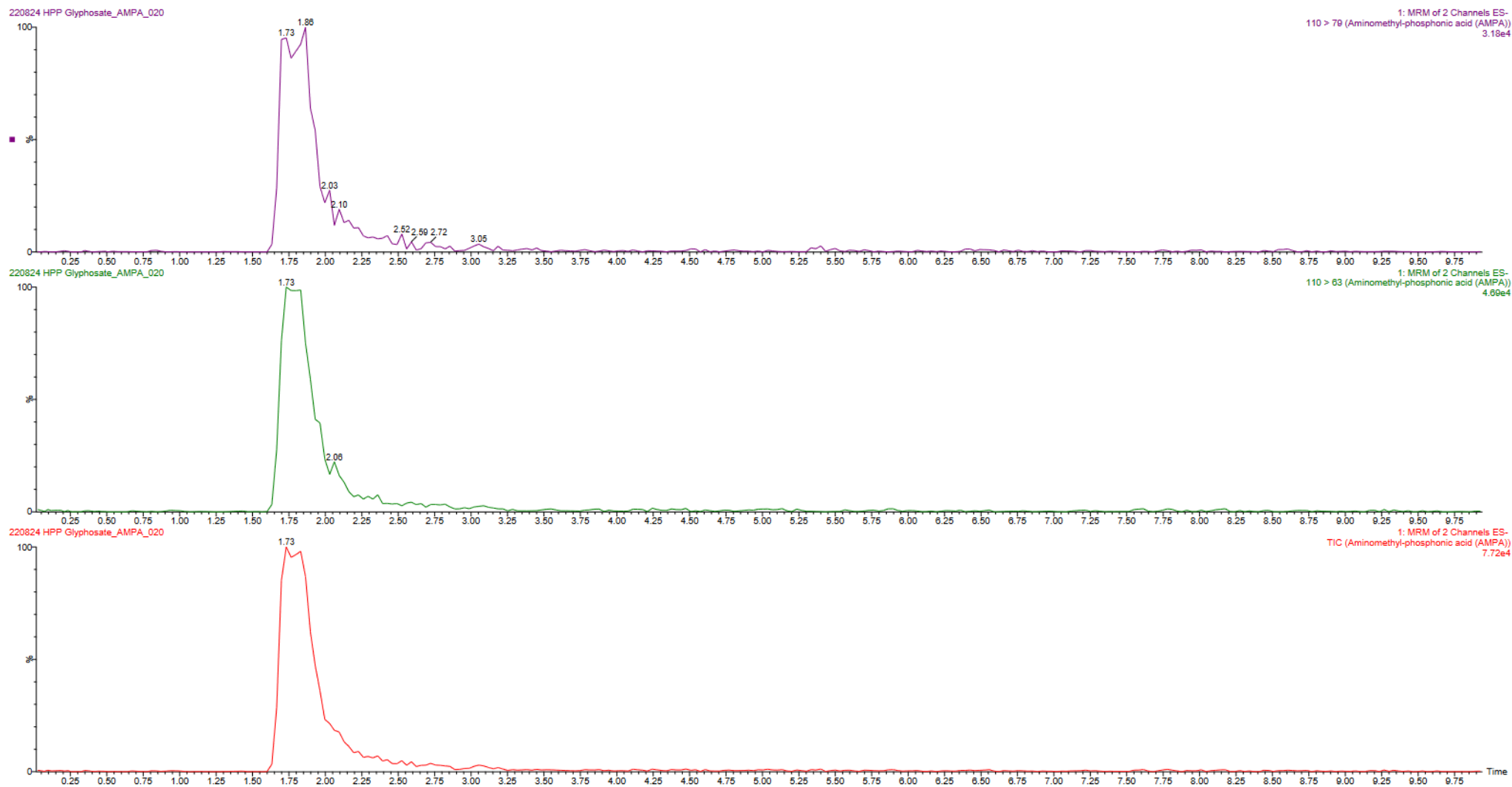


Figure 4.17: Chromatogram for AMPA (aminomethylphosphonic acid) in extracted spiked sediment showing the analyte and matrix effect. The peak splitting is present due to high concentrations of AMPA standard, but is still adequate.

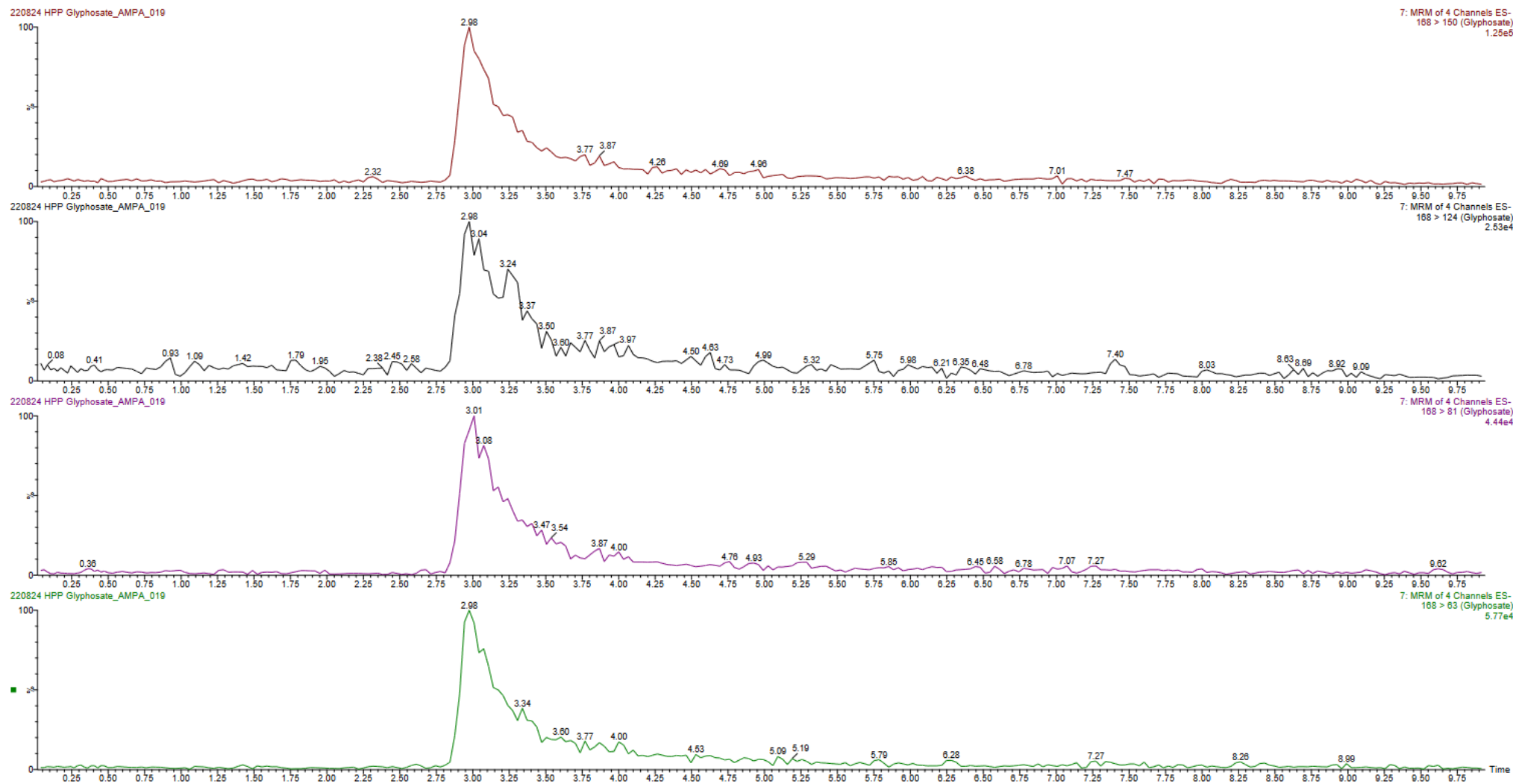


Figure 4.18: Chromatograms of glyphosate in extracted spiked sediment showing the analyte and matrix effect. Peak-shaped tailing and splitting are present and could be due to the presence of chelation during analysis. Four runs indicated by the repeatability of the peaks to validate the analytical method.

The method validation parameters for both the water and sediment/soil extraction and analysis methods produced satisfactory results (Table 4.3 and Table 4.4). The R-squared values were close to one, indicating acceptable linearity. The %RSD was less than 15%, which is appropriate for intraday and interday precision. The % recovery (accuracy) of GLY and AMPA from water was 74% and 67%, respectively. Glyphosate and AMPA were extracted from the sediment in proportions of 76% and 60%, respectively. Although recovery ranges from 60–75%, they are acceptable given the difficulty of removing GLY bound to soil particles (Reddy, 2001). Other studies have extracted GLY and AMPA at high levels (>80%) from soil and water matrices (Hanke *et al.*, 2008; Guo *et al.*, 2019; Lin *et al.*, 2022; Feltracco *et al.*, 2022; Morimoto *et al.*, 2023). Compared to the current study, these studies looked at different technologies and analysis methods. NMISA tested a few extraction methods (see Appendix A), but due to time and financial constraints, a more comprehensive list of methods was not tested.

Table 4.3: Method validation parameters for glyphosate and AMPA (aminomethylphosphonic acid) extraction and the analysis of water

Target compounds	R ²	LOD (µg/L)	LOQ (µg/L)	Inter-day precision (%RSD)	Intra-day precision (%RSD)	Accuracy (%)
Glyphosate	0.99	0.3	1.2	3.5	2.2	74
AMPA	0.99	0.2	1.1	6.5	4.2	67

R² = R square; LOD = limit of detection; LOQ = Limit of quantification; RSD = relative standard deviation.

Table 4.4: Method validation parameters for glyphosate and AMPA (aminomethylphosphonic acid) extraction and analysis of sediment and soil

Target compounds	R ²	LOD (µg/L)	LOQ (µg/L)	Inter-day precision (%RSD)	Intra-day precision (%RSD)	Accuracy (%)
Glyphosate	0.99	0.5	1.7	4.6	3.2	76
AMPA	0.99	0.5	1.5	7.5	6.2	60

R² = R square; LOD = limit of detection; LOQ = Limit of quantification; RSD = relative standard deviation.

4.5.2 The concentration of glyphosate (GLY) and AMPA (aminomethylphosphonic acid) in water, soil, and sediment samples

No GLY or AMPA concentrations were above the LOD in any of the 632 samples analysed in the water, soil and sediment matrices (Appendix C, Table S.21 to Table S.26). The additionally screened compounds include phosphonic acid, fosetyl aluminium, ethephon, 3-methylphosphinico-propionic acid (MPPA), n-acetyl AMPA, n-acetyl GLY, and n-acetyl glufosinate.

4.6 Microbial diversity

In this section, the outcome of the microbial diversity was evaluated within samples potentially affected by GBH. To determine the microbial diversity, specific samples were chosen for the after-spraying event. The site descriptions are given in Table 3.9.

4.6.1 Alpha-diversity

The bacterial diversity within the samples was analysed as a measure of species richness and evenness within the sample. Each sample site was analysed in duplicate, marked with a suffix A or B (e.g., DMDA and DMDB). In total, 21 sites (water and soil) were analysed in duplicate, with 38 successfully analysed samples of the 42 total.

The Chao1 index indicates the species richness, with higher values pointing to higher species richness (Figure 4.19). Site pairs were colour-coded to differentiate between the duplicate sets. Samples such as DMDA, DMDB, X3A, MP4A and MP4B, with ASVs close to 80, showed lower diversity and stood out as outliers. Many sites had ASV counts close to 120–140. The sites M1B, M11B, M11A, M6B, M6A, M7A, M7B, M13B, M13A, M15A, M12A and M12B had ASV counts greater than 160.

The Shannon diversity index measures both the abundance and evenness of the species present, with higher values indicating greater species diversity and evenness. An outlier with low diversity was DMDA and DMDB, with index values below 2.5 (Figure 4.20). All of the sites had a Shannon index greater than 4.0, except for X3A and X3B. Furthermore, the soil sample sites generally had higher diversity compared to the water sites, except for X3B.

Samples with high Chao1 values often also had high Shannon diversity, suggesting a correlation between estimated richness and overall diversity. The Chao1 and Shannon indices highlighted bacterial diversity at multiple sites, highlighting areas of high and low diversity.

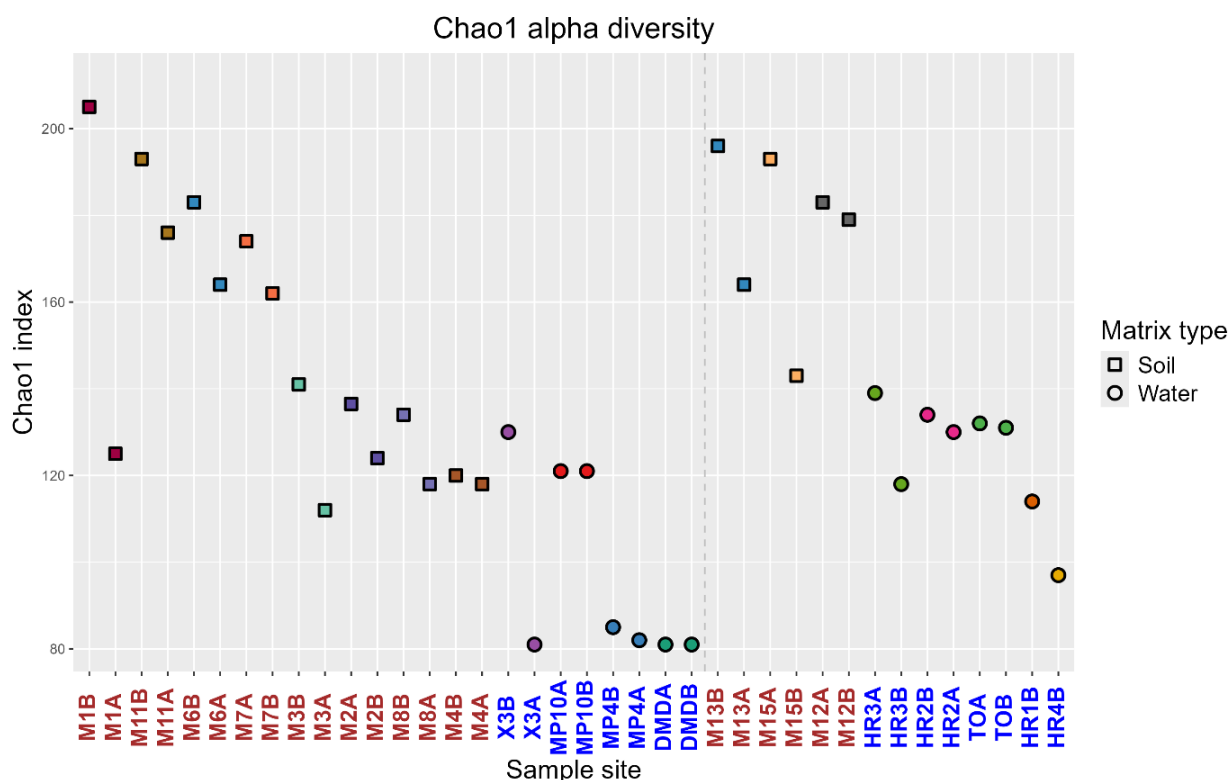


Figure 4.19: Alpha diversity expressed as Chao1 diversity. Blue labels indicate water samples and brown labels indicate soil samples. The dashed line separates the sites between Mpumalanga (MPA) (left) and Vaalharts irrigation scheme (VIS) (right). The pairs are plotted with the highest Chao1 index first (left), followed by the sample with the lowest index.

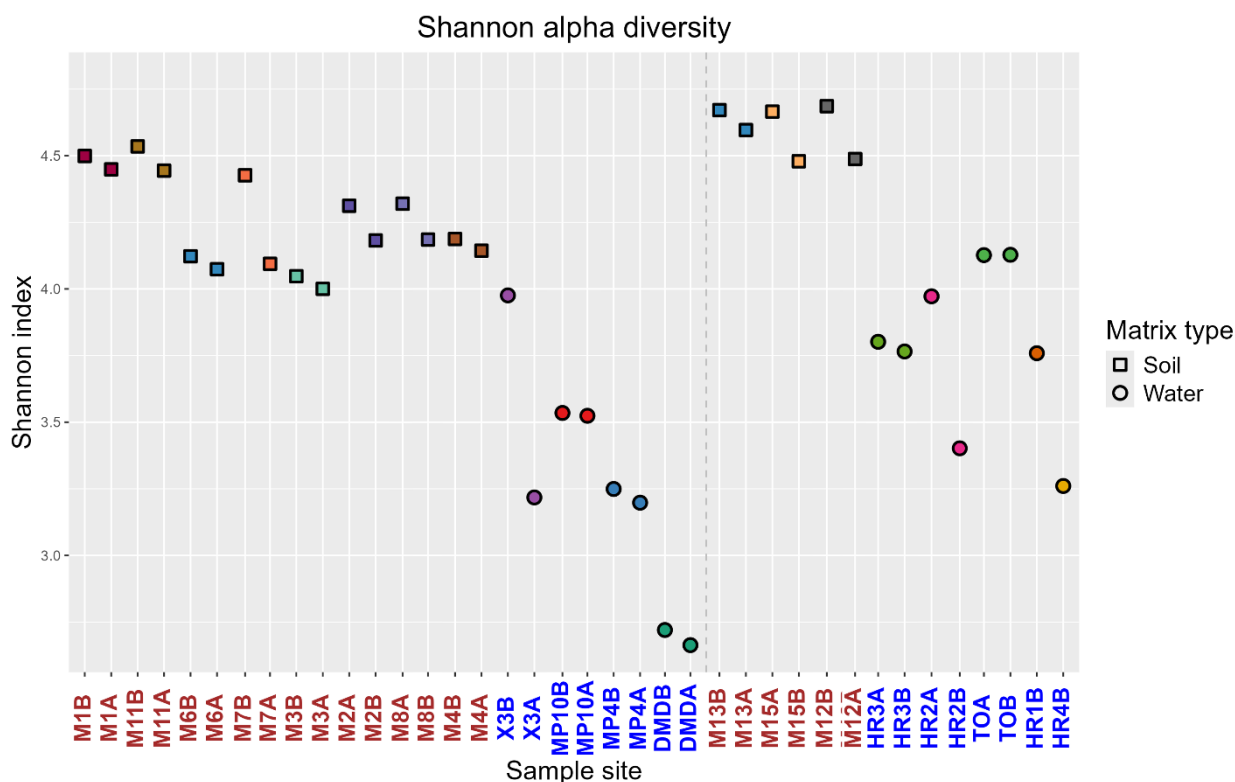


Figure 4.20: Alpha diversity expressed as the Shannon diversity index. Blue labels indicate water samples and brown labels indicate soil samples. The dashed line separates the sites between Mpumalanga (MPA) (left) and Vaalharts irrigation scheme (VIS) (right). The pairs are plotted with the highest Shannon index first (left), followed by the sample with the lowest index.

The four indicators of species diversity: Observed richness, Chao1 index, Shannon diversity index, and evenness differed statistically significantly (Kruskal-Wallis; $p < 0.05$) between soil and water samples for both the Mpumalanga (Figure 4.21) and Vaalharts sites (Figure 4.22).

The analysis of alpha diversity metrics across the Mpumalanga and Vaalharts irrigation schemes reveals distinct trends in microbial communities. In Mpumalanga (Figure 4.21), all four metrics, Observed richness, Chao1, Shannon, and Evenness, were significantly higher in soil samples compared to water samples (Kruskal-Wallis; $p < 0.05$), indicating both greater species richness and a more even distribution of microbial species in the soil. These differences suggest that Mpumalanga's soil hosts a more diverse and evenly distributed microbial community than its corresponding water samples. Similarly, in Vaalharts (Figure 4.22), the soil samples showed significantly higher values for the four-diversity metrics relative to the water samples ($p < 0.05$). This pattern indicates that, like in Mpumalanga, the Vaalharts soil contains a richer and more uniformly distributed microbial population than its corresponding water samples.

Comparison of soil samples between the two regions (Figure 4.23) revealed that the Vaalharts region had significantly higher species richness in terms of Observed and Chao1 metrics (Kruskal-Wallis; $p < 0.05$) compared to Mpumalanga, indicating a greater variety of microbial species. However, the Shannon and Evenness values did not show significant differences, suggesting a similar richness and abundance pattern in the soil of both regions. The comparison of water samples between Mpumalanga and Vaalharts (Figure 4.24) showed no significant differences in any of the alpha diversity metrics. This lack of variation suggests that the species richness and evenness in water samples are consistent between the two regions, showing a relatively consistent microbial community structure.

Although both regions show higher microbial diversity and evenness in soil compared to water samples, Vaalharts soil samples exhibited marginally higher values across all measures (Observed, Chao1, Shannon, Evenness). This suggests that the microbial communities in the Vaalharts soil may be slightly more diverse and evenly distributed than those in the Mpumalanga soil.

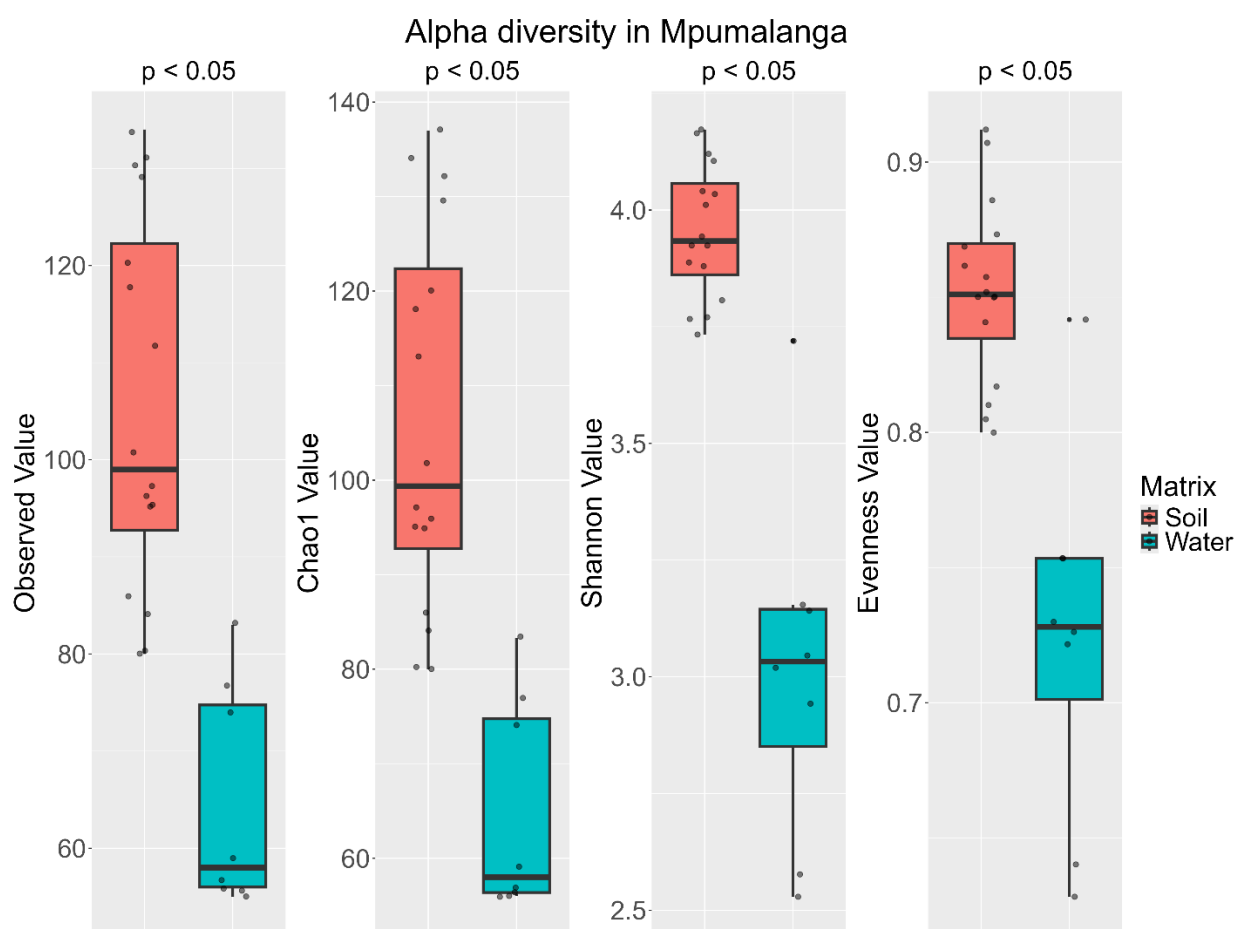


Figure 4.21: Alpha diversity of the water and soil samples expressed as boxplots. The data were rarefied, and Kruskal-Wallis was applied. Four metrics were used to describe the richness and evenness of the species in the respective matrices, such as Observed, Chao1, Shannon, and Evenness. The horizontal black line in each box represents the median value, highlighting central tendencies for each metric, while the vertical lines (whiskers) indicate the range of variability within the data, excluding outliers.

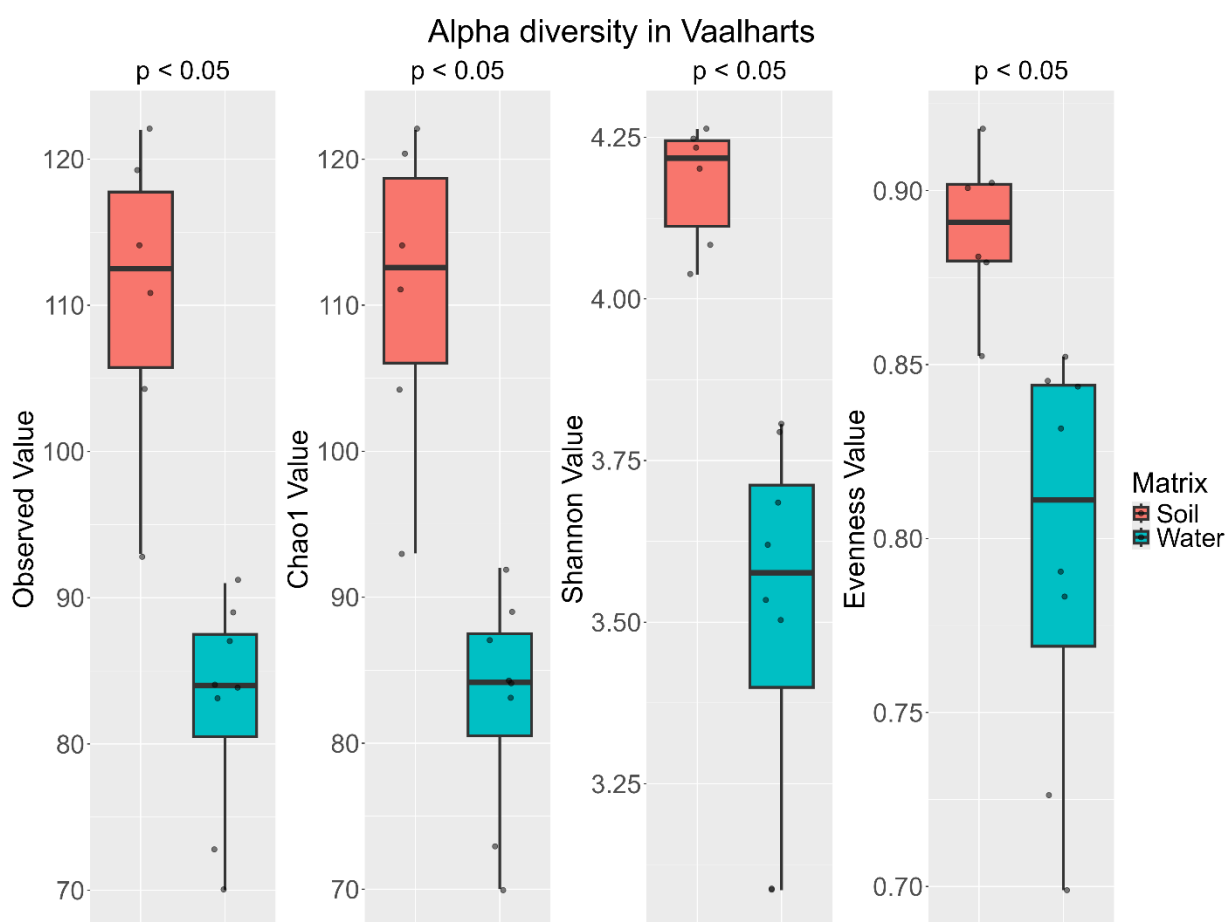


Figure 4.22: Alpha diversity of the Vaalharts irrigation scheme's water and soil samples expressed as boxplots. The data were rarefied, and Kruskal-Wallis was applied. Four metrics were used to describe the richness and evenness of the species in the respective matrices, such as Observed, Chao1, Shannon, and Evenness. The horizontal black line in each box represents the median value, highlighting central tendencies for each metric, while the vertical lines (whiskers) indicate the range of variability within the data, excluding outliers.

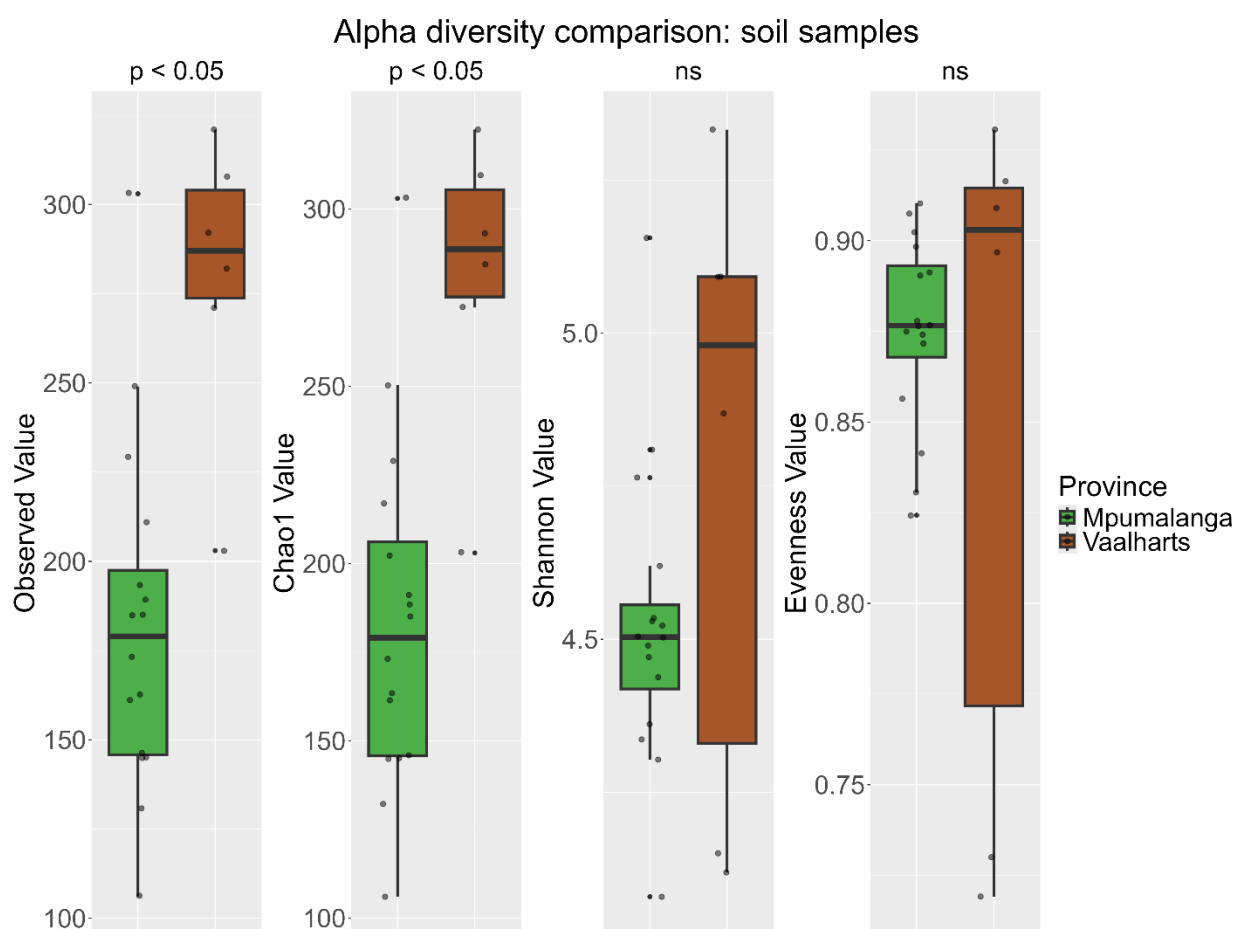


Figure 4.23: Alpha diversity of the soil samples of the Mpumalanga and Vaalharts irrigation scheme expressed as boxplots. The data were rarefied, and Kruskal-Wallis was applied. Four metrics were used to describe the richness and evenness of the species in the respective matrices, such as Observed, Chao1, Shannon, and Evenness. The horizontal black line in each box represents the median value, highlighting central tendencies for each metric, while the vertical lines (whiskers) indicate the range of variability within the data, excluding outliers.

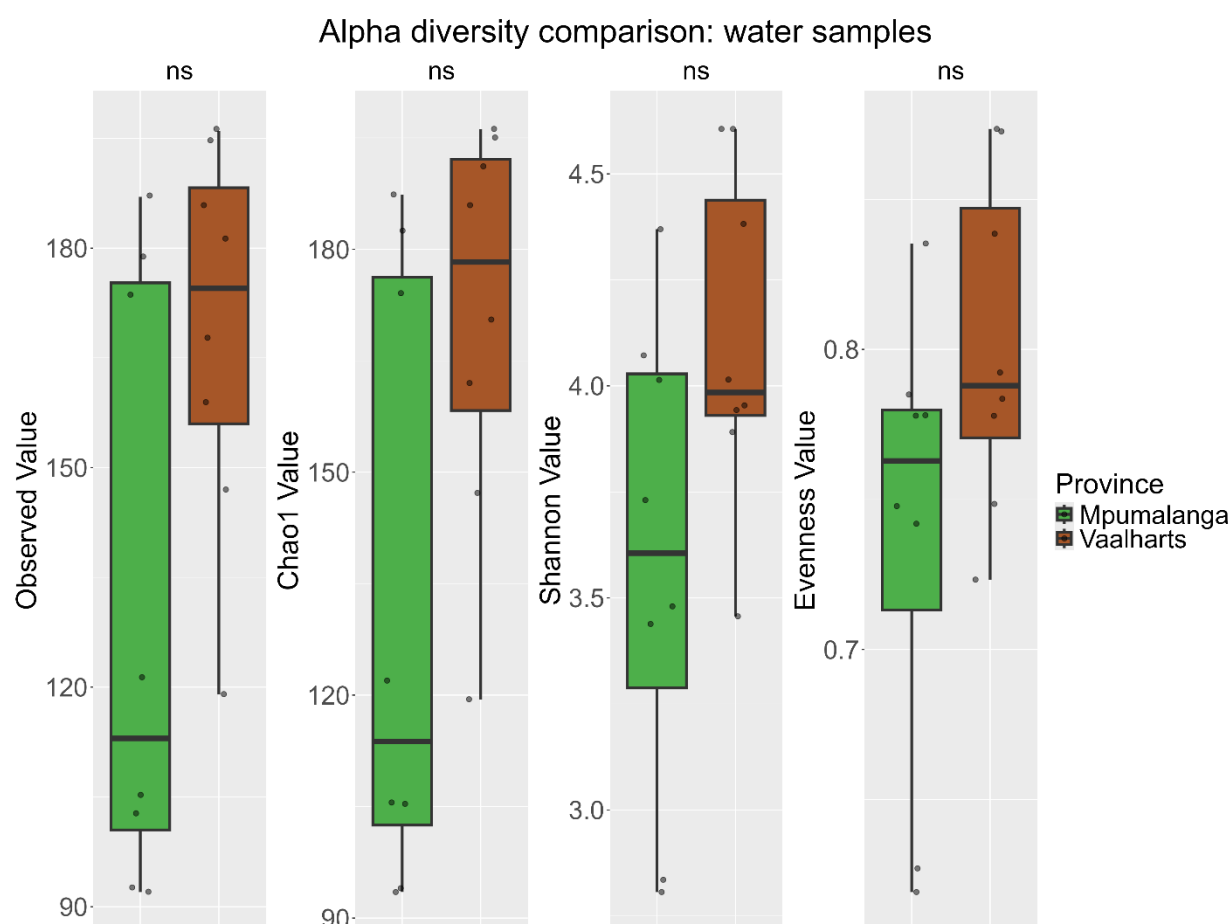


Figure 4.24: Alpha-diversity of the water sites of the Mpumalanga and Vaalharts irrigation scheme expressed as boxplots. The data were rarefied, and Kruskal-Wallis was applied. Four metrics were used to describe the richness and evenness of the species in the respective matrices, such as Observed, Chao1, Shannon, and Evenness. The horizontal black line in each box represents the median value, highlighting central tendencies for each metric, while the vertical lines (whiskers) indicate the range of variability within the data, excluding outliers.

4.6.2 Beta-diversity

Beta diversity is the measure of species differences between two or more samples from different locations. The variation in the composition of the microbial community (Figure 4.25) was illustrated using non-metric multidimensional scaling (NMDS). The percentage values (48.4% for NMDS1 and 51.75% for NMDS2) indicate the proportion of variation explained by each axis. The stress value of 0.109, which indicates how well the multidimensional data are represented in the two-dimensional plot, should generally be below 0.2, indicating a good fit. The ANOSIM R-value of 1 represents total separation, and 0 represents no separation at all. The separation between the groups is statistically significant as indicated by an R-value of 0.66 (Kruskal-Wallis; $p < 0.05$).

The microbial communities in the soil and water samples are clearly distinguished from one another, with the soil samples grouped on the right side of the plot, while the water samples are on the left (Figure 4.25). The soil groups show some separation between the Mpumalanga and Vaalharts samples, with the Vaalharts soil samples clustering close together within the spread-

out Mpumalanga soil samples, but there are three clear clusters for the water groups. These clusters indicate that the water samples from the two regions are more similar.

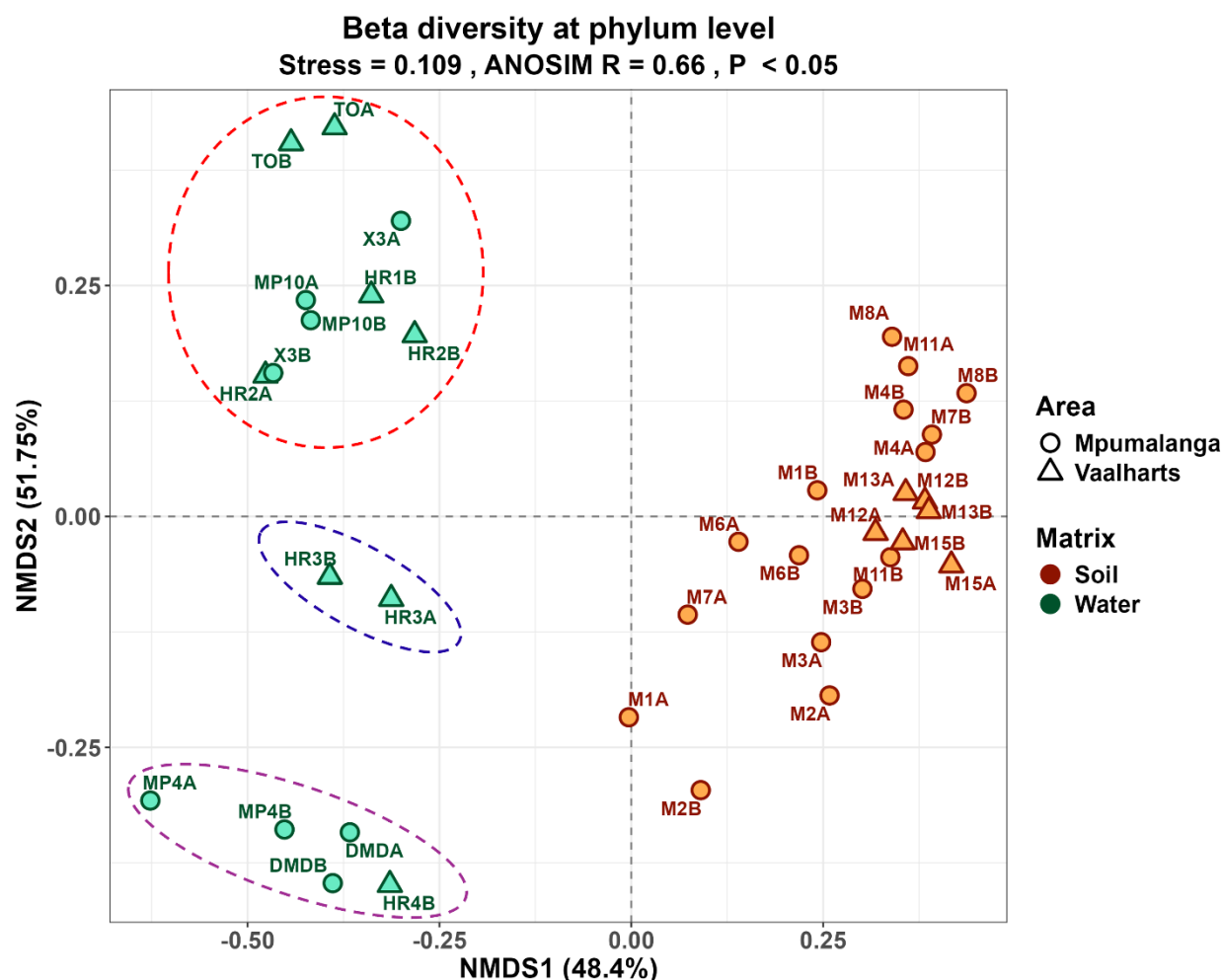


Figure 4.25: A non-metric multidimensional scaling (NMDS) showing the beta diversity between the sample regions' water and sediment matrices of the sample regions based on the taxonomic level. Molecular analysis of similarities (ANOSIM) was used to analyse the group similarities with the Bray-Curtis dissimilarity distance distribution, with a correlation of $R=0.66$.

4.6.3 Abundance

The samples were hierarchically grouped according to the Bray-Curtis dissimilarity index, as shown by the dendrograms in Figures 4.26 and Figure 4.27. Using an index that goes from 0 (complete similarity) to 1 (complete dissimilarity), the compositional variations in species abundances among the samples were evaluated. The relative abundance of bacterial taxa in the soil and water samples, respectively, is also shown in Figures 4.26 and Figure 4.27. A distinct sample is represented by each bar stacked in the figures, with each bar's colour corresponding to a different phylum. The total height is normalised to 1.0 (100%), suggesting that the proportional composition of the bacterial communities, rather than their absolute counts, is represented by the graph.

The Bray-Curtis dendrogram shows three distinct clusters (Figure 4.26), indicating compositional differences across the samples. While the top six phyla dominate the relative abundance profiles, Bray-Curtis dissimilarity reflects variation across the full community, including less abundant taxa, likely resulting from differences in both dominant and sub-dominant phyla. The samples in cluster 1 (red and brown) are highly similar to each other, with Proteobacteria being the predominant taxon. However, cluster 3 (blue) exhibits a greater proportional representation of various phyla compared to cluster 2. Cluster 2 also has more Actinobacteriota, Chloroflexi, and Firmicutes than cluster 3, but not as many Proteobacteria as cluster 1. The sites with the most similar relative abundance were M4A and M4B (which is not a surprise since they were from the same site), followed by M11B and M12A as a close pair, and M3A and M3B (again from the same sample). Samples with a higher prevalence of the Bacteroidota and Cyanobacteria taxa were displayed in Cluster 3 (blue).

The Bray-Curtis dissimilarity analysis identified four clusters in the water samples (Figure 4.27). The highest relative abundance of Bacteroidetes was found in DMDA and DMDB (both from the same site) in the red cluster. A notable observation is the prevalence of Proteobacteria at sites MP4A and MP4B. The samples in cluster 2 (green) have relatively more abundance of Proteobacteria than any of the other sites. The samples in cluster 3 (brown) have a higher abundance of Cyanobacteria, with Bdellovibrionota having the highest abundance at HR2A and X3B of the left-most branch of the brown cluster. For the HR1B, MP10A, and MP10B, the Verrucomicrobiota was highest in the fourth cluster (blue).

The phyla Proteobacteria and Actinobacteria were consistently the most prevalent in soil samples (Figure 4.26), reaching from 28–100% and comprised 72% of the bacterial community of M13B. Site M2B had the smallest fraction of 57–100% and comprised 52% at the same site. In most water samples (Figure 4.27), Proteobacteria and Bacteroidota had the highest proportion at DMDB, ranging from 18–100% comprising 82% of the bacterial community. Site HR1B had the smallest diversity of Proteobacteria and Bacteroidota, 63–100% consisting of 37% of the site. To a lesser extent, other taxa contributed to the overall structure of the community in soil and water samples. Verrucomicrobiota, Cyanobacteria and Chloroflexi are some of these taxa.

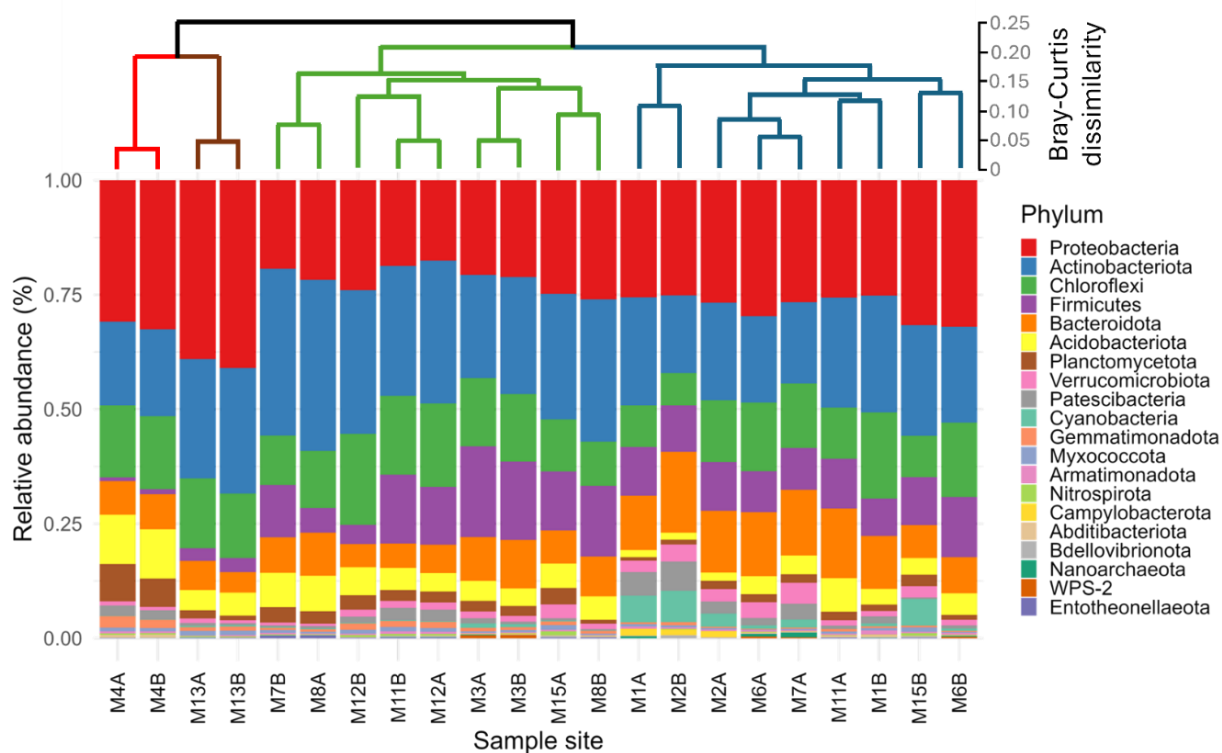


Figure 4.26: Bray-Curtis dissimilarity dendrogram indicating the related bacterial communities per site on the phylum taxonomic level of the soil samples. Relative abundances are expressed as a proportional percentage of the overall community of the top 20 most abundant phyla.

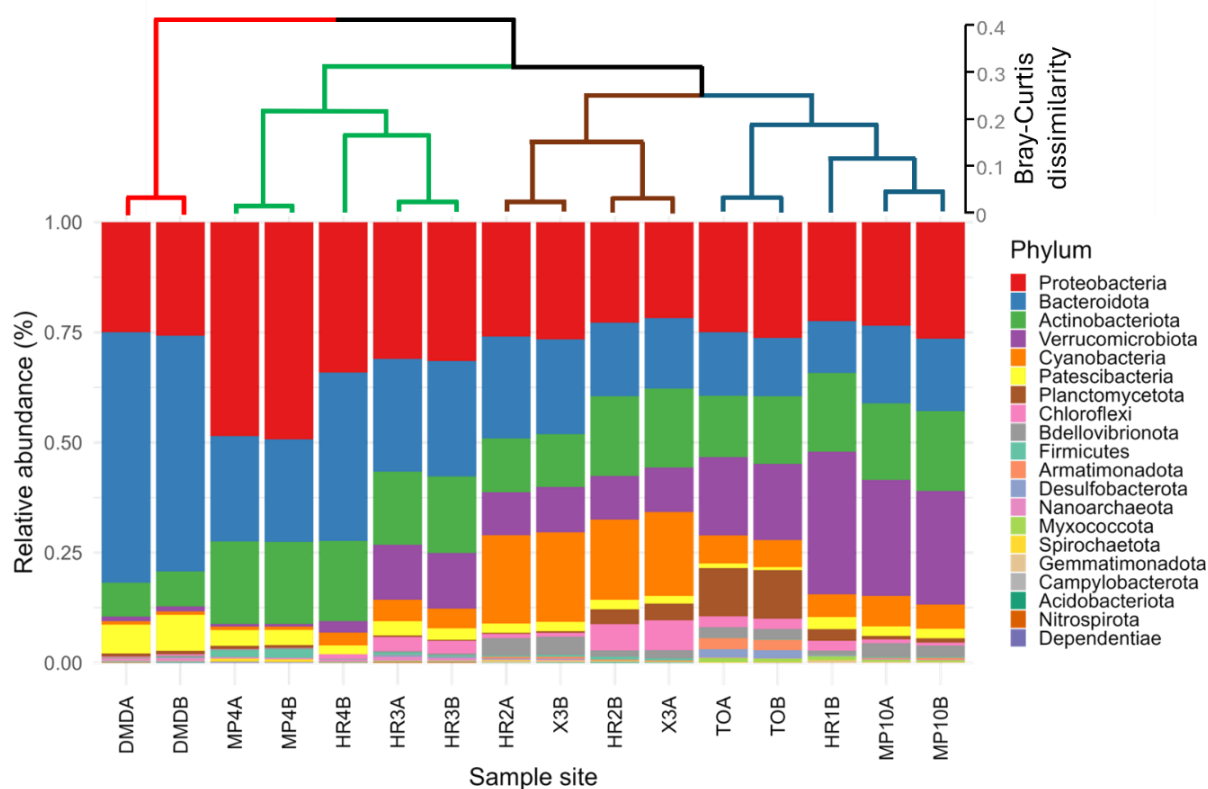


Figure 4.27: Bray-Curtis dissimilarity dendrogram indicating the related bacterial communities per site on the phylum taxonomic level of the water samples. Relative abundances are expressed as a proportional percentage of the overall community of the top 20 most abundant phyla.

4.6.3.1 Bacterial abundance of glyphosate degraders

A subset of the identified sequence counts was associated with bacterial species known for degrading GLY according to the literature (Table 4.5). As previously mentioned, the 16S sequencing identified the organisms to the genus level. The highest count was for the *Flavobacterium* and *Bacillus* genera (44 841 and 43 305, respectively), followed by *Arthrobacter* genera (20 766) and *Pseudomonas* genera counts (10 493). There was not enough information to rule out any of the other *Arthrobacter*, *Bacillus*, *Flavobacterium*, or *Pseudomonas* genera as non-GLY-degrading bacteria; therefore, it is worth mentioning. Other genera, such as *Ochrobactrum* and *Achromobacter*, showed much lower counts, 138 and 32, respectively.

Table 4.5: List of environmental bacteria capable of degrading glyphosate and the bacterial counts obtained in the 16S sequencing of the current study

Bacterial organism	Source	Outcome	16S sequence counts****	Reference
<i>Flavobacterium</i> spp.	Isolated from GLY-contaminated industrial-activated sludge (USA)	Focused on GLY degrading capabilities	44 841	Balthazor and Hallas (1986)
<i>Achromobacter</i> spp.	Isolated from GBH-applied commercial soil (Russia)	The microbes degraded 60% of GLY (500 mg/L) via the sarcosine pathway.	32	Ermakova <i>et al.</i> (2017)
<i>Bacillus cereus</i> *	Isolated from GLY-polluted soil (China)	The microbes degraded 94% of extremely high concentration GLY (6 000 mg/L) within 5 days.	0	Fan <i>et al.</i> (2012)
<i>Ochrobactrum intermedium</i>	Isolated from GLY-contaminated soil (Pakistan)	The microbe is capable of completely degrading GLY 500 mg/L. completely within 4 days.	138	Firdous <i>et al.</i> (2018)
<i>Acetobacter</i> spp.	Isolated from GLY-contaminated rice field (Nigeria)	The microbes tolerated up to 250 mg/mL of GLY and utilised carbon and phosphorus as the sole source.	3	Moneke <i>et al.</i> (2010)
<i>Pseudomonas fluorescens</i> **			0	
<i>Arthrobacter atrocyaneus</i> ***	Isolated from their laboratory (Germany)	Focused on GLY degrading capabilities	0	Pipke and Amrhein (1988)
<i>Bacillus subtilis</i> *	Isolated from wheat, maize, rice & sugarcane GLY-treated agricultural soil (India)	Microbes are capable of degrading 87% of GLY (250 mg/L) via the sarcosine metabolic pathway.	0	Singh <i>et al.</i> (2019)
<i>Rhizobium leguminosarum</i>			0	
<i>Streptomyces</i> spp.			3 990	

Bacterial organism	Source	Outcome	16S sequence counts****	Reference
<i>Bacillus subtilis</i> *	Obtained from the rhizosphere of a pepper plant (China)	Microbes can degrade up to 65% of the initial GLY concentration.	0	Yu <i>et al.</i> (2015)
<i>Pseudomonas</i> spp.	Isolated from GLY-contaminated soil (China)	Three bacterial strains capable of degrading 81% of GLY (500 mg/L).	10 493	Zhao <i>et al.</i> (2015)

*Many *Bacillus* genera counts (43 305) but none that were species-specific. **Many *Pseudomonas* genera counts (10 493) but none that were species-specific. Many. ****Arthrobacter* genera counts (20 766) but none that were species-specific. ****16S sequence counts are for all the sites of the current study.

4.7 Risk assessment

Proteobacteria, Actinobacteriota and Firmicutes were among the most abundant phyla in water and soil samples (Figure 4.26 and Figure 4.27). The pathogens listed in Table 3.10 are among the mentioned phyla. Six of the eleven pathogens modelled in the QMRA belong to Proteobacteria such as *Klebsiella*, *Pseudomonas*, *Legionella*, *Salmonella*, *Escherichia* and *Campylobacter*. The Firmicutes phylum has 4 pathogens, which include *Bacillus*, *Staphylococcus*, *Clostridium* and *Enterococcus*, and finally, in the Actinobacteriota phylum, there is *Mycobacterium*. Together, these three phyla supply the entire pathogen pool carried forward into the QMRA.

4.7.1 Dose and exposure assessment

The probability of pathogenic bacteria causing infections is expressed as the risk of infection when 10%, 50%, and 90% of pathogenic bacteria are inhaled or ingested. Sometimes, the risk of infection is 100% even if 10% of the pathogenic bacteria are ingested or inhaled, depending on the virulence of the pathogen. The bacterial pathogen dose/ concentrations per site are expressed in Appendix D, Table S27 to Table S29. Pathogens that cause infections by being ingested (Figure 4.28), such as *Escherichia coli*, *Clostridium* spp. and *Campylobacter* spp., had the lowest probable infection rates. However, pathogens such as *Enterococcus* spp., *Salmonella* spp. and *Pseudomonas* spp. presented higher probable infection risks at three, five, and nine of the 20 sites (chosen for eDNA sequencing), respectively. In contrast, the inhaled airborne pathogens in Figure 4.29, *Legionella* spp., had a 100% probability of infection at 10 sites. *Mycobacterium* spp. consistently showed a high probability of infection at all sites, except HR4.

The QMRA results are shown in Figure 4.30 for *Klebsiella* spp. *Bacillus* spp. and *Staphylococcus* spp. in various sites revealed distinct patterns in the risk of infection depending on the site category (VIS or MPA) and the transmission route (inhalation or ingestion). In general, infection risks through ingestion are low, particularly for *Klebsiella* spp. and *Staphylococcus* spp., where probabilities are close to zero at all sites. However, the route of inhalation presents higher risks,

particularly for *Bacillus* spp. and *Staphylococcus* spp. at MPA sites, where the probability of infection reaches 0.25 and 1.0, respectively. The results consistently show that MPA sites have higher infection risks compared to VIS sites, highlighting the difference between these areas. *Bacillus* spp. and *Klebsiella* spp. poses a higher risk via inhalation at MPA sites, compared to ingestion. The findings suggest that probable infection by inhalation is more likely for these pathogens, compared to ingestion, especially at MPA sites.

In both water and soil samples (Figure 4.31), pathogens that are primarily related to ingestion, such as *Salmonella* spp., *Enterococcus* spp., *Campylobacter* spp., and *Clostridium* spp., show low infection probabilities. However, for waterborne pathogens, the probability of infection is nearly 1.0 (100%) for *Bacillus* spp., *Pseudomonas* spp., *Legionella* spp., and *Mycobacterium* spp. The wide uncertainty range of *Bacillus* spp. variability (ingested) in water samples points to a possible impact of changing conditions on infection risk. Pathogens that can be inhaled or consumed, such as *Staphylococcus* and *Klebsiella* species, also exhibit consistently low infection probabilities, indicating that they pose a lower risk than pathogens that are only associated with inhalation.

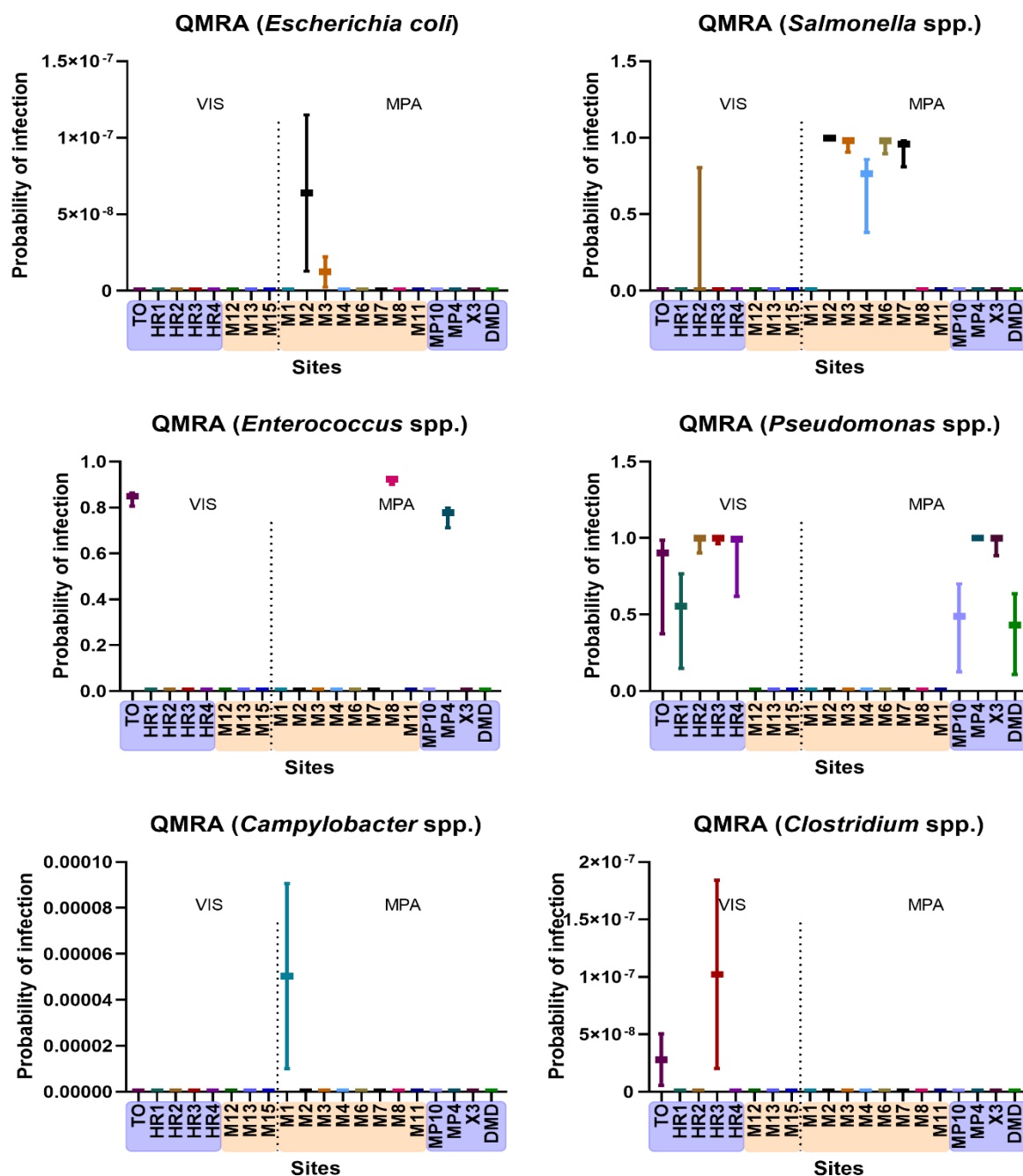


Figure 4.28: The risk of Quantitative Microbial Risk Assessment (QMRA) infection between sites compares the probable infection due to exposure to various pathogens when ingesting contaminated water or soil, where 1.0 represents a 100% probability of infection. The bars represent 10%, 50% and 90% of the total dose of bacteria at their respective sites when ingested. The sites are divided according to Vaalharts irrigation scheme (VIS) and Mpumalanga (MPA) and distinguish the water sites (blue) and the soil sites (light brown).

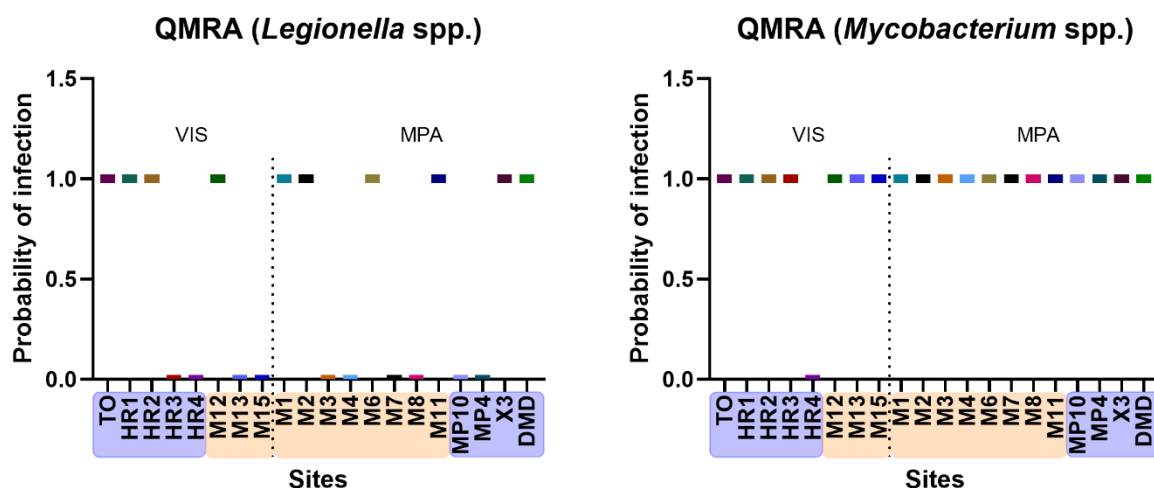


Figure 4.29: The risk of Quantitative Microbial Risk Assessment (QMRA) infection at different sites by comparing the probable risk of infection from exposure to various pathogens when inhaling aerosols from water or soil, where 1.0 represents a 100% probability of infection. The bars represent 10%, 50% and 90% of the total dose of bacteria at their respective sites when inhaled. The sites are divided according to Vaalharts irrigation scheme (VIS) and Mpumalanga (MPA) and distinguish the water sites (blue) and the soil sites (light brown).

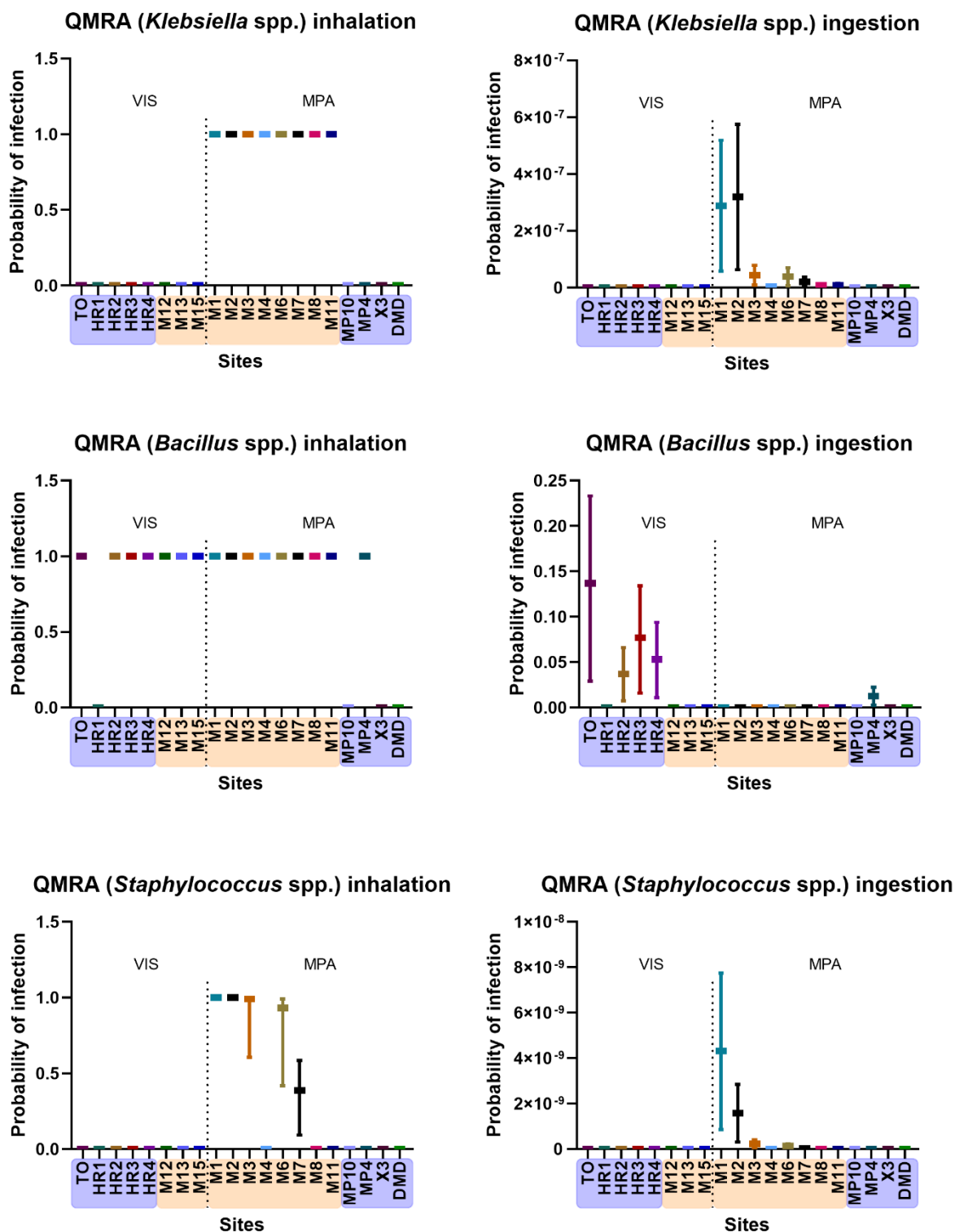


Figure 4.30: Infection risk between sites comparing the risk of probable infection by inhalation (left) and ingestion (right) transmissions. The bars represent 10%, 50% and 90% of the total dose of bacteria at their respective sites when ingested or inhaled. The 1.0 represents a 100% probability of infection. The sites are divided according to Vaalharts irrigation scheme (VIS) and Mpumalanga (MPA) and distinguish the water sites (blue) and the soil sites (light brown).

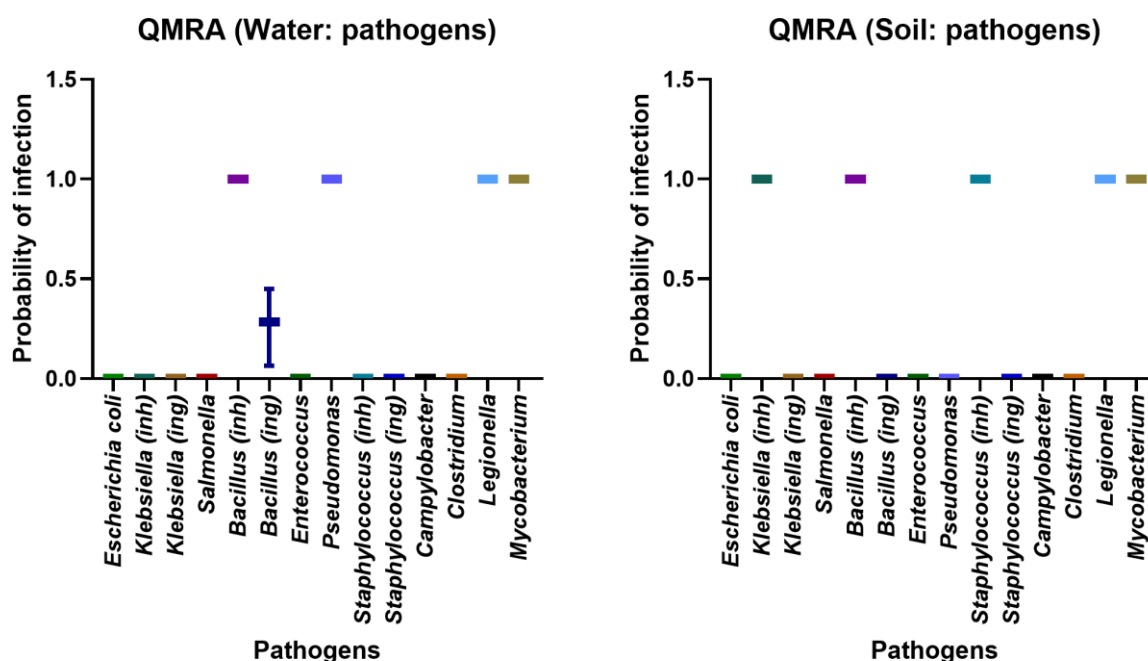


Figure 4.31: Quantitative Microbial Risk Assessment (QMRA) of water and soil-borne pathogens that are primarily ingested (*Escherichia coli*, *Salmonella* spp., *Enterococcus* spp., *Campylobacter* spp. and *Clostridium* spp.), inhaled (*Legionella* spp. and *Mycobacterium* spp.) and both ingested (ing) and inhaled (inh) (*Klebsiella* spp, *Bacillus* spp. and *Staphylococcus* spp.). The infection risk is expressed as a percentage, where 1.0 represents a 100% probability of infection.

4.7.2 Risk analysis

The risk associated with each site is shown in Figure 4.32. Probable infection risks based on pathogen ASV counts per site indicate a strong contrast between the VIS and MPA areas. All MPA sites, except for MP10, X3 and DMD have a probable risk of infection from 0.75–1.0 (75–100%), whereas, in the VIS area, there are only two sites with a higher probability of infection greater than 0.75 (>75%) and the rest are low.

The annual risk shown in Figure 4.33 shows the probability of infection for 365 days of exposure. The risk associated with all soil sites and three of the four water sites of MPA shows a probability of 1.0 (100%). Within the VIS area, three water sites TO, HR2 and HR3, had a probability of infection, and one site was close to 1.0 (100%).

The average probability of infection of all 11 pathogens per site (Figure 4.34) shows an interesting trend when comparing the VIS and MPA areas. The VIS area appears to have a lower probability of infection compared to the MPA area. The sites to be noted at VIS are TO and HR2, and the soil site M12. In MPA, the notable soil sites are M2 and M6. The site M2 has the highest probability of infection at 0.42 (42%).

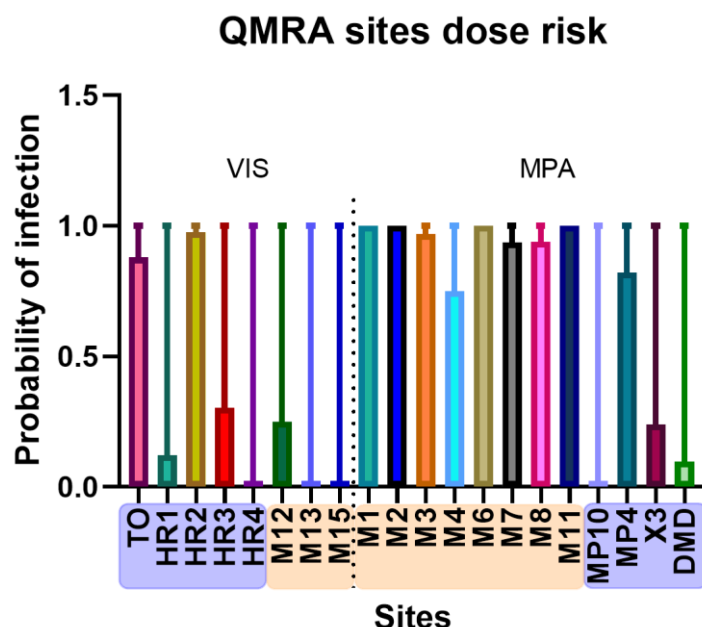


Figure 4.32: Quantitative Microbial Risk Assessment (QMRA) infection risk between sites by comparing probable infection risks based on pathogen ASV counts at each site. The infection risk is expressed as a fraction of one, where 1.0 represents a 100% probability of infection. The sites are divided according to Vaalharts irrigation scheme (VIS) and Mpumalanga (MPA) and distinguish the water sites (blue) and the soil sites (light brown).

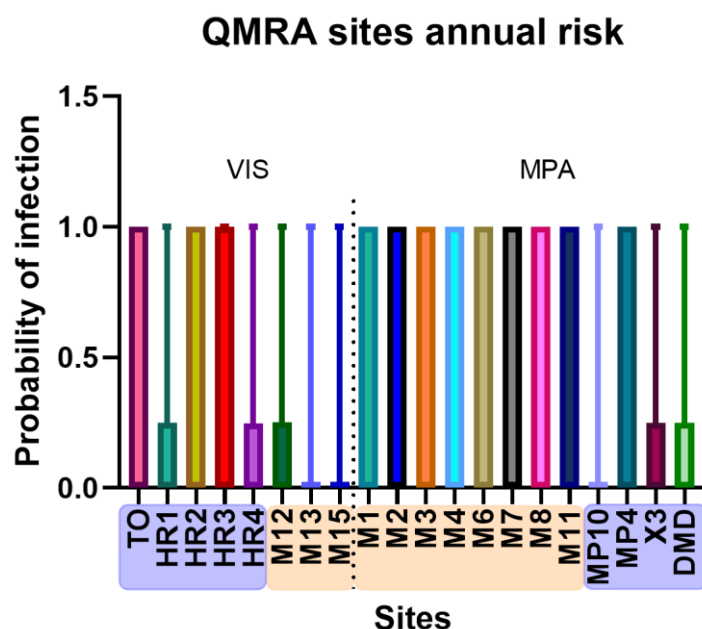


Figure 4.33: Annual infection risk in 365 days between sites comparing probable infection risks based on the average risk at each site. The infection risk is expressed as a fraction of one, where 1.0 represents a 100% probability of infection. The sites are divided according to Vaalharts irrigation scheme (VIS) and Mpumalanga (MPA), and also distinguish the water sites (blue) and soil sites (light brown).

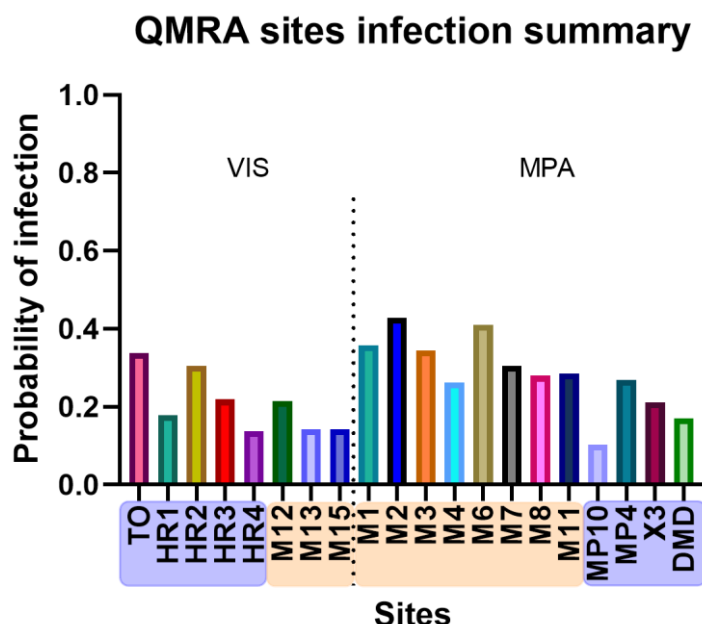


Figure 4.34: Summary of the annual risk of Quantitative Microbial Risk Assessment (QMRA) infection at all sites, comparing the probable infection based on the average risk at each site. The infection risk is expressed as a fraction of one, where 1.0 represents a 100% probability of infection. The sites are divided according to Vaalharts irrigation scheme (VIS) and Mpumalanga (MPA), and also distinguish the water sites (blue) and soil sites (light brown).

The results showed that GLY and AMPA were never detected in any of the water, soil, or sediment samples. This unexpected result led the study to focus on why the chemicals were absent despite intensive herbicide use mapped across GBH application districts. Water-quality and soil-texture analyses revealed no consistent physico-chemical “fingerprint” of GLY release. The pedotransfer of GLY ($K_d \approx 70 \text{ L/kg}$) suggested GLY mobility except at 5 of the 46 sites, and correlations between TOC and CEC were weak ($r_s = 0.26$ ($p = 0.0909$)). Microbial sequencing showed alpha diversity was higher in Mpumalanga, while beta diversity grouped communities by matrix (soil versus water) rather than by region. Finally, the QMRA showed that inhalation posed the greatest annual infection risk, especially at VIS water sites. Together, these findings create a paradox: heavy reported GBH use, yet no quantifiable levels of GLY or AMPA, paired with diverse microbial communities and probable pathogen infection risks, pointing to rapid GLY breakdown conditions.

CHAPTER 5: DISCUSSION

Agrochemicals, particularly GBHs, are widely used in the South African agricultural sector to reduce crop losses and improve weed management. However, despite their effectiveness, these chemicals, especially GLY and its primary metabolite AMPA, raise concerns about their potential to spread beyond targeted agricultural sites through runoff and leaching. Once in the environment, GLY and AMPA can accumulate in soil, water, and sediment, where they may persist and pose risks to non-target ecosystems and organisms. This study investigated the environmental concentrations of GLY and AMPA in various matrices in key agricultural and non-agricultural regions of South Africa. Unfortunately, the results revealed no detectable GLY or AMPA concentrations in any of the samples. The discussion chapter will try to expand on the reasons why there were no quantifiable levels of GLY or AMPA despite extensive sampling events and the precautions to preserve the target compounds in the collected samples. The behaviour of GLY, such as its sorption, degradation, and leachability, varies greatly depending on the environment (e.g., temperature, UV radiation, pH, humidity and microbial activity), clay and organic carbon content, and cation exchange capacity (Campanale *et al.*, 2024; Parus *et al.*, 2023; Zaller *et al.*, 2021). A geospatial analysis was used to map the distribution of the GBH application, highlighting areas of intensive use and potential contamination hotspots.

In addition to chemical analysis, this study also explored the microbial diversity within these GBH-treated environments, employing 16S rRNA gene sequencing to assess changes in the structures of the microbial community. Since no target compounds could be quantified, the selection of sites for microbial analysis was based on adapted criteria (see Table 3.9), and aim 3 (see Section 1.4 in Chapter 1) had to be adapted. A quantitative microbial risk assessment (QMRA) was performed to identify and characterise the potential health risks associated with microbial changes in areas potentially affected by GLY (and now other agricultural chemicals), focusing on pathogenic bacteria and also possible pollution-degrading bacteria.

5.1 Glyphosate-based herbicide (GBH) application maps

The geospatial analysis conducted in this study using ArcGIS revealed clear spatial patterns in GBH application across South Africa, particularly with the cultivation of maize, soybean, wheat, and sunflower. The maps showed regional variation, with consistently high usage observed in the Free State Province, especially along the Vaal River. In 2017, a single magisterial district reported GBH applications of up to 1 753 tonnes. By 2019, this intensive use had expanded, with three magisterial districts in the Free State applying between 405 and 1 065 tonnes. These spatial trends highlight agricultural zones with elevated herbicide use and signal areas where environmental contamination risks may be concentrated.

Several regions appeared with no GBH application data, marked in white on the maps. This absence likely reflects a lack of survey responses or missing data collection rather than true non-use. One such area was the Vaalharts region, which was included as a sampling site in this study based on personal communication and informal reports of GBH use. The decision to sample here despite the lack of official records illustrates the limitations of current monitoring systems and the need for ground-truthing in future mapping efforts.

The Vaal River basin stands out as a particularly relevant focus for herbicide risk. As South Africa's third-longest river (1 300 km) and the primary water source for roughly 60% of South Africa and 20 million people, or roughly 45% of its population, the Vaal River flows through the Vaal Triangle (a highly industrialised area), serving as the foundation of the economy (Ololade, 2018). Over the last 20 years, it has become clear that pollution is becoming a bigger threat to the Vaal River (Chokwe *et al.*, 2019). Historically, this river has been heavily polluted with industrial waste, heavy metals, organochlorine pesticides, dioxin-like compounds, and herbicides such as atrazine and terbuthylazine (Bouwman *et al.*, 2008; Burger, 2008; Quinn *et al.*, 2009; Wepener *et al.*, 2011; Rimayi *et al.*, 2016). Most of this contamination is concentrated in the Vaal Barrage, which functions as a collection zone for sewage and industrial effluent (Tempelhoff, 2009). These maps (Figure 4.1 and Figure 4.2) show another agricultural non-point source pollutant of an already polluted river.

Global modelling efforts further underscore the relevance of this region. Zhang *et al.* (2024) estimated that in 2020 alone, globally, 880 tonnes of GLY and 4 090 tonnes of AMPA entered rivers, more than half of which originated from maize and soybean cultivation. This aligns closely with the dominant crop types in the Free State, strengthening the case for targeted monitoring of pollution in the Vaal catchment.

Given these findings, the absence of detectable GLY and AMPA in such high-risk zones raises critical questions about their environmental behaviour. It suggests that degradation processes such as photodegradation, sorption, or microbial activity may be more active than expected under local conditions. However, the possibility of missed detection due to environmental variability or analytical limitations cannot be ruled out. Further research is therefore essential to clarify the degradation dynamics and potential long-term ecological consequences of GBHs in South African agricultural landscapes.

Monitoring pesticides on a geospatial scale remains a major challenge due to the vast number of registered compounds, each with distinct chemical properties that require specialised analytical techniques. This complexity is further intensified by the high costs of residue analysis and the need for frequent, time-sensitive sampling to capture application timing, dosage and field techniques (Di Guardo and Finizio, 2018). As a result, most pesticide surveillance efforts in South

Africa remain fragmented or non-existent, particularly for specific compounds such as GLY and AMPA.

Geospatial mapping offers a valuable approach to identifying regions where environmental and human exposure risks may intersect. For example, a study by Andrade-Rivas *et al.* (2023) combined pesticide usage and population density data to show that 28% of Ecuador's population lives within areas of high pesticide application, covering approximately 512 km². Similarly, Figure 5.1 in the present study shows partial overlap between high GBH-use zones and densely populated areas in South Africa, suggesting potential exposure risks that merit further investigation. Despite this, geospatial analysis of pesticides remains underutilised in South Africa. As far as is known, Dabrowski (2022) worked on all pesticide use in South Africa, but the current study is the first to focus on GLY and AMPA only. Dabrowski (2022) used data from 2014 and did not generate compound-specific maps detailed enough to support targeted monitoring.

Even though GLY and AMPA were not detected in the current samples, the identification of high-use zones, such as those in the Free State bordering the Vaal River, provides an essential foundation for future environmental surveillance. These mapped areas can serve as strategic focal points for monitoring programmes, especially given the poor water quality compliance historically reported in the Vaal River catchment (DWS, 2021). When combined with known pollution loads and population pressures, the potential risks associated with GBH use become even more pressing.

The spatial patterns of GBH application (Figure 4.1 and Figure 4.2) revealed in this study gain added significance when viewed against ongoing global and local debates. Internationally, Roundup® remains under scrutiny for potential carcinogenic and endocrine-disrupting risks (Van Bruggen *et al.*, 2018; Van Bruggen *et al.*, 2021; Rawat *et al.*, 2023), which makes it important to identify South African regions of intensive use. Locally, GLY use extends beyond agriculture into invasive alien plant eradication under the WfW programme (Babalola & Van Wyk, 2018), particularly in catchment areas where aquatic weeds such as water hyacinth are targeted. This extends the relevance of the application maps, as they also capture zones where non-agricultural use may coincide with vulnerable aquatic ecosystems.

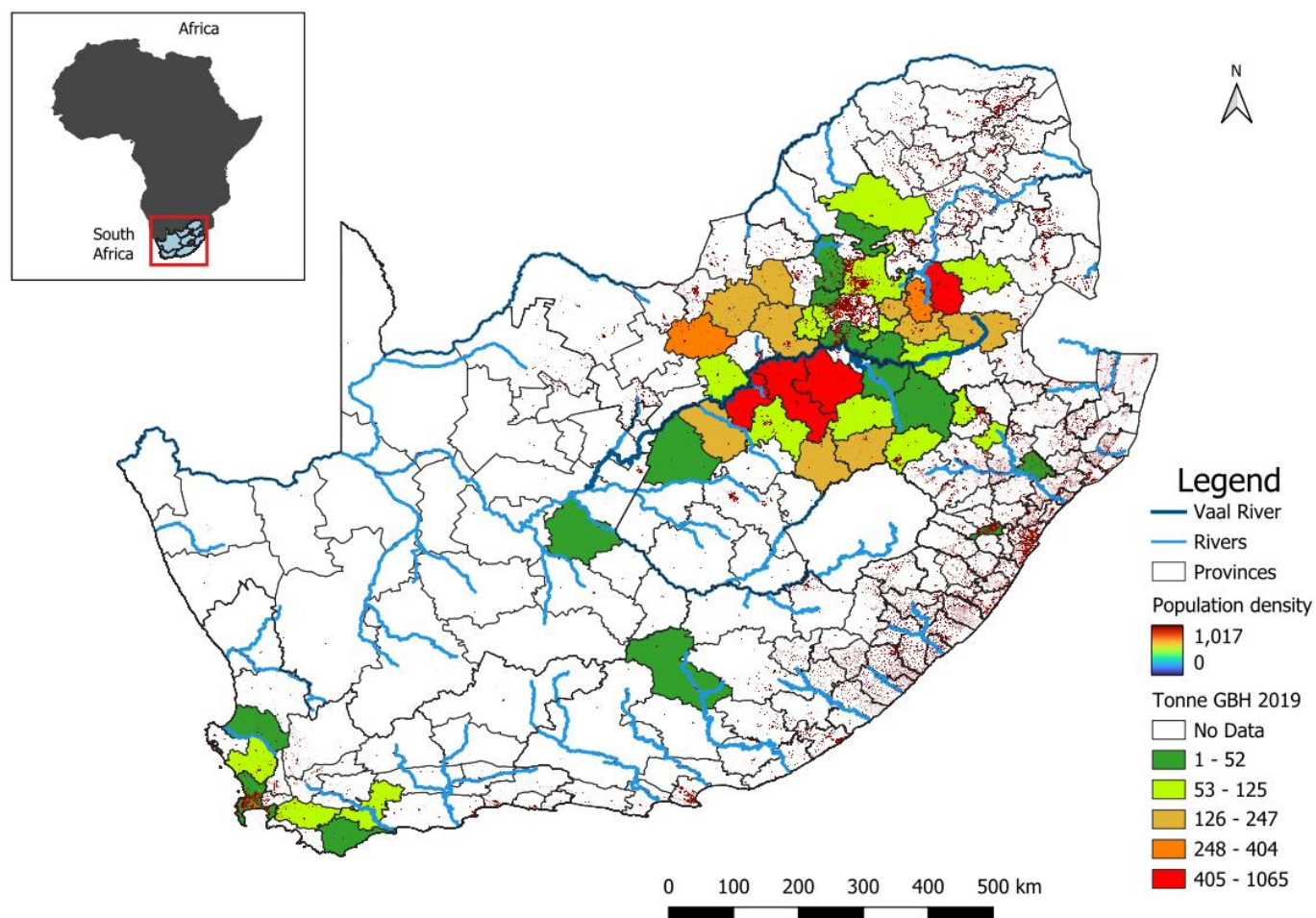


Figure 5.1: The tonnes glyphosate-based herbicide applied in 2019 overlaid with South Africa's 2020 population density data (Schiavina *et al.*, 2023).

5.2 Environmental and climatic conditions affecting glyphosate's (GLYs) fate

The main aim of this study was to quantify GLY and AMPA in the soil, sediment and water of the selected study areas since the areas are known for GBH use. A limited selection of soil and water characteristics was measured. These include nutrients (NH_4^+ , NO_3^- , NO_2^- , SO_4^{2-} , PO_4^{3-} , Cl^-) and physico-chemical characteristics (pH, EC, temperature, turbidity, TH, TSS) and percentage composition of sand, silt, clay, TOC, and CEC. These were quantified because they might explain the varying levels of the expected GLY and AMPA concentrations and their behaviour. The adsorption coefficient K_d was calculated based on the CEC and %Clay. However, after no quantifiable levels of GLY and AMPA were found, these same soil and water characteristics are investigated in this section for their role in explaining this unexpected phenomenon. Together with these characteristics, the role of UV radiation, temperature, soil moisture, and microbial activity will be discussed.

5.2.1 Impact of UV (ultra violet) radiation on glyphosate (GLY) degradation

The UV index can be categorised as low (0–2), moderate (3–5), high (6–7), very high (8–10) and extreme (11+) (Barco-Jiménez *et al.*, 2021). According to Wright and Norval (2021), the average UV index in South Africa in summer ranges from 9–11+ and in winter from 2–6. In contrast, the average UV indices for northern, central, and southern Europe range from 1.1–5.0 (Vitt *et al.*, 2020). The closest UV monitoring station to the VIS sites is 338 km south thereof at De Aar, and it showed a mean UV-B index of 8.7–8.4 during the 2020–2021 agricultural growing season and was classified as very high (8–10) (SAWS, 2024). The closest monitoring station for both MPA and NAS is in Pretoria (a distance of 140 km to MPA and 180 km to NAS, respectively). For both MPA and NAS, the mean UV-B was 6.7–6.8 and categorised as high (6–7).

Ultraviolet radiation is divided into three groups: UV-A (λ 315–400 nm), UV-B (λ 280–315 nm), and UV-C (λ 100–280 nm) (Barco-Jiménez *et al.*, 2021). UV-A has longer wavelengths and lower energy, which limits its role in the direct breakdown of GLY compared to UV-B and UV-C. However, UV-A can still contribute to indirect photodegradation by generating reactive oxygen species (ROS), such as hydroxyl radicals (Phaniendra *et al.*, 2015), which react with GLY and accelerate its breakdown (Papagiannaki *et al.*, 2020). UV-B has more energy than UV-A and can directly cause photolysis, where GLY molecules absorb UV-B energy, breaking down chemical bonds and enhancing degradation rates (Papagiannaki *et al.*, 2020). UV-B is toxic to life but is mostly absorbed by the ozone layer, preventing it from reaching the earth's surface. Ultraviolet-C, the most energetic form of UV radiation, can rapidly degrade GLY. However, UV-C is almost entirely absorbed by the earth's ozone layer (Barco Jiméñez *et al.*, 2021), meaning it does not contribute to GLY degradation in natural environments. As a result, only UV-A and UV-B are factored into the calculation of the UV index (Barco-Jiménez *et al.*, 2021; Snyder *et al.*, 2020). With the high and very high UV-B radiation recorded by SAWS (2024) for South Africa, there is a likelihood that UV-B irradiation would degrade GLY at the sample sites.

The studies listed in Section 2.6.4 of Assalin *et al.* (2010), Balah *et al.* (2017), and Lund-Høie and Friestad (1986) have shown the breakdown of GLY, even when tested the UV-C, which does not contribute to the breakdown of GLY, were all lab-based. The study by Vidal *et al.* (2015) used UV in tandem with peroxide to determine the decrease of GLY toxicity, but the current study did not study peroxides at the sample sites. The most relevant study was done by Papagiannaki *et al.* (2020), who concluded that UV irradiation may help reduce GLY concentration and decrease overall toxicity in environmental settings.

A higher UV index corresponds to stronger UV-B radiation, suggesting that GLY degradation would be more efficient in regions with a high and very high UV index, as previously discussed. This indicates that the rate of degradation of GLY could be faster in regions with more intense UV-B exposure, such as the VIS area, followed by the MPA and NAS areas. Although the studies

mentioned above are lab-based and do not focus on water, sediment, or soil, it remains true that not even GLY's metabolite AMPA was found in the samples, which is most likely attributed to UV irradiation.

5.2.2 Influence of temperature on glyphosate (GLY) behaviour and degradation

Temperature shapes the environmental behaviour of GLY by influencing its sorption, degradation and leachability, interacting with soil properties such as TOC, CEC and microbial activity (Campanale *et al.*, 2024; Parus *et al.*, 2023). Warmer temperatures accelerate both abiotic (e.g., sorption, photolysis, thermolysis) and biotic (primarily microbial) degradation pathways, generally reducing GLY persistence in soil (Singh *et al.*, 2020). Conversely, studies have shown that GLY and AMPA tend to persist longer in cooler, drier soils due to slower degradation rates (Bento *et al.*, 2016).

In South African agricultural topsoils, mean daytime temperatures often range between 21.3°C and 21.6°C of 10–80 cm topsoil (Myeni *et al.*, 2025) in a 2015–2020 data period. The warmer topsoil temperature conditions likely enhanced degradation processes, which may explain the absence of detectable GLY and AMPA residues in this study's samples. This is supported by previous findings in VIS, where similar temperatures were recorded 12 years earlier (Malherbe, 2013) and align with recent SAWS (2024) data for the 2020–2021 growing season. The MPA (Ermelo station) had a mean of 22°C, while both VIS (Vaalharts station) and NAS (Potchefstroom station) had a mean of 27°C, suggesting VIS and NAS have higher GLY breakdown potential.

The wide range of recorded water temperatures (10–35°C) during the sampling period (Figure 4.3) further reflects moderate to high environmental temperatures, which could accelerate both degradation and nutrient leaching. If higher temperatures did enhance GLY breakdown, then long-term regional warming may have shortened the environmental half-life of GLY, further contributing to its absence in the current study. Notably, even samples collected during cooler winter conditions (~10°C) showed no detectable GLY or AMPA, suggesting that other degradation mechanisms may still be active under lower temperatures.

Given GLY's widespread use in South African agriculture, these findings point to the importance of considering temperature effects on assessing herbicide persistence. If regional temperatures continue to rise due to climate change, degradation rates may increase further, potentially requiring adjustments in application timing to maintain herbicidal efficacy. Higher temperatures could also influence weed resistance patterns. Okumu *et al.* (2019) found that GLY-treated weed species (*Sorghum halepense*, *Lolium rigidum*) exhibited higher shikimate accumulation and greater susceptibility at cooler temperatures ($\leq 15^{\circ}\text{C}$), indicating that GLY efficacy may decline under warmer conditions. This suggests a future need for adaptive management strategies to sustain effective weed control under shifting climatic conditions.

5.2.3 Microbial activity and its impact on glyphosate's (GLYs) environmental fate

Glyphosate degrades more rapidly in the environment when mesophilic bacteria are active, as they function optimally at temperatures between 20–40°C, when their metabolic capacity is most active (Muskus *et al.*, 2022). As noted in Section 5.2.2, the water and sediment temperatures at the sampling sites ranged from 10–35°C, conditions conducive to microbial activity and potentially contributing to the absence of detectable GLY and AMPA in soil and water samples.

Glyphosate-tolerant plant growth–promoting rhizobacteria (PGPR), including *Serratia liquefaciens*, *Klebsiella variicola*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Enterobacter ludwigii*, play dual roles in promoting plant health and degrading GLY, particularly under axenic, sandy conditions (Mohy-Ud-Din *et al.*, 2023). All of these, except *Serratia*, were detected in the current study (Table 4.5). These strains belong to the phylum Proteobacteria, the most abundant phylum across all sampled matrices (Figure 4.26 and Figure 4.27). Genera such as *Bacillus*, *Pseudomonas*, and *Serratia* are known to improve root growth, nutrient uptake and stress resilience while contributing to GLY degradation (Mohy-Ud-Din *et al.*, 2023). In the current study, *Bacillus* and *Pseudomonas* were detected (Table S.29), suggesting a potential role in GLY degradation under field conditions.

Microbial diversity in water and soil varied across sites with differing GBH application levels. Mohy-Ud-Din *et al.* (2023) demonstrated that bacterial degradation of GLY was effective at moderate concentrations (100 mg/kg), with 87.1–98.6% degraded after 28 days. At higher concentrations (200 mg/kg), degradation efficiency dropped significantly to 36.7–40.7%, likely due to stress-induced limitations in bacterial adaptability. The findings resemble those of Langerica-Fuentes *et al.* (2024), who reported degradation of 60–85% of GLY in microcosms (15 mg/kg) in Germany within 7 days, yet observed no major shifts in total bacterial abundance between treated and untreated systems.

Sites with high GBH applications (248–404 tonnes), including soil sites M2 and M3 (Table 3.9), showed a negative association with GLY-degrading bacteria such as *Pseudomonas*, *Ochrobactrum*, and *Achromobacter*, alongside lower overall diversity (Figure 4.19). In contrast, sites with lower GBH input (53–125 tonnes, e.g., M6, M7, and M15) showed stronger associations with these bacteria and higher Chao1 diversity values (Figure 4.27), suggesting moderate GLY levels may support microbial utilisation of GLY as a nutrient source. Interestingly, the upstream site M1, with intermediate GBH input (126–247 tonnes), had the strongest negative association with GLY-degrading bacteria yet exhibited the highest Shannon diversity among the soil sites. This may indicate that even moderate GLY levels can suppress sensitive degraders if exposure exceeds a threshold. In contrast, downstream site MP4, despite high GBH input (248–404 tonnes), showed strong associations with degraders such as *Acetobacterium*, *Streptomyces*, and *Bacillus*, suggesting potential community adaptation. The water reference site TO (Table 3.9)

exhibited a weaker association with GLY-degrading bacteria and a stronger one with nutrient availability, implying that bacteria at this site may preferentially utilise readily available nutrients over GLY. Upstream site MP10 also showed limited association with GLY-degrading bacteria, reinforcing the idea that agrichemical input drives community composition. These patterns collectively indicate that GBH exposure may shape microbial diversity through both suppression and selective stimulation of taxa with degradation potential.

The overall bacterial community composition differed between high- and low-GBH application sites. Soil samples generally showed higher microbial diversity than water samples, as reflected in Shannon index values (Figure 4.20), except at a few lower-diversity sites (e.g., M11–M15). This difference likely results from variations in nutrient availability, pH and the ionic strength of soil particles (Pereira *et al.*, 2019). Shifts in microbial communities and diversity may also be brief, driven by short-term exposure to GLY. As Wang *et al.* (2023b) noted, GLY application can initially stimulate microbial activity and alter species composition through selection against sensitive taxa.

The beta diversity (Figure 4.25) further showed a notable microbial community between sites. High-application sites such as M2, M3 and DMD were compositionally distinct from lower-application sites like M6, M7 and X3. Reference sites (HR1, TO) also displayed distinct microbial profiles compared to downstream sites (HR4, MP10, DMD), reinforcing the possible influence of agrichemicals on microbial diversity.

The most abundant bacterial phyla in water samples were Proteobacteria, Bacteroidota, Actinobacteriota, Verrucomicrobiota and Cyanobacteria. In soil, the dominant phyla included Proteobacteria, Actinobacteriota, Chloroflexi, Firmicutes and Bacteroidota. Figure 5.2 shows a clear separation between upstream sites (TO, HR1, HR2, MP10) considered less affected by agrichemicals and downstream sites (HR3, HR4, DMD, MP4), assumed to be exposed to higher agrichemical input. A similar pattern is evident in Figure 5.3, which separates sites by soil and sediment. In Figure 5.3, TOC and silt contribute to variation along the y-axis, while sand, clay and TOC contribute to the x-axis. Fewer bacteria were associated with soil-dominated textures, likely due to greater nutrient leaching in these environments (see Section 5.3.1 for further interpretation).

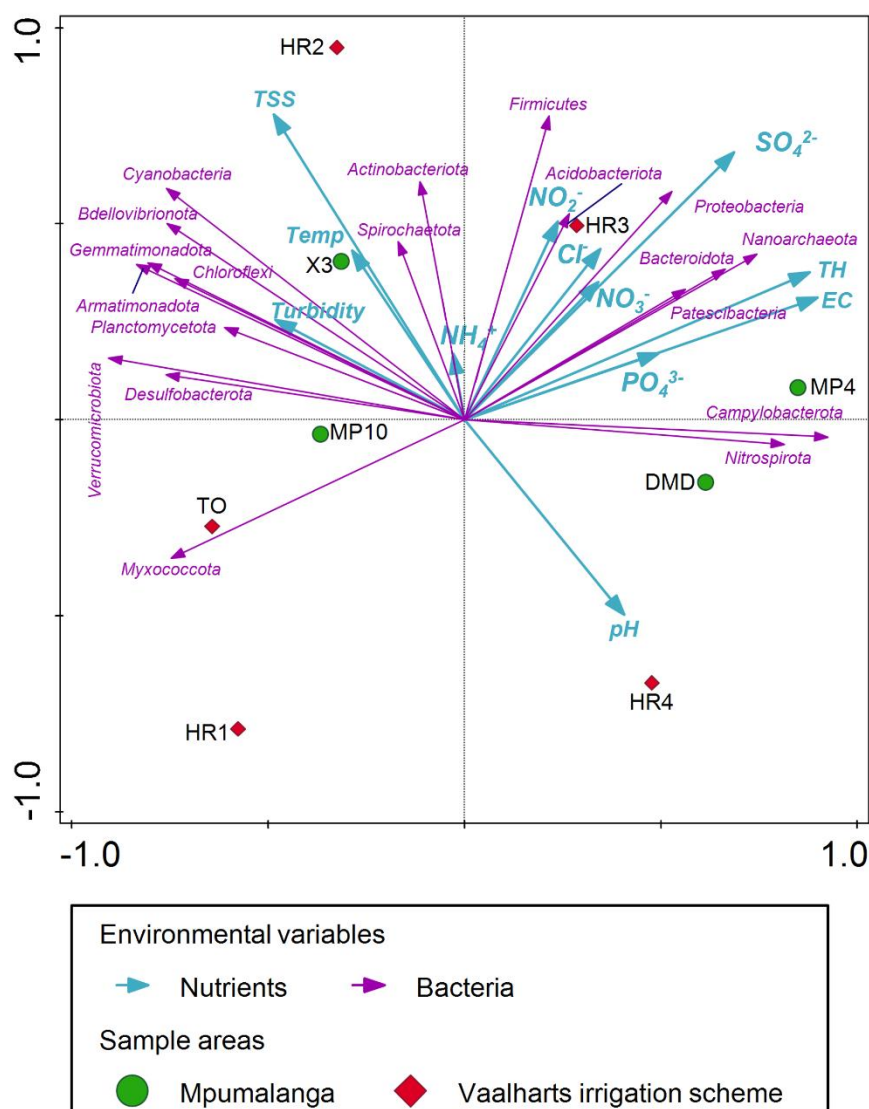


Figure 5.2: Principal component analysis (PCA) biplot of the post-herbicide application sampling event's water quality variable measurements. The biplot accounts for 57.69% of the variation, with 38.55% on the first axis (Factor 1) and 19.15% on the second axis (Factor 2). Nutrients (NH_4^+ , NO_3^- , NO_2^- , SO_4^{2-} , PO_4^{3-} , Cl^-) and physico-chemical (pH, electrical conductivity (EC), temperature, turbidity, total hardness (TH), total suspended solids (TSS)) are examples of water quality variables. Bacteria detected by 16S sequencing are highlighted in purple, representing the top 20 most abundant phyla. The displayed sites correspond to the locations where these phyla were found.

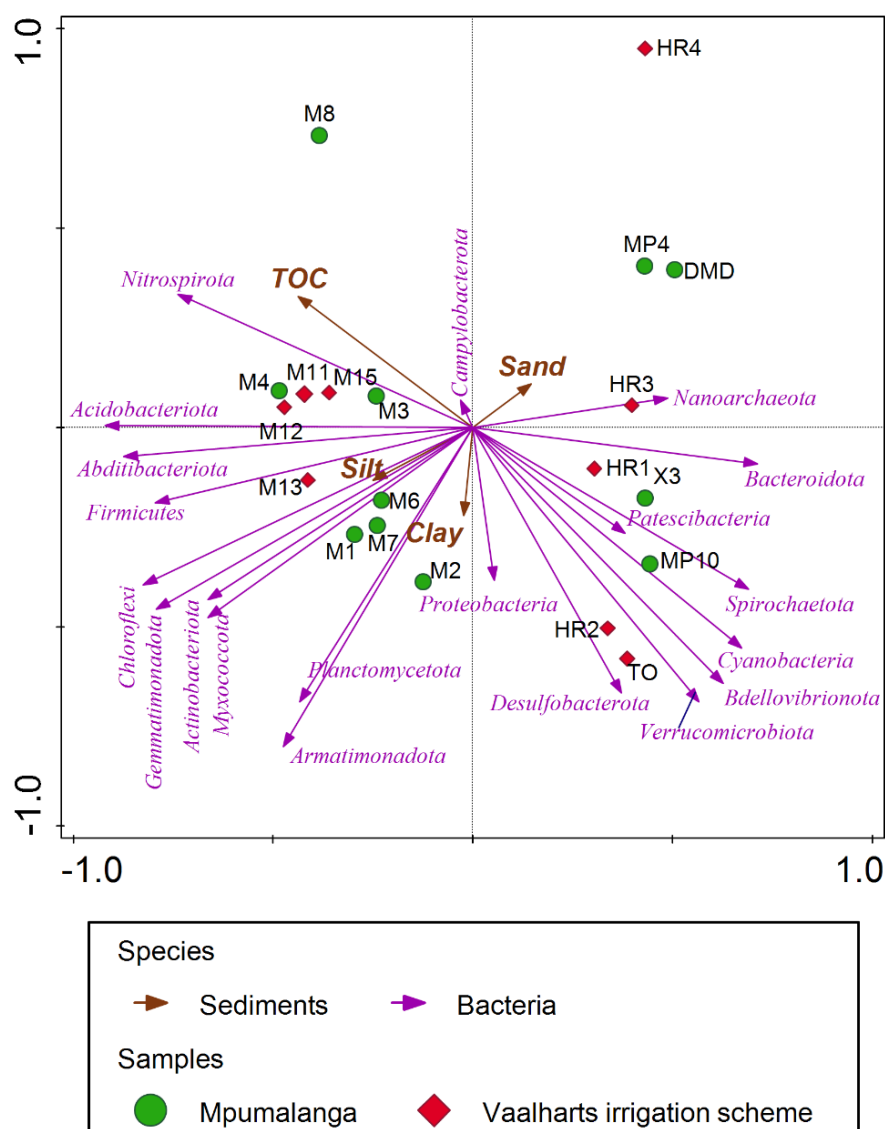


Figure 5.3: Principal component analysis (PCA) biplot of the post-herbicide application sampling event's sediment quality variable measurements. The biplot accounts for 52.50% of the variation, with 34.72% on the first axis (Factor 1) and 17.78% on the second axis (Factor 2). The percentage composition of clay and total organic content, is shown due to possible glyphosate sorption. Bacteria detected by 16S sequencing are highlighted in purple, representing the top 20 most abundant phyla. The displayed sites correspond to the locations where these phyla were found.

Firmicutes are generally more GLY-resistant than Proteobacteria, while Actinobacteriota are among the most GLY-sensitive phyla (Rainio *et al.*, 2021). In the current study, Firmicutes taxa such as *Bacillus*, *Streptococcus*, *Staphylococcus*, *Listeria*, and *Clostridium* (CII category: resistant) were present but did not show increased relative abundance at high-application sites. This suggests that additional ecological or physicochemical factors may be limiting their proliferation despite their resistance classification. Proteobacteria, which includes both GLY-sensitive (CI) and resistant (CII) genera, were abundant across nearly all sites regardless of GBH levels (Figure 4.26 and Figure 4.27). Sensitive genera include *Enterobacter*, *Burkholderia*,

Shewanella, *Neisseria*, *Francisella*, *Klebsiella* and *Yersinia*, while resistant taxa include *Xanthomonas*, *Brucella*–*Ochrobactrum*, *Pseudomonas*, *Campylobacter*, *Acinetobacter* and *Legionella*. The persistence of Proteobacteria across varied GBH conditions suggests that GBH exposure is only one of several drivers shaping community composition. Within Actinobacteriota, which includes highly sensitive genera such as *Corynebacterium*, *Bifidobacterium*, *Propionibacterium* and *Mycobacterium*, no consistent abundance pattern emerged. Even at low-application sites like M6–M8, relative abundance varied, suggesting that single-study resistance profiles do not necessarily predict community-level outcomes in complex natural environments. This underlines the need for caution when extrapolating laboratory findings to field settings.

The relative abundances of phyla and genera also reflected patterns consistent with microbial adaptation. Sites with high GBH application (M2, M3, M4, DMD: 248–404 tonnes) showed a greater representation of possible resistant Firmicutes and Proteobacteria, particularly *Pseudomonas* and *Bacillus* spp. (Table 4.7; Figure 4.26 and Figure 4.27). In contrast, low-application sites (M6, M7, M8: 53–125 tonnes) exhibited relatively higher abundances of Actinobacteriota, a phylum classified as highly sensitive to GLY. The reference site TO, located upstream from major agricultural activity, showed fewer GLY-resistant taxa and a greater representation of GLY-sensitive phyla, reinforcing the impact of GBH use on microbial structure and potential functional capacity for degradation. Similar trends were reported by Aslam *et al.* (2024), who found that GLY exposure increased the abundance of Gram-negative bacteria, including Acidobacteriota and Proteobacteria. In the present study, Acidobacteriota, Bacteroidota, and Proteobacteria were also dominant in soil samples after spraying (Figure 4.26), supporting the hypothesis that GLY was likely present and degraded, even if not chemically detected.

As discussed in Section 2.5.3, GLY was originally patented as an antibiotic. Its role as a selective agent raises concerns about the mobilisation of antibiotic resistance genes (ARGs), especially in environments where GLY exposure co-occurs with ARG carriers, such as those found in untreated manure. This combination could facilitate gene transfer from harmless environmental microbes to opportunistic pathogens (Liao *et al.*, 2021). With the widespread use of GBHs, some bacterial groups may adapt through micro-evolutionary mechanisms of the adaptive efflux pump (Bote *et al.*, 2019b; Kurenbach *et al.*, 2018; Rainio *et al.*, 2021), potentially leading to multi-antibiotic resistance. These mechanisms include reduced GLY sensitivity via increased EPSP synthase production, enzymatic degradation, detoxification pathways and changes in cellular uptake or efflux capacity (Hertel *et al.*, 2021).

A study by Liao *et al.* (2021) found that GLY had the strongest effect on ARG bacteria abundance compared to other herbicides, enhancing resistance to antibiotics such as trimethoprim and kasugamycin and increasing mobile genetic elements such as plasmids, integrases and transposases. Similar findings by Kurenbach *et al.* (2015) and Van Bruggen *et al.* (2021) linked

GLY exposure to enhanced resistance in *Escherichia coli* and *Pseudomonas aeruginosa*, both of which pose public health risks.

Glyphosate in the environment can select GLY-resistant strains, potentially increasing the prevalence of opportunistic pathogens in water and soil systems. Over time, this selection may not only reduce overall microbial diversity but also promote traits that compromise ecological function and human health. These risks are particularly relevant in areas where water quality is already poor, or where exposure routes such as irrigation, animal contact or drinking water are unregulated. The potential for GBH exposure to drive antibiotic resistance development, promote the survival of opportunistic pathogens and alter microbial community function raises serious questions about impacts on public health. These risks are particularly relevant in water-stressed regions where agricultural runoff and human exposure pathways meet. These emerging concerns will be further explored in the context of Quantitative Microbial Risk Assessment (QMRA) in Section 5.4.1, where the presence of potentially resistant pathogens and their implications for human health are assessed.

5.3 Soil and physico-chemical interactions affecting glyphosate's (GLYs) behaviour

Glyphosate's behaviour in the environment is governed by interactions between soil, water, and environmental conditions, which influence its adsorption, mobility, and degradation. In soils, factors such as CEC, TOC, and soil texture determine their retention, while in water, physical and chemical properties affect their transport and availability. External influences, including moisture levels and microbial activity, further shape its persistence. This section examines these interconnected processes to better understand GLY's fate in the environment.

5.3.1 Factors influencing glyphosate (GLY) adsorption

The adsorption and mobility of GLY in soil are strongly influenced by CEC, a measure of a soil's ability to retain positively charged ions (Singh *et al.*, 2024). Soils with higher CEC can bind more ions, affecting how GLY interacts with metal ions in the matrix. This binding reduces GLY mobility, making it less likely to leach into groundwater or nearby aquatic systems. Soil analysis from the current study (Figure 4.5) indicated that sites with high TOC, such as PAN1, also had higher CEC values, supporting stronger GLY retention and likely contributing to the absence of detectable GLY in water and sediment samples. Further analysis of CEC values (Figure 4.11) follows this trend. Sites with lower CEC typically had K_d values below 100 L/kg, consistent with weaker adsorption and greater potential for GLY leaching. This aligns with Dollinger *et al.* (2015), who found that low CEC soils promote GLY desorption due to reduced binding site availability. In contrast, high CEC values generally support stronger retention and reduced GLY mobility.

The CEC of the current study is slightly lower (1.6–2.2 meq/100 g) (Figure 4.11) than that of Siwale *et al.* (2023), 3.40–4.15 cmol(+)/kg, who studied soils in the Vaalharts area. (NOTE: 1 cmol(+)/kg is the same as 1 meq/100 g). The only exception in the study is in SD and DSD, where the CEC is 6.5–8.8 meq/100 g.

Despite strong CEC and TOC retention potential at some sites, such as PAN1, GLY was not detected in sediment samples. This suggests that adsorption alone cannot explain GLY's fate, but rather other processes, including microbial degradation or chemical breakdown, likely contributed. As supported by Odukkathil and Vasudevan (2013) and Harju *et al.* (2021), TOC and soil particle size strongly influence the bioavailability of xenobiotics, with soils functioning as both sinks and sources of pollutants. The PCA results before herbicide application (Figure 4.7) showed that TOC and TSS were highly correlated with PAN1, while clay and silt were dominant at other Mpumalanga sites (MD, MP6, MP4). This suggests that sites with higher TOC, such as PAN1, have greater pollutant retention potential. However, GLY was still undetected at PAN1, implying that either adsorption was too strong for extraction or degradation occurred before sampling.

The sand-dominant sites like VIS and M9 (Figure 4.6) have lower water retention and higher leaching potential, consistent with previous findings (Borggaard and Gimsing, 2008). In clay- and TOC-rich sites, such as PAN1, demonstrated stronger retention, as reflected by higher K_d values (Figure 4.14), although still below the 100 L/kg benchmark that typically indicates strong adsorption. The post-spray PCA (Figure 4.8) confirmed PAN1's strong association with TOC and PO_4^{3-} , reinforcing its role as a pollutant sink, further reducing extractability due to adsorption. This is further supported by the harvest PCA (Figure 4.10), which linked increased TOC to potential GLY retention. Similar outcomes were reported by Parus *et al.* (2023), who noted that strong sorption to organic matter may reduce GLY bioavailability for microbial degradation, potentially extending its environmental half-life. So even if GLY's half-life was extended, the logical conclusion would be that it should have been detected in the chemical analysis.

While the correlation between CEC and TOC (Figure 4.13) showed a weak positive trend ($r = 0.20$, $p = 0.4561$), PCA results (Figure 4.12) confirmed a general association between CEC and TOC. Although TOC likely plays a secondary role compared to clay content in determining GLY retention, its contribution cannot be dismissed, especially in high-TOC, high-CEC soils where GLY may remain tightly bound and unavailable for detection.

In addition to CEC and TOC, pH plays a central role in determining GLY's adsorption behaviour. The pH influences the ionisation state of GLY, which in turn affects its interaction with soil and sediment components (Romano *et al.*, 2021). At low pH (<2.0), GLY's amino group is protonated, resulting in a net positive charge that promotes electrostatic attraction to negatively charged counterions. Between pH 2.0 and 5.6, deprotonation of the phosphonic acid group begins, allowing interaction with monovalent cations. As pH increases between 5.6 and 10.6,

deprotonation of the carboxylate group increases GLY's negative charge, facilitating binding to divalent cations such as Ca^{2+} and Mg^{2+} . At pH values above 10.6, all functional groups are deprotonated, favouring interactions with trivalent cations like Fe^{3+} and Al^{3+} (Romano *et al.*, 2021). The soil surfaces also become more negatively charged at higher pH levels, leading to increased electrostatic repulsion of anionic compounds like GLY (Bi *et al.*, 2023). Another study agrees that higher pH reduces GLY sorption due to this repulsion (Dollinger *et al.*, 2015). In the current study, the average pH across sampling sites was 7.7 (Table 4.1), a range at which soil surfaces are predominantly negatively charged. Under these conditions, GLY is primarily in its anionic form, making it more mobile and less likely to bind strongly to soil particles (Kovács *et al.*, 2024).

The sediment pH was not directly measured, the assumption that sediment pH mirrors water pH (~7.7) suggests a slightly alkaline environment, which favours GLY mobility and weakens retention (Munira *et al.*, 2016). At this pH, both the phosphonic and carboxyl groups of GLY are fully dissociated, further limiting adsorption (Parus *et al.*, 2023).

The ionic interactions with environmental cations and anions could influence GLY behaviour. The NH_4^+ concentrations were highest before and after herbicide application (Figure 4.4), and these ions may have formed weak ionic interactions with GLY, reducing its free mobility. In contrast, other anions present in the system, such as NO_3^- , NO_2^- , SO_4^{2-} , PO_4^{3-} and Cl^- , would likely repel GLY electrostatically due to their similar negative charge (Dollinger *et al.*, 2015). These dynamics further complicate GLY detection, particularly in matrices with fluctuating ionic concentrations.

Glyphosate's ability to form stable complexes with metal ions adds another layer of complexity to its environmental fate. The phosphonate and carboxyl functional groups enable GLY to chelate metals such as Fe, Al, and Ca, significantly reducing their bioavailability and mobility (Borggaard and Gimsing, 2008). These metal-GLY interactions are particularly relevant in areas with high concentrations of amorphous Fe- and Al-oxides, which serve as strong sorbents (Campanale *et al.*, 2024). Once complexed with metals, GLY is more likely to remain in the soil matrix and less likely to leach into water systems.

Soils enriched in variable-charge minerals like goethite and allophane/imogolite are especially effective at sorbing GLY, whereas soils dominated by permanent-charge minerals such as illite, smectite and vermiculite retain less (Campanale *et al.*, 2024). In the Vaalharts region, dominant Hutton soils are known to be rich in Fe-oxides (Verwey and Vermeulen, 2011), which may have enhanced GLY retention and reduced its extractability. Under no-till practices, Fe-oxide formation may be further limited due to minimal oxygen exposure, potentially altering sorption dynamics in ways not captured by standard models. Metal complexation may not only reduce GLY's mobility but also hinder microbial and chemical degradation processes. Insoluble complexes formed through chelation can persist in the environment and resist breakdown, leading to an absence of GLY in water or sediment samples, despite ongoing application.

Soil texture, defined by the relative proportions of sand, silt, and clay, strongly influences GLY retention, mobility, and microbial accessibility (Schoonover and Crim, 2015). Although GLY strongly binds to clay and organic matter, the adsorption can reduce its bioavailability to microbes. Under dry conditions, limited moisture further restricts GLY desorption from soil particles (Schroll *et al.*, 2006). In Figure 4.6, most of the sampled soils were silt-dominated. These soils tend to fall between sandy and clay-rich textures in terms of retention and microbial accessibility. In general, silt-based soils allow for moderate GLY retention while maintaining some degree of bioavailability for microbial degradation. However, the sand-rich sites such as VB, DGP, DSD, M9 and M10 showed K_d values below 100 L/kg during the rainfall event (Figure 4.14), consistent with a high leaching potential and low sorption capacity. Similarly, the pre-spray PCA (Figure 4.7) showed sandy sites such as HR1 and TO on the opposite side of retention variables like TOC and clay. This spatial relationship supports the conclusion that GLY was more likely to leach from sand-dominant soils, consistent with findings from Guijarro *et al.* (2018), who reported stronger sorption in soils with higher TOC, CEC and clay content. Sites like P2 and C2, which had higher clay content, were expected to retain more GLY. Although soil moisture was not directly measured in this study, the dominance of silt soils suggests moderate water retention capacity. This moderate retention likely created conditions that allowed microbial degradation to proceed, especially under the warm temperatures observed (22–27°C).

In conditions of sufficient moisture and organic matter availability, microbial communities become more active, enhancing degradation rates and reducing the present non-sorbed GLY. Post-harvest practices in drier regions of South Africa often include leaving vegetative waste on fields to retain moisture (Van Antwerpen *et al.*, 2021). Crop rotation may further support moisture retention and microbial stability. The dynamic interplay between soil texture, water retention, microbial activity, and land management likely influenced the observed GLY behaviour in the environment.

Temperature accelerates microbial metabolism and abiotic processes such as desorption, while adequate moisture supports microbial growth and access to sorbed GLY. The interaction of these factors is key to understanding why GLY may have been rapidly broken down in the sampled environments. Bento *et al.* (2016) demonstrated that GLY degradation was 8.4 times faster in silty loam loess soil at 30°C compared to 5°C. Degradation rates were also 2.9 and 2.2 times faster in saturated and 60% moisture soils, compared to dry soils (20% moisture), respectively. These findings align with Romano-Armada *et al.* (2019), who found a strong correlation between soil water content and GLY breakdown. Collectively, the results suggest that GLY degrades most rapidly in warm, moist soils and persists longer under cold or dry conditions. This is summarised by Ferreira *et al.* (2021), who ranked degradation conditions as: cold + dry < cold + wet < hot + dry < hot + humid. In the current study, temperatures across sampling sites ranged between 22–27°C, conditions favourable to microbial growth and thereby contributing to degradation. These

moderate-to-warm temperatures likely enhanced degradation rates compared to colder regions, particularly when combined with soil moisture. Supporting this, Suwardji *et al.* (2021) found that GLY partitioning into the soluble phase increased with temperature. At 5–12°C, only 1.4–2.1% of GLY was released into solution, whereas at 22–28°C, up to 52.8% was soluble. In the current study, similar desorption likely occurred, making GLY available for microbial or abiotic degradation.

The K_d values measured in this study (Figure 4.14) were below 100 L/kg for most sites, indicating that GLY was not strongly adsorbed and may have leached or degraded. These results align with findings from Paradelo *et al.* (2015), who noted that GLY exhibits high mobility in loamy soils, especially those with high silt content, as confirmed in this study (Figure 4.6). Skeff *et al.* (2018) similarly reported K_d values of 6.1 L/kg in sandy soils, 55.2 L/kg in silty soils, and 259.5 L/kg in clay-rich soils. The current study's mean K_d of ~70 L/kg falls within this silty range, again suggesting conditions that favour GLY movement and breakdown.

Although retention varies by site, K_d values close to 50 L/kg, such as those seen in MPA and NAS, imply that approximately 2% of applied GLY could remain in the soil (Duke *et al.*, 2012), potentially reaching concentrations (~24 µM) sufficient to impact microbial communities. Yet, the actual chemical detection of GLY was not achieved, likely due to the combination of degradation-enhancing conditions described above.

5.4 Quantitative Microbial Risk Assessment (QMRA)

As previously mentioned, it should be emphasised that not all bacterial genera chosen for this QMRA inherently exhibit pathogenicity or GLY-resistance; however, for this QMRA, they were treated as potential pathogens and pollutant-degrading bacteria reported to be influenced by GLY exposure.

Although the GLY-pathogen-QMRA results indicate the likelihood of infection from different environmental pathogens, no clinical or empirical data are available for comparison with the results of this study and the nature of the unique GLY-pathogen-QMRA. Because this QMRA is unique, it is challenging to validate the results, and, as far as is known, this is the first of its kind. Considering the effects that agricultural practices may have on environmental bacteria and the effects that these bacteria may have on humans, this opens a new area of study.

5.4.1 Quantitative Microbial Risk Assessment (QMRA) and public health impacts

The QMRA results for pathogens showed different patterns in infection risk transmission routes (inhalation/ingestion). The genera identified from literature to be able to degrade GLY and be pathogens (Table 3.10), used for the QMRA, are *Bacillus*, *Klebsiella*, *Staphylococcus*,

Mycobacterium, *Pseudomonas*, *Clostridium*, *Legionella*, *Salmonella*, *Enterococcus*, *Campylobacter* and the species *E. coli*.

Inhalation was the dominant exposure pathway in the analysis (Figure 4.29), where *Legionella* and *Mycobacterium*, both airborne pathogens, had a 100% (1.00) probability of infection at 10/20 sites and 19/20 sites, respectively. Mori and Smith (2023) estimated the risk of *Legionella pneumophila* infection from treated-wastewater spray irrigation up to 7 km away in the USA with a mean probability for low-pressure irrigation ranged from 2.2×10^{-7} – 6.5×10^{-9} (2.2×10^{-5} – 6.5×10^{-7} %), respectively, and for high-pressure irrigation 4.4×10^{-6} – 1.4×10^{-7} (4.4×10^{-4} – 1.4×10^{-5} %) respectively. *Legionella*'s resistance to GLY (Raino *et al.*, 2021; Van Bruggen *et al.*, 2021) could be linked to its prolonged exposure to GLY in the environment. *Mycobacterium* consistently showed high infection probabilities in both water and soil environments at all investigated sites, except for HR4 (Figure 4.29). The environmental ubiquity and resilience of *Mycobacterium* are most likely the reasons for this occurrence, as this organism can occur in water, soil, food, dust, and aerosols (Makovcova *et al.*, 2014).

The ingestion of *Escherichia coli*, *Salmonella*, *Enterococcus*, *Pseudomonas*, *Clostridium*, and *Campylobacter*, however, showed varying results (Figure 4.28). Data from the EFSA show that the most common cause of decreasing effectiveness of antibiotic therapy is infections caused by antibiotic-resistant bacteria of the genera *Salmonella*, *Campylobacter*, *Enterococcus*, and some serotypes of *E. coli* species (Stec *et al.*, 2022). Antwi-Agyei *et al.* (2016) showed that farmers ingested between 2–1 200 *E. coli* cells per day via encountering soil. *Enterococcus* ingestion presented a lower risk compared to *Pseudomonas* and *Salmonella*. *Escherichia coli*, *Clostridium*, and *Campylobacter* showed lower infection probabilities, with one site posing a risk at their respective modelled sites. These results are consistent with research conducted by Goh *et al.* (2015), which demonstrated that *Enterococcus* posed a greater risk than *Salmonella* in water sources in a recreational reservoir in Singapore.

Bacteria capable of infection by both inhalation and ingestion are *Klebsiella*, *Bacillus* and *Staphylococcus*. These pathogens reach full infection probability in surface-water scenarios at several MPA sites (Figure 4.30). The ingestion pathway at MPA sites posed a higher risk for species such as *Klebsiella*, *Bacillus*, and *Staphylococcus* (Figure 4.30), with infection probabilities as high as 1.0 (100%).

Glyphosate may create conditions that may lead to the development of antibiotic resistance, increasing the risk of infection. This is especially true for pathogens such as *Staphylococcus*, which is already well-known to have multi-antibiotic resistance (Chaturvedi *et al.*, 2021). *Bacillus* spp. is known to degrade GLY (Langarica-Fuentes *et al.*, 2024; Manogaran *et al.*, 2018). In this study, a total of 43 305 *Bacillus* spp. counts were recorded, with the highest ASV counts observed at site M6 (7 695 counts) (Appendix D, Table S.27). This suggests that GLY could inadvertently

support the growth of harmful bacteria, increasing the risk of infections. For example, *Bacillus* spp. capable of degrading GLY are often less sensitive or insensitive to it, leading to their accumulation in the environment. Pathogenic *Bacillus* species, such as *Bacillus anthracis*, can cause infection when the bacterium is ingested through animal feed or contaminated crops, or when endospores are inhaled from dust and aerosols (Welkos *et al.*, 2015). The results in Figure 4.30 are in line with the observation that inhalation of pathogens poses a greater risk than ingestion. Additionally, *Klebsiella* spp. is a foodborne pathogen that has been isolated from irrigation water and agricultural soil (Iwu *et al.*, 2022), further showing the potential risks associated with agricultural settings, especially where GLY is used.

Water and soil-borne pathogens can survive for a long time in water and soil if conditions are appropriate. The viability of pathogens can be affected by factors such as humidity, low temperature (specifically in the water column), and low UV radiation (Dungan, 2014; Stec *et al.*, 2022; Xiao *et al.*, 2024). With the high values of the UV-B index discussed in Section 5.2.1, the UV-B index for VIS was higher (8.7–8.4) than that of MPA (6.7–6.8) and this trend is also confirmed in Figure 4.33 where the probability of infection in the VIS is lower than in MPA, indicating that UV-B does indeed affect the viability of environmental pathogens. In the current study, the mean temperature of the water samples of POST-MPA and POST-VIS was 27.1°C (Table 4.1), providing a favourable environment for pathogen growth. At a lower temperature, there would have been less bacterial growth and therefore less pathogenicity. It can be concluded that the MPA area is generally wetter than the VIS area, which is drier. The same trend seen in Figure 4.33 is still applicable, regardless of high temperatures (27.1°C), since there is a more favourable opportunity for pathogen growth.

Bacteria such as *Klebsiella*, *Bacillus*, *Mycobacterium*, *Staphylococcus*, *Pseudomonas* and *Clostridium* are known to depend on the shikimate pathway for the synthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan (Duke *et al.*, 2012). Nutrient enrichment, from agricultural, industrial, anthropogenic, or other sources, may affect sites at MP4 and MP6 in MPA and HR1 in VIS (Table 4.6) and could increase the risks of pathogens in water systems. If there were detectable levels of GLY for the current study, there is a chance that GLY could exert selective pressure on these pathogens that have the shikimate pathway and become resistant to GLY. For example, at the DMD Mpumalanga site, located downstream of agricultural sites where the GBH applications ranged from 248–404 tonnes (according to the map of 2019 shown in Figure 4.2), the expected high levels of GLY runoff can increase the selective pressure of the pathogens and result in infections. Glyphosate should be monitored more stringently in locations where it may be possible for people to be exposed to waterborne aerosols, such as pivot irrigation systems, because of the selective pressure GLY has on bacteria, selecting antibiotic-resistant bacteria.

Site HR1 on the Harts River is located next to an informal settlement where residents may wash their clothes or bathe in the river. Here, the residents have a high probability of infection with *Legionella* and *Mycobacterium*. Iwu *et al.* (2022) found that agricultural soil samples and irrigation water contained a significantly high abundance of *E. coli* O157: H7. Although there was no test done to determine bacterial pathogenicity, such as with the *E. coli* O157: H7 pathogenic strain, there is a small probability of infection for *E. coli* at sites M2 and M3.

The infection probabilities at the VIS sites are still lower than those at the MPA sites, as Figure 4.34 shows, although there is notable variation between the sites. For example, infection risks at VIS sites such as HR2 are almost 1.0 (100%), indicating that outbreaks could occur, even in less intensively farmed areas. Although fewer GLY treatments may have contributed to relatively lower infection risks at other VIS sites, these findings do not completely rule out the possibility of health hazards, particularly in situations where isolated soil or water contamination can result from storm activity or runoff. On the other hand, MPA sites show higher infection probabilities at sites M1–M4 and M6–M8, which may be related to the increase of the pathogens.

Ultimately, both water and soil present significant risks to pathogen transmission, but the key takeaway from Figure 4.31 is the role of environmental factors in amplifying specific risks. Water systems tend to facilitate ingestion-related infections, while soil environments, especially those affected by intensive agricultural practices, pose a higher risk of inhalation-based infections.

There are a few ways to prevent being exposed to aerosols, which could include avoiding irrigation on windy days, notifying residents within 1 or 2 km before irrigation, reducing the exposure of aerosolised *Legionella* (Mori and Smith, 2023) and/or *Mycobacterium* (Masaka *et al.*, 2021). Effective mask-wearing can filter out more than 95% of pathogens, reducing health risks associated with pathogen transmission via inhalation and ingestion (Xiao *et al.*, 2024)

5.5 Detection sensitivity and analytical constraints

Considering the high rates of herbicide application in South Africa, particularly in areas classified as agricultural hotspots by GIS mapping, the lack of detectable GLY and AMPA in the sites of this study was surprising. This result could have several explanations, all of which emphasise the intricate environmental dynamics that control the destiny of GLY and the products of its degradation.

Another probable scenario that could explain the lack of GLY and AMPA in the samples of this study is that GLY and AMPA may react with ammonium that comes from fertiliser. In South Africa, maize farmers use inorganic fertilisers NKP on maize crops (Silwana *et al.*, 2007). These are the nitrogen, potassium and phosphate-containing fertilisers. NKP fertilisers contain these main elements in the form of ammonium nitrate (NH_4NO_3), ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$),

monoammonium phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$) and diammonium phosphate (DAP) ($(\text{NH}_4)_2\text{HPO}_4$) (Ejigu *et al.*, 2024) and GBH adjuvants (Klátyik *et al.*, 2023; Mueller and Steckel, 2019), such as ammonium sulphate. Biodegradation can mineralise these fertilisers and create free NH_4^+ . The NH_4^+ concentrations in the water samples of this study had an overall mean of 0.3 mg/L (Table 4.1). The elevated NH_4^+ measured in the current study reflects a combination of external fertiliser inputs and *in situ* release from nitrogen-rich adjuvants, providing the reactant required for GLY/AMPA–ammonium complexation. In GBHs, the salts of GLY, for example GLY-isopropylammonium salt, -diammonium salt, and -trimethylsulfonium salt, are used to increase the solubility of the a.i. in water (Klátyik *et al.*, 2023). This is yet another source of ammonia that could chelate with GLY (Soltani *et al.*, 2016; Thelen *et al.*, 1995), making detection difficult.

The present study used the Quick Polar Pesticides Extraction (QuPPE) protocol as the primary sample preparation step before LC-MS/MS analysis of GLY. Although QuPPE is recognised for its efficiency and broad applicability, its non-selective extraction also pulls out other compounds from the sample and leads to contamination of the chromatographic and detection systems (Gundermann *et al.*, 2025). To reduce this problem, a matrix-matched calibration curve was used. But even with this correction, the residual matrix load and some of the polar pesticides might not have been fully recovered, resulting in <LOD. Another reason GLY is so challenging to analyse is its zwitterionic nature, which allows the molecule to chelate readily with trivalent and quadrivalent metal ions (Gundermann *et al.*, 2025), see Section 2.6.3. These metal-GLY complexes can exist in several stoichiometries and charge states, altering the compound's solubility, chromatographic behaviour and ionisation efficiency in LC-MS/MS workflows. Unless a targeted pretreatment, such as metal-complex dissociation or selective derivatisation, is applied, these complexes remain in the extract, masking free GLY, reducing recovery, and resulting in <LOD.

A critical review of GLY analytics by Valle *et al.* (2019) highlights why laboratory non-detections, such as those obtained in this study, remain common despite intensive GBH use. Their survey of various published protocols showed that almost every high-performance technique, LC–MS/MS, GC–MS after FMOc derivatisation, capillary electrophoresis, and ^{31}P -NMR, still requires extensive clean-up and/or derivatisation because the parent molecule is highly polar and poorly ionised. The typical GLY method detection limits are between 0.02 and 1 $\mu\text{g/L}$ (0.3 $\mu\text{g/L}$ for the current study) for water and 1–10 $\mu\text{g/kg}$ (0.5 $\mu\text{g/kg}$ (reworked from 0.5 $\mu\text{g/L}$ assuming the solution is water) for the current study) for soils/sediments, depending on sample preparation. Importantly, the authors emphasise that no mature field-deployable sensor yet combines sub- $\mu\text{g/L}$ sensitivity with real-time response, a gap that they term as the “glyphosate paradox”; the world's most-used herbicide is still “one of the hardest to determine.” Valle *et al.* (2019)'s findings, combined with the current study's data, suggest that the non-detection of GLY/AMPA may reflect a combination of degradation/adsorption processes and current analytical ceilings.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 General conclusion

The study aimed to quantify GLY and AMPA levels in soil, sediment, and water samples from South African agricultural and non-agricultural settings. Unfortunately, GLY and AMPA levels were not detected at any of the sampled sites, which was unexpected. This absence calls on environmental processes like photodegradation, adsorption to soil particles, or microbial degradation to help explain GLY's environmental fate. The results indicate that GLY and AMPA may degrade or become mobilised more quickly under environmental conditions, underscoring the need for additional study into their degradation pathways and long-term ecological implications.

The study's first objective was to create geospatial maps of GBH applications in South Africa. These maps (Figure 4.1) provided an overview of GBH use patterns across different agricultural regions, offering valuable insights into potential high-risk areas for contamination. It showed that the Free State Province had the highest GBH use and that the Vaal River has been negatively impacted as a result. The use maps showed a clear GBH "hot-spot" template for later sampling and exposed the paradox that, despite this heavy use, no residues were detected.

The second objective was to determine GLY and AMPA concentrations in environmental samples before, during and after herbicide use. While rigorous sampling and sensitive analytical methods were applied, no residues were detected (<LOD) in any of the 632 total samples, underscoring the analytical and environmental challenges in tracking GLY under real-world conditions. The factors influence the degradation of GLY, which could lead to non-detections. As the study covered only one growing season, the results cannot account for temporal variation.

The third objective was to evaluate microbial diversity in surface waters and soils to see if changes in community composition could be used as indirect indicators of GLY exposure. While no chemical residues were found, changes in the microbial community were observed, possibly due to GBH exposure. There were clear differences in microbial soil and water diversities. The findings indicate that the microbial diversity shift (measurable changes in the community's structure) can serve as valuable ecological indicators with degradation potential, suggesting that microbial analyses can offer early warning indicators of herbicide exposure or even indicate prior exposure, even when GLY concentrations fall below detection limits.

The fourth objective was to conduct a Quantitative Microbial Risk Assessment (QMRA) to evaluate possible public health risks associated with microbial populations exposed to GLY. QMRA identified an increased risk of infection by *Klebsiella* spp., *Bacillus* spp., and *Staphylococcus* spp., raising concerns about the possible indirect effects of herbicide applications

on the structures of the microbial community. *Legionella* and *Mycobacterium* showed a 100% infection probability at 10/20 and 19/20 sites, respectively. There was a higher risk of infection for the MPA sites than at VIS sites.

6.2 Study limitations

While this study provides valuable information about the potential mobility of GLY to explain the absence in the samples as well as about the associated dynamics of the microbial community, some limitations must be recognised. These constraints stem from methodological, environmental and analytical factors that may have influenced the results.

One of the main limitations of this study is the absence of GLY and AMPA detectable in all collected samples. This unexpected result may be attributed to rapid microbial degradation, strong sorption on soil and sediments, or photodegradation. Although these factors suggest that no GLY and AMPA concentrations were detected under experimental conditions, they limit data-driven conclusions about their distribution and potential accumulation. In addition, the study was limited to a single growth season, which meant that seasonal or interannual variations in GLY concentrations could not be assessed.

Another notable limitation is related to sampling strategies and timing. The study design included sampling before, during and after the application of herbicides, as well as after a rainfall event. Given the dynamic nature of agricultural practices and environmental conditions, the timing of the sampling may not be consistent with peak GLY concentrations. Although the sampling was carried out as close as possible to the mentioned events, better communication could have been made with farmers to determine better sampling times rather than assumptions. Herbicide application is influenced by factors such as weather conditions, irrigation patterns and crop cycles, which may result in missing detection windows. In addition, the study was based on surface soil sampling, but GLY can behave differently at different soil depths due to leaching, root absorption, or microbial degradation at subsurface levels.

The study used LC-MS/MS, a highly sensitive technique, and even though the LOD and LOQ were lower compared to previous studies, no GLY and AMPA were detected in this study. In addition, the storage time between sample collection and analysis may affect the sample integrity. The samples should ideally be lyophilised (freeze-dried) immediately after the collection to preserve GLY stability and prevent degradation, to ensure that analysis is carried out within 1–2 months of the sampling to minimise potential losses due to degradation during storage.

Microbial community analysis offered indirect evidence instead of direct results. However, changes to the microbial community may also result from other environmental variables such as nutrient levels, pH, temperature, and other pollutants, which were not fully controlled in this study.

Although microbial shifts were detected, the absence of direct GLY measurements prevents confirmation of an actual link. The detection of microbial taxa known to degrade GLY suggests prior exposure, but without quantitative residue data, conclusions about GLY's specific impact remain uncertain.

Another limitation is the uncertainty of the QMRA. QMRA is based on bacterial taxa identified at the genus level, while pathogen risk models are usually species-specific. This means that some identified bacteria may not be pathogenic or may have different resistance mechanisms than those assumed in the model. The exposure hypotheses used in QMRA were based on literature values instead of direct field measurements of pathogenic concentrations. This introduces a degree of uncertainty in risk estimates, as actual levels of human exposure may differ from model predictions. In addition, QMRA did not account for seasonal variations that could significantly influence microbial dynamics, as temperature and environmental conditions affect pathogen survival and proliferation.

Finally, the study was geographically limited to only two sites in South Africa with known GBH applications, which means that the results may not be fully representative of all agricultural regions where GBH might also be used.

6.3 Recommendations and future studies

The following recommendations outline future research directions that could address the knowledge gaps identified in this study.

6.3.1 Long-term and seasonal monitoring of glyphosate (GLY) and AMPA (aminomethylphosphonic acid)

The lack of detectable GLY and AMPA levels was found in this study, requiring future research to focus on long-term monitoring over multiple growing seasons to capture potential seasonal or interannual variations in the persistence of GLY. Seasonal fluctuations in temperature, precipitation and agricultural practices can affect GLY degradation, sorption and leakage. Expanding the monitoring to cover two hot and cold seasons will provide a more comprehensive picture of how environmental conditions affect the fate of GLY.

6.3.2 Multi-matrix sampling and deeper soil analysis

The study analysed mainly surface water, sediment and soil samples. However, GLY can persist in different environmental matrices beyond those evaluated. Future studies should include:

- Groundwater sampling to evaluate the potential for GLY leaching and long-term accumulation in subsurface water reserves.

- Vegetation sampling to investigate GLY uptake by crops and non-target plants, assessing potential risks to food safety and ecosystem health.
- Deeper soil sampling to determine whether GLY is retained or degraded at various depths, improving our understanding of its mobility in different soil profiles.

6.3.3 Refining analytical methods for ultra-low glyphosate (GLY) detection

Despite using a highly sensitive LC-MS/MS approach, it is possible that GLY was present at concentrations below the detection limits. Future studies should refine analytical methods by:

- Lowering the detection limits (LOD and LOQ) to capture trace levels of GLY and AMPA.
- Implementing immediate sample lyophilisation (freeze-drying) of soil and sediment samples to preserve analyte integrity and minimise degradation between collection and analysis.
- Consider non-spectroscopic analytical techniques: biosensors such as surface plasmon resonance (SPR), horseradish peroxidase (ES-HRP), cyanobacterium sensors, algal biosensors, chlorophyll fluorescence-based techniques, isolated photosystem II (PSII) analytical techniques and enzyme-linked immunosorbent assay (ELISA).
- Consider refining the extraction method for different matrices.

6.3.4 Linking microbial community changes to glyphosate (GLY) exposure

The microbial community analysis in this study suggested potential GLY-associated shifts, but a direct causal link between GLY exposure and microbial changes remains unconfirmed. Future research should:

- Use controlled laboratory experiments where microbial communities are exposed to known concentrations of GLY to confirm its effects on microbial diversity and function.
- Explore functional gene expression analyses to determine whether microbial taxa linked to GLY degradation show upregulation of specific metabolic pathways in response to herbicide exposure.
- Compare microbial responses between agricultural and non-agricultural environments to differentiate GLY-driven changes from other environmental stressors.
- Future monitoring strategies should combine direct chemical analyses with microbial community screening to strengthen ecological and human health risk assessments.

6.3.5 Expanding the Quantitative Microbial Risk Assessment (QMRA) Framework

The QMRA conducted in this study was based on bacterial genera rather than species, introducing uncertainty in pathogen risk modelling. Future QMRA studies should:

- Identify bacteria at the species level to improve the accuracy of risk assessments and differentiate between non-pathogenic and pathogenic strains.
- Compare risk assessments across different climate conditions (hot vs. cold seasons) to determine how temperature and environmental factors influence pathogen survival and infection risks.
- Conduct exposure assessments using direct field measurements rather than literature-based assumptions to enhance risk estimation accuracy.

6.3.6 Assessing the impact of agricultural practices on glyphosate (GLY) fate

Future research should examine how conventional vs. conservation agriculture practices influence GLY degradation, mobility, and potential accumulation. This could include:

- Tilled versus no-tilled fields to evaluate how soil disturbance affects GLY retention and microbial degradation rates.
- Conservation vs. conventional farming systems to determine whether alternative weed management strategies influence GLY persistence.
- Irrigated vs. rainfed cropping systems to assess whether irrigation enhances GLY transport from surface to groundwater.
- Future studies should assess how minimal fertilizer use in regenerative canola systems of the Western Cape affects GLY and AMPA fate in soils.

6.3.7 Investigating potential glyphosate-metal interactions in soil and water

Glyphosate has been shown to form complexes with metal ions such as iron, calcium, and aluminium, which can affect its mobility and bioavailability. Future studies should explore:

- How soil CEC influences GLY sorption and desorption dynamics with other free ions.
- Whether metal-complex formation affects microbial degradation rates, particularly in soils with high mineral content.

6.3.8 Assessing the toxicity of glyphosate (GLY) and AMPA (aminomethylphosphonic acid) in South African contexts

While international studies have explored the ecotoxicological effects of GLY and its metabolites, limited data exist for South African ecosystems. Future research should:

- Build on Mensah *et al.* (2013)'s South African water quality guidelines for GBH benchmarks by testing GLY formulations for chronic, sub-lethal effects across a wider range of aquatic and terrestrial taxa; soil invertebrates, amphibians, and crop-edge plants, under realistic (not lab-based) multi-stress conditions.
- Investigate human health risks by assessing GLY exposure levels in agricultural workers and communities near high-use areas.
- Explore the impact of GLY formulations, including surfactants, on non-target organisms, as certain additives may increase toxicity beyond that of GLY alone.

Future research should be based on the findings of this study by expanding monitoring efforts, improving analytical methods and exploring the broader ecological and health effects of GLY use. The resolution of these knowledge gaps will contribute to a more comprehensive understanding of GLY's environmental fate and better management practices for its sustainable use in agriculture.

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APPENDICES

Here is a breakdown of the supplementary information and data for this study. Each appendix will give its respective details on the contents therein.

Appendix A: Supplementary to Chapter 3, NMISA report.

Appendix B: Supplementary for Chapter 4 water, soil and sediment raw data.

Appendix C: Supplementary data for Chapter 4, chemical analysis raw data.

Appendix D: Supplementary for QMRA calculated data.

Appendix A: NMISA report

Measurement of Glyphosate and Aminomethylphosphonic acid (AMPA) in water and soil

17 November 2022

1. Scope and short description

The purpose of this report is to provide feedback on the extraction and analysis method developed for highly polar pesticides (HPPs), Glyphosate and Aminomethylphosphonic acid (AMPA), in water and soil. Spiked samples were used to develop and evaluate the extraction and analysis method. The analysis and extraction methods were based on and modified from the EURL-SRM QuPPE method for highly polar pesticides in fruit and vegetables (6.1) and an article utilising ASE extraction (6.2). An optimised water and soil method was achieved with acceptable recovery (60–120%).

2. Instrumental analysis

The identification and quantification were performed on a Waters ultra-high-pressure liquid chromatograph coupled to a mass spectrometer. The limit of detection and limit of quantification were evaluated with volumetric preparation of a calibration curve and could be improved with the application of gravimetric procedures. The achieved LOD/LOQ is: Glyphosate – 0.5/1.8 ng/g; AMPA – 0.5/1.7 ng/g.

Table 1: Column and basic method specifications

C18 Column	Hypercarb 2.1 x 100 mm; 5 µm; 40°C
Sample Temp	Sample temperature: 4°C
Injection volume	Injection volume: 10 µL
Mobile phase A	1% Acetic Acid, 5% Methanol in MilliQ water
Mobile phase B	1% Acetic Acid in Methanol
Run time	30 minutes

Table 2: Solvent gradient table

Time (min)	Flow (mL/min)	% A	% B	% C	% D	Curve
Initial	0.200	100	10	0	0	Initial
10.00	0.200	70	30	0	0	6
11.00	0.400	70	30	0	0	6
18.00	0.400	70	30	0	0	6
19.00	0.400	10	90	0	0	6
22.00	0.400	10	90	0	0	6
22.10	0.200	100	0	0	0	6
30.00	0.200	100	0	0	0	6

Table 3: Wash solvents composition

Seal wash	90 % MilliQ water: 10% Methanol
Purge solvent	90 % MilliQ water: 10% Methanol
Strong needle wash	5 % MilliQ water: 5% Isopropanol: 90% Methanol

LC-MS/MS conditions are optimised for all compounds and included chromatographic separation, precursor and product ion identification for specific MRM transitions and associated cone voltage (CV) and collision energy (CE) for selected HPPs. All compounds are analysed in negative ion mode. MRM transitions and MS parameters can either be obtained from literature or through experimental optimisation. A matrix-matched standard will also be run to ensure that no matrix-specific interferences will influence quantification. The MRM transitions developed for the HPPs currently analysed by LC-MS/MS are summarised in **Table 4**.

Table 4: Optimal MS/MS parameters for the analysis of HPPs by LC-MS/MS

Compound	Precursor Ion (Da)	Product Ion (Da)	Cone voltage (CV)	Collision energy (CE)
Phosphonic acid	81	63	25	13
Phosphonic acid	81	79	25	11
Fosetyl aluminium	109	63	20	16
Fosetyl aluminium	109	81	20	10
Aminomethylphosphonic acid (AMPA)	110	63	35	13
Aminomethylphosphonic acid (AMPA)	110	79	35	14
Ethephon	143	79	15	7
Ethephon	143	107	15	7
Ethephon	145	107	15	7
3-Methylphosphinico-propionic acid (MPPA)	151	107	20	14
3-Methylphosphinico-propionic acid (MPPA)	151	133	20	11
N-Acetyl AMPA	152	63	30	15
N-Acetyl AMPA	152	110	20	17
Glyphosate	168	63	25	18
Glyphosate	168	81	25	18
Glyphosate	168	124	25	9
Glyphosate	168	150	25	9
Glufosinate	180	85	30	16
Glufosinate	180	95	30	16
N-Acetyl glyphosate	210	150	25	13
N-Acetyl glyphosate	210	192	25	9
N-Acetyl glufosinate (NAG)	222	69	30	15
N-Acetyl glufosinate (NAG)	222	136	30	20

Optimal separation of the two compounds of interest was achieved (Figures 1 and 2). The peak shape and separation within the spiked water (Figures 5 and 6) and sediment samples (Figures 7 and 8) were acceptable and met the recovery requirements.

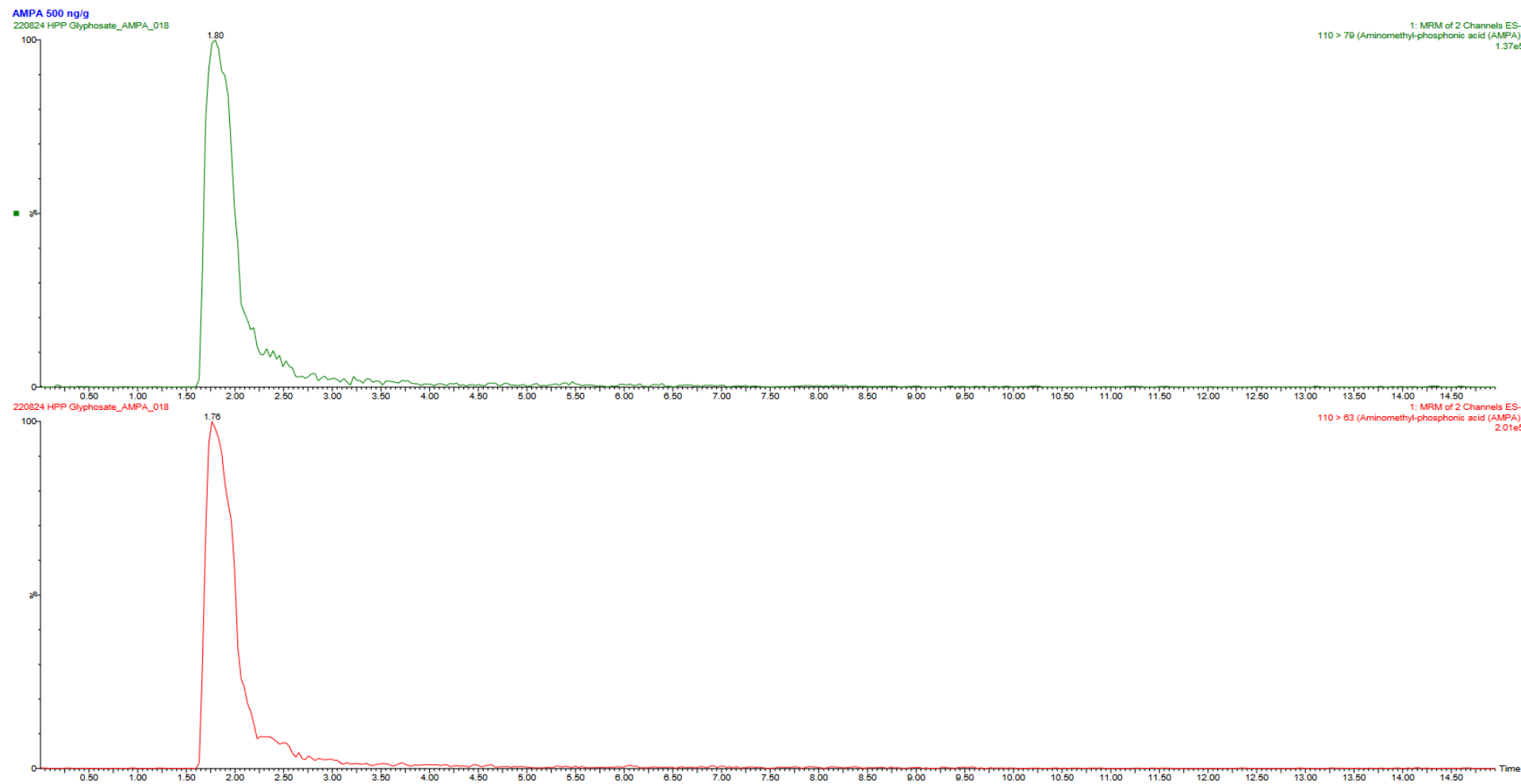


Figure 1: Optimal separation achieved for AMPA. The AMPA is eluting at 1.5 – 2.5 minutes. The peak in the image is slightly overloaded leading to broad spread. However, this will be less pronounced in environmental samples as the concentration will be lower and the matrix will be present.

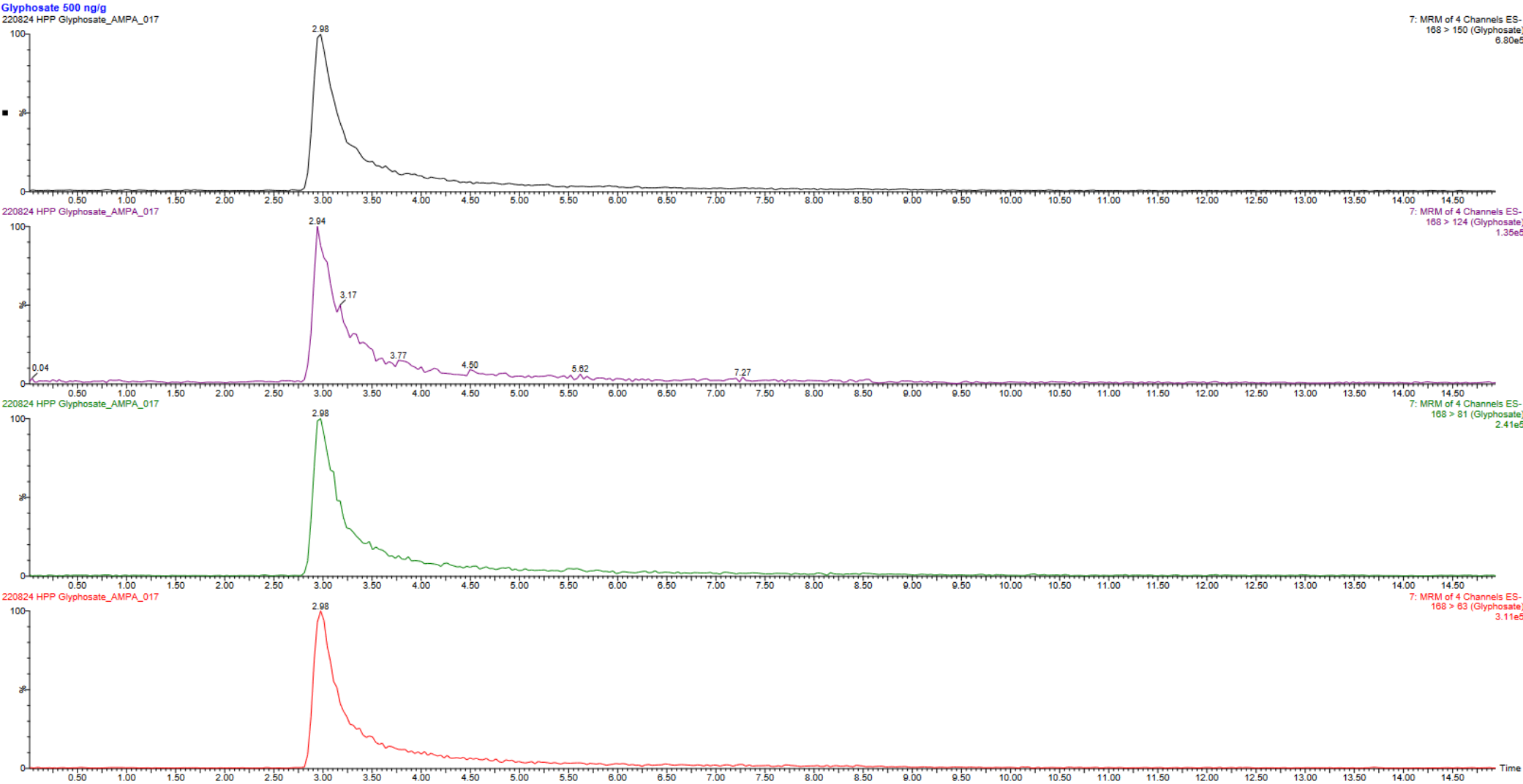


Figure 2: Optimal separation achieved for Glyphosate

Glyphosate is eluting between 2.94 and 2.98 minutes. Peak tailing was observed, possibly due to chelation of glyphosate with metal ions in the LC system. This is a normal occurrence for Glyphosate and is acceptable. Literature has indicated that the tailing can be lessened by injecting 20 injections of matrix before analysing samples (6.1) and replacing the frit before each analysis batch.

The internal standard (Glyphosate-2- ^{13}C) was evaluated to confirm if it was adequate to use during the extraction process. The standard was injected on the UHPLC coupled to the Sciex QTRAP MS/MS. Unfortunately, when energy is applied to the compound, the native of interest Glyphosate is formed and will interfere with the quantification of the compound of interest (Figure 3). Due to the positioning of the carbon label (**Figure 4**) during fragmentation both the isotope and native have fragment with identical masses. Unfortunately, the mass is the quantification mass and therefore there will be an interference during quantification. Other masses cannot be used for the quantification as the response is significantly lower in matrix and will not provide a constant mass fraction for quantification. Another HPP compound Glufosinate, can be evaluated as an alternative to this internal standard.

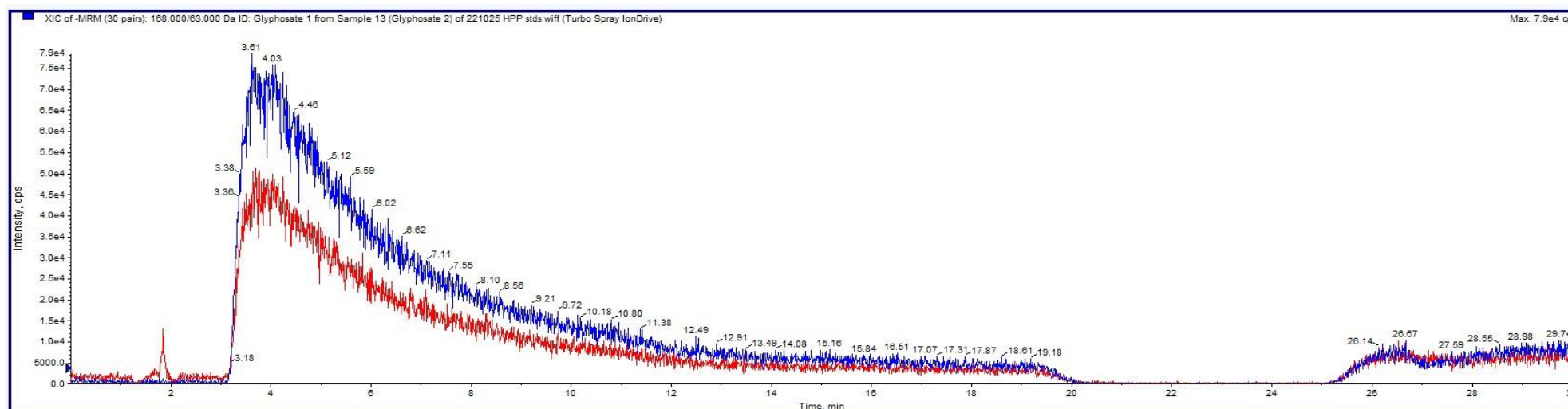


Figure 3: Similar m/z values were achieved when analysing the Glyphosate-2- ^{13}C compared to Glyphosate (m/z 168 > 63)

(a)

(b)

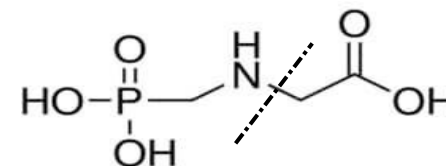
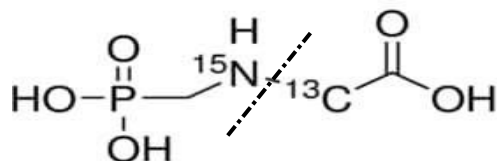


Figure 4: Chemical structure of Glyphosate-2- ^{13}C (a) and Glyphosate (b)

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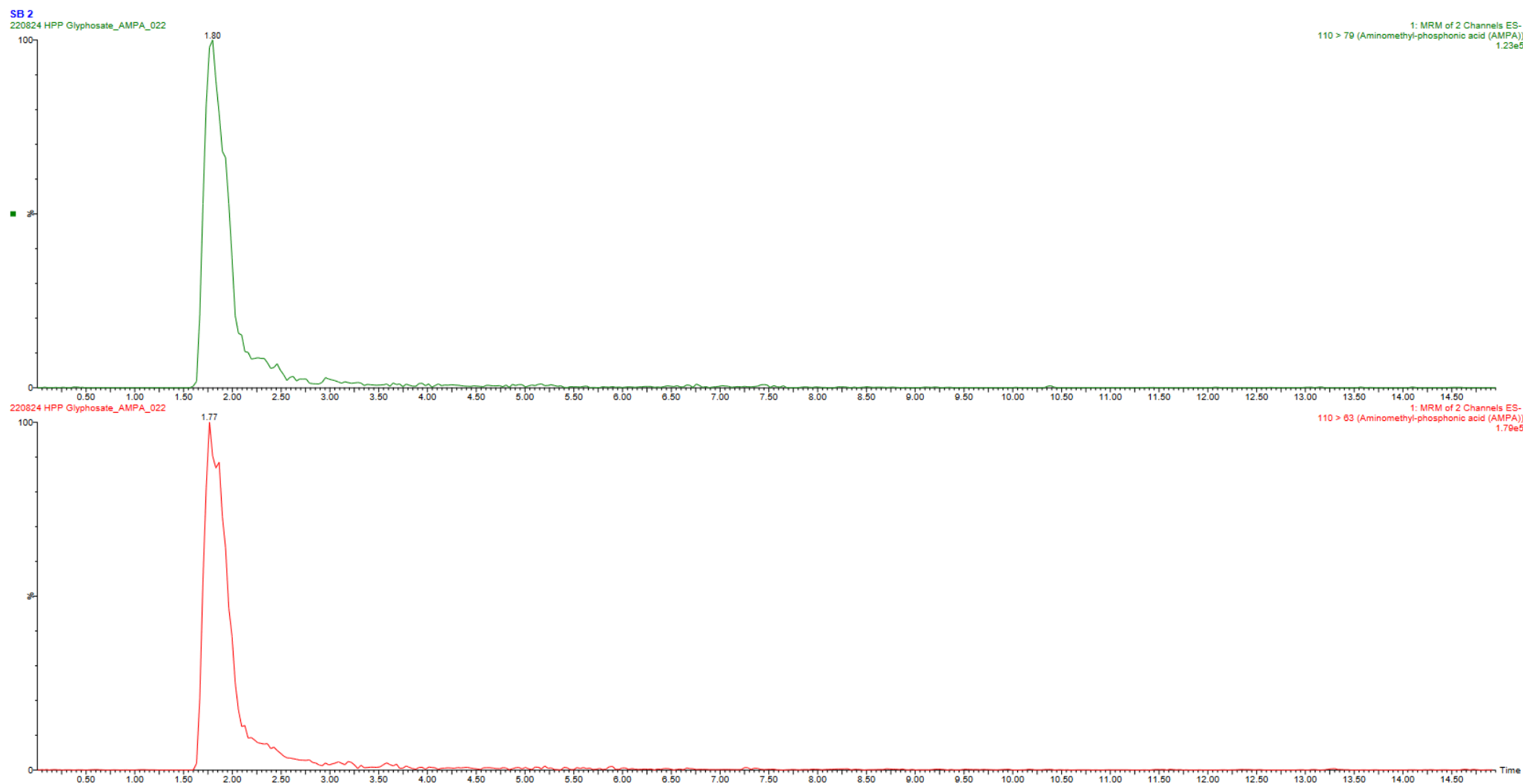


Figure 5: Extracted MRM for AMPA in the spiked water extract. Evaluation of the AMPA from water indicated no interference with the selected m/z values was detected after extraction. Optimal recovery was achieved and peak shape is adequate.

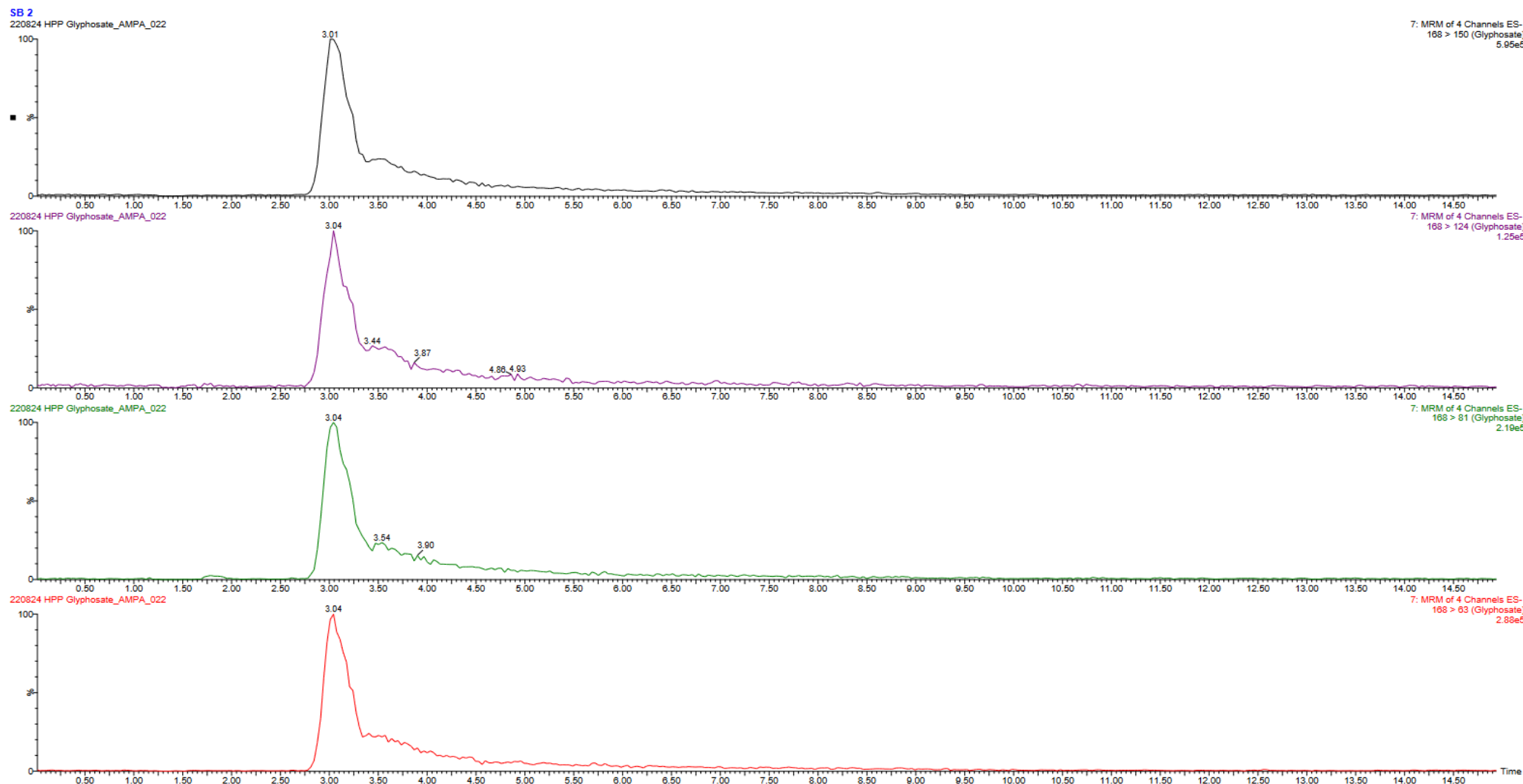


Figure 6: Extracted MRM for Glyphosate in the spiked water extract. Evaluation of the Glyphosate from water indicated no interference with the selected m/z values in the presence of matrix after extraction. Optimal recovery was achieved and the peak shape is adequate. Four mass transitions were evaluated to ensure no interference is present and to evaluate the peak shape of minor m/z masses. Two transitions were selected after extraction evaluation for further analysis

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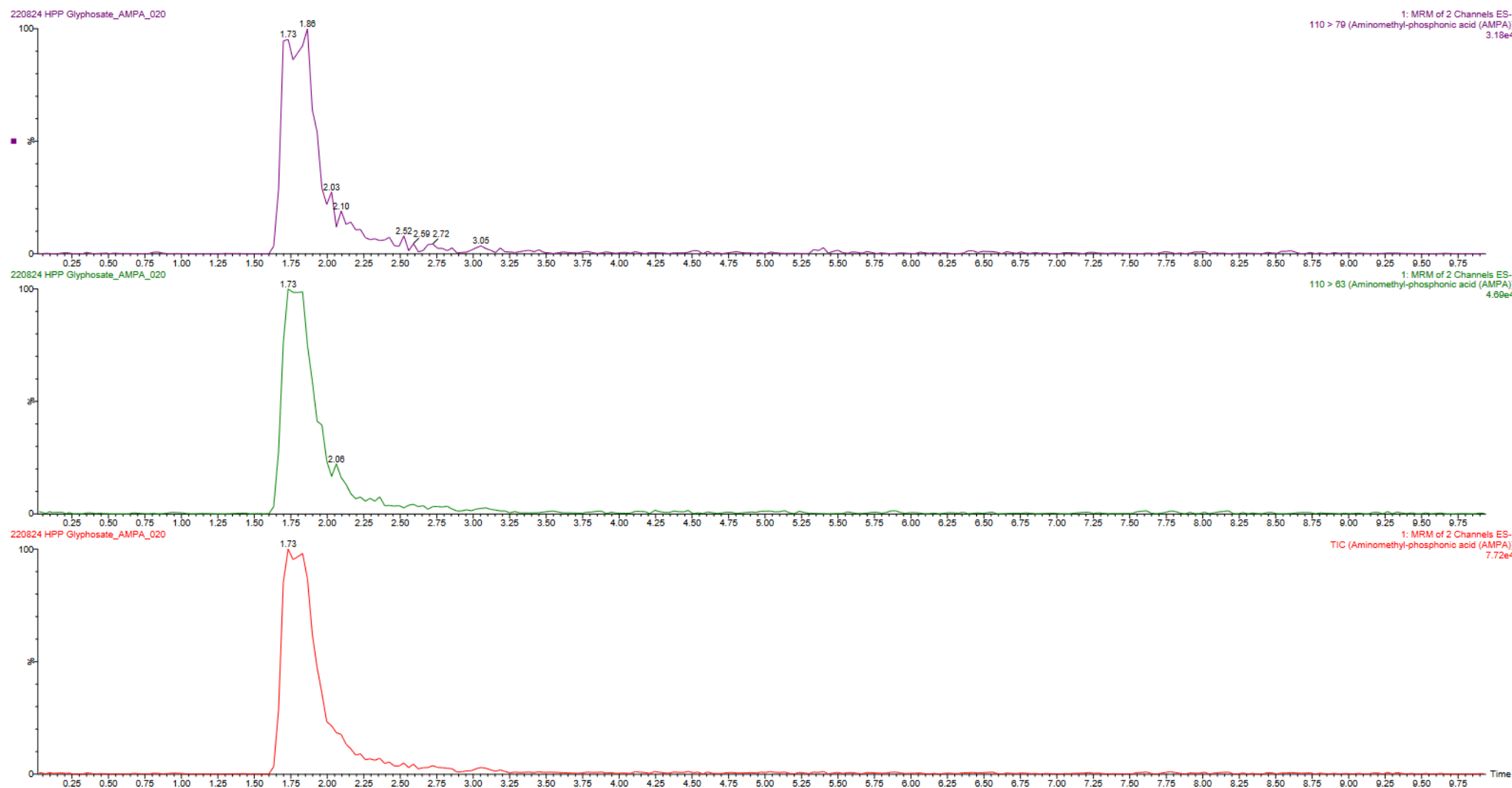


Figure 7: Extracted MRM for AMPA in the spiked soil extract. Evaluation of the AMPA from sediment indicated no interference with the selected m/z values detected in the matrix after extraction. Acceptable recovery was achieved (60–120%). Peak splitting is present due to high concentration but this should not be a problem in the environmental samples as lower concentrations are expected.

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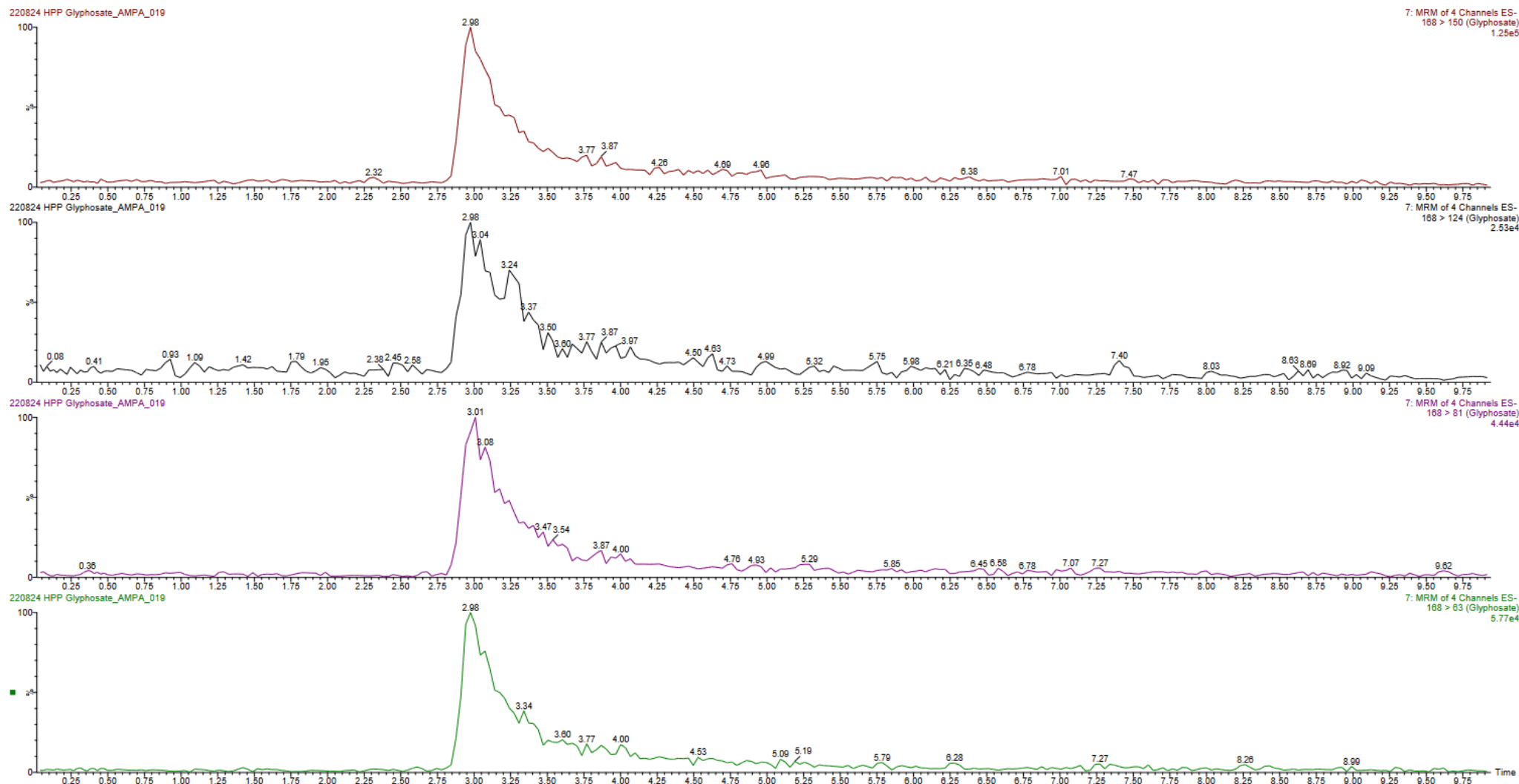


Figure 8: Extracted MRM for Glyphosate in the spiked soil extract

Evaluation of the Glyphosate from sediment indicated no interference with the selected m/z values detected in the matrix after extraction. Peak shape tailing and splitting are present and could be due to the presence of chelation during analysis. This will be prevented by ensuring a clean frit is inserted before every analysis batch and matrix injected to ensure no active sites in the LC system.

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3. Extraction of water

The extraction procedure was adapted from the EURL-SRM QuPPE method for highly polar pesticides in fruit and vegetables. The samples were spiked at a known concentration with the compounds of interest and the recovery achieved was evaluated. Optimal recovery between 80 - 110% was achieved with the selected method.

1. Clearly mark 50 mL centrifuge tubes with sample labels and wrap with foil to prevent exposure to light.
2. Bring sample to room temperature, vortex and weigh 10 mL into centrifuge tube.
3. Add internal and/ or native standards if required. For the quality control sample, a concentration of 500 ng/g is advised. Vortex to ensure optimal equilibration.
4. Add 10 mL Acidified Methanol (1% Formic acid)
5. Vortex for 2 minutes
6. Ultrasonicate samples for 15 minutes
7. Centrifuge for 10 minutes at 6000 g, at 4 °C
8. Filter the sample with a 0.22 µm PTFE filter and inject.

4. Extraction of soil

Multiple extraction methods were evaluated to achieve optimal recovery. Three different techniques were evaluated: accelerated solvent extraction (ASE); Quick method for the analysis of numerous highly polar pesticides in food and plant origin via LC-MS/MS involving simultaneous extraction with methanol (QuPPE-Method); and Potassium hydroxide (KOH) digestion (**Table 5**). Optimal recovery was only achieved with method 3 at 60%. Direct injection was done with each method where possible. In this case, an aliquot from the extract is taken and injected on the LC before evaporation to evaluate if it is possible to detect any compounds. In most cases, no pesticides could be detected, as the extract was too diluted.

1. The extraction that provided the best results (between 58 – 63% recovery) is recommended. This is the ASE extraction with Methanol, 0.1% Formic Acid, 2% Water (Method 3; Table 5). This method is detailed below.
2. Clearly mark 50 mL centrifuge tubes with sample label and wrap with foil to prevent exposure to light.
3. Thoroughly mix dried sample and weigh 10 g into centrifuge tube.
4. Mix hydromatrix/ diatomaceous earth with the sample. The cell volume must be fully used and packed consistently (similar density) to ensure repeatable solvent volumes with extraction on the ASE. Therefore, the amount of hydromatrix/ diatomaceous earth to mix with the sample depends on the size of the cell used.
5. Place two cellulose filters in a precleaned marked extraction cell and fill it with hydromatrix/ diatomaceous earth to cover the filter.
6. Transfer the sample to the extraction cell.
7. Add the required internal and/ or native standard. For the quality control sample, a concentration of 1000 ng/g is advised.
8. Fill the remainder of the cell with hydromatrix/ diatomaceous earth, and hand tighten the top end cap onto the cell body. Note – no filter required on top is required.

Table 5: Extraction methods evaluated for soil

#	Sample mass (g)	Extraction Solvent	After extraction	Outcome
Modified QuPPE method for dry foods				
1	5	Water and acidified Methanol (1% Formic acid) with additional freeze step	• Direct injection Evaporation to dryness and reconstitution in 5% Methanol	• None of the pesticides detected - likely due to the evaporation process
2	5	Acidified Methanol (1% Formic acid)	• Direct injection Evaporation to dryness and reconstitution in 5% Methanol	• None of the pesticides detected - likely due to the evaporation process
Extraction with potassium hydroxide (KOH)				
3	2	0.2 M KOH	Could not inject, further clean-up required by SPE	Requires additional clean-up
ASE				
The following ASE parameters were used for all extractions (6.2): Temperature 110°C, static time 6 min, cycles 3, purge volume 80%, purge time 120 s				
4	10	2:1 Ethyl acetate: Acetone	• Direct injection • Evaporation to dryness and reconstitution in 5% Methanol	• None of the pesticides could be detected • Loss of analyte was likely due to evaporation • The extract must be evaporated as ethyl acetate and acetone cannot be injected into the column. These solvents will damage the column packaging material
5	10	Methanol	• Direct injection • Evaporation to dryness and reconstitution in 5% Methanol	• Low recoveries achieved (10 – 40%) • Likely due to the evaporation process • Due to the volume extraction, evaporation is required to make sure that the pesticides are within the quantification range.
6	10	Methanol, 0.1% Formic Acid, 2% Water	• Direct injection • Evaporation to 1 mL	• Recoveries between 58 – 63% achieved • Increase in recovery could be due to the addition of water. Water acting as an analyte protectant during evaporation. • For this method to work, it is crucial that extracts are not evaporated to dryness. The water phase will remain as the methanol will evaporate faster. • This is the suggested method for extraction.

5. Preparation of quality control samples

Quality control samples will need to be prepared with each batch to ensure recovery and degradation can be evaluated to correct for this deviation in the final data. Three samples of each matrix per batch will be spiked with a known concentration before extraction, and three samples will be extracted, but only spiked after extraction (**Table 6**). This is known as spike before (SB) and spike after (SA) quality control samples. Additionally, a matrix blank will also be added to evaluate if any analytes of interest are present in the matrix used for the spike samples.

Table 6: Quality control sample summary

Matrix	Sample type	Samples	End concentration (ng/g)
Spike before (SB)			
Water	MilliQ water	Three samples spiked as described in section 3.3	500
Soil	Any soil sample	Three samples spiked as described in section 4.6	1000
Spike after (SA)			
Water	MilliQ water	Three samples spiked after extraction before evaporation into 50 mL centrifuge tube	500
Soil	Any soil sample	Three samples spiked after extraction prior to evaporation 50 mL centrifuge tube	1000
Blank			
Water	MilliQ water	No spike, but follows the extraction procedure	0
Soil	Any soil sample	No spike, but follows the extraction procedure	0

5. Important notes

A multitude of factors can influence the analysis and extraction of HPP. Precaution must be taken to prevent loss of analyte. Please note that the following factors must be taken into account.

1. Samples must be stored away from light and frozen to prevent breakdown.
2. Upon thawing, samples should be extracted and analysed within 14 days
3. Ensure all standards and samples are not exposed directly to glass and stored in polypropylene or other suitable containers to prevent interaction with glass surface.
4. Standards and samples must not be exposed to light.
5. Standards must be made in 10% Acetonitrile in water to prevent breakdown and are not stable for longer than 2 weeks.
6. Evaporation to dryness is not advised as this led to substantial loss of analytes.
7. Samples must be stored away from light at room temperature and analysed within 14 days. This was for fruit samples in a mixture of acidified Methanol and water. We would not advise longer than 7 days.

8. Sample analysis

The samples were received as extracts and evaporated in a Genevac as described in section 3 and 4. After evaporation, the samples were filtered and injected for analysis. An 11-point calibration curve was prepared by spiking blank soil and blank water, predetermined to be blank. These samples were enriched with native and internal standard. A linear regression was applied, where S_0 was defined as the x-intercept. The LOD was defined as three times the standard deviation at S_0 and the LOQ was defined as ten times the standard deviation at S_0 . Quality control samples were run with every batch to ensure the quality of analytical runs. Recovery was assessed by gravimetrically spiking blank soil and water. Reported results have not been corrected for recoveries.

No samples contained detectable levels of Glyphosate or AMPA. To ensure no problems were encountered during the process or with the instrumentation, additional tests were performed. Additionally, the samples were screened for the following compounds: Phosphonic acid, Fosetyl aluminium, Ethephon, 3-Methylphosphinico-propionic acid (MPPA), N-Acetyl AMPA, N-acetyl glyphosate and N-Acetyl glufosinate. None of the compounds screened for was present in the samples.

9. Recommendations

As no levels were detected, there might be a few things to consider if another sampling opportunity is presented.

1. Samples must be stored away from light and frozen to prevent breakdown.
2. Upon thawing, samples should be extracted and analysed within 14 days
3. Ensure all standards and samples are not exposed directly to glass and stored in polypropylene or other suitable containers to prevent interaction with glass surface
4. Standards and samples must not be exposed to light
5. Standards must be made in 10% Acetonitrile in water to prevent breakdown and are not stable for longer than 2 weeks
6. Evaporation to dryness is not advised as this led to substantial loss of analytes.
7. Samples must be stored away from light at room temperature and analysed within 14 days. This was for fruit samples in a mixture of acidified Methanol and water. We would not advise longer than 7 days.

10. References

EU Reference Laboratories for Residues of Pesticides – Single Residue Methods (EURL-SRM). Quick method for the analysis of highly polar pesticides in food involving extraction with acidified methanol and LC- or IC-MS/MS measurement. Version 12, 2021.

Schafer *et al.*, 2008. Determination of 10 particle-associated multiclass polar and semi-polar pesticides from small streams using accelerated solvent extraction. *Chemosphere* 70 (2008) 1952-1960

Appendix B: Water, soil and sediment raw data

Here is the raw data for the water quality and the soil/sediment. The tables are organised as follows.

1. Water and sediment tables before spraying (Table S-1 to Table S-5)
2. Water and sediment tables after spraying (Table S-6 to Table S-10)
3. Water and sediment tables after rainfall (Table S-11 to Table S-15)
4. Water and sediment tables after harvest (Table S-16 to Table S-20)

Table S.1: Water quality results before spraying in Mpumalanga in the Olifants River catchment.

Water quality parameters	Symbol	RWQO	MD	DMD	MP4	MP6	PAN1	MP10
pH	pH	6.5–8.5	6.75	6.41	7.36	7.06	8.27	9.43
Temperature (°C)	TEMP	N/A	20.6	19.7	22.2	23.3	26.9	20.4
EC (mS/m)	EC	<70	85	105.4	316	260	2320	42.5
Total suspended solids (mg/L)	TSS	N/A	5	3	7	73	371	1
Turbidity (FAU)	TURB	N/A	7	3	12	3	94	11
Ammonium (mg/L)	NH ₄ ⁺	<0.1	0.13	0.10	0.22	0.14	3.44	0.09
Nitrite (mg/L)	NO ₂ ⁻	<4	0.01	0.01	0.01	0.01	0.00	0.01
Nitrate (mg/L)	NO ₃ ⁻	<4	0.90	0.80	0.50	2.30	0.36	0.60
Sulphate (mg/L)	SO ₄ ²⁻	<500	70	77	75	281	233	44
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.125	0.22	0.28	0.27	0.51	0.69	1.30
Chloride (mg/L)	Cl ⁻	N/A	17	6	22	16	2	16
Total hardness (mg/L)	TH	N/A	26	27	113	81	144	48
%Sand	Sand	N/A	38.1	69.52	34.04	11.06	95.38	0
%Silt	Silt	N/A	34.51	29.79	41.14	70.55	3.68	87.97
%Clay	Clay	N/A	26.53	0.05	24.37	17.45	0.79	11.73
%Total organic content	TOC	N/A	0.34	0.33	0.66	0.49	2.83	0.16

RWQO = Resource water quality objectives, MD = Middelburg Dam, DMD = Downstream Middelburg Dam, MP4 = Mpumalanga 4, MP6 = Mpumalanga 6, PAN1 = PAN1, MP10 = Mpumalanga 10. Grey highlighted values exceeded the RWQO.

Table S.2: Water quality results before spraying in Mpumalanga in the Upper Vaal River catchment.

Water quality parameters	Symbol	RWQO	X1	X2	X3	V9	V11	V12	V13	GD	V15
pH	pH	6.5–8.5	8.13	7.83	7.94	7.60	7.45	7.43	7.50	7.25	7.30
Temperature (°C)	TEMP	N/A	13.7	16.8	15.5	18.5	19.7	19.8	18.5	18.9	18.9
Conductivity (mS/m)	EC	<70	65.1	74.2	61	19.4	19.8	19.5	21.6	27.6	28.3
Total suspended solids (mg/L)	TSS	N/A	2	9	34	53	67	63	72	34	29
Turbidity (FAU)	TURB	N/A	5	14	6	37	55	54	67	37	26
Ammonium (mg/L)	NH ₄ ⁺	<0.073	0.30	0.16	1.02	0.19	0.13	0.19	0.16	0.11	0.33
Nitrite (mg/L)	NO ₂ ⁻	<0.858	0.01	0.01	0.15	0.02	0.03	0.03	0.00	0.02	0.03
Nitrate (mg/L)	NO ₃ ⁻	<0.858	1.23	1.00	2.50	1.10	1.20	0.50	1.30	1.00	1.00
Sulphate (mg/L)	SO ₄ ²⁻	<500	52	62	61	37	38	46	44	56	60
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.02	0.20	0.99	1.12	0.22	0.23	0.98	0.59	0.60	0.90
Chloride (mg/L)	Cl ⁻	N/A	21	15	19	12	10	18	10	12	12
Total hardness (mg/L)	TH	N/A	151	177	104	36	35	39	36	44	46
%Sand	Sand	N/A	17.06	34.32	7.67	83.38	69.02	44.87	69.42	32.97	30.09
%Silt	Silt	N/A	58.06	45.15	83.9	14.37	24.22	38.57	27.09	57.86	60.41
%Clay	Clay	N/A	13.58	19.55	6.27	1.48	5.78	16.31	3.45	8.63	9.02
%Total organic content	TOC	N/A	0.99	0.93	0.8	1.65	1.46	1.26	0.66	1.5	0.15

RWQO = Resource water quality objectives, X1 = Life Water River, X2 = Life Water River, X3 = Life Water River, V9 = Vaal River 9, V11 = Vaal River 11, V12 = Vaal River 12, V13 = Vaal River 13, GD = Grootdraai, V15 = Vaal River 15. Grey highlighted values exceeded the RWQO.

Table S.3: Water quality results before spraying Vaalharts irrigation scheme in the Lower Vaal River catchment.

Water quality parameters	Symbol	RWQO	TO	HR1	C1	HR2	C2	MIC	HR3	HR4	SD	DSD
pH	pH	6.5–8.5	5.8	6.8	6.8	7.04	6.81	6.9	7.00	7.02	8.09	7.46
Temperature (°C)	TEMP	N/A	22.7	21.0	-	23.3	23.5	20.7	23.2	18.0	22.2	22.4
Conductivity (mS/m)	EC	<111	24.5	21.8	106.7	95.3	217	62.5	140	131	111.3	111.4
Total suspended solids (mg/L)	TSS	N/A	71	15	26	5	43	12	28	10	14	351
Turbidity (FAU)	TURB	N/A	40	6	9	12	2	19	14	28	13	26
Ammonium (mg/L)	NH ₄ ⁺	<0.07	0.10	0.09	0.18	0.83	0.30	0.07	0.32	0.21	0.25	0.29
Nitrite (mg/L)	NO ₂ ⁻	<1	0.01	0.04	0.02	0.04	0.04	0.03	0.15	0.04	0.01	0.02
Nitrate (mg/L)	NO ₃ ⁻	<1	1.80	1.70	5.20	3.90	8.60	2.10	4.70	7.90	2.20	3.30
Sulphate (mg/L)	SO ₄ ²⁻	<200	13	16	189	180	248	114	226	228	205	198
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.025	0.23	0.11	0.21	0.14	0.40	0.29	0.25	0.43	0.22	0.27
Chloride (mg/L)	Cl ⁻	N/A	15	19	68	83	220	42	118	136	96	86
Total hardness (mg/L)	TH	N/A	54	54	180	153	190	87	101	176	169	175
%Sand	Sand	N/A	13.02	71.48		28.24			50.61	56.02	51.14	91.82
%Silt	Silt	N/A	58.45	27.89		70.09			42.79	42.32	42.64	7.89
%Clay	Clay	N/A	25.39	0.34		1.17			6.2	1.32	4.62	0.24
%Total organic content	TOC	N/A	0.67	0.24		0.28			0.35	0.54	1.16	1.04

RWQO = Resource water quality objectives, TO = Taung Overflow, HR1 = Harts River 1, C1 = Canal 1, HR2 = Harts River 2, C2 = Canal 2, MIC = Main Inflow Canal, HR3 = Harts River 3, HR4 = Harts River 4, SD = Spitskop Dam, DSD = Downstream Spitskop Dam. Grey highlighted values exceeded the RWQO.

Table S.4: Water quality results of non-agricultural sites in the Upper Vaal River catchment

Water quality parameters	Symbol	RWQO	VB	DGP	MM	TRE	GG	BT
pH	pH	6.5–8.5	6.06	7.21	7.09	8.11	7.77	7.42
Temperature (°C)	TEMP	N/A	26.5	25.9	25.3	29.7	24.9	29.3
Conductivity (mS/m)	EC	<111	68	95.1	103.9	63.5	74.4	63.6
Total suspended solids (mg/L)	TSS	N/A	43	3	14	52	38	7
Turbidity (FAU)	TURB	N/A	8	6	11	16	4	8
Ammonium (mg/L)	NH ₄ ⁺	<0.073	4.24	0.13	0.14	0.27	0.31	0.23
Nitrite (mg/L)	NO ₂ ⁻	<4	0.30	0.02	0.03	0.02	0.04	0.03
Nitrate (mg/L)	NO ₃ ⁻	<4	2.10	0.80	1.00	1.00	0.70	1.50
Sulphate (mg/L)	SO ₄ ²⁻	<500	103	143	163	124	124	84
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.125	0.77	0.56	0.72	0.27	0.20	0.18
Chloride (mg/L)	Cl ⁻	N/A	44	48	79	32	41	48
Total hardness (mg/L)	TH	N/A	137	143	228	143	179	161
%Sand	Sand	N/A	95.51	93.69	14.98	51.36	11.6	-
%Silt	Silt	N/A	2.66	6.26	56.62	38.73	74.83	-
%Clay	Clay	N/A	1.55	0	25.89	8.94	12.98	-
%Total organic content	TOC	N/A	1.13	2.23	0.88	1.21	0.31	-

RWQO = Resource water quality objectives, VB = Vaal Barrage, DGP = Downstream Golfcourse Potchefstroom, MM = Mooiriver Mall, TRE = Tuscan Ridge Estate, GG = Gimmie Gat, BT = Botanical Gardens. Grey highlighted values exceeded the RWQO.

Table S.5: Soil particle size and total organic carbon (%TOC) before spraying sampling event

	Sites	TOC (%)	Sand (%)	Silt (%)	Clay (%)
Vaalharts Irrigation Scheme	TO	0.7	13	58.5	25.4
	HR1	0.2	71.5	27.9	0.3
	C1	-	-	-	-
	HR2	0.3	28.2	70.1	1.2
	C2	-	-	-	-
	MIC	-	-	-	-
	HR3	0.4	50.6	42.8	6.2
	HR4	0.5	56	42.3	1.3
	SD	1.2	51.1	42.7	4.6
	DSD	1	91.8	7.9	0.2
Mpumalanga	MD	0.3	38.1	34.5	26.5
	DMD	0.3	69.5	29.8	0
	MP4	0.7	34	41.2	24.4
	MP6	0.5	11.1	70.6	17.4
	PAN1	2.8	95.4	3.7	0.8
	MP10	0.2	0	88	11.7
	X1	1	17.1	58.1	13.5
	X2	0.9	34.3	45.1	19.5
	X3	0.8	7.7	83.9	6.3
	V9	1.6	83.4	14.3	1.4
	V11	1.5	69.1	24.3	5.8
	V12	1.3	44.9	38.6	16.3
	V13	0.7	69.4	27.1	3.4
	GD	1.5	32.9	57.8	8.6
	V15	0.2	30.1	60.4	9
Non-agricultural	VB	1.1	95.5	2.6	1.5
	DGP	2.2	93.7	6.2	0
	MM	0.9	15	56.7	25.9
	TRE	1.2	51.3	38.7	8.9
	GG	0.3	11.6	74.9	12.9
	BT	-	-	-	-

Grey highlighted values represent the highest composition of the sand, silt or clay. "-" no data available due to inaccessibility of site.

Table S.6: Water quality results post-herbicide application in Mpumalanga in the Olifants River catchment

Water quality parameters	Symbol	RWQO	MD	DMD	MP4	MP6	PAN1	MP10
pH	pH	6.5–8.5	8.14	8.02	8.59	7.57	8.65	7.38
Temperature (°C)	TEMP	N/A	30.9	26.2	24.4	23.1	28.6	22.7
Conductivity (mS/m)	EC	<111	107.3	110.4	301.1	131.7	1656	40.7
Total suspended solids (mg/L)	TSS	N/A	7	8	1	17	9436	13
Turbidity (FAU)	TURB	N/A	5	3	2	4	76	13
Ammonium (mg/L)	NH ₄ ⁺	<0.1	0.06	0.14	0.15	0.12	4.21	0.08
Nitrite (mg/L)	NO ₂ ⁻	<4	0.01	0.02	0.05	0.01	0.06	0.01
Nitrate (mg/L)	NO ₃ ⁻	<4	24.9	4.5	21.7	2.70	12.6	25
Sulphate (mg/L)	SO ₄ ²⁻	<500	195	136	217	243	231	36
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.125	4.16	0.04	2.24	0.04	4.59	0.05
Chloride (mg/L)	Cl ⁻	N/A	16	17	22	18	243	38
Total hardness (mg/L)	TH	N/A	221	244	239	322	230	104
%Sand	Sand	N/A	20.05	35.86	16.23	16.45	35.04	10.26
%Silt	Silt	N/A	34.47	60.83	55.91	68.08	48.3	82.02
%Clay	Clay	N/A	44.79	2.78	26.27	15.02	15.43	7.08
%Total organic content	TOC	N/A	0.43	0.46	0.54	0.73	1.55	0.4

RWQO = Resource water quality objectives, MD = Middelburg Dam, DMD = Downstream Middelburg Dam, MP4 = Mpumalanga 4, MP6 = Mpumalanga 6, PAN1 = PAN1, MP10 = Mpumalanga 10. Grey highlighted values exceeded the RWQO

Table S.7: Water quality results post-herbicide application in Mpumalanga in the Upper Vaal River catchment

Water quality parameters	Symbol	RWQO	X1	X2	X3	V9	V11	V12	V13	GD	V15
pH	pH	6.5–8.5	7.49	7.46	7.19	7.00	7.35	7.22	7.42	7.60	7.42
Temperature (°C)	TEMP	N/A	27.10	27.40	27.7	27.5	25.9	28.3	26.2	21.9	23.2
Conductivity (mS/m)	EC	<70	60.50	76.30	56.4	22.2	20.4	20.2	27.6	26.6	27
Total suspended solids (mg/L)	TSS	N/A	9.00	14.00	22	77	3	68	104	157	11
Turbidity (FAU)	TURB	N/A	7.00	18.00	24	67	73	81	69	74	19
Ammonium (mg/L)	NH ₄ ⁺	<0.073	0.14	0.09	0.86	0.18	0.10	0.47	0.04	0.00	0.04
Nitrite (mg/L)	NO ₂ ⁻	<0.858	0.01	0.01	0.11	0.02	0.05	0.02	0.06	0.11	0.01
Nitrate (mg/L)	NO ₃ ⁻	<0.858	23.4	21.10	5.50	2.00	6.10	6.5	23.8	3.00	24
Sulphate (mg/L)	SO ₄ ²⁻	<500	56.00	60.00	85	48	53	44	59	59	62
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.02	0.04	0.07	0.65	0.08	0.08	0.18	0.05	0.15	0.06
Chloride (mg/L)	Cl ⁻	N/A	28.00	30.00	31	7	10	14	8	7	8
Total hardness (mg/L)	TH	N/A	197.00	234.00	119	53	52	53	69	65	67
%Sand	Sand	N/A	4.68	70.72	2.91	17.04	63.13	99.75	99.01	76.72	40.74
%Silt	Silt	N/A	75.06	18.95	74.25	71.74	29.99	0.39	0.7	18.91	53.71
%Clay	Clay	N/A	19.63	9.74	22.11	10.64	6.49	0	0.15	3.78	4.62
%Total organic content	TOC	N/A	0.29	1.03	0.78	0.48	1.07	2.37	2.26	1.71	0.38

RWQO = Resource water quality objectives, X1 = Life Water River, X2 = Life Water River, X3 = Life Water River, V9 = Vaal River 9, V11 = Vaal River 11, V12 = Vaal River 12, V13 = Vaal River 13, GD = Grootdraai, V15 = Vaal River 15. Grey highlighted values exceeded the RWQO

Table S.8: Water quality results post herbicide application in the Vaalharts irrigation scheme in the Lower Vaal River catchment

Water quality parameters	Symbol	RWQO	TO	HR1	C1	HR2	C2	MIC	HR3	HR4	SD	DSD
pH	pH	6.5–8.5	7.94	8.38	7.73	7.53	7.74	7.99	7.75	7.84	8.18	8.18
Temperature (°C)	TEMP	N/A	26.7	28.1	33.0	31.5	30.2	26.5	28.3	26.0	24.5	24.7
Conductivity (mS/m)	EC	<111	33.1	27.6	77.8	101	226	66.6	194.2	168.2	123.1	120.4
Total Total suspended solids (mg/L)	TSS	N/A	14	4	114	39	1	5	11	2	4	2
Turbidity (FAU)	TURB	N/A	9	17	70	31	4	34	33	25	42	55
Ammonium (mg/L)	NH ₄ ⁺	<0.07	0.06	0.12	0.03	0.10	0.12	0.33	0.11	0.25	0.07	0.25
Nitrite (mg/L)	NO ₂ ⁻	<1	0.00	0.01	0.08	0.03	0.05	0.01	0.24	0.02	0.00	0.01
Nitrate (mg/L)	NO ₃ ⁻	<1	5.70	1.30	6.30	7.20	8.30	0.90	7.60	6.60	2.20	6.90
Sulphate (mg/L)	SO ₄ ²⁻	<200	15	20	165	183	65	128	147	51	204	215
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.025	0.02	0.13	0.05	0.05	0.11	0.03	0.22	0.07	0.03	0.09
Chloride (mg/L)	Cl ⁻	N/A	13	5	66	86	231	50	205	182	141	128
Total hardness (mg/L)	TH	N/A	56	47	137	153	110	101	201	179	173	178
%Sand	Sand	N/A	48.8	6.45	9.27	1.82	-	-	35.76	27.1	3.07	1.47
%Silt	Silt	N/A	43.81	82.43	66.68	54.33	-	-	51.96	65.36	47.92	72.4
%Clay	Clay	N/A	6.99	9.79	22.78	42.13	-	-	11.64	6.33	46.34	24.41
%Total organic content	TOC	N/A	0.41	0.79	0.22	1.03	-	-	0.56	1.3	2.03	1.07

RWQO = Resource water quality objectives, TO = Taung Overflow, HR1 = Harts River 1, C1 = Canal 1, HR2 = Harts River 2, C2 = Canal 2, MIC = Main Inflow Canal, HR3 = Harts River 3, HR4 = Harts River 4, SD = Spitskop Dam, DSD = Downstream Spitskop Dam.
Grey highlighted values exceeded the RWQO.

Table S.9: Water quality results of the non-agricultural sites in the Upper Vaal River catchment

Water quality parameters	Symbol	RWQO	VB	DGP	MM	TRE	GG	BT
pH	pH	6.5–8.5	8.07	6.05	6.66	7.00	8.88	7.9
Temperature (°C)	TEMP	N/A	23.8	25.1	25.7	26.7	28.7	25.2
Conductivity (mS/m)	EC	<111	69	90.9	90.1	45.9	72	48.8
Total Total suspended solids (mg/L)	TSS	N/A	158	4	29	19	39	3
Turbidity (FAU)	TURB	N/A	7	15	9	10	14	6
Ammonium (mg/L)	NH ₄ ⁺	<0.073	0.15	2.94	0.41	0.13	0.14	0.13
Nitrite (mg/L)	NO ₂ ⁻	<4	3.10	4.00	2.50	2.40	1.70	1.30
Nitrate (mg/L)	NO ₃ ⁻	<4	0.03	0.28	0.05	0.06	0.01	0.01
Sulphate (mg/L)	SO ₄ ²⁻	<500	106	152	152	166	88	59
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.125	0.09	0.84	0.77	1.16	0.10	0.14
Chloride (mg/L)	Cl ⁻	N/A	33	54	62	60	44	35
Total hardness (mg/L)	TH	N/A	154	118	217	204	85	132
%Sand	Sand	N/A	94.12	76.02	37.37	30.12	36.28	-
%Silt	Silt	N/A	4.59	19.59	37.55	48.14	46.1	-
%Clay	Clay	N/A	0.89	3.75	24.02	20.79	16.29	-
%Total organic content	TOC	N/A	1.83	3.43	2.47	1.36	2.18	-

RWQO = Resource water quality objectives, VB = Vaal Barrage, DGP = Downstream Golf course Potchefstroom, MM = Mooiriver Mall, TRE = Tuscany Ridge Estate, GG = Gimmie Gat, BT = Botanical Gardens. Grey highlighted values exceeded the RWQO.

Table S.10: Soil particle size and total organic carbon (%TOC) post herbicide application

	Sites	TOC (%)	Sand (%)	Silt (%)	Clay (%)
Vaalaharts Irrigation Scheme	TO	0.4	48.8	43.8	6.9
	HR1	0.8	6.4	82.4	9.8
	C1	0.2	9.3	66.7	22.8
	HR2	1	1.8	54.3	42.1
	C2	-	-	-	-
	MIC	-	-	-	-
	HR3	0.6	35.7	51.9	11.6
	HR4	1.3	27.1	65.3	6.3
	SD	2	3.1	47.9	46.4
	DSD	1.1	1.5	72.4	24.4
Mpumalanga	MD	0.4	20.1	34.5	44.8
	DMD	0.5	35.8	60.8	2.7
	MP4	0.5	16.2	55.9	26.3
	MP6	0.7	16.5	68	15
	PAN1	1.5	35	48.3	15.4
	MP10	0.4	10.2	82	7.1
	X1	0.3	4.7	75.1	19.7
	X2	1	70.7	18.9	9.8
	X3	0.8	2.9	74.3	22.1
	V9	0.5	17	71.7	10.6
	V11	1.1	63.2	30	6.5
	V12	2.4	99.8	0.4	0
	V13	2.3	99	0.7	0.1
	GD	1.7	76.7	18.9	3.8
	V15	0.4	40.7	53.7	4.6
Non-agricultural	VB	1.8	94.1	4.6	0.9
	DGP	3.4	76.1	19.6	3.8
	MM	2.5	37.3	37.5	24.1
	TRE	1.4	30.1	48.1	20.7
	GG	2.2	36.3	46.1	16.3
	BT	-	-	-	-

Grey highlighted values represent the highest composition of the sand, silt or clay. "-" no data available due to inaccessibility of site.

Table S.11: Water quality results after a rain event in Mpumalanga in the Olifants River catchment

Water quality parameters	Symbol	RWQO	MD	DMD	MP4	MP6	PAN1	MP10
pH	pH	6.5–8.5	7.86	8.02	7.87	7.95	7.30	7.60
Temperature (°C)	TEMP	N/A	21.1	20.7	21.5	20.6	17.8	18.5
Conductivity (mS/m)	EC	<111	77.3	108.4	191.2	117.5	1010	36.6
Total Total suspended solids (mg/L)	TSS	N/A	405	5	13	5	32	35
Turbidity (FAU)	TURB	N/A	42	25	26	42	43	32
Ammonium (mg/L)	NH ₄ ⁺	<0.1	0.13	0.04	0.04	0.13	0.05	0.12
Nitrite (mg/L)	NO ₂ ⁻	<4	0.06	0.02	0.31	0.02	0.60	1.70
Nitrate (mg/L)	NO ₃ ⁻	<4	0.80	0.70	0.90	1.10	0.60	1.70
Sulphate (mg/L)	SO ₄ ²⁻	<500	197	257	158	237	278	42
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.125	0.08	0.34	0.05	0.66	0.08	0.12
Chloride (mg/L)	Cl ⁻	N/A	18	18	20	19	22	18
Total hardness (mg/L)	TH	N/A	180	228	188	164	202	10
%Sand	Sand	N/A	47.49	65.59		25.82	0.24	5.82
%Silt	Silt	N/A	46.71	33.63		62.13	57.97	78.46
%Clay	Clay	N/A	5.12	0.44		11.25	40.14	10.51
%Total organic content	TOC	N/A	0.9	0.73		0.51	1.77	0.31

RWQO = Resource water quality objectives, MD = Middelburg Dam, DMD = Downstream Middelburg Dam, MP4 = Mpumalanga 4, MP6 = Mpumalanga 6, PAN1 = PAN1, MP10 = Mpumalanga 10. Grey highlighted values exceeded the RWQO.

Table S.12: Water quality results after a rain event in Mpumalanga in the Upper Vaal River catchment

Water quality parameters	Symbol	RWQO	X1	X2	X3	V9	V11	V12	V13	GD	V15
pH	pH	6.5–8.5	7.41	7.49	7.50	7.50	7.33	7.53	6.90	7.10	5.08
Temperature (°C)	TEMP	N/A	20.1	21.2	20.0	22.7	22.8	22.4	23.3	21.1	24.0
Conductivity (mS/m)	EC	<70	56.3	44.6	44.8	26.5	26.9	28.3	30.6	21.9	20.2
Total Total suspended solids (mg/L)	TSS	N/A	105	60	129	98	94	110	44	77	109
Turbidity (FAU)	TURB	N/A	26	45	45	50	6	16	31	47	41
Ammonium (mg/L)	NH ₄ ⁺	<0.073	0.41	0.08	0.06	0.09	0.05	0.09	0.25	0.05	0.08
Nitrite (mg/L)	NO ₂ ⁻	<0.858	0.50	2.00	1.00	0.70	0.70	0.50	0.17	1.00	0.60
Nitrate (mg/L)	NO ₃ ⁻	<0.858	0.5	2.00	1.00	0.70	0.70	0.50	0.70	1.00	0.60
Sulphate (mg/L)	SO ₄ ²⁻	<500	54	50	58	37	38	48	79	47	32
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.02	0.45	0.35	0.39	0.31	1.26	0.20	0.19	0.65	0.28
Chloride (mg/L)	Cl ⁻	N/A	25	17	19	14	18	17	15	15	14
Total hardness (mg/L)	TH	N/A	73	80	100	44	38	51	5	18	7
%Sand	Sand	N/A	16.41	11.48	5.83	80.12	37.98	83.74	8.23	9.1	0.39
%Silt	Silt	N/A	70.1	66.84	83.55	17.23	48.61	13.81	59.54	87.39	65.34
%Clay	Clay	N/A	12.75	20.38	9.17	2.25	11.49	2.04	30.43	3.2	30.24
%Total organic content	TOC	N/A	0.19	2.06	0.46	1.18	1.56	1.66	0.9	0.7	0.24

RWQO = Resource water quality objectives, X1 = Life Water River, X2 = Life Water River, X3 = Life Water River, V9 = Vaal River 9, V11 = Vaal River 11, V12 = Vaal River 12, V13 = Vaal River 13, GD = Grootdraai, V15 = Vaal River 15. Grey highlighted values exceeded the RWQO.

Table S.13: Water quality results after a rain event in the Vaalharts irrigation scheme in the Lower Vaal River catchment

Water quality parameters	Symbol	RWQO	TO	HR1	C1	HR2	C2	MIC	HR3	HR4	SD	DSD
pH	pH	6.5–8.5	6.89	6.10	6.87	6.70	6.91	6.84	6.48	6.50	7.19	6.64
Temperature (°C)	TEMP	N/A	24.2	26.8	29.2	26.1	30.5	26.3	26.0	23.0	23.1	30.5
Conductivity (mS/m)	EC	<111	24.5	21	128.6	64.8	159.2	40.2	94.6	97.5	45.8	37.6
Total Total suspended solids (mg/L)	TSS	N/A	75	56	83	155	164	215	155	399	97	153
Turbidity (FAU)	TURB	N/A	-	45	51	38	65	61	53	56	67	25
Ammonium (mg/L)	NH ₄ ⁺	<0.07	0.09	0.25	0.16	0.41	0.06	0.08	0.16	0.13	0.18	0.14
Nitrite (mg/L)	NO ₂ ⁻	<1	0.22	1.4	0.31	0.18	0.15	0.08	0.11	0.12	0.11	0.19
Nitrate (mg/L)	NO ₃ ⁻	<1	1.10	14.00	10.00	0.60	3.40	1.20	1.80	1.90	1.00	4.10
Sulphate (mg/L)	SO ₄ ²⁻	<200	44	46	202	78	177	90	130	150	63	67
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.025	0.13	2.60	0.31	0.05	0.04	0.05	0.06	0.08	0.06	0.08
Chloride (mg/L)	Cl ⁻	N/A	17	20	14	24	24	21	18	21	22	23
Total hardness (mg/L)	TH	N/A	5	5	301	101	151	5	29	114	211	78
%Sand	Sand	N/A	5.88	28.08	-	5.66	1.19	-	10.14	0.87	4.85	27.17
%Silt	Silt	N/A	93.59	71.64	-	53.72	38.84	-	76.94	61.15	41.73	62.29
%Clay	Clay	N/A	0.14	0.33	-	39.18	59.45	-	11.95	36.44	50.1	9.38
%Total organic content	TOC	N/A	0.34	0.23	-	1.08	0.21	-	0.43	2.06	1.66	2.36

RWQO = Resource water quality objectives, TO = Taung Overflow, HR1 = Harts River 1, C1 = Canal 1, HR2 = Harts River 2, C2 = Canal 2, MIC = Main Inflow Canal, HR3 = Harts River 3, HR4 = Harts River 4, SD = Spitskop Dam, DSD = Downstream Spitskop Dam.

Grey highlighted values exceeded the RWQO

Table S.14: Water quality results after a rain event of the non-agricultural sites in the Upper Vaal River catchment

Water quality parameters	Symbol	RWQO	VB	DGP	MM	TRE	GG	BT
pH	pH	6.5–8.5	7.85	7.97	8.15	8.46	8.66	8.54
Temperature (°C)	TEMP	N/A	22	20.9	21.4	21.1	21.6	19.5
Conductivity (mS/m)	EC	<111	70.3	77.5	72	71.4	71.4	59.4
Total Total suspended solids (mg/L)	TSS	N/A	25	7	0	43	90	31
Turbidity (FAU)	TURB	N/A	48	46	1	52	49	8
Ammonium (mg/L)	NH ₄ ⁺	<0.073	1.52	0.09	0.08	0.05	0.05	0.04
Nitrite (mg/L)	NO ₂ ⁻	<4	0.02	0.03	0.02	0.35	0.03	0.01
Nitrate (mg/L)	NO ₃ ⁻	<4	1.00	0.90	1.70	0.7	2.00	0.70
Sulphate (mg/L)	SO ₄ ²⁻	<500	73	133	127	74	80	43
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.125	0.18	0.38	1.79	0.33	0.72	0.72
Chloride (mg/L)	Cl ⁻	N/A	21	25	26	18	17	21
Total hardness (mg/L)	TH	N/A	222	181	183	79	120	67
%Sand	Sand	N/A	33.87	39.62	26.33	13.29	42.68	-
%Silt	Silt	N/A	43.56	44.71	44.62	43.65	52.23	-
%Clay	Clay	N/A	21.31	11.78	24.67	39.39	4.46	-
%Total organic content	TOC	N/A	1.82	3.53	3.05	3.14	0.32	-

RWQO = Resource water quality objectives, VB = Vaal Barrage, DGP = Downstream Golfcourse Potchefstroom, MM = Mooiriver Mall, TRE = Tuscany Ridge Estate, GG = Gimmie Gat, BT = Botanical Gardens. Grey highlighted values exceeded the RWQO

Table S.15: Soil particle size and total organic carbon (%TOC) after the rain sampling event

	Sites	TOC (%)	Sand (%)	Silt (%)	Clay (%)
Vaalaharts Irrigation Scheme	TO	0.3	5.9	93.5	0.1
	HR1	0.2	28	71.6	0.3
	C1	-	-	-	-
	HR2	1.1	5.6	53.8	39.2
	C2	0.2	1.2	38.9	59.5
	MIC	-	-	-	-
	HR3	0.4	10.2	76.9	11.9
	HR4	2.1	0.9	61.1	36.5
	SD	1.7	4.8	41.7	50.1
	DSD	2.4	27.1	62.3	9.4
Mpumalanga	MD	0.9	47.4	46.8	5.1
	DMD	0.7	65.6	33.6	0.4
	MP4	-	-	-	-
	MP6	0.5	25.8	62.1	11.2
	PAN1	1.8	0.2	58	40.1
	MP10	0.3	5.8	78.4	10.5
	X1	0.2	16.4	70.1	12.8
	X2	2.1	11.5	66.9	20.3
	X3	0.5	5.8	83.6	9.2
	V9	1.2	80.1	17.2	2.3
	V11	1.6	37.9	48.6	11.5
	V12	1.7	83.8	13.8	2
	V13	0.9	8.2	59.6	30.4
	GD	0.7	9.2	87.4	3.2
	V15	0.2	0.4	75.2	30.2
Non-agricultural	VB	1.8	33.8	43.5	21.3
	DGP	3.5	44.6	44.7	11.7
	MM	3.1	26.3	44.6	24.7
	TRE	3.1	13.3	43.7	39.4
	GG	0.3	42.7	52.2	4.4
	BT	-	-	-	-

Grey highlighted values represent the highest composition of the sand, silt or clay. "-" no data available due to inaccessibility of site.

Table S.16: Water quality results at the end of the maize growing season in Mpumalanga in the Olifants River catchment

Water quality parameters	Symbol	RWQO	MD	DMD	MP4	MP6	PAN1	MP10
pH	pH	6.5–8.5	8.80	8.63	8.50	8.20	8.30	8.25
Temperature (°C)	TEMP	N/A	17.7	16.6	13.1	12.3	10.9	7.3
Conductivity (mS/m)	EC	<111	104.5	102.9	262	179.5	1146	39
Total suspended solids (mg/L)	TSS	N/A	22	15	136	6	307	93
Turbidity (FAU)	TURB	N/A	14	40	1	3	46	10
Ammonium (mg/L)	NH ₄ ⁺	<0.1	0.10	0.07	0.05	0.09	0.03	0.04
Nitrite (mg/L)	NO ₂ ⁻	<4	0.05	0.01	0.01	0.06	0.01	0.02
Nitrate (mg/L)	NO ₃ ⁻	<4	1.7	0.7	1.20	2.20	0.20	0.2
Sulphate (mg/L)	SO ₄ ²⁻	<500	314	238	234	230	280	46
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.125	0.10	0.71	0.09	0.03	0.03	0.09
Chloride (mg/L)	Cl ⁻	N/A	49	55	115	41	141	39
Total hardness (mg/L)	TH	N/A	218	200	215	187	18	78
%Sand	Sand	N/A	53.49	44.14	1.96	8.66	2.44	0.9
%Silt	Silt	N/A	41.6	54	49.71	80.06	69.54	81.16
%Clay	Clay	N/A	4.76	1.57	42.49	11.08	27.19	17.3
%Total organic content	TOC	N/A	0.83	1.05	0.44	0.57	10.57	0.24

RWQO = Resource water quality objectives, MD = Middelburg Dam, DMD = Downstream Middelburg Dam, MP4 = Mpumalanga 4, MP6 = Mpumalanga 6, PAN1 = PAN1, MP10 = Mpumalanga 10. Grey highlighted values exceeded the RWQO.

Table S.17: Water quality results at the end of the maize growing season in Mpumalanga in the Upper Vaal River catchment.

Water quality parameters	Symbol	RWQO	X1	X2	X3	V9	V11	V12	V13	GD	V15
pH	pH	6.5–8.5	8.22	8.18	7.82	8.40	8.42	8.09	8.02	7.85	7.25
Temperature (°C)	TEMP	N/A	6.1	12.7	12.1	18.1	13.2	11.4	13.0	12.9	12.0
Conductivity (mS/m)	EC	<111	58.6	54.7	51.9	39.8	41.6	42.9	42.8	20.9	22
Total suspended solids (mg/L)	TSS	N/A	108	13	52	22	105	12	20	35	49
Turbidity (FAU)	TURB	N/A	10	25	22	8	31	10	23	27	25
Ammonium (mg/L)	NH ₄ ⁺	<0.1	0.02	0.05	0.04	0.08	0.03	0.05	0.06	0.05	0.05
Nitrite (mg/L)	NO ₂ ⁻	<4	0.03	0.02	0.03	0.04	0.01	0.01	0.01	0.01	0.03
Nitrate (mg/L)	NO ₃ ⁻	<4	0.80	0.1	4	0.9	1.4	0.4	1.6	0.7	0.4
Sulphate (mg/L)	SO ₄ ²⁻	<500	56	55	78	46	65	64	57	38	43
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.125	0.08	0.05	0.03	0.09	0.05	0.17	0.06	0.07	0.04
Chloride (mg/L)	Cl ⁻	N/A	32	44	74	100	66	54	54	48	35
Total hardness (mg/L)	TH	N/A	120	127	91	59	69	81	72	35	41
%Sand	Sand	N/A	2.34	6.94	2.73	5.76	44.03	6.7	2.92	6.34	1.76
%Silt	Silt	N/A	67.27	72.84	85.88	90.91	52.79	80.3	33.63	75.13	76.82
%Clay	Clay	N/A	29.76	18.58	11.28	2.15	2.74	12.38	61.08	18.29	21.37
%Total organic content	TOC	N/A	0.77	1.72	1.67	0.15	0.31	5.31	0.37	6.02	0.12

RWQO = Resource water quality objectives, X1 = Life Water River, X2 = Life Water River, X3 = Life Water River, V9 = Vaal River 9, V11 = Vaal River 11, V12 = Vaal River 12, V13 = Vaal River 13, GD = Grootdraai, V15 = Vaal River 15. Grey highlighted values exceeded the RWQO.

Table S.18: Water quality results at the end of the maize growing season in the Vaalharts irrigation scheme in the Lower Vaal River catchment

Water quality parameters	Symbol	RWQO	TO	HR1	C1	HR2	C2	MIC	HR3	HR4	SD	DSD
pH	pH	6.5–8.5	7.30	8.11	8.40	8.18	8.18	8.71	8.41	8.36	8.56	8.46
Temperature (°C)	TEMP	N/A	15.3	16.1	17.6	16.4	16.4	20.8	15.6	12.4	12.2	14.5
Conductivity (mS/m)	EC	<111	30	33.1	170.3	94.7	94.7	42.8	190.1	173	72.5	80.8
Total suspended solids (mg/L)	TSS	N/A	23	4	150	73	94	61	94	91	51	119
Turbidity (FAU)	TURB	N/A	33	8	10	23	1	33	8	12	17	17
Ammonium (mg/L)	NH ₄ ⁺	<0.07	0.05	0.05	0.05	0.09	0.05	0.14	0.07	0.09	0.05	0.05
Nitrite (mg/L)	NO ₂ ⁻	<1	0.02	0.06	0.02	0.02	0.03	0.02	0.29	0.08	0.02	0.03
Nitrate (mg/L)	NO ₃ ⁻	<1	1.50	1.10	2.10	2.30	4.80	1.30	2.00	2.20	1.50	1.40
Sulphate (mg/L)	SO ₄ ²⁻	<200	26	4	291	145	297	60	212	200	70	80
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.025	0.09	0.03	0.06	0.03	0.03	0.06	0.08	0.02	0.08	0.04
Chloride (mg/L)	Cl ⁻	N/A	32	22	190	121	231	41	252	211	159	83
Total hardness (mg/L)	TH	N/A	65	67	265	235	287	79	291	277	145	159
%Sand	Sand	N/A	12.95	3.49		0.63	1.56	17.99	0	3.1	12.9	56.42
%Silt	Silt	N/A	69.27	54.63		48.72	58.93	55.44	56.64	66.51	67.52	41.84
%Clay	Clay	N/A	17.18	41.83		50.22	37.95	24.7	43.31	26.34	19.09	1.69
%Total organic content	TOC	N/A	1.21	1.02		0.66	0.56	0.2		1.38	9.05	0.59

RWQO = Resource water quality objectives, TO = Taung Overflow, HR1 = Harts River 1, C1 = Canal 1, HR2 = Harts River 2, C2 = Canal 2, MIC = Main Inflow Canal, HR3 = Harts River 3, HR4 = Harts River 4, SD = Spitskop Dam, DSD = Downstream Spitskop Dam.

Grey highlighted values exceeded the RWQO

Table S.19: Water quality results at the non-agricultural sites in the Upper Vaal River catchment.

Water quality parameters	Symbol	RWQO	VB	DGP	MM	TRE	GG	BT
pH	pH	6.5–8.5	9.20	8.98	9.44	9.23	9.46	9.18
Temperature (°C)	TEMP	N/A	12.8	13.8	13.3	12.4	13.8	10.9
Conductivity (mS/m)	EC	<111	71.1	73.3	78.8	77.1	55.3	58.8
Total suspended solids (mg/L)	TSS	N/A	45	45	49	22	39	1333
Turbidity (FAU)	TURB	N/A	38	32	9	14	23	45
Ammonium (mg/L)	NH ₄ ⁺	<0.073	0.09	1.79	0.05	0.05	0.05	0.05
Nitrite (mg/L)	NO ₂ ⁻	<4	0.01	0.19	0.01	0.01	0.01	0.01
Nitrate (mg/L)	NO ₃ ⁻	<4	0.20	1.30	0.60	1.00	0.90	1.70
Sulphate (mg/L)	SO ₄ ²⁻	<500	101	111	111	111	103	89
Ortho-phosphate (mg/L)	PO ₄ ³⁻	<0.125	0.06	0.91	0.13	0.13	0.10	0.08
Chloride (mg/L)	Cl ⁻	N/A	66	98	101	71	84	112
Total hardness (mg/L)	TH	N/A	181	112	176	70	32	31
%Sand	Sand	N/A	40.22	-	2.47	31.73	19.68	-
%Silt	Silt	N/A	49.03	-	60.59	54.17	71.14	-
%Clay	Clay	N/A	10.7	-	36.55	13.41	8.59	-
%Total organic content	TOC	N/A	1.27	-	6.39	1.05	0.22	-

RWQO = Resource water quality objectives, VB = Vaal Barrage, DGP = Downstream Golfcourse Potchefstroom, MM = Mooiriver Mall, TRE = Tuscany Ridge Estate, GG = Gimmie Gat, BT = Botanical Gardens. Grey highlighted values exceeded the RWQO

Table S.20: Soil particle size and total organic carbon (%TOC) after harvest season

	Sites	TOC (%)	Sand (%)	Silt (%)	Clay (%)
Vaalaharts Irrigation Scheme	TO	1.2	12.9	69.3	17.2
	HR1	1	3.5	54.6	41.8
	C1	-	-	-	-
	HR2	0.7	0.6	48.7	50.2
	C2	0.6	1.6	58.9	37.9
	MIC	0.2	18	55.4	24.7
	HR3	-	0	56.6	43.3
	HR4	1.4	3.1	66.5	26.4
	SD	9.1	12.9	67.6	19.1
	DSD	0.6	56.4	41.8	1.7
Mpumalanga	MD	0.8	53.5	41.6	4.8
	DMD	1.1	44.1	54.1	1.6
	MP4	0.4	2	49.7	42.5
	MP6	0.6	8.7	80.1	11.1
	PAN1	10.6	2.4	69.5	27.2
	MP10	0.2	0.9	81.2	17.3
	X1	0.8	2.3	67.3	29.7
	X2	1.7	7	72.9	18.6
	X3	1.7	2.7	85.9	11.2
	V9	0.1	5.8	90.9	2.2
	V11	0.3	44	52.8	2.7
	V12	5.3	6.7	80.3	12.4
	V13	0.4	2.9	33.7	61
	GD	6	6.3	75.1	18.3
	V15	0.1	1.7	76.8	21.3
Non-agricultural	VB	1.3	40.3	49.1	10.7
	DGP	-	-	-	-
	MM	6.4	2.5	60.6	36.6
	TRE	1	31.8	54.2	13.4
	GG	0.2	19.7	71.1	8.6
	BT	-	-	-	-

Grey highlighted values represent the highest composition of the sand, silt or clay. "-" no data available due to inaccessibility of site.

Appendix C: Chemical analysis raw data

Here is the raw data for the quantification of GLY and AMPA:

1. GLY in water samples (Table S.21)
2. AMPA in water samples (Table S.22)
3. GLY in sediment samples (Table S.23)
4. AMPA in sediment samples (Table S.24)
5. GLY in soil samples (Table S.25)
6. AMPA in soil samples (Table S.26)

Table S.21: Quantification results of glyphosate in the water samples

	Sites	Before spraying	After herbicide application	After a rain event	After harvest
Mpumalanga	Downstream Middelburg Dam	<LOD	<LOD	<LOD	<LOD
	Middelburg Dam	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 4	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 6	<LOD	<LOD	<LOD	<LOD
	PAN1	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 10	<LOD	<LOD	<LOD	<LOD
	Life Water River 1	<LOD	<LOD	<LOD	<LOD
	Life Water River 2	<LOD	<LOD	<LOD	<LOD
	Life Water River 3	<LOD	<LOD	<LOD	<LOD
	Vaal River 9	<LOD	<LOD	<LOD	<LOD
	Vaal River 11	<LOD	<LOD	<LOD	<LOD
	Vaal River 12	<LOD	<LOD	<LOD	<LOD
	Vaal River 13	<LOD	<LOD	<LOD	<LOD
	Grootdraai	<LOD	<LOD	<LOD	<LOD
	Vaal River 15	<LOD	<LOD	<LOD	<LOD
Vaalharts Irrigation Scheme	Taung Outflow	<LOD	<LOD	<LOD	<LOD
	Harts River 1	<LOD	<LOD	<LOD	<LOD
	Canal 1	<LOD	<LOD	<LOD	<LOD
	Harts River 2	<LOD	<LOD	<LOD	<LOD
	Canal 2	<LOD	<LOD	<LOD	<LOD
	Main Inflow Canal	<LOD	<LOD	<LOD	<LOD
	Harts River 3	<LOD	<LOD	<LOD	<LOD
	Harts River 4	<LOD	<LOD	<LOD	<LOD
	Spitskop Dam	<LOD	<LOD	<LOD	<LOD
	Downstream Spitskop Dam	<LOD	<LOD	<LOD	<LOD
Non-agricultural	Vaal River Barrage	<LOD	<LOD	<LOD	<LOD
	Downstream Potchefstroom Country Club	<LOD	<LOD	<LOD	<LOD
	Mooi River Mall	<LOD	<LOD	<LOD	<LOD
	Tuscany Ridge Estate	<LOD	<LOD	<LOD	<LOD
	Gimmie Gat	<LOD	<LOD	<LOD	<LOD
	NWU Botanical Gardens	<LOD	<LOD	<LOD	<LOD

LOD = limit of detection

Table S.22: Quantification results of AMPA in the water samples

	Sites	Before spraying	After herbicide application	After a rain event	After harvest
Mpumalanga	Downstream Middelburg Dam	<LOD	<LOD	<LOD	<LOD
	Middelburg Dam	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 4	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 6	<LOD	<LOD	<LOD	<LOD
	PAN1	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 10	<LOD	<LOD	<LOD	<LOD
	Life Water River 1	<LOD	<LOD	<LOD	<LOD
	Life Water River 2	<LOD	<LOD	<LOD	<LOD
	Life Water River 3	<LOD	<LOD	<LOD	<LOD
	Vaal River 9	<LOD	<LOD	<LOD	<LOD
	Vaal River 11	<LOD	<LOD	<LOD	<LOD
	Vaal River 12	<LOD	<LOD	<LOD	<LOD
	Vaal River 13	<LOD	<LOD	<LOD	<LOD
	Grootdraai	<LOD	<LOD	<LOD	<LOD
	Vaal River 15	<LOD	<LOD	<LOD	<LOD
Vaalharts irrigation scheme	Taung Outflow	<LOD	<LOD	<LOD	<LOD
	Harts River 1	<LOD	<LOD	<LOD	<LOD
	Canal 1	<LOD	<LOD	<LOD	<LOD
	Harts River 2	<LOD	<LOD	<LOD	<LOD
	Canal 2	<LOD	<LOD	<LOD	<LOD
	Main Inflow Canal	<LOD	<LOD	<LOD	<LOD
	Harts River 3	<LOD	<LOD	<LOD	<LOD
	Harts River 4	<LOD	<LOD	<LOD	<LOD
	Spitskop Dam	<LOD	<LOD	<LOD	<LOD
	Downstream Spitskop Dam	<LOD	<LOD	<LOD	<LOD
Non-agricultural	Vaal River Barrage	<LOD	<LOD	<LOD	<LOD
	Downstream Potchefstroom Country Club	<LOD	<LOD	<LOD	<LOD
	MooiRiver Mall	<LOD	<LOD	<LOD	<LOD
	Tuscany Ridge Estate	<LOD	<LOD	<LOD	<LOD
	Gimmie Gat	<LOD	<LOD	<LOD	<LOD
	NWU Botanical Gardens	<LOD	<LOD	<LOD	<LOD

LOD = limit of detection

Table S.23: Quantification results of glyphosate in sediment samples

	Sites	Before spraying	After herbicide application	After a rain event	After harvest
Mpumalanga	Downstream Middelburg Dam	<LOD	<LOD	<LOD	<LOD
	Middelburg Dam	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 4	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 6	<LOD	<LOD	<LOD	<LOD
	PAN1	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 10	<LOD	<LOD	<LOD	<LOD
	Life Water River 1	<LOD	<LOD	<LOD	<LOD
	Life Water River 2	<LOD	<LOD	<LOD	<LOD
	Life Water River 3	<LOD	<LOD	<LOD	<LOD
	Vaal River 9	<LOD	<LOD	<LOD	<LOD
	Vaal River 11	<LOD	<LOD	<LOD	<LOD
	Vaal River 12	<LOD	<LOD	<LOD	<LOD
	Vaal River 13	<LOD	<LOD	<LOD	<LOD
	Grootdraai	<LOD	<LOD	<LOD	<LOD
	Vaal River 15	<LOD	<LOD	<LOD	<LOD
Vaalharts irrigation scheme	Taung Outflow	<LOD	<LOD	<LOD	<LOD
	Harts River 1	<LOD	<LOD	<LOD	<LOD
	Canal 1	<LOD	<LOD	<LOD	<LOD
	Harts River 2	<LOD	<LOD	<LOD	<LOD
	Canal 2	<LOD	<LOD	<LOD	<LOD
	Main Inflow Canal	<LOD	<LOD	<LOD	<LOD
	Harts River 3	<LOD	<LOD	<LOD	<LOD
	Harts River 4	<LOD	<LOD	<LOD	<LOD
	Spitskop Dam	<LOD	<LOD	<LOD	<LOD
	Downstream Spitskop Dam	<LOD	<LOD	<LOD	<LOD
Non-agricultural	Vaal River Barrage	<LOD	<LOD	<LOD	<LOD
	Downstream Potchefstroom Country Club	<LOD	<LOD	<LOD	<LOD
	Mooi River Mall	<LOD	<LOD	<LOD	<LOD
	Tuscany Ridge Estate	<LOD	<LOD	<LOD	<LOD
	Gimmie Gat	<LOD	<LOD	<LOD	<LOD
	NWU Botanical Gardens	<LOD	<LOD	<LOD	<LOD

LOD = limit of detection

Table S.24: Quantification results of AMPA in sediment samples

	Sites	Before spraying	After herbicide application	After a rain event	After harvest
Mpumalanga	Downstream Middelburg Dam	<LOD	<LOD	<LOD	<LOD
	Middelburg Dam	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 4	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 6	<LOD	<LOD	<LOD	<LOD
	PAN1	<LOD	<LOD	<LOD	<LOD
	Mpumalanga 10	<LOD	<LOD	<LOD	<LOD
	Life Water River 1	<LOD	<LOD	<LOD	<LOD
	Life Water River 2	<LOD	<LOD	<LOD	<LOD
	Life Water River 3	<LOD	<LOD	<LOD	<LOD
	Vaal River 9	<LOD	<LOD	<LOD	<LOD
	Vaal River 11	<LOD	<LOD	<LOD	<LOD
	Vaal River 12	<LOD	<LOD	<LOD	<LOD
	Vaal River 13	<LOD	<LOD	<LOD	<LOD
	Grootdraai	<LOD	<LOD	<LOD	<LOD
	Vaal River 15	<LOD	<LOD	<LOD	<LOD
Vaalharts irrigation scheme	Taung Outflow	<LOD	<LOD	<LOD	<LOD
	Harts River 1	<LOD	<LOD	<LOD	<LOD
	Canal 1	<LOD	<LOD	<LOD	<LOD
	Harts River 2	<LOD	<LOD	<LOD	<LOD
	Canal 2	<LOD	<LOD	<LOD	<LOD
	Main Inflow Canal	<LOD	<LOD	<LOD	<LOD
	Harts River 3	<LOD	<LOD	<LOD	<LOD
	Harts River 4	<LOD	<LOD	<LOD	<LOD
	Spitskop Dam	<LOD	<LOD	<LOD	<LOD
	Downstream Spitskop Dam	<LOD	<LOD	<LOD	<LOD
Non-agricultural	Vaal River Barrage	<LOD	<LOD	<LOD	<LOD
	Downstream Potchefstroom Country Club	<LOD	<LOD	<LOD	<LOD
	Mooi River Mall	<LOD	<LOD	<LOD	<LOD
	Tuscany Ridge Estate	<LOD	<LOD	<LOD	<LOD
	Gimmie Gat	<LOD	<LOD	<LOD	<LOD
	NWU Botanical Gardens	<LOD	<LOD	<LOD	<LOD

LOD = limit of detection

Table S.25: Quantification results of glyphosate in soil samples

	Sites	Before spraying	After herbicide application	After a rain event	After harvest
Mpumalanga	Maize 1	<LOD	<LOD	<LOD	<LOD
	Maize 2	<LOD	<LOD	<LOD	<LOD
	Maize 3	<LOD	<LOD	<LOD	<LOD
	Maize 4	<LOD	<LOD	<LOD	<LOD
	Maize 5	<LOD	<LOD	<LOD	<LOD
	Maize 6	<LOD	<LOD	<LOD	<LOD
	Maize 7	<LOD	<LOD	<LOD	<LOD
	Maize 8	<LOD	<LOD	<LOD	<LOD
	Maize 9	<LOD	<LOD	<LOD	<LOD
	Maize 10	<LOD	<LOD	<LOD	<LOD
	Maize 11	<LOD	<LOD	<LOD	<LOD
Vaalharts irrigation scheme	Maize 12	<LOD	<LOD	<LOD	<LOD
	Maize 13	<LOD	<LOD	<LOD	<LOD
	Maize 14	<LOD	<LOD	<LOD	<LOD
	Maize 15	<LOD	<LOD	<LOD	<LOD
	Pecan 1	<LOD	<LOD	<LOD	<LOD
	Pecan 2	<LOD	<LOD	<LOD	<LOD
	Pecan 3	<LOD	<LOD	<LOD	<LOD
	Pecan 4	<LOD	<LOD	<LOD	<LOD

Table S.26: Quantification results of AMPA in soil samples

	Sites	Before spraying	After herbicide application	After a rain event	After harvest
Mpumalanga	Maize 1	<LOD	<LOD	<LOD	<LOD
	Maize 2	<LOD	<LOD	<LOD	<LOD
	Maize 3	<LOD	<LOD	<LOD	<LOD
	Maize 4	<LOD	<LOD	<LOD	<LOD
	Maize 5	<LOD	<LOD	<LOD	<LOD
	Maize 6	<LOD	<LOD	<LOD	<LOD
	Maize 7	<LOD	<LOD	<LOD	<LOD
	Maize 8	<LOD	<LOD	<LOD	<LOD
	Maize 9	<LOD	<LOD	<LOD	<LOD
	Maize 10	<LOD	<LOD	<LOD	<LOD
	Maize 11	<LOD	<LOD	<LOD	<LOD
Vaalharts irrigation scheme	Maize 12	<LOD	<LOD	<LOD	<LOD
	Maize 13	<LOD	<LOD	<LOD	<LOD
	Maize 14	<LOD	<LOD	<LOD	<LOD
	Maize 15	<LOD	<LOD	<LOD	<LOD
	Pecan 1	<LOD	<LOD	<LOD	<LOD
	Pecan 2	<LOD	<LOD	<LOD	<LOD
	Pecan 3	<LOD	<LOD	<LOD	<LOD
	Pecan 4	<LOD	<LOD	<LOD	<LOD

LOD = limit of detection

Appendix D: QMRA calculated data

Here is the calculated data of the probability of infection for the QMRA

1. The pathogen dose (amount of pathogens) per site (Table S.27)
2. The calculated probability data per site (Table S.28)
3. The calculated probability data for water and soil (Table S.29)

Table S.27: QMRA pathogen concentration (dose) per site

Phylum	Patho- gen	Total	DMD	HR 1	HR 2	HR 3	HR 4	M 11	M 12	M 13	M 15	M 1	M 2	M 3	M 4	M 6	M 7	M 8	MP 10	MP 4	TO	X3
Actino- bacteriota		3458																				
	Myc	3458	301	19	720	20	0	217	154	140	119	267	213	119	120	128	91	40	24	22	21	723
Firmicutes		46176																				
	Bac	43305	0	0	9	19	13	5905	3400	1716	3173	3716	2268	5866	399	7695	6625	2463	0	3	35	0
	Clo	2302	0	0	0	22	0	91	58	32	108	594	224	582	23	434	118	10	0	0	6	0
	Ent	23	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0	0	0	0	0
	Sta	546	0	0	0	0	0	0	0	0	0	376	138	19	0	11	2	0	0	0	0	0
Proteo- bacteria		13773																				
	Cam	17	0	0	0	0	0	0	0	0	0	17	0	0	0	0	0	0	0	0	0	0
	E.coli	80	0	0	0	0	0	0	0	0	0	0	67	13	0	0	0	0	0	0	0	0
	Kleb	3005	0	0	0	0	0	34	0	0	0	1187	1315	181	13	160	84	31	0	0	0	0
	Leg	167	2	2	36	0	0	7	9	0	0	4	71	0	0	3	0	0	0	0	17	16
	Pseu	10493	21	30	432	610	180	391	465	3234	238	1997	439	65	62	53	24	133	25	1606	87	401
	Sal	11	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0
	Site total		324	51	1197	671	193	6645	4097	5122	3638	8158	4758	6845	617	8484	6944	2677	49	1631	166	1140

Myc = *Mycobacterium* spp.; Bac = *Bacillus* spp.; Clo = *Clostridium* spp.; Ent = *Enterococcus* spp.; Sta = *Staphylococcus* spp.; Cam = *Campylobacter* spp.; E. coli = *Escherichia coli*; Kleb = *Klebsiella* spp.; Leg = *Legionella* spp.; Pseu = *Pseudomonas* spp.; Sal = *Salmonella* spp. Blue coloured site names are water sites, and light brown coloured site names are soil sites.

Table S.28: The calculated probability of infection per site at the 0.3, 0.5 and 0.9 concentrations

Mode	Organism	Model	K	Alpha	N ₅₀	DMD			HR1			HR2			HR3			HR4		
						0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9
Ing	E.coli	β-P		1.55E-01	2.11E+06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Inh	Kleb	Ex	1.62E-06			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ing						0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ing	Sal	β-P		1.09E+00	1.34E+07	0	0	0	0	0	0	0	0	8.04E-01	0	0	0	0	0	0
Inh	Bac	Ex	1.65E-05			0	0	0	0	0	0	1	1	1	1	1	1	1	1	1
Ing						0	0	0	0	0	0	7.56E-03	3.72E-02	6.60E-02	1.59E-02	7.70E-02	1.34E-01	1.09E-02	5.33E-02	9.39E-02
Ing	Ent	β-P		1.60E-01	5.99E+04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ing	Pseu	Ex	1.05E-04			1.07E-01	4.31E-01	6.37E-01	1.49E-01	5.53E-01	7.65E-01	9.02E-01	1	1	9.62E-01	1	1	6.19E-01	9.92E-01	1
Ing	Sta	Ex	7.64E-08			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Inh						0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ing	Cam	β-P		1.44E-01	8.90E+02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ing	Clo	Ex	1.82E-11			0	0	0	0	0	0	0	0	0	2.05E-08	1.02E-07	1.84E-07	0	0	0
Inh	Leg	Ex	5.99E-02			1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Inh	My	Ex	6.93E-04			1	1	1	1	1	1	1	1	1	1	1	1	0	0	0

Mode	Organism	Model	K	Alpha	N ₅₀	M11			M12			M13			M15			M1		
						0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9
Ing	E.coli	β-P		1.55E-01	2.11E+06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Inh	Kleb	Ex	1.62E-06			1	1	1	0	0	0	0	0	0	0	0	0	1	1	1
Ing						1.65E-09	8.26E-09	1.49E-08	0	0	0	0	0	0	0	0	0	5.77E-08	2.88E-07	5.19E-07
Ing	Sal	β-P		1.09E+00	1.34E+07	0	0	0	2.3878E-11	1.1939E-10	2.14903E-10	0	0	0	0	0	0	0	0	0
Inh	Bac	Ex	1.65E-05			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ing						2.92E-06	1.46E-05	2.63E-05	1.68E-06	8.41E-06	1.51E-05	8.49E-07	4.25E-06	7.64E-06	1.57E-06	7.85E-06	1.41E-05	1.84E-06	9.20E-06	1.66E-05
Ing	Ent	β-P		1.60E-01	5.99E+04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ing	Pseu	Ex	1.05E-04			1.23E-06	6.16E-06	1.11E-05	1.46E-06	7.32E-06	1.32E-05	1.02E-05	5.09E-05	9.17E-05	7.50E-07	3.75E-06	6.75E-06	6.29E-06	3.15E-05	5.66E-05
Ing	Sta	Ex	7.64E-08			0	0	0	0	0	0	0	0	0	0	0	0	8.62E-10	4.31E-09	7.76E-09
Inh						0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
Ing	Cam	β-P		1.44E-01	8.90E+02	0	0	0	0	0	0	0	0	0	0	0	0	1.01E-05	5.04E-05	9.07E-05
Ing	Clo	Ex	1.82E-11			4.97E-14	2.48E-13	4.47E-13	3.16E-14	1.58E-13	2.85E-13	1.74E-14	8.74E-14	1.57E-13	5.90E-14	2.95E-13	5.31E-13	3.24E-13	1.62E-12	2.92E-12
Inh	Leg	Ex	5.99E-02			1	1	1	1	1	1	0	0	0	0	0	0	1	1	1
Inh	My	Ex	6.93E-04			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Ing = ingestion; Inh = inhalation; E. coli = *Escherichia coli*; Kleb = *Klebsiella* spp.; Sal = *Salmonella* spp.; Bac = *Bacillus* spp.; Ent = *Enterococcus* spp.; Pseu = *Pseudomonas* spp.; Sta = *Staphylococcus* spp.; Cam = *Campylobacter* spp.; Clo = *Clostridium* spp.; Leg = *Legionella* spp.; Myc = *Mycobacterium* spp.; β-p: Beta-Poisson; Ex: Exponential; K: infection probability per dose; Alpha: the shape of dose-response variability; N₅₀: the dose to the median response.

Mode	Organism	Model	K	Alpha	N ₅₀	M2			M3			M4			M6			M7		
						0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9
Ing	E.coli	β-P		1.55E-01	2.11E+06	1.28E-08	6.39E-08	1.15E-07	2.48E-09	1.24E-08	2.23E-08	0	0	0	0	0	0	0	0	0
Inh	Kleb	Ex	1.62E-06			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ing						6.39E-08	3.20E-07	5.75E-07	8.80E-09	4.40E-08	7.92E-08	6.32E-10	3.16E-09	5.69E-09	7.78E-09	3.89E-08	7.00E-08	4.08E-09	2.04E-08	3.67E-08
Ing	Sal	β-P		1.09E+00	1.34E+07	9.88E-01	9.98E-01	9.99E-01	9.06E-01	9.82E-01	9.90E-01	3.81E-01	7.65E-01	8.58E-01	8.94E-01	9.79E-01	9.89E-01	8.10E-01	9.60E-01	9.78E-01
Inh	Bac	Ex	1.65E-05			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ing						1.12E-06	5.61E-06	1.01E-05	2.90E-06	1.45E-05	2.61E-05	1.98E-07	9.88E-07	1.78E-06	3.81E-06	1.90E-05	3.43E-05	3.28E-06	1.64E-05	2.95E-05
Ing	Ent	β-P		1.60E-01	5.99E+04	1.38E-07	6.92E-07	1.25E-06	0	0	0	0	0	0	0	0	0	0	0	0
Ing	Pseu	Ex	1.05E-04			1.38E-06	6.91E-06	1.24E-05	2.05E-07	1.02E-06	1.84E-06	1.95E-07	9.76E-07	1.76E-06	1.67E-07	8.35E-07	1.50E-06	7.56E-08	3.78E-07	6.80E-07
Ing	Sta	Ex	7.64E-08			3.16E-10	1.58E-09	2.85E-09	4.35E-11	2.18E-10	3.92E-10	0	0	0	2.52E-11	1.26E-10	2.27E-10	4.58E-12	2.29E-11	4.13E-11
Inh						1	1	1	6.06E-01	1	1	0	0	0	4.17E-01	9.32E-01	9.92E-01	9.33E-02	3.87E-01	5.86E-01
Ing	Cam	β-P		1.44E-01	8.90E+02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ing	Clo	Ex	1.82E-11			1.22E-13	6.12E-13	1.10E-12	3.18E-13	1.59E-12	2.86E-12	1.25E-14	6.28E-14	1.13E-13	2.37E-13	1.18E-12	2.13E-12	6.44E-14	3.22E-13	5.80E-13
Inh	Leg	Ex	5.99E-02			1	1	1	0	0	0	0	0	0	1	1	1	0	0	0
Inh	My	Ex	6.93E-04			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Mode	Organism	Model	K	Alpha	N ₅₀	M8			MP10			MP4			TO			X3		
						0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9	0.1	0.5	0.9
Ing	E.coli	β-P		1.55E-01	2.11E+06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Inh	Kleb	Ex	1.62E-06			1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ing						1.51E-09	7.53E-09	1.36E-08	0	0	0	0	0	0	0	0	0	0	0	0
Ing	Sal	β-P		1.09E+00	1.34E+07	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Inh	Bac	Ex	1.65E-05			1	1	1	0	0	0	1	1	1	1	1	1	0	0	0
Ing						1.22E-06	6.10E-06	1.10E-05	0	0	0	2.53E-03	1.26E-02	2.25E-02	2.91E-02	1.37E-01	2.33E-01	0	0	0
Ing	Ent	β-P		1.60E-01	5.99E+04	9.02E-01	9.24E-01	9.31E-01	0	0	0	7.12E-01	7.78E-01	7.98E-01	8.06E-01	8.50E-01	8.63E-01	0	0	0
Ing	Pseu	Ex	1.05E-04			4.19E-07	2.09E-06	3.77E-06	1.26E-01	4.89E-01	7.01E-01	1	1	1	3.73E-01	9.03E-01	9.85E-01	8.84E-01	1	1
Ing	Sta	Ex	7.64E-08			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Inh						0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ing	Cam	β-P		1.44E-01	8.90E+02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ing	Clo	Ex	1.82E-11			5.44E-15	2.73E-14	4.92E-14	0	0	0	0	0	0	5.58E-09	2.79E-08	5.02E-08	0	0	0
Inh	Leg	Ex	5.99E-02			0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
Inh	My	Ex	6.93E-04			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Ing = ingestion; Inh = inhalation; E. coli = *Escherichia coli*; Kleb = *Klebsiella* spp.; Sal = *Salmonella* spp.; Bac = *Bacillus* spp.; Ent = *Enterococcus* spp.; Pseu = *Pseudomonas* spp.; Sta = *Staphylococcus* spp.; Cam = *Campylobacter* spp.; Clo = *Clostridium* spp.; Leg = *Legionella* spp.; Myc = *Mycobacterium* spp.; β-p: Beta-Poisson; Ex: Exponential; K: infection probability per dose; Alpha: the shape of dose-response variability; N₅₀: the dose to the median response.

Table S.29: The calculated probability of infection by ingesting contaminated water or soil at the 0.3, 0.5 and 0.9 concentrations.

Mode	Organism	Model	K	Alpha	N ₅₀	Water			Soil		
						0.1	0.5	0.9	0.1	0.5	0.9
Ing	E.coli	β-P		1.55E-01	2.11E+06	0	0	0	1.53E-08	7.63E-08	1.37E-07
Inh	Kleb	Ex	1.62E-06			0	0	0	1	1	1
Ing	Sal	β-P		1.09E+00	1.34E+07	0	0	0	1.46E-07	7.30E-07	1.31E-06
Ing	Bac	Ex	1.65E-05			1	1	1	2.39E-11	1.19E-10	2.15E-10
Ing	Ent	β-P		1.60E-01	5.99E+04	6.44E-02	2.83E-01	4.51E-01	1	1	1
Ing	Pseu	Ex	1.05E-04			0	0	0	2.14E-05	1.07E-04	1.93E-04
Ing	Sta	Ex	7.64E-08			1	1	1	1.38E-07	6.92E-07	1.25E-06
Ing	Cam	β-P		1.44E-01	8.90E+02	0	0	0	2.24E-05	1.12E-04	2.01E-04
Ing	Clo	Ex	1.82E-11			0	0	0	1.25E-09	6.26E-09	1.13E-08
Inh	Leg	Ex	5.99E-02			0	0	0	1	1	1
Inh	My	Ex	6.93E-04			0	0	0	1.01E-05	5.04E-05	9.07E-05
						2.60E-08	1.30E-07	2.34E-07	1.24E-12	6.21E-12	1.12E-11
						1	1	1	1	1	1
						1	1	1	1	1	1

Ing = ingestion; Inh = inhalation; E. coli = *Escherichia coli*; Kleb = *Klebsiella* spp.; Sal = *Salmonella* spp.; Bac = *Bacillus* spp.; Ent = *Enterococcus* spp.; Pseu = *Pseudomonas* spp.; Sta = *Staphylococcus* spp.; Cam = *Campylobacter* spp.; Clo = *Clostridium* spp.; Leg = *Legionella* spp.; Myc = *Mycobacterium* spp.; β-p: Beta-Poisson; Ex: Exponential; K: infection probability per dose; Alpha: the shape of dose-response variability; N₅₀: the dose to the median response.

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