Glyphosate displacement from New Zealand soils and its effect on non-target organisms

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GLYPHOSATE DISPLACEMENT FROM NEW ZEALAND SOILS AND ITS EFFECT ON NON-TARGET ORGANISMS

ABSTRACT

Glyphosate (GlyP) is the most commonly used herbicide worldwide; it is retained in the soil and is decomposed by soil microorganisms. The main degradation product of GlyP is Aminomethyl-phosphonic acid (AMPA). Phosphorus and GlyP are antagonistic anions that compete for the soil's reaction sites; P accumulation in the soil can increase GlyP translocation through the environment and increase its bioavailability. Residual GlyP and AMPA accumulation in the soil has generated concerns about their potential toxicity to non-target organisms such as crops and soil microorganisms. GlyP *in situ* remediation has therefore emerged as an option to reduce the residence time of the herbicide in soil.

Laboratory experiments were carried out in order to elucidate the effect of the interaction between soil chemical and physical properties, and phosphorus addition on GlyP sorption to soil surfaces. The results of the GlyP-AMPA batch adsorption-desorption experiment demostrated that the Kd and fixation of GlyP and AMPA in the soil was proportional to the Al-Fe oxy-hydroxides content of the soil, in the following order Allophanic>Brown>Pallic. In another experiment, phosphorus addition to soil reduced GlyP adsorption, which demonstrated that phosphate will occupy the same soil reaction sites as GlyP. These results suggest that due to the stability of the bond formed between Al oxy-hydroxides and P, Al oxy-hydroxides will fix GlyP; while the higher reactivity of Fe oxy-hydroxides will facilitate the exchange of phosphate by GlyP. A column leaching experiment demonstrated that the interaction between the physical and chemical characteristics of the soil will influence water infiltration and solubilisation of GlyP. Phosphorus addition to the columns enhanced GlyP's vertical displacement through the soil and AMPA detection in the leachate. The Pallic soil with a poor physical structure had reduced GlyP vertical displacement. In contrast, the free-drained Brown soil had higher AMPA percolation regardless of the P addition. The Allophanic soil had the lowest GlyP percolation risks, despite the fact that P addition increased AMPA detection at the bottom of the column. However, AMPA was undetected in the Allophanic soil's leachate. A soil induced respiration (SIR) experiment demonstrated that GlyP (variable doses) did not affect soil microorganism

respiration, while Agave amendments were used as an exogenous source of carbon and triggered soil respiration (Agave applied had 0.382 mg TC/g soil and control C applied was 1.25 mg C/g soil). The SIR ratio values observed in the soils were as follows Allophanic>Pallic>Brown, and were inversely proportional to the total dissolved carbon concentration in soil extracts. These results demonstrate that the greater Al-Fe oxy-hydroxide content of the Allophanic soil protected organic matter from mineralisation enabling greater microbial activity over the GlyP molecule. The P adsorption-desorption experiment using Agave powder demonstrated that Agave constituents desorbed phosphorus from soil surfaces, which might help in the desaturation of P from soil, while increasing its bioavailability.

Glasshouse experiments using Roundup doses and Agave amendment applied to the soil of white clover potted plants were carried out in order to elucidate the potential for GlyP degradation in soil and the biochemical responses of white clover plants. The results demonstrated that Agave amendment attenuated the translocation of GlyP to white clover shoots for a Roundup dose of 90 kg a.i./ha. The chemical constituents of Agave, 12 hrs after GlyP application to the soil, enhanced GlyP degradation to AMPA in soil at the 15 kg GlyP treatment. A similar improved GlyP degradation was observed during three days of evaluation at the 7.5 kg dose. The biochemical responses of white clover shoots demonstrated an increase of gallic acid and tartaric acid accumulation proportional to the increasing Roundup doses. This suggested that Roundup alone, and in combination with Agave amendments, exerted oxidative stress on the plants. Alternatively, the herbicide could have affected the EPSPS enzyme disrupting the carbon cycle. These results demonstrate that the white clover metabolic disruption caused by the Roundup treatments of 7.5 and 15 kg/ha, expressed through tartaric acid and gallic acid, was alleviated at the third day of evaluation.

The results of this thesis can support decision-making for the implementation of strategies which could mitigate glyphosate and AMPA displacement from New Zealand farmland; as example, it may encourage the prevention of phosphorus accumulation in the farmland. In addition, these results can encourage the development of further research related to the potential use of Agave amendments for glyphosate remediation, and help in the understanding of the effects of the herbicide on the metabolism of non-target organisms.

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1. INTRODUCTION AND STUDY AIM

Glyphosate (GlyP) is the most used herbicide worldwide in the agricultural sector. The main degradation product of GlyP is aminomethylphosphonic acid (AMPA), and in the United States AMPA has been detected in a greater number of environmental samples than GlyP (Scribner et al., 2007). GlyP and phosphate are anions that compete for the soil's Al-Fe oxy-hydroxides. Hence, P accumulation in soils may increase GlyP bioavailability and transportation risks out of farmland (Gimsing et al., 2004; Munira et al., 2018).

GlyP disrupts the shikimate pathway of target plants; however, its specificity has been challenged because it can also reduce the efficiency of enzymes in other living organisms (Modesto & Martinez, 2010; Fan et al., 2016). Post-sowing management practices for pasture production of white clover can include the use of herbicides for weed control to ensure its adequate root establishment (Sandral et al., 1997). The extended use of GlyP has generated concerns about its accumulation in the soil and potential toxicity to non-target organisms. This has encouraged assessment of its chemical effects on both soil microorganisms and crops, as well as the risk of potential transfer into the food chain (Tong et al., 2017). The development of GlyP remediation *in situ* techniques has emerged as a solution to reduce GlyP's residence time in the environment (Zhan et al., 2018).

Taking into consideration the negative environmental consequences of glyphosate displacement beyond farmland and its potential toxicity on non-target organisms, the aim of this thesis was to contribute to understanding of GlyP sorption on New Zealand soils and its effects on non-target organisms. This thesis is comprised of two experimental parts. In the first, laboratory experiments were conducted to assess the potential for displacement of GlyP from three contrasting New Zealand soils. This work was designed to elucidate GlyP transportation risks from farmland caused by the interaction of GlyP with soil properties and phosphate. The second experimental part investigates opportunities for the remediation of residual GlyP in soil. Agave plant extracts were used as soil amendments, in combination with variable rates of Roundup, and applied to soil planted with white clover. The degradation of GlyP to

AMPA and the metabolic responses of white clover was quantified in order to elucidate the potential toxicity caused by GlyP to white clover plants.

The research conducted in this thesis will be useful for future estimates of the potential loses of GlyP and its degradation product AMPA from farmland as a function of the chemical and physical characteristics of soils that are influenced by farm management. The results of this thesis could help in decision-making for farm management strategies that mitigate the displacement of the herbicide from the soil. This research has expanded the knowledge of the effect of GlyP in non-target organisms such as soil microorganisms and white clover for pasture production. In addition, this thesis has assessed the potential use of Agave amendments for *in situ* GlyP remediation.

2. LITERATURE REVIEW

Background

Glyphosate (GlyP) is a commercial post-emergence organophosphate herbicide widely used in agricultural systems with a chemical formula N-phosphonomethyl glycine: $^{-}HO_3PCH_2NH_2\ ^{+}CH_2COOH$. GlyP is mainly absorbed through the leaves of target weeds, and its mechanism of action is the inactivation of the enzyme 5-enolpyruvylshikimate3-phosphate synthase (EPSPS). This enzyme participates in the shikimate pathway, which is essential for the biosynthesis of aromatic compounds and is only present in microorganisms and plants. GlyP binds to the enzyme's active site competing with phosphoenolpyruvate, inactivating the functionality of EPSP (Schönbrunn et al., 2001).

GlyP has three functional groups, amino, carboxylic and phosphonate, each of which can be a source of nutrients for soil microorganisms. GlyP has a half-life of 47 days in the soil; GlyP decomposition is through microbial mineralisation that occurs immediately after application (Haney, Senseman, & Hons, 2002). The primary degradation product of GlyP is the molecule Aminomethylphosphonic acid (AMPA) (Figure 2.1) and the potential mobilization of GlyP and its degradation products out of farmland has raised concerns around the world. In the United States, AMPA has been detected in surface waters at a greater concentration than GlyP (Scribner et al., 2007).

Figure 2.1 Molecule of glyphosate and its main degradation product aminomethylphosphonic acid (Zhan et al., 2018).

Chemically, AMPA can be classified as a phosphonate; a chemical group composed of a phosphonic acid in which the basic structure is the C-P moiety. Phosphonates are synthesised for industrial purposes due to their chelating effect. Phosphonates can interfere with enzymes and DNA synthesis in living organims. Absorption of phosphonates and GlyP in the human body is relatively low; if absorption occurs, the kidneys excrete the metabolites. Both GlyP and AMPA has been detected on human urine, and although the levels detected are below the maximum limits established by the World Health Organization, its potentially harmful effects on human health cannot be disregarded (Nowack, 2003; Zhan, Feng, Fan, & Chen, 2018).

The widespread use of organophosphate herbicides has generated concerns due to the possible recalcitrance of the C-P moiety, which is decomposed only under phosphate-limiting conditions, and by specific soil microorganisms which possess the C-P lyase enzyme capable of break the C-P bond (Hamer, Egli, Snozzi, & European Federation of Biotechnology, 1989). The persistence of the C-P moiety in soil can increase its bioavailability and this can represent a risk to non-target organisms, such as soil microorganisms and plants. For example, GlyP can affect the enzyme functionality of nitrogen-fixing bacteria in the soil (Fan et al., 2017). Plant uptake and redistribution of GlyP or its metabolites has been actively studied by research groups around the world (Tong et al., 2017). This is because plants cannot metabolise the herbicide. Instead, GlyP is stored in leaf vacuoles as a mechanism to reduce its toxicity (Schrübbers, Valverde, Strobel, & Cedergreen, 2016).

In the USA, after the development of GlyP-resistant crops in 1998, GlyP usage increased exponentially every year and there is evidence of its presence in soil and water. The USGS in a 2007 survey detected GlyP concentrations in soil samples from 1 to 476 µg/kg, while for surface waters concentrations ranged from 0.03 to 427 µg/L (Scribner et al., 2007). In New Zealand, the maximum residue limit of GlyP is 0.01 mg/kg in fruit, while for other foods the limit is 0.1 mg/kg. New Zealand follows the recommendations of the World Health Organization which state that the acceptable daily intakes must not exceed the consumption of 1 mg GlyP/kg of body weight. In 2016 MPI analysed GlyP residues in wheat, finding that 26 out of 60 samples were above the limit of 0.1 mg/kg (MPI, 2017).

2.1 Factors that influence glyphosate adsorption-desorption in the soil

- Aluminium and iron oxy-hydroxides concentration

Soil chemical composition is a function of the influence of climate on soil parent material, as this determines the extent of weathering of mineral fractions. Volcanic soils have aluminium-rich minerals such as allophane; aluminium and iron oxy-hydroxides are key soil components which determine the soil's anion retention capacity, and this soil property is useful to predict the soil's capacity to buffer P loads, and knowledge helps to establish effective P fertilisation schemes. Analysis of the diverse Al and Fe extractable fractions of a soil can help to elucidate its anion retention capacity (Metson & Blakemore, 1978; Saunders, 1965).

GlyP adsorption-desorption in the soil is driven by GlyP's phosphonic acid group, which has similar interactions with soil particles as phosphate. GlyP primarily reacts with the surfaces of Al and Fe oxy-hydroxides, which reduce GlyP mobility through the soil profile. Phosphate has an antagonistic reaction with GlyP for soil reaction sites. Phosphate has a stronger affinity for Al-Fe oxy-hydroxides, and this effect can increase the displacement of GlyP from the soil surface (Sprankle, Meggitt, & Penner, 1975a).

When the soil's Al-Fe oxy-hydroxides content is insignificant, other soil characteristics may lead the GlyP adsorption; such as, CEC, clay composition, texture, pH and organic matter content. Studies have associated low CEC with reduced GlyP retention. Sandy soil has reduced reaction sites and hence lower CEC, and simultaneously has course texture, which may increase GlyP displacement risks. In contrast, a clay soil with greater CEC and fine texture would retain greater GlyP (Zhao, Zhang, Gong, Zhang, & Zhang, 2009).

- Clay type

The types of clay minerals in soil can influence GlyP's absorptivity. Clays saturated with Al and Fe retain higher amounts of the GlyP molecule, compared to clays saturated with Na and Ca (Sprankle et al., 1975a). Calcium and magnesium have a higher affinity for water molecules due to their greater hydration enthalpy, in

comparison to Al and Fe with lower hydration enthalpy. On clay structures, Ca^{2+} can form weak structures of two bonds, while Al^{3+} and Fe^{3+} can form trigonal stable structures, and it is on the edges of Al and Fe structures of clay minerals that the herbicide can be retained (Meunier, 2005). The greater hydration enthalpy and lower electronegativity of calcium-based clays can influence GlyP holding capacity, and higher solubilisation of the herbicide can be expected in soils dominated by such clays. Sedimentary soils have high amounts of Ca and Mg clays and this negatively influences the potential for GlyP adsorption to constituent soil particles, in comparison to volcanic soils with greater Al-Fe clays, which can strongly retain anions (Sprankle et al., 1975a).

In soils, trivalent aluminium is more abundant and stable than trivalent iron. Iron, when it is present as Fe^{2+}, it can be interchanged from clay structures by Mg^{2+}. Thus the displacement of Fe from the clay structures may reduce GlyP holding capacity, due to the higher hydration enthalpy of Mg, which can release the herbicide to the soil solution easier than Fe (Velde, 1995).

- Organic matter

GlyP can adsorb onto organic matter (OM) surfaces through a sequence of deprotonation of GlyP's phosphonic group, then hydrogen bonding to OM phenolic groups. OM's hydrophobicity restrains its capacity to retain GlyP due to the anionic behaviour of GlyP. However, OM is a significant vector for GlyP retention in sandy soils, which have reduced CEC (Albers, Banta, Hansen, & Jacobsen, 2009).

Glyphosate sorption onto biochar generated through the pyrolysis of woody biomass is related to the temperature of pyrolysis treatment. Wood biochar has alkaline pH, and biomass prepared at 900 °C has the highest potential for GlyP retention, even under alkaline conditions GlyP desorption is generally expected. Thus, biochar amendment of soil can lead to GlyP immobilisation (Hall et al., 2018).

- pH

GlyP has three functional groups: carboxylic, amino and phosphonic groups. GlyP sorption-desorption onto soil surfaces shows a strong dependecy on pH, which is dominated by the phosphonic group. The isoelectric point of GlyP is at a pH of 2.6; at pH< 2 the chemical structure has a cationic charge while at pH >2.6 the negative charge becomes dominant. Alkaline conditions (pH>9) strongly reduce GlyP adsorption in soils (Sprankle et al., 1975a; Zhao et al., 2009)GlyP's amino group, glycine, can have a cationic charge between 2.2 and 9.6 pH, with a predominant zwitterionic form between 3.3 to 8.9 pH (Sprankle et al., 1975a).

The phosphonic group has chelating properties and can form complexes with solubilised metals such as Fe^{3+} and Al^{3+} which reduces both GlyP mobility and metal toxicity (Kaur et al., 2017). The phosphonic group has a high affinity for Al and Fe oxy-hydroxides, with the effect being greater at pH<4.6, and this is the major reason for GlyP retention under most environmental conditions.

- Methods for adsorption-desorption analysis of glyphosate-AMPA in soils

Batch adsorption-desorption experiments are laboratory techniques used to understand the sorption mechanisms of agrichemicals in soils. The method analyses the chemical interactions between soil particles and the agrochemical tested. Methodology consists of the addition of a solution of the agrichemical to soil, with the slurry shaken under controlled conditions. Thereafter, the agrichemical can be desorbed from the soil with a range of extractants depending on the purposes of the study. The agrichemical can be measured from the saturation solution, extractant and solid phase (Okada et al., 2016).

The sorption coefficient (Kd) is a value that helps to estimate the agrichemical retention capacity of soils or growing media. It is calculated considering the amount of agrochemical adsorbed in the media divided by the residual agrochemical found in the solution (Hall et al., 2018). Column leaching experiments are helpful in analysing the interactions between soil chemical and physical properties in the downward displacement of agrochemicals through columns packed with soil. Water infiltration

and solubilisation are key factors that lead to the percolation of agrochemicals through the soil profile (Katagi, 2013).

2.2 Glyphosate biodegradation

Soil microorganisms (SoMo) are the major contributors of GlyP breakdown through enzymatic lysis, which happens when enzymes synthesised by SoMo cleave specific bonds of the GlyP's molecule yielding a diverse range of degradation products (Zhan et al., 2018). Chemical breakage of the GlyP molecule can also happen, but this process is not as significant as degradation effected by SoMo (Duke, 2011). The main degradation product of GlyP is aminomethylphosphonic acid (AMPA), which is the product of the rupture of the C-N bond by the enzyme glyphosate oxidoreductase (GOX), also yielding glyoxylate. The second metabolite is sarcosine, which is the product of the C-P rupture by the enzyme C-P lyase, which yields phosphate as well. Degradation of GlyP to AMPA is favoured because the covalent bond C-P of the phosphonic moiety is stable, preserving the integrity of the AMPA molecule (Figure 2.2). The rupture of the C-P bond by SoMo will be favoured under P limiting conditions because this is a more accessible form of phosphate for SoMo when it is available, rather than the phosphonic moeity. Degradation of the C-P bond requires the specific C-P lyase enzyme, and this enzyme has been identified from Pseudomonas sp., Rhizobium spp., Streptomyces sp. and other fungi strains (Duke, 2011

Figure 2.2 The main enzymatic routes for GlyP biodegradation (Zhan et al., 2018)

2.3 Effect of glyphosate on soil microorganisms

The widespread use of GlyP in farmlands throughout the world has led to concerns about the possible adverse effects of GlyP on SoMo. The results from research into the effect of GlyP on SoMo are often contradictory, due to different environmental and experimental variables used in its evaluation. For example, studies that have evaluated the *in vitro* effect of GlyP on cultures of fungi as compared to soil microcosms have shown different effects of GlyP on the same strain of microorganisms. The available data suggests that soil has a strong buffer capacity to reduce GlyP toxicity to SoMo, while the herbicide applied to *in vitro* cultures can have a direct and fast negative effect on fungi growth (Wardle & Parkinson, 1990).

Microbial biomass carbon (MBC) and soil respiration (MSR) are techniques which can be used to assess the response of a whole SoMo community to GlyP. Studies using both techniques have demonstrated that after the use of high GlyP rates, the herbicide does not negatively affect SoMo, and can even enhance the proliferation of SoMo, suggesting a beneficial effect. More detailed techniques, such as PCR with molecular markers for analysing specific SoMo comunities, can help elucidate the response of specific SoMo communities to any GlyP effect. Studies have demonstrated that the herbicide can cause an imbalance of the structure of SoMo communities enhancing populations of saprophytic microbes while reducing the occurrence of N-fixing bacteria and the efficiency of nitrogenase, an enzyme responsible for atmospheric N fixation (Fan et al., 2017). Fungi strains can use GlyP as a source of carbon, nitrogen and phosphorus. *Aspergillus* sp., a soil borne fungi with pathogenic activity on crops, can synthesise enzymes that decompose GlyP (Carranza, Barberis, Chiacchiera, & Magnoli, 2017).

Soil induced respiration (SIR) is a laboratory technique that helps to assess the responses of SoMo to soil pollutants using exogenous C sources. The Microresp® system is useful for evaluating SIR, where, after incubation, soil respiration is measured as CO_2 emitted by SoMo, which is trapped in a Cresol-Agar reagent placed in a microwell plate; the reaction with the CO_2 causes a colour change in the Cresol-Agar reagent that is measured through absorbance using a UV spectrophotometer. In most cases, this technique will detect potential detrimental effects of soil pollutants

due to a reduction of soil respiration, which may indicate a reduction of SoMo proliferation (Berard et al., 2014; Wakelin et al., 2016).

In studies that have analysed SoMo responses to GlyP rates by SIR, the use of GlyP at recommended commercial rates demonstrates a minor or null effect on SoMo respiration; only high GlyP doses of around 79 mg/kg soil, which is 10 times above the recommended dose, will negatively affect SoMo respiration. In this particular study, the effect of GlyP on SoMo respiration was related to physical and chemical features of the soils used, where the soil's anion retention capacity might play a significant role in reducing GlyP bioavailability (Nguyen et al., 2018). Similarly, studies that have analysed glucose mineralisation using different types of herbicides, combined and alone, but not including glyphosate, have demonstrated that SIR responses are highly related to soil texture, with a null effect of herbicide treatments on SIR recorded (Mendes et al., 2017).

The periodic use of GlyP can change the structure of soil microbial communities, therefore, farm management can influence microbial communities and GlyP mineralisation. Soils receiving regular applications of GlyP through many crop seasons can have higher microbial activity compared to background soils, due to the adaptation and proliferation of GlyP resistant strains. However, the regular use of GlyP can impair microbial communities, reducing bacteria populations, while enhancing fungi prevalence (Araújo, Monteiro, & Abarkeli, 2003). This same response to GlyP use has been observed in forest soils (Ratcliff, Busse, & Shestak, 2006). Soil microbial biomass can be afected under excesive GlyP doses. In one study, the use of ten times the maximum recommended GlyP rate reduced the cultivable bacteria and fungi, with gram-negative bacteria communities dominating in the induced conditions (Liu et al., 2018).

2.4 Potential recalcitrance of phosphonates

Phosphonates can be synthesised by microorganisms as a defence mechanism. For example, *Streptomyces* sp. synthesise the phosphonate phosphinothricin, which inhibits glutamine synthetase, providing antibiotic and phytotoxic effects in the rhizosphere (Ternan et al., 1998).

In controlled conditions, phosphonates can be used for human health as antitumor agents and antibiotics. Similarly, phosphonates have been used for the synthesis of modified nucleic acids with inhibitory enzyme effects for DNA therapy and biotechnology (Shen & Hong, 2018).

AMPA is a phosphonate and belongs to a class of chemicals called organophosphates with a distinctive C-P covalent bond. Phosphonates are synthesised for industrial purposes due to useful chelating properties of this family of chemicals. Phosphonates are present in nature and some marine organisms synthesise them under P restricted conditions in order to retain P within biological tissues. Phosphorous covalently bound with C is much more stable than P as the phosphate molecule (Ternan et al., 1998).

The chelating properties of phosphonates rely on the number of phosphonic groups. AMPA possess one phosphonic group which forms weak M^+-complexes, in comparison to EDTMP which has four phosphonic groups forming strong M^+-complexes. Phosphonates usually are present in the environment complexed with cations (Nowack, 2003). The occurrence of AMPA in the rhizosphere may be related to farm management and soil characteristics, particularly related to phosphorus fertilisation and cultivation practices. Although GlyP is immediately decomposed after reaching the soil, the possible recalcitrance of AMPA has raised concerns due to the chemical stability of the phosphonic moiety and the preference of SoMo to uptake phosphate rather than C-P (Ternan et al., 1998)

Despite the low adsorption of GlyP and AMPA in the human body, GlyP's toxicity in the ecosystem and in human health is under scrutiny. This is due to the extended use of herbicides in farmland. The main concerns are the possible affinity of GlyP and AMPA to different enzymes, not only EPSPS; but also, its possible interference during DNA encoding (Ternan et al., 1998; Shen & Hong, 2018). Furthermore, the presence of GlyP or its metabolites into the food chain have encouraged research around the plant uptake of GlyP and AMPA (Tong et al., 2017).

Due to the low toxicity of the GlyP molecule to humans, it has been used in the improvement of mass spectrophotometry to detect phosphonic-based chemical weapons (Wagner, Wetzel, Kern, & Kingston, 2012).

Taking into consideration the potential persistence of AMPA in the soil, in conjunction with the periodical GlyP applications that are part of the farm management, and its

emerging use in the chemical industry, a key aim of this thesis was to elucidate the soil characteristics that influence the adsorption-desorption process of GlyP and AMPA, and their potential vertical displacement out of the root zone.

2.5 Factors of farm management that influence the occurrence of glyphosate

- Phosphate fertilisation

Intensive farm management has led to the accumulation of phosphorus in the soil through mineral fertilisation or manure application. The use of manure as a source of phosphorus can underestimate the total amount of P applied, because the requirements of manure used as fertiliser are based on ready-available P for crops. However, manure contains great amounts of organic P forms, which are not immediately available for crops, and thus, significant amounts of P remain in agricultural fields. When mineralisation occurs, the organic forms of P stored in the soil contribute to the long-term P losses to water bodies (Hooda et al., 2001; Pizzeghello, Berti, Nardi, & Morari, 2011). In New Zealand, land treatment of farm dairy effluent (FDE) on paddocks is an efficient way to improve soil nutrient pools and enhance crop productivity, and this helps to reduce the nutrient surpluses of dairy farms. FDE requires adequate management in order to reduce potential nutrient losses to water bodies and to reduce nutrient saturation of the soils. Paddock rotation, deferred irrigation and the use of aerobic ponds are crucial elements for appropriate land treatment of FDE (Houlbrooke, Horne, Hedley, Hanly, & Snow, 2010; Monaghan et al., 2010).

Phosphate and GlyP are antagonistic anions, GlyP absorption onto soil surfaces is influenced by phosphate availability. Both anions compete for reaction sites with phosphate having greater affinity for Al Fe oxy-hydroxides than GlyP (Sprankle, Meggitt, & Penner, 1975b; Zhao et al., 2009). Therefore, high phosphate fertilisation rates can saturate soil reaction sites. Regardless of pH, phosphate saturation may influence the displacement of GlyP from soil surfaces and increase GlyP bioavailability (Munira, Farenhorst, Flaten, & Grant, 2016).

Phosphate fertilisation can therefore increase the phytotoxicity of residual GlyP in the soil, particularly in soils with low P retention/buffer capacity. Pot experiments have

demonstrated that residual GlyP can reduce lupin shoot biomass after P application (Rose, van Zwieten, Claassens, Scanlan, & Rose, 2018).

Phosphate fertilisation is indispensable for crop production. The overuse of P fertilisers to overcome the natural P retention in soils has led to environmental issues such as P displacement from soil to water bodies. Of more relevant concern to this thesis is the relationship between P saturated soils and increased GlyP phytotoxicity and plant uptake. Research into the interactions between GlyP and phosphorus in New Zealand soils is essential to reduce the potential phytotoxicity of GlyP, and to mitigate the risk of this transfer to the food chain.

- Crop management

Perennial crops receiving multiple GlyP applications through the seasons can be subject to metabolic stress as GlyP disrupts plant metabolism. Studies on coffee plants receiving single or multiple GlyP doses demonstrated that GlyP application timing affected plant leaves to a greater extent than rates; however, high rates and multiple GlyP applications led to GlyP accumulation in the plant. The effect of GlyP on perennial crops is related to plant phenology and crop management practices. During the rainy season there is greater weed occurrence. Simultaneously, this is also a time of active crop growth. Therefore, the use of non-selective herbicides during active growth of the crop can be detrimental to yield as the herbicide affects the biomass production of young developing leaves (Schrübbers et al., 2016).

The use of herbicides in non-tillage systems is indispensable for weed control. Soil structure in non-tillage systems is unaffected by ploughing, which promotes greater fissures and macropores (Pires et al., 2017; Isik et al., 2017). Hence, in non-tillage systems, greater GlyP usage and greater macropores would promote GlyP vertical displacement, while ploughing in tillage systems disaggregates the structure of the topsoil, which enhances water infiltration at the beginning of the crop establishment (Naderi-Boldaji & Keller, 2016). Therefore, the potential for GlyP vertical displacement would be higher if herbicides are applied close to the time of ploughing.

Glyphosate is used for white clover pasture production as pre- and post-sowing weed control. After sowing, GlyP helps to secure white clover root establishment during the

early stages of the crop development, because weeds can interfere in this plant reaching its optimal root structure (Lewis, Lucas & Moot 2017). In addition, GlyP spray-topping at low doses during crop growth reduces white clover overgrowth, which reduces undesired dead biomass (Casey, Brown & Stevens, 2000). GlyP spray-topping also increases pasture digestibility (Gatford et al., 1999). Therefore, the extended use of GlyP as part of crop management may influence the metabolism of non-target plants, while simultaneously, residual GlyP and its degradation products may accumulate in the soil. Taking into consideration the importance of white clover as forage in the New Zealand dairy industry, the effect of GlyP on white clover plants was investigated as part of this thesis.

2.6 Glyphosate interaction with organisms

Glyphosate can form insoluble metal complexes in the soil, and these can be detrimental for plant nutrition because they reduce the availability of cationic essential trace elements (Kaur et al., 2017). On the other hand, GlyP-metal complexation can help in the immobilisation of potential hazardous cations such as cadmium. Research has shown that Cd toxicity to the earthworm *Eisenia fetida* is alleviated with increased GlyP doses, due to reduced Cd bioavailability associated with GlyP application to soil (Zhou et al., 2014). However, in contrast, GlyP-Cu complexes are toxic to aquatic organisms (Hansen & Roslev, 2016).

Polyethoxylated tallow amine (POEA) is a surfactant used in several formulations of herbicides including the commercial formula Roundup™; POEA enhances the penetration of the GlyP molecule through the plant cuticle. POEA has toxic effects on aquatic organisms; it disrupts the cell membranes of respiratory organs (Brausch, Beall, & Smith, 2007). Although it is recognised that exposure to GlyP-based herbicides below the maximum threshold is safe for human health, recent research has shown that POEA may be a toxic compound against human cells, alone or in conjunction with GlyP (Mesnage, Bernay, & Séralini, 2013).

The major recognised activity of GlyP is the inactivation of the enzyme EPSP synthase which is only present in microbes and plants. This represents a minor risk for mammals. However, the specificity of GlyP's mechanisms of action has been

challenged, with some evidence demonstrating disruption in human cells (Mesnage et al., 2013). Laboratory trials in fish have shown a toxic effect of GlyP due to the inactivation of the enzymes superoxide dismutase and glutathione peroxidase, leading to lipid peroxidation in the liver and brain dysfunction due to inhibition of the enzyme acetylcholinesterase which alters the nervous system (Modesto & Martinez, 2010). Studies have found a possible correlation between the seasonal application of herbicides and congenital disabilities for agricultural workers who use herbicides in the Red River Valley, Minnesota USA (Garry et al., 2002).

Glyphosate disrupts the plant metabolism of both sensitive plants and genetically modified GlyP-resistant plants, although the extent of affect is different. GlyP-resistant plants can overcome the stress induced by GlyP due to genes of microbes inserted to the plant's genome. The genetic insertion allows the plant to synthesise isozymes, which have the same function as EPSPS. Therefore, despite EPSPS being inactive, the isozymes take on EPSPS' function, nullifying GlyP disruption in the shikimate pathway (Green, 2007). GlyP-resistant soybean has a symbiosis with Rhizobacteria, and this association yields significant amounts of N for crop growth. However, studies on GlyP-resistant soybean have demonstrated that foliar application of herbicide reduces the total *Rhizobium* nodule mass and the activity of the microbial enzyme nitrogenase. Herbicide use also affects soybean root biomass, the N content and the chlorophyll content. (Fan et al., 2017).

After the inactivation of the EPSPS enzyme by GlyP; accumulation of precursor metabolites occurs upstream of the shikimate pathway, and this leads to the depletion of secondary metabolite products of the EPSPS synthesis. The primary carbon cycle is compromised as well, as the synthesis of metabolites is stopped (Zabalza, Orcaray, Fernandez-Escalada, Zulet-Gonzalez, & Royuela, 2017).

2.7 Glyphosate and AMPA transportation pathways from the soil

Residual glyphosate (GlyP) and AMPA are strongly held in the soil, however, lateral flow is the major GlyP displacement pathway, which is aggravated if rainfall occurs after herbicide application (Scribner et al., 2007; Borggard & Gimsing, 2008). GlyP vertical flow is unlikely to happen as the herbicide is retained in deeper soil layers.

However, soil characteristics, such as coarse texture and low anion retention capacity can facilitate GlyP's vertical distribution through the soil profile (Sprankle, Meggitt & Penner 1975a; Zhao et al., 2009). In addition, geological features such as cracks and unconformities in deeper strata may promote the downward distribution of the herbicide and its degradation products, increasing the risks of contamination of underground water (Rendon-von Osten & Dzul-Caamal, 2017).

2.8 Glyphosate remediation

Despite the relatively low persistence of GlyP in the soil, its extended use has led to the gradual accumulation of the herbicide in the environment, and this has encouraged a search for remediation techniques which reduce its presence and assumed toxicity in a range of media. In sludge treatment plants, different physical-chemical techniques have been used to decompose GlyP and other recalcitrant pollutants; but the primary approach for GlyP remediation is the use of GlyP-degrading microorganisms, which is the most reliable *in situ* technique to reduce GlyP persistence in the environment (Zhan et al., 2018).

In field soil, native SoMo can promote GlyP degradation, especially where strains are adapted and specialised to its degradation due to the regular use of herbicide (Araújo et al., 2003). Factors that facilitate SoMo proliferation in the soil are pH, temperature, GlyP concentration, and inoculation of biomass (Zhan et al., 2018). Soil carbon sources are beneficial for SoMo proliferation; adequate levels of soil organic matter boost GlyP biodegradation (Haney et al., 2002). Application of exogenous carbon sources to the soil may contribute to GlyP biodegradation, due to increasing demands for P and N by SoMo, which can facilitate the breakdown of the phosphonic acid C-P moiety.

- Bioremediation potential of Agave by-products

Agave leaves in Mexico are by-products of the Tequila industry, and in Australia are a potential bioethanol feedstock due to their high levels of water-soluble sugars and low lignin content (Corbin et al., 2015). Agave contains organic acids, such as succinic acid which has *in vitro* anthelmintic effect against nematodes in goats (Santos et al., 2017). Organic acids are excreted by SoMo and roots in order to enhance nutrient

uptake; organic acids have an antagonistic relationship with phosphate and other anions for soil reaction sites (Bhatti, Comerford & Johnston 1998). Agave leaves possess significant amounts of secondary metabolites, such as phenols, and, to a major extent, saponins (Ribeiro, Barreto, & Coelho, 2015)

Phenols (PhE) are compounds widely synthesized in the plant kingdom. These compounds have diverse functions in plants and are involved in plant protection against pathogens and herbivores. These compounds also have antioxidant functions during oxidative stress (Viladomat & Bastida, 2015). Phenols are organic acids able to form complexes with metals which can interact in the soil displacing anions from the mineral fraction (Kováčik, Klejdus, Hedbavny, & Zoń, 2011).

Saponins are biosynthesized in plants and microorganisms; these compounds protect plants against pathogens. Saponins have surfactant activity and thus can desorb contaminants from the soil matrix. For example, cadmium and phenanthrene have been shown to be desorbed from soils using plant-derived saponins (Song, Zhu, & Zhou, 2008), although for Cd, soil texture will strongly influence contaminant mobility when using saponins (Gusiatin & Klimiuk, 2012). Saponins can affect the lipid stability of a microorganisms' membrane cell, and this is a concern during soil bioremediation with surfactant-microbes systems. However, with controlled surfactant doses, the impact on microbes activity can be mitigated (Smułek et al., 2017).

Agave constituents may have similar functions as exudates of SoMo and plant roots in order to enhance nutrient acquisition in the rhizosphere. A key hypothesis of this thesis is that the by-products of agave leaves after industrial processes have potential use in the remediation of glyphosate or other soil pollutants. As GlyP remediation relies on SoMo breakdown, agave could be a carbon source for enhancing SoMo proliferation. At the same time, saponins, organic acids and phenols could displace GlyP or AMPA molecules from the soil's mineral fraction, facilitating biodegradation, reducing the potential for accumulation of these contaminants in the food chain.

The *Agave* genera has naturalised species in New Zealand. *Agave attenuata* and *A. americana* are used for ornamental purposes. Also, there are young *Agave tequilana* plantations in the Golden Bay region for spirit production (pers. com. Terry Knight, TeKiwi Co.) In the medium-term (5 years) these NZ agave plantations represent a potential source of saponins and phenols for bioremediation of contaminated soils.

The target material of the Agave plantations is the stem, where most of the free and complex sugars are stored. Commercial stem sizes require an average time of growth of at least 5 years. However, Agave crop management requires annual leaf pruning to maintain a plantation's health and increase productivity, and this represents a significant annual yield of biomass (Corbin et al., 2015).

2.9 Glyphosate determination from environmental samples

The peculiarity of the GlyP molecule hinders its assessment, especially in complex samples, where the matrix effect can interfere with GlyP determination. The optimum method used for GlyP detection depends on the type of sample and the concentration required for detection. For soil samples, the steps of extraction, purification and derivatisation are required prior to GlyP detection (Koskinen, Marek, & Hall, 2016).

GlyP is strongly adsorbed to soil surfaces requiring strong alkaline solutions to desorb it, which also can remove humic substances. Both, GlyP and humic compounds have an absorbance in a similar wavelength. When GlyP is detected using the UV-Vis spectrophotometric method, the results can be an overestimate of GlyP amounts in environmental samples if corrections are not taken (Waiman, Avena, Garrido, Fernández Band, & Zanini, 2012)

Alkaline solutions such as NaOH and KOH at a concentration of 0.1-1M have been used to extract GlyP from soil samples. Sodium tetraborate 40 mM instead of 0.1 M KOH can be used to reduce the interference of humic substances extracted. After GlyP extraction, purification with non-polar solvents can reduce the interference of hydrophobic fractions on soil organic matter (Waiman et al., 2012).

High-performance liquid chromatography is a reliable and the most suitable cost-effective method for GlyP detection. HPLC coupled to fluorescence detection for GlyP analysis requires a derivatisation step, and this is due to the absence of a chromophore group in the GlyP molecule. Derivatisation is a reaction that links a protecting molecule with GlyP. This synthesis yields a new molecule, which enables the detection of GlyP on HPLC. The most used derivatising agent for GlyP's detection is FMOC-Cl (9-fluorenyl methoxycarbonyl chloride), which binds to GlyP's amino group; this reaction is carried out in alkaline conditions. FMOC-Cl has been widely

used for the protection and detection of amino acids. The aromatic group of the FMOC-Cl moiety is a chromophore. Chromophores adsorb energy at certain wavelength of the UV-Vis spectra then reemit the energy, which can be measured by conventional fluorescent and MS detectors (Koskinen et al., 2016).

2.10 Specific aims of this thesis

Due to the widespread use of GlyP in agricultural systems, and its competition with phosphate for the adsorption onto the soil surfaces, concerns have been raised over the potential for GlyP displacement beyond the farmland. Hence, part of the aims of this thesis were to analyse the sorption of GlyP onto three representative soils orders of New Zealand using laboratory techniques. Specifically, the aims of the first experimental part of this thesis (Chapter 4) were to 1) evaluate the sorption of GlyP and AMPA onto soils; 2) evaluate the effect of phosphate in the sorption of GlyP onto soils; 3) evaluate the vertical displacement of GlyP through columns packed with disturbed topsoil; 4) evaluate the effect of GlyP and Agave amendments in soil respiration; and 5) evaluate the effect of Agave leaf powder in the desorption of phosphate from soils

The accumulation of GlyP in the soil, and its potential toxic effects on non-target organism, has encouraged both evaluation of the responses of non-target organisms to GlyP, and the development of remediation techniques which can reduce the residence time of the herbicide. Due to the characteristics of Agave plant constituents, waste from the Agave plant possesses potential for use in GlyP remediation from soils. Therefore, the aims of the second experimental part of this thesis (Chapter 5) were to: 1) evaluate the effect of Roundup doses and Agave amendments in the degradation of GlyP in the soil of white-clover potted plants; and 2) evaluate the effect of Roundup doses and Agave amendments on the metabolic responses of one-month old white clover potted plants.

3 MATERIALS AND METHODS

The materials and methods outlined in this chapter were the standard methods commonly used across the two experimental chapters of this thesis. If changes to these standard methods were adopted, this is outlined in the materials and methods section of each experimental chapter. In the first section of this chapter the sampling procedures and analytical methods used for the physical and chemical characterisation of the soil samples collected from three different locations of New Zealand is presented.

Thereafter, the analytical procedures used for the assessment of GlyP-AMPA in soil samples using an RP-HPLC system is described. Finally, the analytical procedures followed for the metabolomic characterisation of plant samples used for this thesis are described.

3.1 Soil sampling locations

Soil representative of three New Zealand soil orders was collected from three field locations in New Zealand. At each location composite soil samples were collected in transects, with at least three transects per location collected, although the number of sampled transects depended on the field topography. The organic layer was removed and the topsoil (7.5 cm depth) was collected with a spade taking aproximately 2 kgs of fresh soil per subsample.

The soils were air-dried until constant weight. The subsamples were homogenised with a mortar and pestle and passed through a 2 mm sieve. The soils were stored in plastic bags at room temperature until use.

The soil identification was performed taking into consideration the soil properties of the layer collected and using the geographical coordinates of the sampling sites referenced on the database of the soils orders of the S-Maps website (Landcare Research, 2019).

Perch-Gley Pallic soil

The Perch-Gley Pallic soil belongs to the order of Pallic soils. Pallic soils have a low iron content and as a consequence possess a pale colour. These soils have a weak physical structure and low permeability making them prone to waterlogging (Landcare Research, 2019). Topsoil (7.5 cm depth) was collected in the Massey University, Palmerston North research farm, near Sheep Farm Road. Soil (about of 40 kg of dry soil) was collected from near the Manuka bush plantation at this location (Figure 3.1).

Orthic Brown (Fill Anthropic) soil

Orthic Brown soil belongs to the order of Brown soils, with an intermediate development, these soils are common on slopes or young land surfaces. The brown colour is caused by iron oxides weathered from parent material (Landcare Research, 2019). Anthropic soils are derived from human disturbances; such as urban, industrial and mining activities; therefore, the normal properties of the natural soil are destroyed. Fill anthropic soils are formed by the deposition of inorganic material such as soil, metals, debris and so forth. Nevertheless, if the properties of the Anthropic soil have been recovered to the characteristics of any soil order (not including Raw or Recent orders), then the soil cannot be classified as Anthropic soil (Hewitt, 2010).

Topsoil (7.5 cm depth) was collected at the proximity of the North-eastern creek located at the Mt Rinopai sub-catchment. Soil (about 20 kg of dry soil) was collected from under wild Manuka bush. The sampling site was a hillside in the proximity to the SH60 Takaka-Collingwood Highway and Tukurua Road, between Parapara and Onekaka, Golden Bay, New Zealand (Figure 3.2). The UTM coordinates are E 1574067; N 5489628.

The landowner of the sampling site mentioned that in the past, at the base of the hill were deposited tailings coming from an iron mine, a creek nearby the collection site had reddish staining on the bed, which implied the presence of Fe oxides. Although, the collection of the sub-samples was performed uphill at the manuka bush away of the tailings, the degree of anthropic disturbance in that area, such as the extent of the deposition of the tailings, is unknown and this explains the description of this soil as Orhic Brown (Fill Anthropic).

The Northwest of the Nelson region, Takaka-Collingwood valley, has a significant mineral richness with economic importance, including asbestos deposits, nickeliferous sulphide and quartz-carbonate rocks (Hunter, 1977). At the Parapara River area in the Richmond Hill has been reported iron sulphide minerals, such as pyrrhotite and pyrite (Grapes & Challis, 1999). The Parapara area in the Collingwood district was under gold mining since the late XIX until the early XX century (Newport, 1980).

Taking into consideration the anthropic influence in the sampling area, special care has been taken to classify this soil. Tailings possess high concentrations of minerals in the soil and drainage; up to 129,000 mg Fe /L was found in mine water of Sherridon mine as described by Lindsay et al. (2015). Sulphide oxidation reaction leads to the precipitation, dissolution and formation of secondary minerals, which generate acidic conditions that deplete the neutralization capacity of the soil (Lindsay et al., 2015). The above mentioned conditions are beyond characteristics observed in the sampling area of the manuka bush. In the sampling site there was a well formed organic horizon, and the chemical and physical properties matched the properties of a restored Brown soil if anthropic disturbance occurred (Tables 4.1, 4.2 and 4.3).

Following this discussion, the soil is classified as a Brown soil (either recovered or natural). Anthropic disturbance at the base of the hill might have influenced the conditions of the sampling area. Therefore, care must be taken when comparing the results obtained in this thesis with other soils of the Brown order due to the potential for anthropic disturbance.

Orthic Allophanic

Orthic Allophanic soil belongs to the order of Allophanic soils, which are dominated by allophane minerals possessing high anion retention capacity, low density and high porosity (Landcare Research, 2019). Topsoil (7.5 cm depth) was collected from non-cultivated area on a dairy farm at Hawera in the Taranaki region (Figure 3.3).

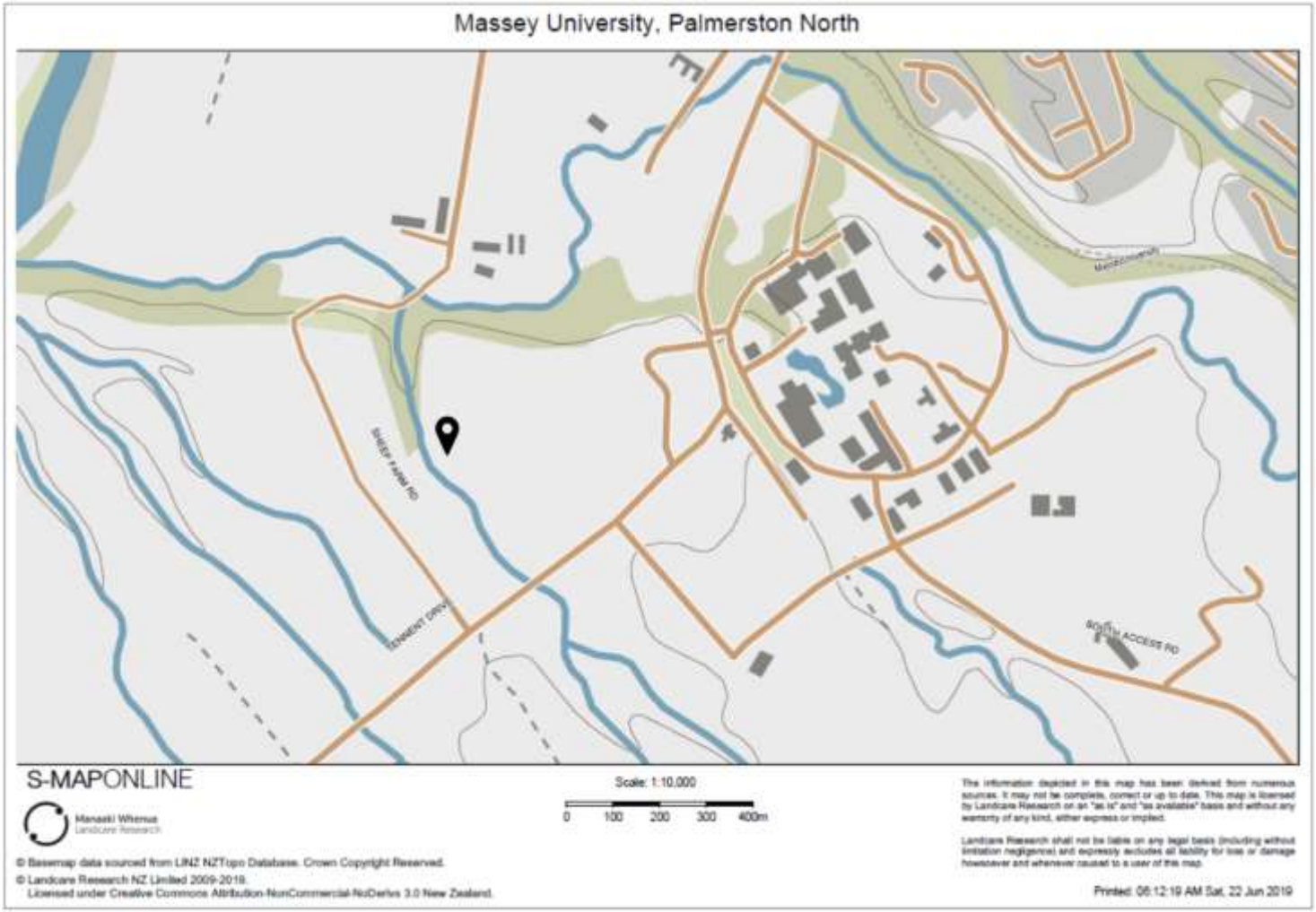

Figure.3.1 Collection site of Perch-Gley Pallic soil at Massey University, Palmerston North.

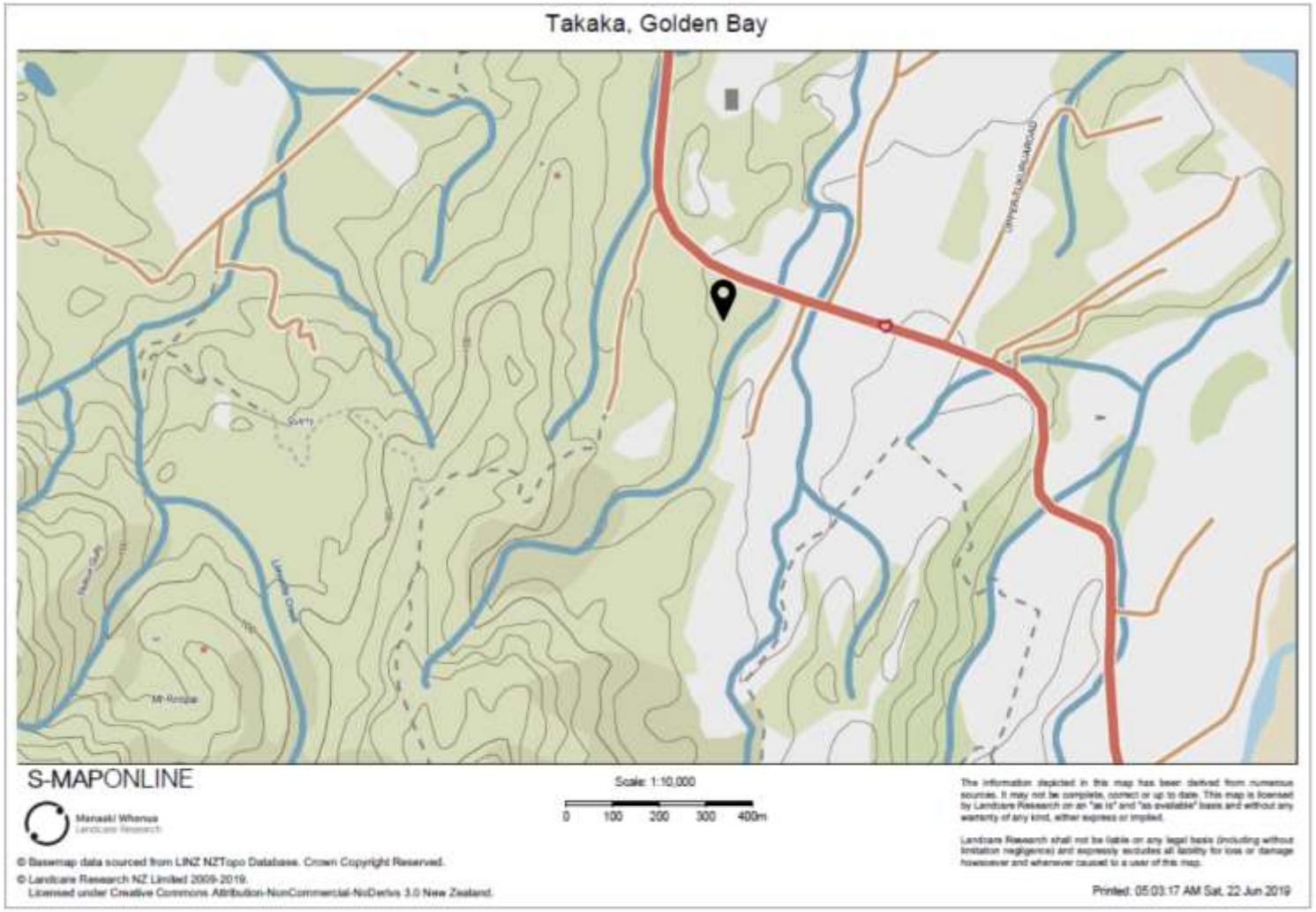

Figure 3.2 Collection site of Orthic Brown soil at Takaka, Golden Bay.

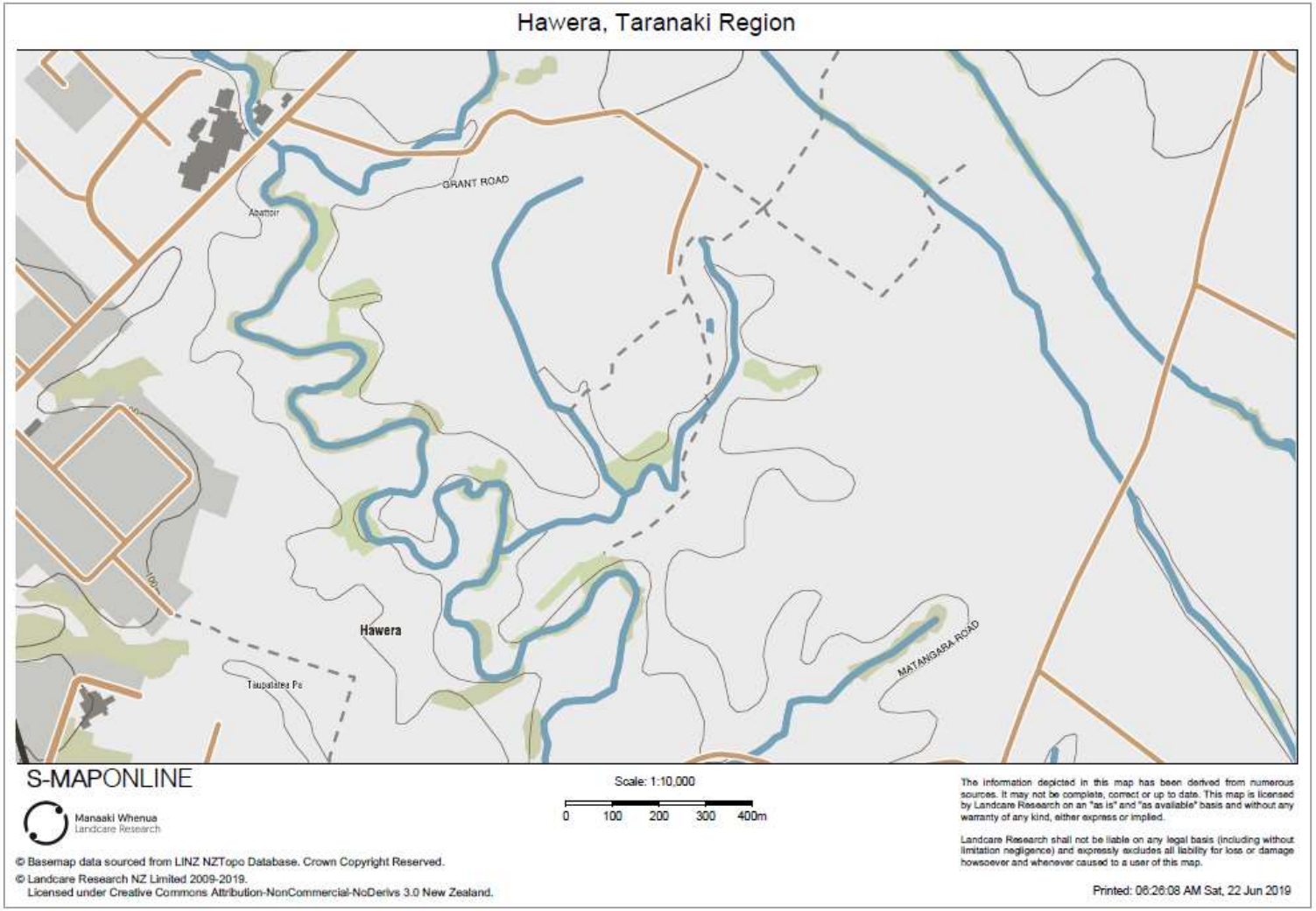

Figure 3.3 Collection zone of Orthic Allophanic soil at the Taranaki region.

3.2 Chemical analysis of soil samples

Air-dried 2mm sieved soils were analysed for base cations, CEC, dithionite and oxalate extractable Al-Fe concentration, P retention and total P concentration according to the standard methods for chemical analysis of soils described by Blakemore et al. (1987). Blanks and controls soils were used to check the accuracy of the methods. The methods used are briefly described as follows:

Cation exchange capacity (Ammonium acetate pH 7): One gram of soil was mixed with 2 g of acid-washed silica and placed into a leaching tube with filter paper at the bottom. The mixture was leached with Ammonium acetate pH 7 using a peristaltic pump, until 50 mL of leachate was collected. The pH of this leachate was measured. Thereafter, 2 mL of Strontium-Caesium (20,000 mg/L) solution was placed in each sample to reduce cation interference during measurement, then the leachate was analysed by MP-AES 4200 (Agilent, Germany) for quantification of Ca, Mg, K and Na against standard solutions. Results were expressed as meq/ 100g soil.

Oxalate and dithionite extractable Fe-Al

The dithionite extraction dissolves iron oxides from soil constituents, primarily amorphous forms, but partly the crystalline forms. Thus, the dithionite Fe extraction estimates Fe oxides available in the soil, such as hydrated forms of Fe which are a product of mineral weathering, and partially measures the primary minerals that come from the parent material. The oxalate extraction dissolves aluminium and iron amorphous forms and minimum amounts of crystalline forms (McKeague & Day, 1965).

Al-Fe Oxalate extraction: the acid oxalate extractant was prepared with 16.2 g of ammonium oxalate and 10.8 g oxalic acid dissolved in 1 L of deionised water at pH 3. The extraction was carried out with a soil:solution (w/v) ratio 1:100 in the dark, shaking for 4 hrs in an end-over shaker. The solution was filtered through Whatman No. 42. One mL of the extract was dissolved in 9 mL of diluent solution, prepared as follows:

22 mL HCl conc. and 1.41 g CsCl were dissolved in 1 L of deionised water (DI). Fe and Al concentrations were measured using MP-AES against Al-Fe standard solutions at a concentration range of 5 to 50 µg/mL. After adjusting the dilution factor of the samples, the results were expressed as a percentage of Fe and Al in the sample.

Al-Fe dithionite-citrate extraction: the citrate extractant was prepared by dissolving 220 g of trisodium citrate in one L of DI water. One gram of soil plus one gram of sodium dithionite and 50 mL of citrate extractant were placed in 100 mL containers, and the solution was shaken for 16 hrs in an end-over-end shaker. Thereafter, 5 drops of superfloc 0.2% were added and the suspension was shaken again for a few seconds. The solution was subsequently filtered through Whatman No. 42. One mL of the extract was diluted in 19 mL of DI water and left to settle for 2 days. The concentration of Al and Fe in solution was then measured by MP-AES against standard solutions from 5 to 50 µg/mL concentration. After adjusting the dilution factor of the samples, the results were expressed as percentage similar as the oxalate method.

Soil pH ($CaCl_2$): Ten grams of soil were stirred with 25 mL of 10mM $CaCl_2$ and the slurry was left to stand overnight. The pH was subsequently measured from the stable supernatant with a pH meter (Hanna Instruments).

Total P: One gram of soil and 10 mL of Aqua regia (3:1 HCl:HNO_3) were placed in 50mL digestion tubes, capped with a glass funnel. Digestion was carried out in digestion blocks stepping up the temperature to 80 °C over ten minutes, then to 90 ºC after one hour, then increased to 130 ºC and maintained for 4 hours. Thereafter, the temperature was increased to 150 ºC and the glass funnels were removed to evaporate the solution until approximately 1 mL was residual. The digest solution was made to 50 mL with DI water and mixed by vortex. One mL of the digest solution was diluted (1:10) with 9 mL of 2% HNO_3 and analysed for P concentration with MP-AES.

P retention: Two grams of soil and 20 mL of KH_2PO_4 (1,000 mg P/L at pH 4.5) were placed in centrifuge tubes and shaken overnight in an end-over-end shaker. After

shaking, the tubes were centrifuged at 8,000 rpm for 5 minutes then the supernatant was filtered with Whatman 42 and collected for analysis. The P concentration of the solution was analysed with the Vanadomolybdate reagent at 466 nm absorbance. The results were expressed as P retention %; where 0% P retention is equals to the concentration of 1,000 mg P/L and 100% P retention is no residual P in solution.

Total C and N: subsamples of air-dried soil (sieved 2mm), were finely ground with a mortar and pestle, then 120 mg of soil was placed in a tin foil cup and a similar amount of Wolframium reagent was added. The cup was wrapped and compacted, then the C and N concentration quantified using an Elementar analyser (Vario MACRO, Germany) for total C and N analysis.

Dissolved organic carbon: The organic matter water-extractable (OMWE) fraction of the soil was analysed according to the work of Chantigny et al. (2014). The total dissolved carbon and total dissolved nitrogen concentration in the aqueous fraction was measured. The aqueous extraction was performed using a soil:extractant ratio of 1:5 (w/v), respectively. Six grams of soil were placed into centrifuge tubes, then 30 mL of DI water was added. The suspension was shaken for one hour in an end-over shaker then centrifuged at 8,000 rpm for 10 minutes. The supernatant was filtered through Whatman 42; and collected in glass vials for analysis with the Shimadzu TOC-L Analyser.

3.3 Physical analysis of soil samples

Bulk density: Soils were packed with regular vibration into pre-weighted glass columns of 17.67cm^3 volume. The packed column was weighed, and the weight of the column deducted to quantify soil weight. Bulk density was calculated according to Equation 3.1.

Bulk density (g/cm^3) = Soil weight (g) / column volume (cm^3) Equation 3.1

Porosity and solid particle %: Soil porosity was calculated theoretically from the bulk density divided by the average mineral particle density of the soils assumed to be 2.65 g/cm^3 (Landry, Dousset, Fournier, & Andreux, 2005), using Equation 3.2 (Nimmo, 2013):

Porosity % = [1-(Bulk density/Particle density)] * 100 Equation 3.2

The solid particle % was then calculated using Equation 3.3:

Solid Particle % = 100%-Porosity% Equation 3.3

Maximum water holding capacity (MWHC %): Maximum water holding capacity was calculated on a weight basis. Ten grams of dry soil were placed in a pre-weighted plastic container. The soil in the container was saturated with a known amount of water. Free water was drained, and the container with soil was weighed. MWHC percentage was calculated according to Equation 3.4:

%MWHC = [(Dry soil g /fully drained soil g)] *100 Equation 3.4

Texture: Texture was calculated with the micro-pipette method according to the work of Miller & Miller (1987). Four grams of soil were placed in 50 mL plastic containers with 40 mL of dispersant solution (10 mL of 1M NaOH dissolved in one litre of DI water). The slurry was shaken in an end over end shaker for 16 hrs. Thereafter, the slurry was left stand for one hour before 2.5 mL was collected at a depth of 2.5 cm from the suspension, which corresponded to the clay fraction. The solution collected with the pipette was placed into a pre-weighted aluminium dish and dried in an oven at 106º C until constant weight. Using blanks, the weight of the salts of the dispersant solution were subtracted from the sample weight. The percentage of clay was calculated according to the aliquot taken and the total soil.

Sand was calculated by passing the slurry through a 53 µm sieve. The excess water was decanted and the solid was oven dried to quantify sand content. Silt was calculated by the difference between the sand and clay content.

3.4 Glyphosate and AMPA extraction and purification from soil samples

Glyphosate and AMPA extraction from soil samples was performed according to Sun et al. (2017). Briefly, 20 mL of extractant sodium phosphate (0.03 mol/L) and trisodium citrate (0.01 mol/L) at pH 12.5 were added to 2 grams of soil in centrifuge tubes, and shaken for 30 min in an end-over-end shaker. The suspension was centrifuged at 8,000 rpm for 5 min. The supernatant was filtrated through Whatman 42. The pH of the supernatant was adjusted to 9 with 1M HCl before the purification step.

Purification was achieved through fractionating by affinity according to the method of Sun et al. (2017). Ten mL of supernatant were added to a separation funnel, then 10 mL of n-Hexane were added, and the sample was gently shaken three times. The sample was left to settle until two phases were formed. The organic phase was discarded. Ten mL of the aqueous fraction were placed in glass vials for derivatisation.

3.5 Analysis of glyphosate and AMPA by RP-HPLC

FMOC-Cl pre-column derivatisation: Following the method of Sun et al. (2017), 10 mL of purified aqueous solutions were placed in glass vials, and mixed with 1.2 mL of $Na_2B_4O_7$ 0.05 mol/L at pH 9 and 2 mL of FMOC-Cl (1g FMOC-Cl/L ACN - the derivatisation reagent). Samples were gently shaken and allowed to stand at room temperature for one hour. Thereafter, the reaction was stopped by removing the excess of FMOC-Cl by fractionation. To achieve this, 10 mL of the sample and 10 mL of diethyl ether were placed in a separation funnel, and gently shaken until the suspension settled into two phases. The organic phase was discarded. After removing the excess FMOC-Cl, 2 mL of sample were filtered through a 0.22 µm PTFE membrane syringe filter and placed into a 2-mL HPLC vial for measurement by HPLC.

Standard Curve: Stock solutions of glyphosate and AMPA compounds at 50 mg/L were prepared in mili-Q water. Final dilutions for the standard curve were prepared from 0.004 mg/L to 1 mg/L. Ten mL of each standard solution was directly derivatised using FMOC-Cl, and filtered through a PTFE 0.22 µm syringe filters, after stopping the derivatisation reaction with ethyl ether, and the standards were placed in 2-mL HPLC vials.

RP-HPLC Programme: The separation of glyphosate and AMPA was carried out in a Phenomenex Luna C-18 (2) column (5 µm particle size, pore size 100 Å. Length x internal diameter 150 x 4.6 mm; with guard cartridge C18 4x 3 mm at temperature 25 C and flow rate of 1 mL/min). The measurement was carried out by following the method of Druart, Delhomme, de Vaufleury, Ntcho, and Millet (2011), the mobile phase was a gradient programme of solvent (A): ACN and buffer (B): Phosphoric acid 0.2%. The organic solvent (A) increased from 10% to 45% in 32 min, then decreased from 45% to 10% in 3 min. Total run time was 35 minutes per sample and the sample injection volume was 20 µL. Glyphosate and AMPA were analysed with a fluorescent detector set to an excitation of 260 nm and at an emission of 310 nm.

Individual standard solutions of GlyP and AMPA were injected to identify the retention time (RT) of each compound, and combined standard solutions were used to assess the separation efficiency of the method. After 35 minutes of run per sample, glyphosate eluted first with a RT of 24.59±0.03 minutes (n=5±SD), followed by AMPA with 26.39±0.02 minutes (n=5±SD), the last peak corresponded to FMOC-OH, which is product of the reaction between FMOC-Cl and water (Figure 3.4). Druart et al. (2011) found a RT of 17.4 and 21.3 minutes for glyphosate and AMPA respectively. It has been reported that when using alkaline solutions for GlyP and AMPA extraction from soil samples, organic matter can be extracted which can interfere with the detection of the target compounds (Miles & Moye, 1988; Waiman et al., 2012).

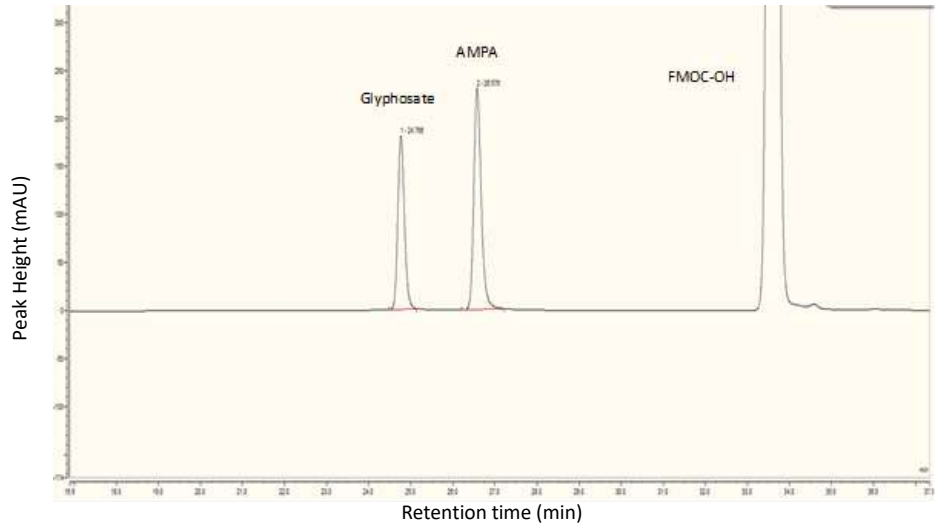

Figure 3.4. Standard solution Glyphosate-AMPA at 0.8 mg/L concentration

However, the purification step carried out in this thesis nullified the organic matter interference for GlyP and AMPA detection. Furthermore, stopping the derivatisation reaction with a non-polar solvent enabled more effective purification of the sample by removing hydrophobic organic matter. The method used for this work was adequate for GlyP and AMPA detection from soil samples because the separation between GlyP and AMPA peaks was optimum; no coelution or undefined peaks were detected. Impurities coming from soil did not interfere with GlyP and AMPA detection (Figure 3.5).

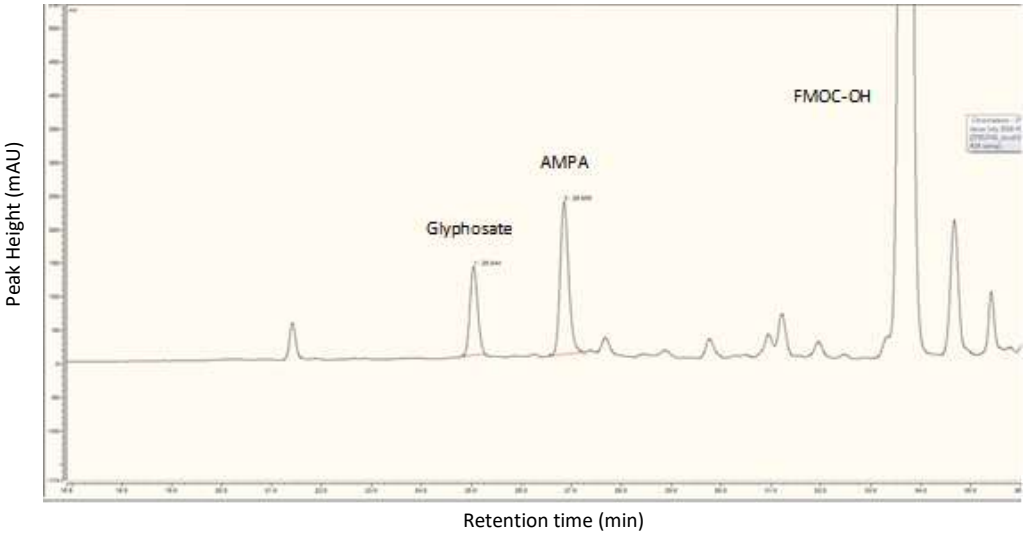

Figure 3.5 Chromatogram of Glyphosate and AMPA extracted from Allophanic soil

3.6 Data analysis of glyphosate adsorption-desorption batch experiments

The results for GlyP or AMPA concentration residual in equilibrium solution and extracted from the solid phase were used to calculate the theoretical value for GlyP and AMPA adsorbed and fixed, and Kd values using Equations from 3.5 to 3.7:

1) Glyphosate or AMPA adsorbed in soil % (A) = (B) added% – (C) remaining in solution % Equation 3.5

2) Glyphosate or AMPA fixed in soil % = adsorbed in soil% (A) – (D) extracted alkaline solution% Equation 3.6

3) Kd = adsorbed in soil% (A) / (C) remaining in solution%

Equation 3.7

(A) Glyphosate or AMPA added%: The 100% of glyphosate added corresponded to the total concentration of the equilibrium solution used relevant to each experiment. For example, in Chapter 4.2.1, a volume of 20 mL of an equilibrium solution GlyP-AMPA at 1 mg/L was used; then, the 100% added corresponded to 20 mg of GlyP-AMPA.

(B) Glyphosate or AMPA remaining in solution% =

$$\frac{\text{(X mg GlyP or AMPA residual in solution detected by HPLC)}}{\text{(X mg total concentration of the solution)}} * 100$$

(C) Glyphosate or AMPA extracted with alkaline solution % =

$$\frac{\text{(X mg GlyP or AMPA extracted detected by HPLC)}}{\text{(X mg total concentration of the solution)}} * 100$$

3.7 Analysis of metabolites in plant samples

Phenolic compounds: Gallic acid, trans-caffeic acid and vanillic acid measurements by RP-HPLC: Stock solutions of 1 mM gallic acid, 1mM vanillic acid and 0.5 mM of trans-caffeic acid were prepared and diluted to 0.04, 0.1, 0.4, 0.6, 0.8 mM for HPLC analysis.

The samples were measured by RP-HPLC according method explained by Mattila & Kumpulainen (2002). The stationary phase of the system was a Phenomenex Luna C-18 (2) column (5 µm particle size, pore size 100 Å. Length x internal diameter 150 x 4.6 mm; with guard cartridge C18 4x 3 mm) with the column temperature and flow rate controlled at 25 ºC and 0.7 mL/min. The mobile phase was a gradient elution of 50mM H_3PO_4 pH 2.5 (A) and acetonitrile (B): 0-5 min 95% A, 5-35min 50% A, 35-40min 50%

A, 40-42 min 95% A. The Run time was 42 minutes per 20 µL sample injection. The phenolic compounds were measured with PDA-UV detector and data acquisition at 254, 270, 280 and 329 nm.

Organic acids: oxalic acid, citric acid, malic acid, tartaric acid, succinic acid, fumaric acid measurement by RP-HPLC: The samples were measured by RP-HPLC according to the work described by Cawthray (2003). The system was consisted of a Phenomenex Luna C-18 (2) column (5 µm particle size, pore size 100 Å. Length x internal diameter 150 x 4.6 mm; with guard cartridge C18 4x 3 mm) as the stationary phase. The column temperature and flow rate were 25 ºC and 1 mL/min and the sample injection volume was 20 µL. The mobile phase was an isocratic elution of phosphate buffer at pH 2.5. (KH_2PO_4 25 mM pH adjusted with H_3PO_4) and the run time of a sample was 15 minutes. The organic acids were measured with PDA-UV detector data acquisition at 210nm.The standards used were analytical grade: oxalic acid, citric acid, malic acid, tartaric acid, succinic acid, fumaric acid. The concentration of the standards used for the standard curve was 0.04, 0.1, 0.4, 0.6, 0.8 and 1 mM.

3.7.1 Agave leaf powder characterisation

Agave leaf powder aqueous extract preparation: *Agave attenuata* naturalised plants of different ages were collected from a residential area in Palmerston North, New Zealand (ornamental plants). The leaves were removed from the stem, washed with soapy water and disinfested by immersion them in a 1% hypochlorite solution for 1 minute. Thereafter, the leaves were finely chopped and placed in an oven at 60 ºC until the weight become constant. Then, the leaves were ground with an herbage disk mill and the leaf powder was stored at room temperature for further analysis.

Agave leaf aqueous extract (AgvE) was prepared using agave leaf powder and DI water, at a ratio of 1:30 (w/v). The mixture was shaken overnight in an end-over-end shaker and at the end the solution was centrifuged at 8,000 rpm for 5 min. The supernatant was filtered through Whatman 41 paper and collected for further analysis. The aqueous extract was stored at 4 ºC, for a maximum of 24 hours, while in use. After that time, it was discarded, and fresh extract was prepared as required.

Water-extractable phenolic acids and total phenolic acids determination of Agave leaf powder by colorimetric method and HPLC: Water-extractable phenolic acids were measured directly from the AgvE without any further dilutions. Total phenolic acids were measured as follows: one gram of Agave leaf powder and 30 mL of 50% ethanol were mixed and sonicated for 30 minutes, then the solution was centrifuged at 8,000 rpm for five minutes. The supernatant was filtered through Whatman 41 paper. The obtained solution was analysed directly without any further dilutions. Phenolic acids were measured with the Folin-Ciocalteu reagent as described by Magalhaes, Santos, Segundo, Reis, and Lima (2010). Briefly, 50 µL of sample and 50 µL of the Folin-Ciocalteu reagent at a ratio of 1: 20 DI water (v/v) were added in to a 96 micro-well plate . Then 100 µL of NaOH (0.175 M) was added to the same mixture and the plate was incubated for 10 min at 30 ºC. Thereafter, the absorbance was measured at 740 nm in a microplate reader Vis spectrophotometer. Standard curve points for the concentrations of 0.04 mM, 0.1, 0.4, 0.6, 0.8 and 1mM were prepared using an analytical grade standard gallic acid reagent . The results were expressed as mg of gallic acid.

The Agave aqueous extract was filtered through a PTFE 0.22 µm syringe filter and analysed according to the HPLC system described in Chapter 3.7.

Water-extractable organic acids and total oxalate concentration of Agave leaf powder: The extraction of organic acids and total oxalate was performed according to the work of Williams et al. (2016), with modifications. Water-extractable organic acids were analysed as follows: One mL of Agave leaf aqueous extract (AgvE) was dissolved in 9 mL of phosphate buffer at pH 2.4 (KH_2PO_4 20 mM; pH adjusted with H_3PO_4). The solution was shaken and filtered through a syringe filter (PTFE 0.22 µm) and placed in a HPLC vial for organic acid analysis by RP-HPLC according to Chapter 3.7.

The total oxalate concentration of the agave powder was measured as follows. In a 50 mL digestion tube, 0.5 g of Agave leaf powder was taken and mixed with 5 mL of HCl (25%). The tube was placed in a digestion block and capped with a glass funnel. The temperature was stepped up to 80 ºC over 10 minutes and maintained at this

temperature for 120 minutes. Thereafter, the glass funnel was removed to allow the solution to evaporate to approximately 1 mL. The solution was made up to 100 mL with phosphate buffer. An aliquot of 2 mL was filtered with a syringe filter (PTFE 0.22 µm) and placed in a HPLC vial for organic acids analysis by RP-HPLC according to Chapter 3.7.

Dissolved carbon and nitrogen: Dissolved organic carbon, inorganic carbon, total carbon and total nitrogen were analysed from the aqueous extract of the Agave plant material at a ratio of 1:30 (w/v), extracted as previously explained. The supernatant was filtered through Whatman 42 and diluted 1:1 with DI water placed in a glass vial and analysed with Shimadzu TOC analyser.

3.8 Statistical analysis

Statistical analysis per factor and/or per treatment (interaction of levels) where relevant, was performed using ANOVA and mean analysis with Tukey's test ($p<0.05$) using the software SAS ver. 9.4. Graphs were plotted using Sigmaplot ver. 11.

4 GLYPHOSATE DISPLACEMENT FROM NEW ZEALAND SOILS

4.1 Introduction and Aim

The extensive use of glyphosate (GlyP) in the agricultural sector has led to global concerns about its potential movement from farmland into the wider environment. The main degradation product of GlyP is aminomethylphosphonic acid (AMPA), and both molecules have an anionic charge which determines their sorption in the soil. Soil characteristics such as Al-Fe oxy-hydroxide content influences the amount of GlyP that will adsorb onto soil surfaces (Sprankle, Meggitt & Penner, 1975a; Zhao et al., 2009). Farm practices determine the amount of herbicide that will be added to soil as a function of both the timing and rates of GlyP used (Pires et al., 2017; Lewis et al., 2017). Furthermore, as phosphorus and GlyP compete for soil reaction sites, P fertilisation also has a direct effect on GlyP accumulation in soil. Phosphate has a greater affinity for soil surfaces than GlyP and this increases GlyP bioavailability through displacement of GlyP residues from the soil surface (Munira et al., 2016). A research hypothesis described in this chapter is that soil characteristics, in particular Al-Fe oxy-hydroxides content and P-sorption capacity of a soil, may influence GlyP displacement from soil.

Surface runoff is the main pathway for the transportation of GlyP from farmland due to the strong binding of the pesticide to soil particles. However, GlyP leaching may occur under conditions of coarse soil texture, reduced anion retention capacity and unconformities in the soil strata that may facilitate the preferential flow of the herbicide and its degradation products through the profile. Glyphosate leaching will increase the risk of contamination of groundwater (Borggaard & Gimsing, 2008; Zhao et al., 2009).

Batch adsorption-desorption and column leaching experiments are complementary laboratory techniques that can be useful to understand the interaction between chemical and physical characteristics of a soil, and the potential for displacement of agrichemicals from the soil (Katagi, 2013; Okada et al., 2016).

The widespread movement of GlyP into the environment is problematic as studies have also shown that GlyP not only disrupts the enzyme EPSPS of the shikimate pathway of target weeds, but that it can reduce the efficiency of enzymes in living organisms (Modesto & Martinez, 2010; Fan et al., 2016). Even though GlyP is

biodegradable, the herbicide has a residual effect in soil, and due to its potential toxicity to non-target organisms, it is crucial to understand the effect of GlyP on soil microorganisms (SoMo) which are responsible for herbicide decomposition (Zhan et al., 2018). Soil induced respiration (SIR) is a technique that measures the CO_2 released by SoMo respiration; it is an effective tool for assessing the effect of soil pollutants on the SoMo community. In most cases while using SIR, inhibition of SoMo respiration may indicate a detrimental effect caused by soil pollutants (Bérard, Mazzia, Sappin-Didier, Capowiez, & Capowiez, 2014; Wakelin, Cavanagh, Young, Gray, & van Ham, 2016).

Glyphosate remediation techniques are emerging to reduce its residence time in the environment (Zhan et al., 2018). Exogenous sources of carbon can trigger SoMo activity that may enhance GlyP degradation (Nguyen et al., 2018). Agave plant materials is one example of an exogenous source of carbon that contains soluble sugars and anionic secondary metabolites (Corbin et al., 2015; Santos et al., 2017). Agave plant materials could be used as soil amendments for promoting microbial proliferation that accelerate GlyP biodegradation. The anionic constituents of agave biomass may enhance P bioavailability, through the displacement of P from the soil reaction sites. Therefore, another research hypothesis is proposed in this chapter that Agave amendments applied to soil might provide carbon and enhance P bioavailability in rhizosphere, which might promote microbial proliferation measured as increased SIR, which could infer a greater glyphosate mineralisation.

Taking into consideration that biodegradation can reduce the displacement of the herbicide, SIR responses at different GlyP doses were assessed. Treatments included Agave aqueous extract as an exogenous carbon source, in order to elucidate its effect in soil respiration.

The aims of this chapter were to 1) investigate the effect of phosphorus addition to soil and the interaction between the soil physical and chemical properties on the displacement of glyphosate from three contrasting New Zealand soils; and 2) assess the effect of Agave plant by-product amendments on soil induced respiration, in order to elucidate the interactive effect of GlyP and agave amendments on soil microorganism respiration, and likely GlyP degradation.

4.2 Material and Methods

To achieve the aims of this chapter, a series of experiments (five) were designed and performed.

In the first, a batch GlyP-AMPA adsorption-desorption experiment was conducted to elucidate the effect of soil chemical properties, in particular Al-Fe oxy-hydroxides content of soils, on the sorption of GlyP and its main degradation product onto soil surfaces.

In the second, a batch GlyP adsorption-desorption experiment on P saturated soils was conducted to elucidate the effect of phosphorus and soil chemical characteristics on GlyP sorption to soil.

In the third experiment a column leaching experiment was performed to elucidate the interaction between soil chemical, physical characteristics and P addition in the vertical displacement of GlyP and AMPA through the column packed with disturbed soils.

In the fourth experiment the Microresp technique was used in order to elucidate the effect of GlyP doses and Agave aqueous extract in the soil microorganisms respiration.

In the fifth experiment a batch adsorption-desorption experiment on P saturated soils using Agave leaf powder as a soil amendment was performed to elucidate the effect of Agave's constituents on P desorption from soil surfaces.

Each experiment is described and discussed sequentially in this chapter.

4.2.1 Glyphosate and AMPA adsorption-desorption batch experiment

Soils were saturated with a glyphosate-AMPA equilibrium solution, and the residual GlyP-AMPA concentration remaining in the equilibrium solution was measured. GlyP-AMPA from the solid phase were extracted with an alkaline solution and quantified. The amounts of adsorbed and fixed GlyP-AMPA in the solid phase was calculated according to Chapter 3.6 using the values of GlyP-AMPA analysed from the equilibrium solution and extracted from solid phase. The experimental unit was one

soil slurry (two grams of soil/GlyP-AMPA solution) per soil type (n=3). The variable responses evaluated were residual (%), adsorbed (%), Kd, desorbed (%) and fixed (%) GlyP-AMPA in the solid/aqueous phase. Mean total values of the GlyP-AMPA quantified were analysed statistically as explained in Chapter 3.8.

Soil saturation and desorption: Twenty millilitres of glyphosate and AMPA solution (1 mg/L of both chemicals) and two grams of soil were added to centrifuge tubes. The pH changed to 5.0 using either 0.1 M HCl or 0.25 M NaOH, then shaken overnight for 16 hrs on an end-over-end shaker. Thereafter, the tubes were centrifuged at 8,000 rpm for 5 minutes, and the supernatant was filtered through Whatman 42. The supernatant was collected then purified, derivatised and the GlyP-AMPA concentration quantified by HPLC according to the methods described in Chapters 3.4 and 3.5.

4.2.2 Glyphosate batch adsorption-desorption experiment in soil saturated with phosphorus

Phosphate saturated soils were equilibrated with a GlyP solution, and the residual GlyP concentration remaining in the equilibrium solution was measured. GlyP was then extracted from the solid phase with an alkaline solution. The amounts of adsorbed and fixed GlyP in the solid phase were calculated according to Chapter 3.6 using the values of GlyP analysed from the equilibrium solution and extracted from solid phase. The experiment had two factors; three soil types: Pallic, Brown and Allophanic soils. Second factor was phosphorus with two concentration levels: P saturated soils and control (without P). The experimental unit was one soil slurry (2 g soil/equilibrium solution) per treatment (Soil type*Phosphorus); in total six treatments made up in this experiment with 3 replicates. The variable responses evaluated were residual (%), adsorbed (%), Kd, desorbed (%) and fixed (%) of GlyP in the solid/aqueous phase. Mean total values of the herbicide quantified were analysed statistically as explained in Chapter 3.8.

Soil saturation with phosphorus: Soil (2g) and 20 mL of KH_2PO_4 (1,000 mg P/L at pH 4.5) were placed in centrifuge tubes and shaken overnight in an end over shaker. After shaking, the tubes were centrifuged at 8,000 rpm for 5 minutes then the supernatant was filtered with Watman 42 and collected for the determination of P retention %. The residual P concentration in solution was analysed with vanadomolybdate reagent.

Soil saturation with GlyP solution: Soil saturated with P (2 g) was resuspended in 20 mL of a glyphosate solution (10 mg GlyP/L at pH 5), and followed the procedure for glyphosate saturation explained in Chapter 4.2.1. The sequence of extraction, purification, derivatisation and GlyP analysis by RP-HPLC was followed as explained in Chapters 3.4 and 3.5.

4.2.3 Glyphosate column leaching experiment

Glass columns were packed with soil, then leached with a P solution, followed by a GlyP solution. The leachate was collected and analysed; GlyP and AMPA were extracted with alkaline solution and analysed, and the retention and displacement of GlyP and AMPA as a function of added P quantified. Two factors were used; three soil types: Pallic, Brown and Allophanic soils. Second factor, was phosphorus with two concentration levels of P application and control (without P application). The experimental unit was one column per treatment (Soil type*P application n=3); in total six treatments made up this experiment. GlyP and AMPA content was evaluated: the leachate collected (GlyP-AMPA µg/leachate), and soil extraction from 0-5 cm and 5-10 cm depth (GlyP-AMPA µg/column). Mean total values of the herbicide quantified were analysed statistically as explained in Chapter 3.8.

Soil packing and $CaCl_2$ 10 mM initial saturation: The column leaching miscible displacement method described by Katagi (2013) was used for this work. Glass columns of 20 cm longitude x 1.5 cm diameter, with a total volume of 32 cm^3 were used under laboratory conditions. Glass wool fibre was tightly placed at the bottom of

each column, in order to prevent the displacement of the soil out of the column. At the end of each column, a 10 cm long rubber hose was attached to control the flow of the leachate. The soil was packed into the column to 10 cm depth with regular vibration to prevent the formation of macropores for a final effective volume of 17.67cm^3 per column.

Pore volume was calculated from the soil porosity percentage (Table 4.3) and converted to volume basis considering the total volume of 17.67cm^3 using Equation 4.1.

Pore volume % = (Total volume * Porosity)/ 100 Equation 4.1

The pore volume of each column was used to determine the quantity of $CaCl_2$ solution required (2.5 pore volumes) to distribute P and GlyP through the column.

After packing, the soil was saturated with a solution of $CaCl_2$ (0.01M) using a peristaltic pump. The flow of the solution was adjusted using different hose diameters according to each soil type. Preliminary trials demonstrated that the soil saturation rate was faster for the Pallic soil leading to water stagnation, requiring the lowest flow rate of 0.0123 mL/minute, in order to maintain a similar saturation time for the three soils. A flow rate of 0.0246 mL/minute was used for the Brown and Allophanic soils. When the solution reached the glass wool at the bottom of the column, the hose attached at the end of the column was closed to prevent leachate loses. The solution kept flowing until a meniscus formed at the top of the soil, and then the pump was immediately stopped and the hose was opened to let the solution flow. The leachate was collected in a pre-weighted 35 mL plastic container. After saturation with the $CaCl_2$ solution, the hose remained open for the remaining period of the experiment to prevent anoxic conditions. After three days of column saturation at a $CaCl_2$ solution flow rate of 0.0123 mL/minute in all the soil types (0.5 pore volume per column during 12 hours for a total of 2.5 pore volumes/column), the P treatment commenced.

Phosphorus and glyphosate application: The phosphorus application rate per column was calculated according to the maximum single recommended application of 100 kg P/ha for fertiliser use on New Zealand dairy farms (Roberts and Morton, 2016). The Equation 4.2 (OECD/OCDE Guidelines for the testing of chemicals No. 312: Leaching in Soil columns, 2014) was used to determine required P application of 1,767.15 µg P/Column.

$$M\ [\mu g] = \frac{A\ [kg/ha] \bullet 10^9\ [\mu g/kg] \bullet d^2\ [cm^2] \bullet \pi}{10^8\ [cm^2/ha] \bullet 4}$$

where:

M = amount applied per column [µg]

A = rate of application [$kg \cdot ha^{-1}$]

d = diameter of soil column [cm]

π = 3.14

Equation 4.2

A pipette was used to dispense 1.76 mL from a stock solution of 1,000 mg P/L concentration into each of the P treated columns; a similar volume of $CaCl_2$ (0.01M) was applied to the control columns. Thereafter, 2.5 column pore volumes of $CaCl_2$ (0.01M) was leached to distribute the applied P through the column and the leachate was collected in 35 mL plastic containers.

The glyphosate application rate per column was calculated according to the maximum recommended glyphosate application of 7.5 kg a.i./ha stated on the label of the product Glyphosate 540 Ravensdown. Using Equation 4.2, a dose of 132.53 µg GlyP/Column was calculated, and a pipette was used to dispense 2.65 mL from a stock solution at 50 mg GlyP/L concentration into all the columns of both treatments - control and P addition. Thereafter, 2.5 column pore volumes of $CaCl_2$ (0.01M) was leached to distribute the glyphosate through the column, with the leachate collected in 35 mL pre-weighted plastic containers. After full drainage, the leachate collected from each column was weighed, then converted to volume basis. Two mL of leachate were purified and analysed using RP-HPLC in order to quantify residual herbicide concentration in solution according to the procedures of Chapters 3.4 to 3.5.

Glyphosate extraction from soil column samples: After five days of column drainage, the soil was removed with a spatula and divided into two sections based on depth (0-5 cm and 5-10 cm). Each section was air-dried until constant weight, homogenised with a mortar and pestle, and the total soil sample per column section was weighted. GlyP and AMPA were extracted from the soil with an alkaline solution, purified and derivatised according to the procedures of Chapters 3.4 and 3.5, for quantification of the herbicide using RP-HPLC.

4.2.4 Soil induced respiration using glyphosate doses and Agave amendments

In this experiment, GlyP doses and Agave (AgvE) amendments were applied to soil to elucidate its effect on SIR. After incubation, the CO_2 emitted by soil microorganisms was measured by the MicroResp system. The experimental design was two factors. First, three soil types: Pallic, Brown and Allophanic soils. Second factor was GlyP doses and AgvE amendments with 9 levels: GlyP doses of 2.5, 5, 7.5, 15, 20 kg GlyP/ha, and the combination of AgvE amendments with GlyP doses of 7.5 and 20 kg GlyP/ha, including an AgvE control (only AgvE amendment addition) and control (no GlyP and AgvE addition). Each treatment was replicated 3 times, therefore, the total experimental setup had 27 units. Each experimental unit consisted 10 g of treated soil. The variables analysed were the SIR ratio, water-extractable dissolved fractions (mg/L) of total organic carbon (TOC), total carbon (TC), inorganic carbon (IC) and total nitrogen (TN).

Soil pre-incubation for SIR analysis: To activate soil microbial activity, 10 g of dry soil was placed in 35 mL free-draining plastic containers and the moisture content was adjusted to 40% of mean water holding capacity (Table 4.3). The soils were incubated at 23 ºC day/night in total darkness for 7 days. The moisture content was reduced to 35% on the 5^{th} day of the pre-incubation step.

Glyphosate doses and Agave aqueous extract application: The glyphosate doses and carbon applications used in this experiment were calculated following the

agronomical parameters used by Nguyen et al. (2018). In this experiment the parameters used to calculate the GlyP doses were:

- Soil mean bulk density 1.27 g/cm^3
- Glyphosate depth penetration of 5 cm
- Glyphosate 540 Ravensdown maximum recommended dose of 7.5 kg a.i./ha.

According to these parameters, a rate of 118.1 µg Glyphosate/10 g soil was calculated for the experiment as the recommended rate of GlyP to achieve the manufacturers recommendations.

The moisture content of the pre-incubated soils was increased to 50% using a GlyP solution made up to apply variable GlyP doses for varying treatments (Table 4.0). For the treatments that included Agave amendments, the concentration of the GlyP dose required was doubled in order to require just half of the solution, with the other half used for the Agave aqueous extract amendments. After applying GlyP, the samples were incubated at 23 ºC day/night total darkness during 48 hrs.

Table 4.0 Glyphosate doses used for this experiment calculated based on the maximum application rate of 7.5 kg a.i./ha of commercial product Ravensdown 540.

Comm. Rate	Glyphosate doses	
kg a.i./ha	mg/kg	µg/10 g soil
20	31.53	315.3
15	23.65	236.2
7.5*	11.82	118.2
5	7.88	78.8
2.5	3.94	39.4

The Agave amendments (AgvE) were prepared according to the extraction method described in Chapter 3.7.1. The AgvE doses were calculated based on a rate of 1.25 mg of exogenous C applied per g soil, used by Nguyen et al. (2018). The total soluble carbon concentration in the aqueous agave extract was 152.96 mg TC/20mL (n=3) as determined using a Shimadzu TOC- L Analyser. Agave extract contains C in diverse forms; such as primary metabolites like free soluble sugars, and secondary metabolites such as saponins and phenols (Ribeiro et al., 2015) and some of these may be toxic for soil microorganisms in high concentration. Therefore, for the purpose

of this experiment, 0.5 mL of crude AgvE extract was applied per 10 g of soil, which corresponded to 3.82 mg TC/container (0.382 mg TC/g soil). For all treatments with AgvE, the AgvE was applied 24 hrs before the GlyP doses.

All preparations were incubated for 48 hours following the application of the GlyP doses, and the final moisture content of each container calculated by weight (approximately 40% for all treatments). Subsequently, soil induced respiration (CO_2) was measured using the MicroResp system.

MicroResp® System: Approximately three grams of soils at 40% moisture content per treatment were distributed into 8 deep micro 96-wells plates (0.37 g/well). To accurately quantify the moisture of the soil distributed into the 8 wells per treatment, the deep well plate was weighed before and after adding the soil samples.

Glucose at a concentration of 30 mg per well was prepared according to the volume of water required to achieve a final 60% moisture content and applied to the wells as a source of C to initiate the experiment. Then a micro-well plate with Cresol-Agar reagent were tightly placed at the top of the deep well plate using the MicroResp clamp, and incubated for 4.5 hrs at 21 ºC. The colour of the Cresol-Agar reagent changed accordingly to the CO_2 emitted by the soil; thus, the absorbance of the micro-well plate was measured at 470 nm using a micro-well spectrophotometer, before the soil incubation (blank) and after 4.5 hrs of incubation.

Cresol-Agar reagent preparation: To prepare the Cresol Indicator Solution Cresol Red 18.75 mg, Potassium Chloride 16.77 g and Sodium bicarbonate 0.315 g were added to 900 mL of DI water (final volume 1,000 mL),

Agar 3% was prepared by mixing 3 g of Agar in 100 mL of hot DI water, with stirring at 60 ºC until the solution became clear. The agar was transferred to a water bath at 60 ºC. Finally, the Cresol solution and Agar were mixed at a ratio of 1: 2 (Agar: Cresol), keeping the solution at 60 ºC. Agar-Cresol solution (150 µL) was placed in each well, then the microplate was placed into the refrigerator. Before analysis, the micro-well plate was placed into a desiccator with soda lime to stabilise it to room temperature.

Analysis of MicroResp results: The absorbance of the micro-well plates before incubation (blank) was subtracted from the absorbance after 4.5 hrs incubation. The SIR ratio treatment/control was calculated according to Nguyen et al., 2018, where the absorbance of the treatments were divided by the average absorbance of the control soil.

4.2.5 Phosphate adsorption-desorption batch experiment on P saturated soils using Agave aqueous extract.

In this experiment P saturated soils were shaken with a 10mM $CaCl_2$ solution and Agave leaf powder. The supernatant was collected and analysed for P, Al and Fe. The experimental design was two factors. First, three soils: Pallic, Brown and Allophanic soils. Second, was Agave, with two levels: Agave powder addition and control (no Agave addition). The experimental setup consisted of total six treatments with 3 replicates, and each unit contained 2 g soil slurry (2 g soil/$CaCl_2$ solution). The variables evaluated were the concentration (mg/g) of Al, Fe and P desorbed from soil.

Soil P saturation: Two grams of soil and 20 mL of KH_2PO_4 solution (1,000 mg P/L pH 4.5) were placed in centrifuge tubes and the suspension was shaken overnight (16 hrs) on an end-over-end shaker. Thereafter, the slurry was centrifuged at 8,000 rpm for 5 min and the supernatant was filtered with Whatman 42. The residual P concentration in solution was analysed by UV-Vis spectrophotometer 420 nm using vanadomolybdate reagent.

The solid phase was re-suspended in 20 mL of $CaCl_2$ (10mM solution at pH 7) and shaken for one hour, in order to remove the residual soluble P, and weakly adsorbed P from the solid phase. Thereafter, the slurry was centrifuged at 8,000 rpm for 5 min. The supernatant was filtered with Whatman 42, and the concentration of P in solution was analysed by UV-Vis spectrophotometer 420 nm using vanadomolybdate reagent (Appendix 1).

P desorption with Agave powder: The solid phase of the P saturated soil was re-suspended in 30 mL of $CaCl_2$ (10mM at pH 7), with 1 g of Agave leaf powder. The slurry and control soil were adjusted to pH 7.0 with 1 M and 0.5 M NaOH, repectively. Samples of both treatments were spiked with 2 mL of KH_2PO_4 solution (1,000 mg P/L). After 30 min of shaking the slurry was re-adjusted to pH 7.0. Thereafter, the pH was adjusted to pH 7 in every two hrs during a ten-hour period. Once the pH of the solution was relatively stable, the pH was increased to 7.3 ±0.2 to achieve a final pH of 7.0 after a total 16 hrs shaking. Thereafter, the samples were centrifuged at 15,000 rpm for ten minutes. The supernatant was filtered using Whatman 42 and was collected for acid digestion.

Supernatant digestion and MP-AES analysis: The supernatant was digested according to the work of Sumontha et al. (2006) with modifications. Two and half mL of the supernatants were transferred to 50 mL digestion tubes, and mixed with 2 mL of H_2O_2 (30% analytical grade phosphate-free) and 5 mL of HNO_3 (69%). The tubes were placed in a digestion block with glass funnels at the top of each tube and the temperature was increased to 130 ºC over ten minutes, then kept at 130 ºC for 30 minutes. Thereafter, the glass funnels were removed, and the temperature was increased to 190 ºC in order to evaporate the solution to a final volume of 1 mL. The remaining solution was diluted with 25 mL of DI water and homogenised using a vortex mixer. The solution was used directly for analysis of Al, Fe and P by MP-AES.

4.3 Results and Discussion

4.3.1 Soil chemical and physical characterisation

The Allophanic soil had the highest Al and Fe oxy-hydroxide concentration as expressed by the Al and Fe dithionite and oxalate extractable concentration and this was in agreement with the highest P retention. The Brown soil had a similar Fe dithionite-extractable concentration to the Allophanic soil, but reduced Al concentration.

In contrast, the Pallic soil had the lowest Al-Fe oxy-hydroxides concentration and lowest P retention (Table 4.1). The Pallic soil possessed the highest CEC and exchangeable Ca concentration, while, the Allophanic and Brown soils had similar CEC. The Allophanic soil recorded the highest total C and N concentration (Table 4.2). The physical characteristics of the sedimentary soils were similar, while the Allophanic soil possessed the highest WHC of 58% and porosity of 51% (Table 4.3).

Table 4.1 Aluminium and iron dithionite-extractable (%); and oxalate-extractable (%); P retention (%), pH at 10mM $CaCl_2$ ratio 1:2.5 w/v (soil:solution) and Total P (mg/g) concentration of soils used in this study (Mean n=3±SD).

Soil Type	M^+ Dithionite-extractable		M^+ Oxalate-extractable		P retention	pH	Total P
	Al %	Fe %	Al%	Fe%	%	10mM $CaCl_2$	mg/g
Pallic	0.11 ± 0.004	0.48 ± 0.04	0.2 ± 0.03	0.71 ± 0.11	12.7 ± 2.23	5 ± 0.01	0.57 ± 0.03
Brown	0.87 ± 0.021	2.23 ± 0.06	0.76 ± 0.031	1.4 ± 0.05	59.5 ± 2.1	4.06 ± 0.06	0.17 ± 0.03
Allophanic	1.71 ± 0.09	2.2 ± 0.13	2.67 ± 0.146	2.25 ± 0.08	82.2 ± 1.15	4.5 ± 0.03	0.68 ± 0.05

Table 4.2 Base cations, cation exchange capacity (CEC) (meq Cation/100g); total carbon, nitrogen (%) concentration and C:N ratio of soils used in this study (Mean n=3±SD).

Soil Type	meq K/100g	meq Ca/100g	meq Mg/100g	meq CEC/100g	C %	N %	C:N
Pallic	0.95 ± 0.15	6.38 ± 1.24	1.47 ± 0.30	24.21 ± 3.00	2.98 ± 0.03	0.31 ± 0.004	9.70 ± 0.10
Brown	0.50 ± 0.04	1.89 ± 0.34	1.89 ± 0.16	17.41 ± 0.72	4.65 ± 0.08	0.30 ± 0.003	15.64 ± 0.41
Allophanic	0.62 ± 0.03	2.20 ± 0.08	0.72 ± 0.03	17.81 ± 2.24	7.85 ± 0.20	0.84 ± 0.022	9.40 ± 0.05

Table 4.3 Bulk density (g/cm^3), texture (%); solid particle (%), porosity (%) and maximum water holding capacity (MWHC %) of soils used in tis study (Mean n=3±SD).

Soil Type	Bulk density	Texture (%)			%		
	g/cm^3	Sand	Silt	Clay	Solid Particle	Porosity	MWHC
Pallic	1.61 ± 0.02	36.8 ± 0.3	51.8 ± 0.5	11.4 ± 0.6	60.62 ± 0.80	39.38 ± 0.80	44.33 ± 0.91
Brown	1.59 ± 0.03	40.2 ± 0.5	41.4 ± 0.9	18.4 ± 0.5	59.82 ± 1.26	40.18 ± 1.26	41.70 ± 0.47
Allophanic	1.29 ± 0.02	55.8 ± 0.8	38.4 ± 1.3	5.76 ± 0.5	48.77 ± 0.72	51.23 ± 0.72	58.07 ± 0.68

4.3.2 Glyphosate and AMPA adsorption-desorption batch experiment

Glyphosate-AMPA adsorption

After equilibration of the soils with the GlyP-AMPA solution, the residual GlyP and AMPA concentration detected in the solution of the Pallic soil (8.98 and 2.39% respectively) was significantly higher ($p<0.05$) than the concentration found in the solution of the Brown and Allophanic soils (Table 4.4). The Brown and Allophanic soils adsorbed almost all the GlyP and AMPA from the solution with about 99% retention of both compounds. The Kd observed for GlyP and AMPA in the Allophanic soil was significantly higher than in the Brown and Pallic soils (Table 4.4). These results demonstrated that the Allophanic soil had the highest GlyP and AMPA retention capacity. In contrast, the Pallic soil had reduced GlyP and AMPA adsorption, which suggests that the reaction sites of the Pallic soil were saturated with the 1 mg/L glyphosate-AMPA solution, meaning it was possible to detect significant residual amounts of both compounds in the equilibrium solution. In the Brown and Allophanic soils the residual GlyP-AMPA found in the equilibrium solution was minimal.

Soil P retention values (Table 4.1) were proportional to GlyP Kd and AMPA adsorption (Table 4.4), and followed the order Allophanic>Brown>Pallic. Considering that the Al-Fe oxy-hydroxides play a significant role in the soils' P retention, this result coincides with the findings of Gimsing and Borggaard (2002) who found greater glyphosate adsorption in an Al-rich soil in comparison to a soil with a higher clay proportion and CEC. These authors cited this data to emphasise the importance of a soil's Al content for glyphosate retention. In another study, Munira et al. (2018) observed higher glyphosate sorption coefficients in Fe-rich soil, in comparison to calcium carbonate-rich soils. Overall, the current data and literature reports highlight that Al-Fe oxy-hydroxides are key elements for GlyP-AMPA retention in the soil.

Table 4.4 Glyphosate (GlyP) and AMPA adsorption in New Zealand soils. The soil was equilibrated with a glyphosate-AMPA 1 mg/L solution, ratio 1:10 (w/v) at pH 5. The residual % GlyP-AMPA in solution was calculated using the residual results analysed by RP-HPLC. Adsorbed % and Kd values were calculated theoretically using the residual % values see Chapter 3.5 for details.

Soil Type	Glyphosate			AMPA		
	Residual %	Adsorbed %	Kd	Residual %	Adsorbed %	Kd
Pallic	8.98 a	91.01 b	10.14 c	2.39 a	97.6 c	40.84 b
Brown	0.08 b	99.91 a	1267.03 b	1.5 b	98.49 b	65.6 b
Allophanic	0.03 b	99.96 a	2837.89 a	0.37 c	99.62 a	266.66 a
MSD	0.6	0.6	739.7	0.2	0.2	31.28

*Mean values (n=3) in the same column followed by the same letter are not statistically different according to Tukey's test ($p<0.05$). MSD, minimum significant difference.

The Kd of AMPA in the Brown soil was statistically similar to the Pallic soil while the Kd of GlyP for the Brown soil was significantly higher than the Pallic soil. It is relevant to understand the adsorption behaviour of AMPA on both sedimentary soils in order to elucidate the factors involved in the retention of the herbicide and its degradation product. P retention was greater for the Brown soil than the Pallic soil which was in agreement with the greater Al-Fe oxy-hydroxide concentration of this soil (Table 4.1). Thus, the greater GlyP Kd of the Brown soil was a function of its chemical composition.

Differences observed in AMPA's Kd between the sedimentary soils (i.e. the Pallic and Brown soils), can be related to differences of sorption capacity between GlyP and AMPA. Sidoli et al. (2016) observed competition between phosphate, AMPA and glyphosate onto soil surfaces with the relative affinity reported by the authors for the soil's reaction sites being $H_2PO_4^-$>AMPA>GlyP. The authors' suggested that AMPA's greater adsorption was related to its lower molecular weight in comparison to GlyP, which facilitated the adsorption of the AMPA molecule to soil reaction sites. In this experiment, a lower residual amount of AMPA (2.39%) was analysed in the Pallic soil solution than residual GlyP (8.98%) (Table 4.4). This suggested that AMPA had greater affinity for the Pallic soil reaction sites than GlyP.

Glyphosate and AMPA desorption

Glyphosate and AMPA were desorbed from the solid phase using an alkaline extractant. The Allophanic soil had the lowest GlyP and AMPA desorption ($p<0.05$) when compared to the Pallic and Brown soils (Table 4.5). The amount of GlyP extracted from the Pallic and Brown soils was statistically the same at 60 and 62%, respectively, whereas there was a significant difference in the amount of AMPA desorbed from these two soils (82% and 59% for the Pallic and Brown soil respectively). These results suggest that greater AMPA exchangeability occurred in the Pallic soil, which had similar Kd AMPA values as Brown soil, but desorbed greater AMPA than the Brown soil.

Table 4.5 Glyphosate (GlyP) and AMPA desorption in New Zealand soils. GlyP and AMPA were extracted from the solid phase using an alkaline solution ratio 1:10 (w/v). Desorbed % GlyP-AMPA was calculated using the extraction results analysed by RP-HPLC. Fixed % GlyP-AMPA were calculated theoretically see Chapter 3.5 for details.

Soil Type	Glyphosate %				AMPA %			
	Desorbed		Fixed		Desorbed		Fixed	
Pallic	60.26	a	30.8	c	82.12	a	15.48	c
Brown	62.26	a	37.7	b	58.66	b	39.82	b
Allophanic	39.77	b	60.2	a	30.97	c	68.65	a
MSD	3.23		3.12		6.89		6.95	

*Mean values (n=3) in the same column followed by the same letter are not statistically different according to Tukey's test ($p<0.05$). MSD, minimum significant difference.

The amount of fixed GlyP and AMPA was proportional to the Al-Fe oxy-hydroxides concentration of the soil, and followed the order Allophanic>Brown>Pallic, although it was the Al oxy-hydroxide concentration of the volcanic soil that played a significant role in the retention of GlyP and AMPA. The concentration of Al-dithionite in the Allophanic soil was double that in the Brown soil (Table 4.1), and this likely explains the higher fixation of GlyP and AMPA in the Allophanic soil. Gimsing et al. (2002) observed similar results and found a greater sorption coefficient of glyphosate on the tested soil with the highest Al dithionite-extractable and Al oxalate-extractable concentration. In the same study, another soil with the highest Fe dithionite-extractable and Fe oxalate-extractable content showed lower GlyP retention, suggesting that Al

hydrous oxides are the primary determinant of GlyP retention. In the present study, the iron concentration of Brown and Allophanic was similar. It was the greater Al concentration of the Allophanic soil which explained the increased GlyP and AMPA fixation relative to the Brown soil.

The results observed in this experiment helped to elucidate the importance of the soils' characteristics on the GlyP and AMPA adsorption-desorption process. In particular, the soil's Al-Fe oxy-hydroxides concentration played a significant role in the Kd and fixation of the herbicide and its degradation product. These results can help in the assessment of GlyP and AMPA losses from farmland.

4.3.3 Glyphosate batch adsorption-desorption experiment in soils saturated with phosphorus

Glyphosate adsorption in P saturated soils

Mean adsorption of GlyP onto the Pallic soil saturated with phosphorus (Pallic Psat) was 28.07% lower than the control Pallic soil (Pallic Unsat) (Figure 4.1). Similarly, there was a reduction of around 13% of GlyP adsorption onto the Brown Psat and Allophanic Psat soils relative to their respective controls and these results demonstrate that P saturation reduced the soil's capacity to adsorb GlyP. Munira et al. (2018) found similar results on agricultural soils with long-term applications of P fertilisers during an evaluation of the sorption capacity of P and GlyP on Fe-rich and $CaCO_3$-rich soils. They found that P accumulation onto soil surfaces reduced the sorption of either additional P or of GlyP, although P had greater affinity for the soil's reaction sites and was effective in displacing GlyP from the solid phase. The authors observed a correlation between high Olsen P values and low sorption coefficients of GlyP. Gimsing et al. (2002) observed similar results in an adsorption-desorption batch experiment. The authors found significant reduction of glyphosate sorption onto Danish soils when P was added to the soil slurry using 0.1M KCl as a background electrolyte.

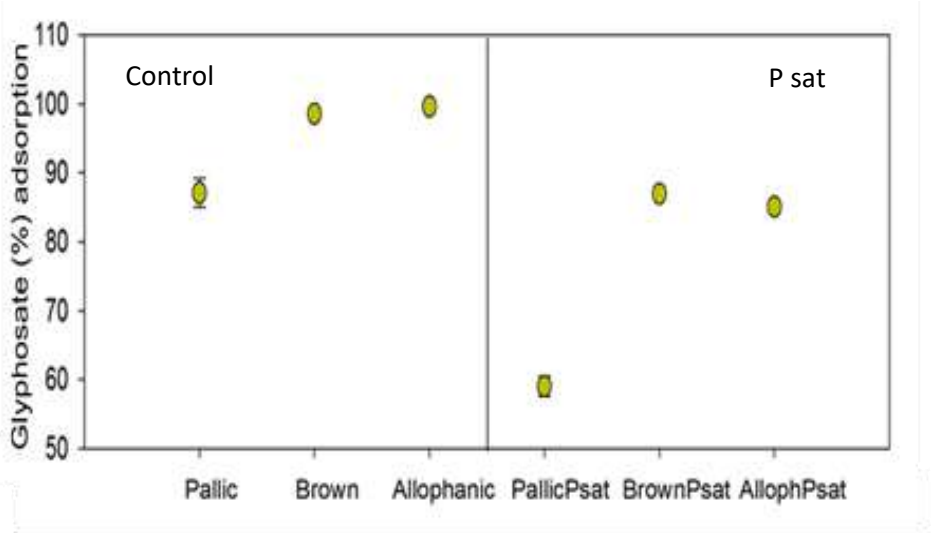

Figure 4.1 Glyphosate (GlyP) adsorption% onto New Zealand soils without P addition (Control) and soil initially saturated with phosphate (P sat) (n=3 ±SE).

Despite soil saturation with P, the capacity of the Brown Psat and Allophanic Psat soils to absorb GlyP was about 27% greater than the Pallic Psat. However, P saturation diminished the sorption coefficient of the Brown and Allophanic soils, which had Kd values similar to the Pallic Unsat soil ranging from 5.71 to 6.9 Kd. This was due to the greater concentration of GlyP remaining in the equilibrium solution as a result of P occupying the soil reaction sites. In contrast, Allophanic Unsat had the highest Kd followed by Brown Unsat at values of 294.23 and 71.53, respectively. The GlyP sorption coefficient in the Unsat soils (Table 4.6) was proportional to the soil's Al-Fe oxalate-extractable concentration as follows Allophanic> Brown> Pallic (Table 4.1).

Phosphorous saturation of the Pallic soil decreased desorption of GlyP by approximately 22% relative to the control soil (47.45% for the control soil; 25.88% for the P saturated soil) (Table 4.6). In contrast, relatively limited change in GlyP desorption as a function of P saturation observed for the Allophanic soil was related to its greater anion retention capacity (Table 4.1); the greatest GlyP fixation was observed for the control Allophanic soil. However, P reduced initial GlyP adsorption by 14%, which reduced significantly the amount of GlyP fixed in the Allophanic Psat by 20% compared to the Allophanic Unsat (Table 4.6).

Table 4.6 Glyphosate (GlyP) residual%, adsorbed%, sorption coefficient (Kd), desorbed (%) and fixed (%) in New Zealand soils, without P addition (Control) and saturated with phosphate (Saturated).

P content	Soil Type	Residual %		Adsorbed %		Kd		Desorbed %		Fixed%	
Control	Pallic	12.88	b	87.12	b	6.9	c	47.45	ab	42.82	bc
	Brown	1.41	c	98.58	a	71.53	b	51.64	a	46.93	bc
	Allophanic	0.34	c	99.66	a	294.2	a	28.46	c	68.02	a
Saturated	Pallic	40.95	a	59.05	c	1.44	c	25.88	c	33.17	c
	Brown	13.02	b	86.97	b	6.68	c	43.99	ab	43.16	bc
	Allophanic	14.91	b	85.08	b	5.71	c	36.89	bc	48.18	b
MSD		2.97		2.97		26.9		11.23		14.15	

*Mean values (n=3) in the same column followed by different letter are statistically significant according to Tukey's test ($p<0.05$). MSD, minimum significant difference.

The Brown Unsat soil had significantly higher GlyP desorption than the Allophanic soil (23.18% greater; Table 4.6) despite the fact that the Brown soil had a similar GlyP adsorption response to the Allophanic soil for both treatments. While, the Brown Psat soil had higher desorption than the Pallic and Allophanic Psat soils (Table 4.6). These results suggested a high reactivity of the Brown soil regardless of its P status. The Fe oxy-hydroxide concentration of the Brown soil was a soil feature that could play a significant role in the GlyP adsorption-desorption process. The higher Fe reactivity in the soil solution relative to Al could help to explain the enhanced GlyP desorption in the Brown soil.

The Brown soil's Fe oxy-hydroxide concentration was similar to the Allophanic soil (Figure 4.2) and therefore the similar GlyP adsorption response between the Brown and Allophanic Unsat soils could be partially due to the Fe concentration. While the reduced GlyP desorption of the Allophanic Unsat soil was due to its greater Al oxy-hydroxide concentration (Table 4.1) which fixed a greater proportion of GlyP onto the solid phase, the Brown soil's Fe oxide reactivity facilitated the GlyP desorption.

The degree of weathering that characterised the pedology of the three soils may have influenced Fe reactivity, as weathering leads to greater surface reaction sites of the Fe minerals to react with phosphate and GlyP (Waychunas, Kim, & Banfield, 2005). The Brown soil was collected from an area with presence of iron sulphide minerals (Grapes & Challis, 1999); furthermore, at the bottom of the sampling site there were

highly weathered old mine tailings. As consequence of mineral weathering of soils' Fe minerals and the sulphide oxidation of the tailings, Fe oxides were clearly visible as red staining deposited in an adjacent creek bed (Chapter 3.1).

The diverse types of Fe minerals present in the environment each have a different affinity for GlyP. Studies have demonstrated that goethite can retain more GlyP than magnetite (Yang, Deng, Yan, Jing, & Zhang, 2018). GlyP desorption experiments from goethite using $CaCl_2$ 0.01 M or DI water as extractants have demonstrated high potential for GlyP desorption from this mineral. Around 60% of the total GlyP sorbed by goethite was desorbed by both extractants and the authors suggested possible GlyP solubilisation risks on Fe-rich soils (Orcelli et al., 2018). Gimising et al. (2002), observed greater GlyP retention exerted (and therefore reduced GlyP desorption) by Al oxides than Fe oxides.

The Fe-dithionite concentration of the soils in this study was proportional to the GlyP adsorption on the P saturated soils (Figure 4.2) and this trend can help to elucidate the role of the Fe oxy-hydroxides in the GlyP adsorption-desorption process on each of the P-saturated soils. Specifically, available research tells us that Al oxy-hydroxides have a greater affinity for the $H_2PO_4^{2-}$ molecule than the GlyP molecule; and that Al is more stable than Fe in the soil solution (Spranke et al., 1975a; Sidoli et al., 2016). Available evidence suggests that the Al oxy-hydroxides remained mostly occupied by phosphorus, while the higher reactivity of the Fe oxy-hydroxides facilitated the substitution of $H_2PO_4^{2-}$ by GlyP (Yan et al., 2016).

GlyP sorption on the Fe oxy-hydroxides can be explained according to diverse chemical reactions that might occur between the solid phase and the herbicide. The strongest bond between Fe and GlyP happens when two iron molecules form a complex with one GlyP molecule through each of the available oxygens of the phosphonic group. In contrast, the weakest bond forms when one iron molecule holds just one GlyP molecule, which is prone to solubilisation. When the phosphate molecule enters the system it may compete or have an additive effect on GlyP adsorption onto Fe-oxides. Competition involves the total displacement of GlyP from the Fe-oxide by phosphate. An additive effect occurs when two phosphates adsorbed in two Fe-oxides form a bridge with a free Fe^{3+}, then the free electron bond to the GlyP molecule (Borggaard et al., 2008).

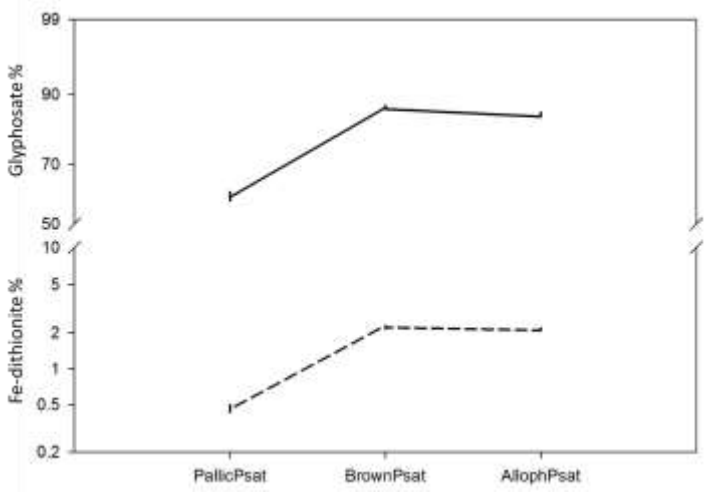

Figure 4.2. Glyphosate adsorption (%) on P saturated soil and the Fe-dithionite concentration (%) of the Pallic, Brown and Allophanic soils.

The results observed in this experiment demonstrate the competition between GlyP and phosphate for adsorption onto soil reaction sites. Phosphorus reduced GlyP adsorption; however, the responses observed in P saturated soils suggested that the higher reactivity of the Fe oxy-hydroxides played a significant role in the GlyP adsorption-desorption process and enabled the exchange of P by GlyP. This research shows that the Al oxy-hydroxide concentration exerts a primary control over GlyP fixation in the solid phase of control soils. The Allophanic soil had the greatest GlyP fixation under both control and P-saturated treatments, whereas the Pallic soil was prone to greater GlyP displacement due to its low anion retention capacity. The Brown soil was susceptible to increased exchangeability between phosphate and GlyP caused by the reactivity of the Fe oxy-hydroxides.

4.3.4 Glyphosate column leaching experiment

In a preliminary leaching trial, the columns packed with the same soil bulk density were irrigated with the same flow rate of 0.0246 mL/minute. The Brown soil had greater water infiltration in comparison to the Pallic soil where water stagnated at the top of the column. This was despite both sedimentary soils possessing similar physical characteristics (Table 4.3). Soil management could therefore affect the soil's particle microstructure. The Pallic topsoil was collected from manuka plantations used as

detainment strips, which retain soil particles displaced from pasture fields. Thus, cultivation and erosion could disrupt the soil structure as the soil particles are subjected to a disaggregation during tillage; and aggregation processes due to water deposition could enhance compaction (Naderi-Boldaji & Keller, 2016). In contrast, the Brown soil was collected from a non-cultivated soil, which preserved a stable structure over time allowing water infiltration through macropores (Katagi, 2013).

The concentration of GlyP and AMPA analysed from the leachate and soil samples was converted to content basis, by multiplying the concentration quantified by the total leachate volume or soil weight. This approach was used because in field conditions the total amount of GlyP and AMPA displaced out of the rhizosphere or deposited to groundwater is the combined effect of water infiltration which is a function of soil physical characteristics, and the GlyP-AMPA sorption process which is a function of chemical characteristics. In this way, use of GlyP-AMPA content helped to elucidate the total movement of the GlyP-AMPA in order to estimate the contamination risks associated with soil characteristics.

Leachate analysis

The Pallic soil with P addition had a significantly ($p<0.05$) higher GlyP content detected in the leachate with value of 0.0146 µg Gly P/Column, in comparison to all control soils and the Allophanic soil with P addition where values ranged from 0.0012 to 0.0015 µg GlyP/Column. These results demonstrated that initially P occupied part of the Pallic soil's reaction sites increasing the immediate downward displacement of GlyP out of the column (Table 4.7).

GlyP degradation to AMPA occurred during the evaluation period because AMPA was detected in the leachate of both treatments of the Brown soil, with values of 0.13 and 0.2 µg AMPA/Column, for the control and P addition treatments respectively. These values were statistically higher than the control and P addition to the Pallic and Allophanic soil treatments (Table 4.7). The interaction between the Brown soil's chemical and physical characteristics influenced GlyP degradation to AMPA, and increased the posterior displacement of AMPA out of the column, regardless of the P application; however, P addition increased at 0.07 µg AMPA/Column the detection of AMPA compared to the control.

The Fe oxy-hydroxides are important chemical constituents of the Brown soil which participated in the sorption process of GlyP. The degradation rate of the GlyP molecules adsorbed to the soil's Al-Fe oxy-hydroxides can be reduced in comparison to solubilised molecules, because the minerals protect them from microbial degradation. However, studies have suggested that GlyP adsorbed in Fe-oxides can lose the exposed carboxyl group due to microbial cleavage, which yields AMPA (Borggaard et al., 2008).

Taking into consideration that AMPA is a hydrophilic compound and the high reactivity of the Fe oxy-hydroxides (Orcelli et al., 2018; Waychunas, Kim, & Banfield, 2005), it is possible that water infiltration through the Brown soil increased AMPA's solubilisation and displacement out of the column. The addition of P almost doubled the amount of AMPA in leachate from the Brown soil, which demonstrated that P occupied the Al-Fe oxy-hydroxides increasing AMPA's displacement from the solid phase (Zhao et al., 2009). The content of AMPA analysed in the leachate of the Brown soil treatments (added P and control) was greater than that of GlyP detected. Similar results were found by Napoli et al. (2015) who found a higher concentration and frequency of AMPA in leachate samples, in comparison to GlyP. This demonstrates the GlyP's high degradation rate and the importance of measuring AMPA and other GlyP's degradation products in environmental samples.

GlyP column extraction

Analysis of the alkaline extract from the soil showed only the presence of AMPA in the soil at the end of the column leaching experiment, not GlyP. The AMPA content of the first 5cm depth of soil was quantified at 79.24 µg/column for the Pallic soil after P addition and was statistically higher than in the Brown and Allophanic soil, with or without P addition (Table 4.7). These results suggested that the Pallic soil's poor structure and the competition between GlyP and P played a significant role in the distribution of the herbicide. At the beginning of the experiment, the Pallic soil's loose structure allowed GlyP distribution through the column, shown by significant GlyP values detected in the leachate. After continuous water addition, compaction occurred locking the GlyP at the top of the column, while the P addition reduced the AMPA adsorption. In contrast, the Allophanic soil's highest anion retention capacity was the

main feature involved in AMPA retention in the solid phase. Similar results have been observed in undisturbed column leaching experiments, were the original GlyP molecule was not detected, only AMPA, which was allocated mostly at the top of the column (Landry et al., 2005; Napoli et al., 2015; Okada et al., 2016).

Table 4.7 Glyphosate, AMPA detection in leachate (µg/column) and AMPA detection (µg/Column) extracted from the sections 0-5cm and 5-10cm of the columns packed with New Zealand soils without P addition (Control) and P addition (P applied) treatments.

Treatment	Soil Type	Leachate µg/Column				Soil extraction AMPA µg/Column			
		GlyP		AMPA		5 cm depth		10 cm depth	
Control	Pallic	0.0014	b	0	b	60.73	ab	10.77	B
	Brown	0.0012	b	0.13	a	45.9	ab	15.59	B
	Allophanic	0.0015	b	0	b	31.39	b	17.85	B
P applied	Pallic	0.0146	a	0.03	b	79.24	a	12.35	B
	Brown	0.0077	ab	0.2	a	51.35	ab	11.26	B
	Allophanic	0.0013	b	0	b	37.32	b	41.08	A
	MSD	0.01		0.073		37.8		32.81	

*Mean values (n=3) in the same column followed by different letter are statistically significant according to Tukey's test ($p<0.05$). MSD, minimum significant difference.

The addition of P to the Allophanic soil increased the detection of AMPA below the 0-5 cm depth, leading to a significantly higher mass of AMPA at the 5-10 cm depth (41.08 µg/Column) (Table 4.7). The Allophanic soil's bulk density was lower, and had about 10% more pore space than the sedimentary soils, and this inferred initially higher water infiltration. The results suggested a synergy between P and the initial soil loose structure, which increased water infiltration and GlyP solubilisation, increasing its displacement to the bottom of the column, which was detected in the leachate as well. The detection of AMPA at the 5-10 cm depth suggested that AMPA was displaced from the upper column's layer as a product of GlyP decomposition; or alternatively, the GlyP initially retained at the 5-10 cm depth was decomposed to AMPA. The highest anion retention capacity and highest WHC (Tables 4.1 and 4.3) was observed for the Allophanic soil, and this would have resulted in strong adsorption of AMPA to the solid phase avoiding its further displacement out of the column. That effect was supported

by the absence of AMPA detected in the leachate of both treatments of the Allophanic soil. Zhao et al. (2009) observed similar results in a glyphosate column leaching experiment where after P application to the columns, GlyP was displaced downwards to the bottom of the column, while in the control soil most of the GlyP was allocated in the top of the column, However, the soil's high anion retention capacity reduced GlyP percolation. Napoli et al. (2015) conducted a GlyP column leaching experiment under outdoor conditions during a 3-year period and did not find extractable GlyP from the soil profile.

The sum of GlyP and AMPA measured in the soil's leachate demonstrated that leachate from the Brown soil (with and without P treatment), had the highest content of herbicide and its degradation product; followed by the Pallic soil with P addition. The sum of the AMPA content extracted from the 0-5 cm and 5-10 cm depth columns' sections was determined. In the control soils the ordering of AMPA concentration Pallic>Brown>Allophanic was seen, which was similar to the trend observed in the AMPA desorption from soils of the Chapter 4.3.1 (Table 4.7). Phosphorus addition increased AMPA extraction in both sections of the column of the Pallic and Allophanic soil (Figure 4.3).

The results observed in this experiment helped to elucidate the effect of the interaction between soil physical and chemical properties on the vertical movement of GlyP and AMPA through the soil profile. Soil physical properties played a significant role in GlyP translocation due to increased water infiltration. The initial loose soil structure allowed the movement of GlyP through the column, which was detected in the leachate of all treatments. The Pallic soil's poor physical structure led to retention of AMPA at the top of the column regardless of the P addition; however, taking into consideration its low anion retention capacity and the results observed, this soil possesses higher herbicide surface runoff risk. In contrast, the free-drained Brown soil in combination with its high Fe oxy-hydroxides reactivity resulted in increased risk of GlyP percolation.

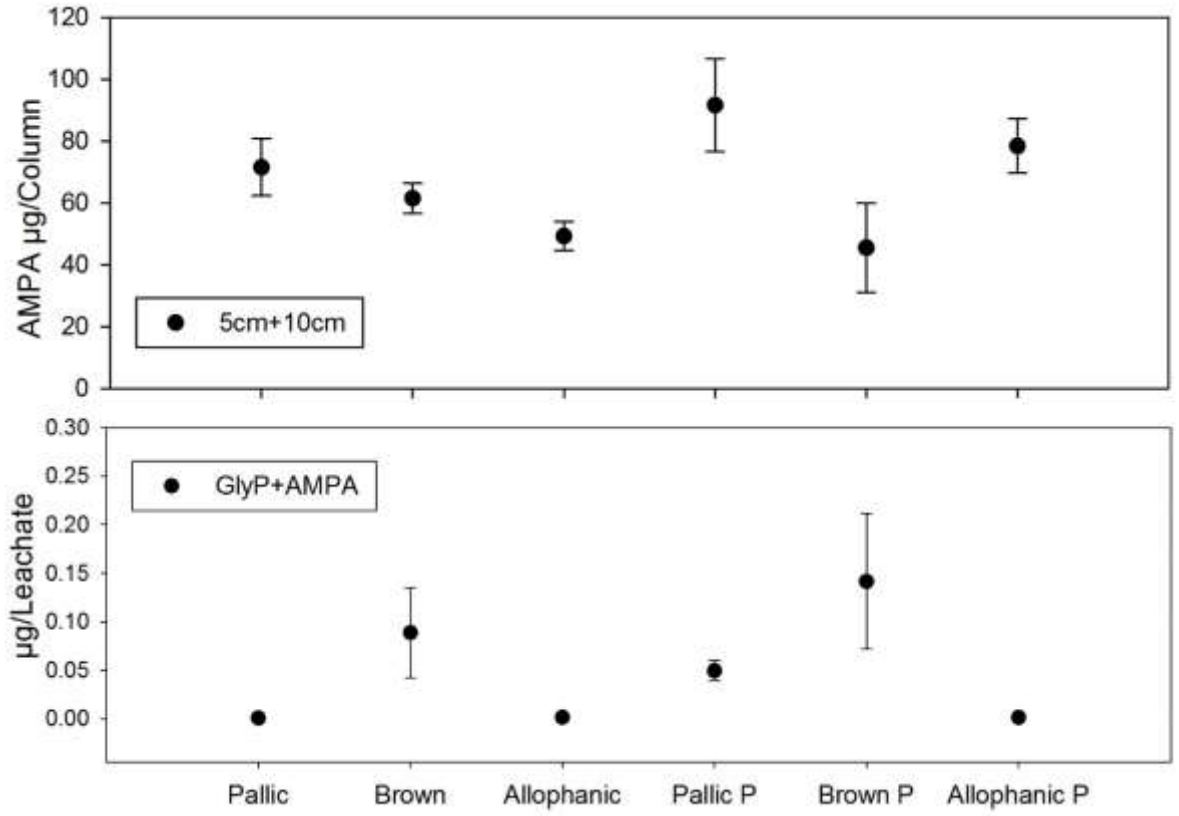

Figure 4.3. Sum of AMPA (µg/Column) detected in both soil column's sections 5 and 10 cm depth. Sum of glyphosate and AMPA (µg/Column) detected in soil column's leachate. Mean (n=3± SE).

Phosphate addition to the columns resulted in a six-fold increase in GlyP movement and almost doubled the mass of AMPA leached from the Brown soil, compared to the control soil. Higher P affinity for soil reaction sites increased AMPA retention at the top of the Pallic soil column, and P addition increased GlyP displacement through the Allophanic soil column. However, the absence of AMPA in leachate from the Allophanic soil (with and without added P) suggested greater retention of the herbicide and its degradation product by the Allophanic soil regardless of the P addition. Overall, these results helped to clarify the importance of the soils chemical and physical characteristics in GlyP displacement.

4.3.5 Soil induced microbial respiration using glyphosate dose and Agave amendments

4.3.4.1 Soil induced respiration

Soil induced respiration (SIR) is a method that can be used to analyse the amount of carbon dioxide emitted by soil microorganisms in response to GlyP doses and Agave amendments. The SIR ratio used in this work was calculated by dividing the treatment response by the control (soil without treatment). The reason for using this ratio was to focus on the effect of the treatments rather than the combined respiration product or soil organic matter mineralisation that may be caused by the treatments.

The factorial analysis demonstrated that the Allophanic soil had the greatest SIR ratio of 1.78, followed by the Pallic soi (1.32) and the Brown soil (1.18) ($a<0.05$) (Table 4.8). These results demonstrated that the physical and chemical properties of the Allophanic soil had a greater influence on microbial activity after GlyP treatments than soil organic matter mineralisation. This comment is substantiated by the fact that in calculating the SIR ratio, respiration from soil organic matter is not considered, with respiration solely that induced by the treatments.

Table 4.8 Factorial analysis per soil type of dissolved organic carbon fractions (TOC, total organic carbon; TC, total carbon; IC, inorganic carbon) and total nitrogen (mg/L) from Pallic, Brown and Allophanic soils under GlyP treatments.

Soil Type	TOC		TC		IC	TN		SIR	
Pallic	46.1	b	47.23	b	1.08	33.8	b	1.32	b
Brown	85.4	a	86.46	a	1.02	7.97	c	1.18	c
Allophanic	42.3	c	43.42	c	1.02	51.11	a	1.78	a
MSD	3.49		3.48		0.17	3.33		0.09	

*Mean values (n=27) in the same column followed by different letter are statistically significant according to Tukey's test ($p<0.05$). MSD, minimum significant difference.

The soil type had greater influence in the response of the SIR ratio, expressed on the factorial results, with a higher mean square (MS) value of 3.2 for the soil type, in comparison to 0.92 for the GlyP doses. These results demonstrated that the

significance of the soil type was greater in the experimental model used in this work (error MS 0.022 and model MS 1.43). A similar response was observed for the presence of total dissolved organic carbon (TOC); the soil type had the strongest influence with an MS value of 10241 in comparison to 38.08 of the GlyP doses (error MS 18.61 and model MS 2078.74) (Appendix). A conclusion of these observations is that the carbon in the soil was significant for the responses of soil respiration and dissolved organic carbon.

The Brown soil had the highest TC of 86.5 mg/L, in contrast, the Allophanic soil had the lowest TC of 43.4 mg/L (Table 4.8). Despite the fact that the Allophanic soil had the greatest total C concentration of 7.85%, DOC was the lowest for this soil (Table 4.2.). These results demonstrated the importance of the soils' Al-Fe oxy-hydroxides concentration in the protection of the organic matter against microbial mineralisation and its effect on GlyP degradation. The soils' total C concentration was proportional to the P retention and Al-Fe oxy-hydroxides concentration of each soil, and followed the order Allophanic>Brown>Pallic (Tables 4.1 and 4.2).

Soils derived from volcanic ash, such as the Allophanic soil, represent a larger global C pool compared to non-volcanic soils. The abundance of reaction sites in volcanic ash soils reduces the potential for organic matter mineralisation, and hence soil respiration. Al-Fe oxy-hydroxides are therefore key elements in the process of C sequestration in soils (Matus et al., 2014). The Allophanic soil's Al-Fe oxy-hydroxides protected organic matter from microbial mineralisation, and a consequence of this was greater microbial activity on the GlyP molecule, which represented a source of new C. In this regard, the ordering of SIR ratio values Allophanic>Pallic>Brown was inversely proportional to the ordering of total dissolved carbon Brown>Pallic>Allophanic.

The SIR ratios observed for the Pallic and Brown soils demonstrated increased microbial activity over the soils' organic matter compared to the Allophanic soil. This may be due to low protection of organic matter by reduced Al-Fe oxy-hydroxides concentration as occurred in the Pallic soil; or greater reactivity of the Fe oxy-hydroxides, which while initially protecting organic matter, led to subsequent solubilisation of labile fractions and mineralisation. In this experiment, the Brown soil had the highest amount of dissolved C, which is related to its higher C pool of 4.65% in comparison to a C pool of 2.98% for the Pallic soil. In this regard, the Pallic soil had

a higher CEC compared to the Brown soil (Table 4.2) which also might influence in C stabilisation and lower carbon solubilisation in the Pallic soil. In non-volcanic soils, clay mineral fractions play a significant role in C stabilisation, where clays with the highest proportion of reactive sites which may reduce soil respiration (Singh, 2017). Taking into consideration that Fe oxy-hydroxides are significant constituents of the Brown soil, these results suggested that the Fe oxy-hydroxides influenced C storage capacity. However, the greater detection of dissolved C in the Brown soil inferred reduced C sequestration due to Fe oxy-hydroxides' reactivity relative to the effect of Al oxy-hydroxides of the Allophanic soil.

These results suggest that in sedimentary soils glyphosate degradation occurred alongside soil organic matter mineralisation. Haney et al. (2002) found a short-term faster GlyP degradation rate where a high amount of organic matter was available. However, long-term, total GlyP degradation rate was not compromised by the soil's organic matter content.

Analysis of the interaction between soil type and GlyP doses (treatment) demonstrated a relationship between the SIR response and the concentration of the GlyP doses (Table 4.9). The sedimentary soils had a SIR increase proportional to the GlyP dose. The lowest SIR was observed at the lowest GlyP dose, then SIR increased accordingly to the GlyP dose; the treatments with AgvE amendment in combination with GlyP doses had the highest SIR response. In both sedimentary soils the SIR response for the maximum recommended commercial dose of 7.5 kg GlyP was statistically similar to the lowest GlyP dose (2.5 kg) and the highest GlyP dose (20 kg) (Table 4.9). These results demonstrated that even the highest GlyP doses did not affect the soil's microbial respiration. Similar results were observed in the work of Wardle & Parkinson (1990). These authors analysed the effect of glyphosate doses on soil microbial biomass carbon and found that rates close to the maximum GlyP recommended doses exerted a minor effect on the microbial balance. Excessive GlyP rates effected a change in soil microorganism communities, favouring fungi proliferation, which overall increased total microbial biomass.

Table 4.9 Statistical analysis of the interaction Soil type*GlyP doses (n=3) response on SIR ratio and factorial analysis of SIR ratio per GlyP dose (n=9).

Rate	Pallic		Brown		Allophanic		Soil	
Agv/20	1.63	a	1.5	a	2.2	ab	1.77	a
Agv/7.5	1.66	a	1.46	a	2.27	a	1.8	a
Agv	1.49	ab	1.16	b	2.18	ab	1.61	ab
20	1.34	bc	1.25	b	1.76	bc	1.45	bc
15	1.29	bc	1.1	bc	1.61	c	1.33	cd
7.5	1.13	cd	1.1	bc	1.52	c	1.25	de
5	1.06	d	0.93	d	1.43	c	1.14	de
2.5	0.96	d	0.94	cd	1.32	c	1.07	e
Control*	0.22		0.25		0.11		0.1792	
MSD	0.21		0.16		0.46		0.19	

Control* =Mean absorbance of SIR measured at 470nm (CO_2 trapped in cresol gel). Mean values (n=9) in the same column followed by different letter are statistically significant according to Tukey's test ($\alpha<0.05$). MSD, minimum significant difference.

AgvE amendments combined with 20 and 7.5 kg GlyP doses for both sedimentary soils yielded SIR ratio values which were statistically higher than the rest of the treatments. These results show that the highest GlyP 20 kg dose alone, and in combination with AgvE, did not affect microbial respiration; whereas an exogenous C source (either Agave or GlyP) improved microbial respiration. Nguyen et al. (2018) found a similar response using glucose as an exogenous C substrate, alongside high doses of the commercial herbicide Roundup. The authors observed that the SIR ratio increased proportional to the concentration of Roundup used, while the maximum recommended commercial rate had no detrimental effect on the SIR ratio.

In the Allophanic soil, a similar SIR ratio increase dependant on GlyP dose was observed; however, a clear statistical difference between the GlyP doses alone from 2.5 to 15 kg was observed; the SIR ratio for treatments with AgvE amendments was significantly higher (Table 4.9). These results suggest that C from the AgvE amendment improved microbial activity, regardless of the Allophanic soil's high capacity to retain organic matter, which indicate that the C fractions in the AgvE amendment were readily-available for soil microorganism uptake. This observation is supported by the fact that the highest concentration of dissolved total carbon (48.27

mg TC/L) was recorded for the AgvE treatment of the Allophanic soil (Figure 4.4). In contrast, for both sedimentary soils, treatment GlyP doses with AgvE reduced dissolved total carbon compared to treatments without AgvE (Figures 4.5 and 4.6). These results suggested that the AgvE constituents in the sedimentary soils were immediately available for soil microorganisms, increasing SIR, and then leading to TC depletion due to microbial uptake.

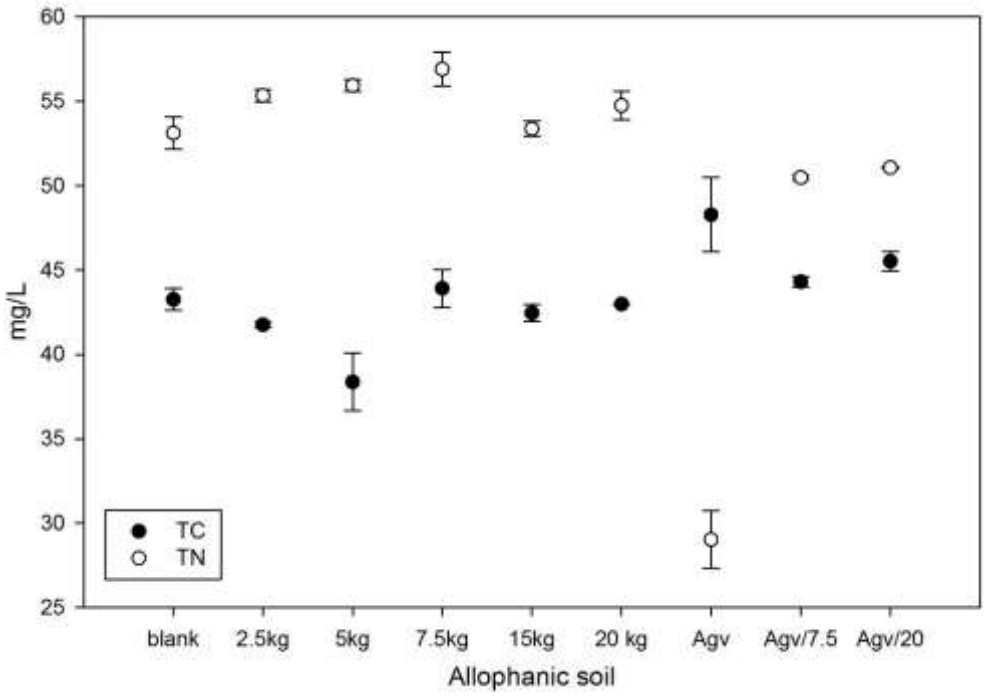

Figure 4.4. Dissolved total carbon (TC) and total nitrogen (TN) concentration (mg/L) of Allophanic soil samples with GlyP doses and Agave amendments applications (n=3± S.E).

The Allophanic AgvE treatment had the lowest dissolved nitrogen value of 29.03 mg TN/L (Figure 4.4). Dissolved N depletion was observed in all the treatments with AgvE amendment of the Allophanic and Brown soils when compared to the other treatments (Figures 4.4 and 4.5). The reduction in TC for both sedimentary soils and reduction in TN reduction for the Brown and Allophanic soils after AgvE amendment suggests enhanced microbial activity as a function of AgvE amendment, which depleted the N and C available in the soil solution. Nitrogen is an indispensable nutrient during soil carbon mineralisation and can become scarce due to microbial acquisition (Hessen et al., 2004). Haney et al. (2002) found similar results; the authors observed a positive correlation between C and N allocated in microbial biomass during glyphosate

degradation. The GlyP degradation capacity of the soils could therefore be related to N availability and the form of organic matter; alongside other soil features such as clay type, organic matter content and exchangeable acidity (Nguyen et al., 2018).

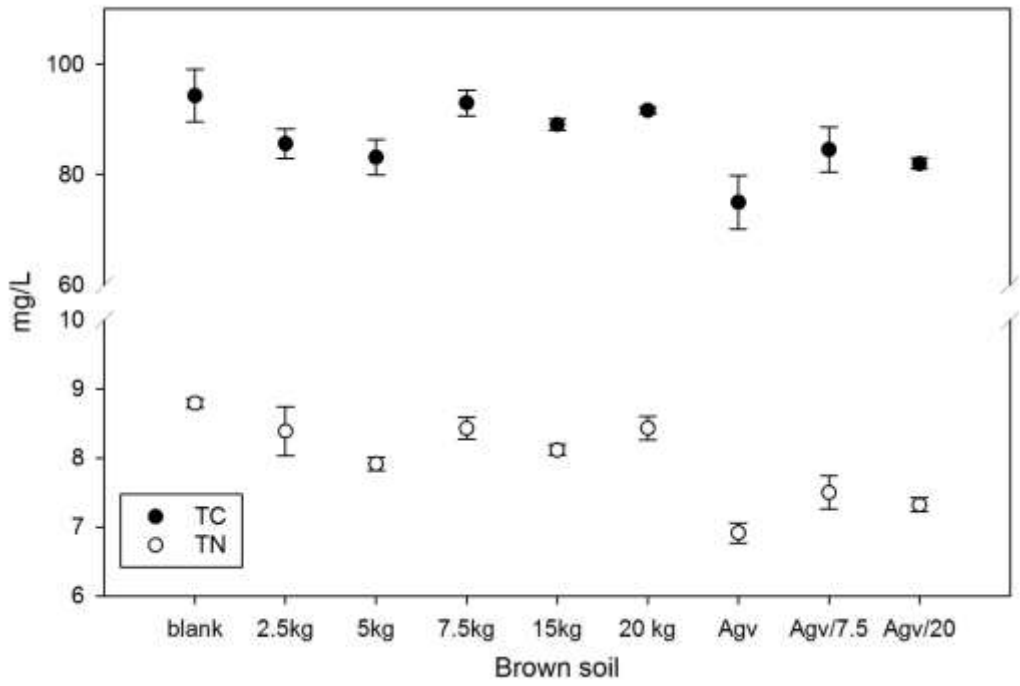

Figure 4.5 Dissolved total carbon (TC) and total nitrogen (TN) concentration (mg/L) of Brown soil samples with GlyP doses and Agave amendments applications (n=3± S.E).

Overall, these results demonstrated a strong influence of the soils' chemical characteristics on SIR responses, specifically on the capacity of soil to stabilise organic matter, which enabled greater microbial activity over the GlyP molecule. The GlyP maximum recommended commercial dose of 7.5 kg did not affect soil respiration, while higher doses increased soil respiration suggesting minimal effect of the herbicide on the soil microorganism community. The AgvE amendment triggered soil respiration which may infer greater GlyP degradation.

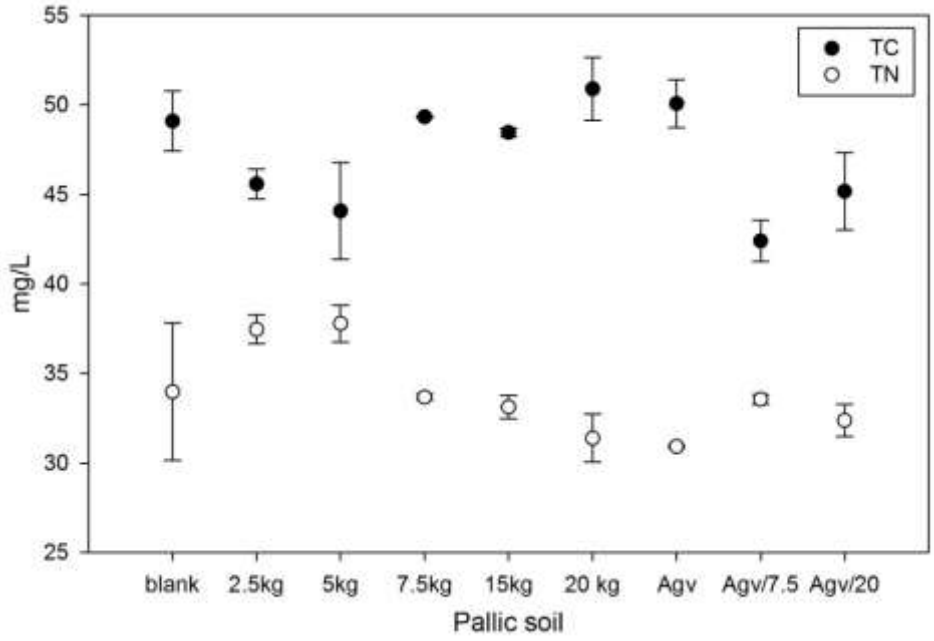

Figure 4.6 Dissolved total carbon (TC) and total nitrogen (TN) concentration (mg/L) of Pallic soil samples with GlyP doses and Agave amendments applications (n=3±S.E).

4.3.6 Phosphate adsorption-desorption batch experiment on P saturated soils using Agave leaf powder.

The Agave leaf powder had constituent P (mean P concentration of 0.35 mg P/g), and after subtracting the P concentration of the Agave powder from the treatments with Agave addition, statistical analysis was carried out. The potential effect of Agave leaf powder to desorb P from soil is summarised in Table 4.10. The P concentration of 0.5 mg P/g soil desorbed from the Brown soil with Agave addition was significantly higher than that measured for the Pallic and Allophanic soil (0.3 and 0.2 mg P/g soil, respectively).

For the control soils (only shaken with 10mM $CaCl_2$), there was no P desorption from the Pallic soil; whereas, low amounts of P were desorbed from the Brown and Allophanic soils, although the results were statistically similar to the Pallic soil. Desorption of P was only promoted by the Agave constituents. Anions known to be present in Agave powder such as organic acids and phenols can interact with $H_2PO_4^-$ desorption (Kovacik, 2011). About 59% of the total phenol concentration of the Agave powder (66.19 mg/g) was water-extractable (39.46 mg/g) (Table 4.11) and this is likely

to have displaced P from the soil's reaction sites. Within the phenolic compounds an average gallic acid (GAL) concentration of 0.112 mg GAL/ g in agave leaf powder was quantified.

Table 4.10. Aluminium, iron and phosphorus (mg/g soil) measured in supernatant; after extraction performed with $CaCl_2$ 10mM solution (Control) and with addition one gram of Agave powder (Agave).

Treatment	Soil type	Al		Fe		P	
Agave	Pallic	0.393	a	0.314	a	0.29	b
	Brown	0.988	b	0.161	b	0.45	a
	Allophanic	2.1	c	0.13	c	0.17	c
Control	Pallic	0.009	d	0.007	d	0	d
	Brown	0	d	0	d	0.04	d
	Allophanic	0.002	d	0	d	0.03	d
	MSD	0.191		0.03		0.08	

Treatments in columns with different letter are statistically significant according to Tukey's test ($p<0.05$).

Significant metal desorption from the soils in the Agave treatment was detected. Fe desorption with Agave powder was significantly higher in the Pallic soil compared to the rest of the treatments, despite the fact that the Brown soil had a higher Fe total concentration than the Pallic soil (Table 4.1). The Allophanic soil with Agave addition had the greatest Al desorption (2.1 mg Al/g soil), followed by the Brown soil and Pallic soil (1 and 0.3 mg Al/g soil respectively) (Table 4.10). Aluminium desorption from the soils was proportional to the oxalate-extractable concentration of the soils (Table 4.1) Allophanic>Brown>Pallic. The standard method used by the NZ Soil Bureau for the analysis of extractable Al and Fe by acid oxalate-extraction uses ammonium oxalate and oxalic acid as reagents for the metal quantification (Blakemore et al., 1987). Oxalic acid is a component of the Agave powder; and would have a similar effect on Al extraction as the reagents used in the standard protocol for Al quantification. The significant desorption of metals from the soil exerted by the Agave powder was a function of a synergistic effect of Agave's constituents such as organic acids, phenols and saponins (Ribeiro et al., 2015). Saponins are biosurfactants which have been

used as an amendment to implement bioremediation strategies for desorption of metals from soils (Cao et al., 2013; Filipkowska & Kuczajowska-Zadrożna, 2016).

Table 4.11. Water-extractable and total concentration of organic acids and phenols extracted from Agave leaves powder. Mean (n=3±S.D).

Extraction Type	Organic acids and phenol concentration (mg/g) of Agave powder			
	Oxalic	Citric	Succinic	Total phenols
Water	4.04 ± 1.18	86.15 ± 0.94	162.92 ± 0.43	39.47 ± 3.22
Total	18.75 ± 1.20	70.02 ± 2.61	161.05 ± 0.33	66.2 ± 8.46

Three organic acids were detected in the Agave powder using HPLC. The water-extractable concentration of oxalic, citric and succinic acids was 4.04, 86.15 and 162.92 mg/g agave powder, respectively (Table 4.11). The total oxalate concentration was almost 5 times the water-soluble concentration. Total oxalate includes free soluble and insoluble oxalic acid and in plant metabolism the acid form is bound to cations, primarily Ca. Oxalic acid is important for ion regulation in plants, due to its ability to form metal complexes (Nakata, 2003). The total concentration of citric and succinic acid was similar to the water-extractable concentration in the agave powder, indicating that all citric and succinic acid in the powder was water-extractable. However, there are analytical limitations with the method used. The determination of total citric and succinic acids was performed using the same extraction process as that for total oxalate and the heat of the process may have denaturalised citric acid converting total acid to water soluble acid. Thus, the total oxalate extraction method was specific for oxalic acid determination, but may have not be suitable for the measurement of total citric and succinic acid (Williams, 2016).

S,S-Ethylenediamine-N,N-disuccinic Acid (EDDS), is a chelate that has been bio-synthesised from organic acids and EDTA through bacteria metabolism, in order to produce a less toxic molecule for bioremediation strategies. EDTA is a powerful chelate but can accumulate in the soil due to its low degradation rate (Takahashi et al., 1999). Cao et al. (2013) used EDDS and saponins for the desorption of PCB, Pb and Cu from soil. The authors found a synergetic effect between saponins and EDDS

in the desorption of metal. In this thesis, organic acids and phenols are likely to have played a significant role as weak chelates, interacting with the surfactant activity of saponins in the desorption of metals and P.

P desorption from the soil observed in this work caused by Agave powder was a positive effect. However, the presence of metals in the soil solution is not desirable; in particular Al, which could have toxic effects for microorganisms and plants (Wood & Cooper, 1984; Berenji, Moot, Moir, Ridgway, & Rafat, 2017). A P-metal complexation effect in the soil solution is a dynamic adsorption-desorption process between Al, Fe and P. Under natural conditions the metals can interact with different anions forming complexes, thus reducing their bioavailability; although caution must be taken in extrapolating the results from laboratory work to field scale. Continuous shaking during the batch adsorption-desorption experiment may have damaged the structure of soil particles releasing a greater mass of metals into solution when compared to what may happen in natural conditions (Kookana, Gerritse & Aylmore, 1992). Overall, this experiment was useful for assessing the chemical interactions which occurred between the Agave powder and the soil.

These results helped to elucidate the effect of Agave powder in P desorption, which might promote P uptake in the rhizosphere, facilitating microbial proliferation for GlyP degradation. However, further research is required to clarify the potential presence of metals in the soil solution caused by Agave amendments.

4.4 Conclusion

Soil characteristics played a significant role in the adsorption-desorption process of GlyP and its degradation product AMPA. In particular, the soils' Al-Fe oxy-hydroxide concentration (ordering from least to greatest Allophanic>Brown>Pallic) was proportional to the fixation of both molecules to the soil solid phase. Phosphorus competed with GlyP for soil reaction sites. Phosphorus saturated soils had greater GlyP displacement from the soil surface. The results from this work suggest that P primarily occupied the Al-oxy-hydroxides, while the higher reactivity of the Fe oxy-hydroxides enabled the exchange of P by GlyP. However, additive adsorption between P and GlyP might occur as well.

The interaction of the soils' physical and chemical characteristics, expressed through water infiltration and a soils' P retention capacity, determined the vertical displacement of GlyP through leaching columns. GlyP degradation was observed during the experimental work, and an increase in AMPA was quantified at the top of the columns. Phosphorus addition to the columns enhanced GlyP displacement from the soil reaction sites, which increased AMPA leaching in the sedimentary soils. Phosphorus addition increased AMPA detection at the bottom of the Allophanic soil column. However, the Allophanic soil's high anion retention capacity and high water holding capacity mitigated AMPA leaching regardless of the presence of phosphorus in the column. High water infiltration observed in the Brown soil, together with high reactivity of constituent Fe oxy-hydroxides in this soil, enabled greater solubilisation and AMPA leaching under both treatments of P addition and control. However, P addition almost doubled the amount of AMPA leached. This is of concern, as free-draining soils with high GlyP-AMPA solubilisation might increase GlyP translocation to deeper soil layers or underground water. In contrast, the Pallic soil's poor physical structure enhanced compaction, which could increase GlyP surface runoff under real-life scenarios.

5. EVALUATION OF AGAVE LEAF AQUEOUS EXTRACT USED AS A SOIL AMENDMENT FOR GLYPHOSATE REMEDIATION AND ITS EFFECT ON WHITE CLOVER'S BIOCHEMICAL RESPONSES

5.1 Introduction and Aim

White clover requires adequate root development during the early stages of growth to ensure its establishment. The presence and abundance of weeds is therefore a key factor that can restrict the root growth of white clover. In response, the use of herbicides such as GlyP at pre and post-sowing for weed control is a common pasture management practice (Lewis, Lucas, & Moot, 2017).

The use of GlyP as part of pasture management is not only about weed control. GlyP spray-topping at low doses (250 mL/ha of Roundup, 360 g/L a.i. GlyP) is practiced to reduce pasture growth during late-spring and to inhibit weed competition. This strategy is useful to minimize white clover overgrowth, which can increase the amount of unwanted dead biomass in pasture (Casey, Brown & Stevens, 2000). In addition, pasture spray-topping can increase pasture digestibility after late-spring (Gatford et al., 1999).

The extended use of GlyP in farming systems has led to concerns about its potentially toxic effects on non-target organisms such as soil microorganisms and plants. GlyP inhibits the function of the enzyme 5-enolpyruvylshikimate-3-phosphate-synthase (EPSPS), which is a part of the biosynthetic pathway of aromatic compounds in plants and microorganisms. In glyphosate-sensitive plants, the disruption of EPSPS function can lead to the accumulation or depletion of metabolites synthesized in the shikimate pathway. Gallic acid is a phenolic compound synthesized in the 3-Dehydroshikimate step, its synthesis occurs before the action of EPSPS in the shikimate pathway and thus the inactivation of EPSPS may affect further biosynthesis of gallic acid derivatives. The analysis of biomarkers for sensing the stress in plants is a useful tool to assess the effect of glyphosate in non-target plants. Gallic acid is located upstream of the action of EPSPS; the disruption of gallic acid content can be used as a biomarker to sense the interference of glyphosate in plant metabolism (Zabalza, Orcaray, Fernandez-Escalada, Zulet-Gonzalez, & Royuela, 2017). A hypothesis of this chapter was that residual herbicide in the soil might influence white clover metabolism.

GlyP is biodegradable, but its extended use has led to accumulation in the soil. The main GlyP degradation pathway is the initial microbial breakage through the enzyme Glyphosate oxidoreductase, which yields aminomethylphosphonic acid (AMPA) and glyoxylate. Then, AMPA can lose the amino group through enzymatic lysis producing phosphonoformaldehyde, or alternatively, the cleavage of the C-P bond yields phosphate and methylamine (Zhan, Feng, Fan, & Chen, 2018). Hence, AMPA detection in soil samples only explains part of the total GlyP degradation process. The presence of AMPA provides no information about the fate of the C-P moiety.

High-Performance Liquid Chromatography (HPLC) is the most common technique for the detection of GlyP and AMPA from environmental samples. However, GlyP and AMPA need a derivatization step for their quantification by HPLC. This is because the molecular characteristics of GlyP and AMPA hinder their direct measurement by conventional detectors. FMOC-Cl is a chromophore molecule commonly used as a derivatization reagent for GlyP and AMPA. As a derivitisation agent, FMOC-Cl binds with the amino group of GlyP and AMPA enabling their detection via MS and fluorescence detection (Druart, Delhomme, de Vaufleury, Ntcho, & Millet, 2011).

However, the HPLC-FMOC-Cl method does not detect the tertiary metabolites of GlyP, such as phosphonoformaldehyde, which have already lost the amino group through enzymatic cleavage. This precludes the analysis of tertiary metabolites by FMOC-Cl derivatization. Therefore, phosphonoformaldehyde and other GlyP's tertiary metabolites remain in the soil undetected by conventional methods, and this may represent a potential hazard due to the stability of the C-P molecule in the environment (Zhan et al., 2018).

The phosphonic acid has the C-P moiety, which is stable in alkaline and acid conditions. Phosphonic acid is potentially persistent in the soil because not all soil microorganisms possess the enzyme C-P lyase to cleave this functional unit (Ternan et al., 1998). Under controlled conditions, phosphonic acids can be used for the synthesis of DNA nucleotides for medical purposes. This occurs because phosphonic acid has the ability to take the place of the PO_4^{3-} molecule in DNA (Shen & Hong, 2018). Thus, the potential persistence of the phosphonic moiety in the soil could increase its plant uptake, which might be disadvantageous for the safety of the food chain.

Strategies for GlyP remediation are therefore important to reduce the persistence of this chemical in the environment. There are a diverse range of potential GlyP remediation strategies, however *in situ* biodegradation is considered as the most cost-effective and efficient remediation strategy to reduce the presence of GlyP in the environment (Zhan et al., 2018). The use of plant extracts as soil amendments for GlyP remediation can be feasible, because plant constituents may promote microbial proliferation, which could enhance GlyP degradation. The plant constituents of Agave include soluble sugars, organic and phenolic acids (Ribeiro, Barreto, & Coelho, 2015), which are similar to the compounds excreted by soil microorganisms and plant roots to improve phosphorus uptake from the soil (Bhatti, Comerford & Johnston, 1998). Studies have demonstrated that exogenous organic acids applied to the soil increased phosphorus plant uptake and biomass yields (Bolan et al., 1994).

Taking into consideration the potential for GlyP accumulation in soil and the properties of Agave constituents, a key hypothesis of this experiment is that the use of Agave by-products as soil amendments may reduce GlyP accumulation in the soil. Agave's soluble sugars may enhance microbial growth that would promote GlyP degradation. Agave's organic and phenolic acids may increase phosphate bioavailability, which is a limiting nutrient for soil microorganisms growth. Increasing microbial phosphate uptake would as a consequence reduce GlyP displacement from the soil and eventually facilitate the biodegradation of GlyP's phosphonic moiety. Overall, Agave amendments may help to reduce the GlyP residence time in the soil and its potential toxicity to crops.

In order to elucidate the effect of residual GlyP in the rhizosphere, Roundup doses were applied directly to the soil of one-month old white clover potted plants, in an experiment which simulated the accumulation of the herbicide. Two glasshouse experiments with white clover potted plants were conducted. The aim of the first experiment was to investigate the immediate effect of Agave amendments in combination with four Roundup doses on GlyP degradation in the soil and the metabolic responses of white clover. The second experiment aimed to investigate the effect of application of Agave amendments in combination with two Roundup doses on GlyP degradation in the soil, and the metabolic response of white clover over three days of evaluation.

5.2 Materials and Methods

5.2.1 Glasshouse set-up for two pot experiments

The Pallic soil collected from the top 7.5 cm of the soil described in Chapter 3.1 was used for this experiment. The soil was air-dried and homogenised with a mortar and pestle, and passed through a 4mm sieve. Soil sub-samples (150 g) were placed in black plastic containers of 5cm height with an effective volume of 125 cm^3. The experiment was carried out in a glasshouse at the Plant and Growth Unit, Massey University. The soil moisture content was kept at 80% pot field capacity. The pots were incubated for one week to reactivate microbial activity and to settle air porosity. White clover (*Trifolium repens*) Apex var. seeds were sowed at the end of incubation period at top 0.5 cm depth. The experimental treatments were applied after one-month of the seed germination.

GlyP and AgvE amendments doses: The agronomical parameters used to calculate GlyP doses per treatment using the commercial herbicide Roundup 540, were based according to the work of Nguyen, Rose, Rose, and van Zwieten (2018). Parameters use in the dose calculations were: a soil density of 1.27 ton/m^3; GlyP penetration depth of 50 mm; and a maximum recommended commercial dose of 7.5 kg a.i. /ha of Roundup 540, unit application rate of 12.96 µg GlyP/g soil (1.944 mg GlyP/pot 150g) for the 7.5 kg a.i./ha dose (Table 5.1) was calculated. The unit dose was multiplied by dosing factors (x Times Dose) to increase the rate of applied treatment. GlyP doses (5mL) were applied with a pipette directly to the soil around the stem of one-month old white clover plants.

The AgvE amendments were prepared from Agave leaf powder aqueous extract according to Chapter 3.7.1: AgvE amendments (5mL) were applied with a pipette directly to the soil before the GlyP doses. For treatments without AgvE amendments, water was applied as a control.

Table 5.1. Glyphosate doses applied using the commercial brand Roundup concentrated 540 g a.i./L.

xTimes Dose	kg a.i./ha	mg GlyP/Pot	µg GlyP/g
1	7.5	1.944	12.96
2	15	3.888	25.92
6	45	11.664	77.76
8	60	15.552	103.68
12	90	23.328	155.52

First experiment: Immediate (12 hrs) metabolic response of white clover and GlyP degradation in soil on elevated GlyP doses and AgvE amendment

Four different high GlyP application rates of 15, 45, 60 and 90 kg glyphosate/ha (Table 5.1) were separately applied with and without 5 mL AgvE amendment. In total this experiment consisted of 10 treatments; the GlyP doses (4); GlyP+AgvE (4); AgvE alone (1) and a control without GlyP or AgvE amendment (1). The experimental unit was one plant/pot with four replicates (n=4). White clover shoot biomass was harvested 12 hours after treatment application, and soil from the pots was sampled for GlyP and AMPA analysis.

Second experiment: GlyP degradation in soil and metabolic responses of white clover on two GlyP doses and AgvE amendments over a three-day period of evaluation

Two GlyP doses of 7.5 and 15 kg glyphosate/ha were separately applied with and without AgvE amendments. This experiment consisted of six treatments: GlyP (2); GlyP+AgvE (2); AgvE (1) and a control without GlyP or AgvE amendments (1). The white clover shoots were harvested at 24, 48 and 72-hour intervals after treatment application, and bulk soils of the pots were sampled each day for GlyP, AMPA and dissolved carbon and nitrogen analysis. The experimental unit was one plant/pot with four replicates (n=4) of treatment per sampling day.

5.2.2 Plant, soil sampling and analyses in both experiments

After conclusion of the respective experiment, bulk soils from the pots were air-dried and homogenised with mortar and pestle, then passed through a 2 mm sieve before storage in plastic bags until analysis. GlyP and AMPA ($NaPO_4$ extractable) extraction was performed as described in Chapter 3.4 and 3.5. Dissolved carbon and nitrogen extraction and analysis were performed as described in Chapter 3.2. White clover's fresh shoot biomass was immediately stored at -80 C after collection, then freeze dried and weighed. Thereafter, stored in sealed plastic bags at room temperature until metabolomic analyses were performed.

Glyphosate and AMPA detection from soil samples: GlyP and AMPA were quantified, the extraction and quantification in both experiments were carried out as explained in Chapters 3.4 and 3.5; thereafter, this extraction method will be referred as total extractable GlyP and AMPA ($NaPO_4$ extractable). In addition, only in the first experiment of this Chapter was measured the potential plant-available GlyP and AMPA; briefly, soil extraction was performed by shaking soils solution (soil: extractant 1:10 v/w) with 0.5 M $NaHCO_3$ at pH 8.4 for 30 mins, the following procedures for quantification were similar as explained in Chapters 3.4 and 3.5.

5.2.3 Metabolomics' analysis of white clover plant samples.

Glyphosate and AMPA detection from white clover plant samples from first experiment: Glyphosate and AMPA extraction, purification and derivatization of white clover shoot samples was performed according to the work of Schrübbers, Valverde, Strobel, and Cedergreen (2016) and Tong et al. (2017) with modifications. Approximately 0.5 g of dry biomass was placed into 15 mL Falcon tubes, 9 mL of DI water and 1 mL of 1M HCl was added, and the biomass extracted overnight (16 hrs) in an end-over-end shaker. The solution was centrifuged at 5,000 RPM for 5 minutes. Five mL of supernatant was collected in a Falcon tube and the pH adjusted to 9 with 400 µL of NaOH 1M. The sample was fractionated by adding 2.5 mL of

dichloromethane and shaken by vortex, then left to stand until two phases were formed. The aqueous phase was collected in scintillation vials for derivatization.

Four mL of the aqueous phase was derivatized with FMOC-Cl 17.5 mM diluted in ACN (2.25 g FMOC-Cl/1000 mL ACN). One mL of borate buffer (50mM) and 1 mL of FMOC-Cl 17.5 mM/ACN were added, and gently shaken. The reaction was allowed to stand for 2 hrs, and thereafter the reaction was stopped by fractionating with 2.5 mL of dichloromethane. The non-polar organic phase was discarded, and the aqueous phase was filtered through 0.22 µm PTFE syringe filter and placed into a HPLC vial.

Sequential extraction with MetOH 50% and acetone 70% of plant samples: Plant extract was performed according to Melini et al. (2019) with modifications. About 0.02 g of white clover dry biomass was placed into 2mL Eppendorf vials, and 1mL of MetOH 50% added. The suspension was shaken overnight (16 hrs) in an end-over shaker. The samples were centrifuged at 1,000 RPM for 5 minutes, and 500 µL of supernatant was collected.

After the MetOH 50% extraction, the wet plant biomass was re-suspended in 1mL of 70% acetone, and the samples were shaken overnight (16 hrs) in an end-over shaker. The samples were centrifuged at 1,000 RPM for 5 minutes. One mL of the supernatant was collected and mixed with the MetOH 50% extract. The combined plant extract was diluted 1:1 v/v with MetOH 50%, then was filtered through a PTFE 0.22 µm syringe filter and placed into a HPLC vial. The samples were analysed for phenolic acids and organic acids by HPLC according to the methods outlined in Chapter 3.7.

5.3 Results and Discussion

5.3.1 Effect of GlyP doses and AgvE amendment on GlyP degradation in soil

First experiment: Immediate (12 hrs) GlyP degradation in soil with elevated GlyP doses and AgvE amendment

There was an increase in the total extractable GlyP ($NaPO_4$-extractable) concentration in the soil proportional to the Roundup treatments alone (no AgvE added) (15 kg GlyP to 90 kg GlyP), with the GlyP concentration ranging from 1.07 to 2.93 µg GlyP/g soil (Table 5.2). The GlyP concentration was statistically significant different between the treatments of 15, 45 and 60 kg GlyP. However, the treatments of 60 and 90 kg GlyP were statistically similar in their GlyP concentration (Table 5.2).

From the treatments 15 kg GlyP to 60 kg GlyP there was a significant ($p<0.05$) increase in the total extractable AMPA concentration from 0.64 µg AMPA/g soil at the treatment of 15 kg GlyP, to a maximum AMPA concentration of 2 µg AMPA/g soil at the 60 kg GlyP (Table 5.2). At the highest treatment of 90 kg GlyP the AMPA concentration dropped to 1.42 µg AMPA/g soil, despite the higher rate of GlyP dose. These results suggest that the highest dose of 90 kg/ha might reduce the GlyP degradation to AMPA. This might be a result of the potential toxicity of additives used in Roundup's formulation, which can have greater toxic effects to soil microorganisms than the active ingredient (Nguyen et al., 2018). The Polyethoxylated tallow amine surfactant used in a diverse range of herbicides, including Roundup, is toxic to aquatic organisms due to its ability to disrupt the integrity of cell membranes (Brausch, Beall & Smith, 2007).

If toxicity caused by the highest Roundup dose reduced the concentration of AMPA, then a possible combined toxic effect between Roundup's additives and the AgvE amendments might reduce microbial GlyP degradation to AMPA at higher GlyP doses than the maximum commercial recommended rate (7.5 kg a.i. /ha). A significant reduction of AMPA concentration from 1.36 to 1.01 µg AMPA/g soil was observed for the treatment 45 kg/ha with AgvE amendment (45 kg/AgvE) relative to the 45 kg GlyP /ha treatment. While lower the AMPA concentration observed in the treatments 60

kg/AgvE and 90 kg/AgvE compared to the same doses without AgvE amendment was statistically the same, which can be interpreted as a null effect of the AgvE amendment on GlyP degradation (Table 5.2) at these higher rates. Agave leaves contain significant amounts of saponins (Ribeiro et al., 2015), which can have cytotoxic and molluscicidal effect due to its capacity to disrupt cell membranes (Abdel-Gawad et al., 1999; (Yokosuka & Mimaki, 2009). In combination with GlyP, AgvE's saponins might reduce microbial activity.

Table 5.2. Glyphosate and AMPA concentration (mg/L) extracted from soil with $NaPO_4$ (total-extractable); after 12 hrs of the application of high Roundup doses from 2x to 12x times the maximum recommended dose of 7.5 a.i. kg/ha, alone and combined with AgvE amendment.

	$NaPO_4$-extractable			
Treatment	Glyphosate		AMPA	
90	2.93	ab	1.42	c
90/Agv	3.07	a	1.53	bc
60	2.86	ab	2	a
60/Agv	2.57	bc	1.75	ab
45	2.41	cd	1.36	c
45/Agv	2.33	cd	1.01	d
15	1.07	e	0.64	e
15/Agv	2.01	d	0.97	d
MSD	0.406		0.31	

*Mean values (n=4) in the same column followed by different letter are statistically significant according to Tukey's test ($p<0.05$). MSD, minimum significant difference.

An alternative explanation that contrasts with the combined toxicity exerted by Roundup and AgvE is that at Roundup doses from 45 kg/AgvE to 90 kg/AgvE, where the AgvE did not increase the AMPA concentration compared to the GlyP treatments alone (Table 5.2), microbial proliferation as a result of AgvE amendment increased nitrogen demand. In this scenario, soil microorganisms may have metabolised the amino group from AMPA, which inhibited its detection by the HPLC method used in this work which relies on the derivatisation of the amino group with FMOC-Cl (Druart et al., 2011).

The potential loss of the amino group due to microbial proliferation would similarly reduce the concentration of GlyP in the HPLC-FMOC method used. The treatments 45 kg/AgvE and 60 kg/AgvE had statistically similar GlyP concentrations, and the same pattern was observed between the treatments 45 kg/AgvE and 15 kg/AgvE, which had statistically similar GlyP concentrations, despite the differential rate of GlyP concentration applied. In comparison, the treatments without AgvE amendment from 15 kg GlyP to 60 kg GlyP had an increasing concentration where statistical differences ($p<0.05$) were apparent between the treatments.

This same scenario described for AgvE amendment could potentially have occurred at the highest Roundup dose of 90 kg GlyP. Surplus exogenous C from this dose may have triggered microbial proliferation enhancing the simultaneous degradation of GlyP and AMPA (Wardle & Parkinson, 1990), which was recorded in the data set by a reduction of GlyP and AMPA concentration at the highest GlyP dose, to a similar (GlyP) or lower (AMPA) concentration than the treatment 60 kg GlyP/ha.

These speculations should be considered for future research as AMPA is only a secondary metabolite of GlyP degradation. Potentially hazardous GlyP tertiary metabolites such as phosphonoformaldehyde that lacks the amino group, might remain undetected in the soil by the HPLC-FMOC-Cl method, which is the most commonly used method for research into GlyP in soil (Zhan et al., 2018).

There was a readily apparent effect of the AgvE amendment on the treatment 15 kg in contrast to higher Roundup doses. Soil amendment with AgvE doubled the analysed GlyP concentration ($p<0.05$) from 1.07 µg GlyP/g soil to 2.01 µg GlyP/g (15 kg/AgvE) (Table 5.2). This result suggests that AgvE constituents such as organic acids and phenols may reduce GlyP adsorption to soil surfaces increasing its extractable or solution concentration (Bolan et al., 1994; Kovacik et al., 2011)

The effect of the AgvE amendment on GlyP degradation was clear at the dose of 15 kg/ha where AgvE significantly ($p<0.05$) increased the extractable AMPA concentration in soil by 0.33 µg/g soil, (from 0.97 µg AMPA/g soil to 0.64 µg AMPA/g soil) (Table 5.2). This result suggests that the AgvE constituents (organic acids and sugars) might have promoted soil respiration enhancing GlyP degradation (Nguyen et al., 2018). In conjunction with desorption of GlyP from soil particles effected by AgvE, the treatment increased GlyP degradation to AMPA at this Roundup dose. The data

suggests that for a Roundup dose close to the maximum recommended commercial dose, AgvE is a soil amendment which can improve GlyP degradation to AMPA. However, Roundup rates above the maximum recommended dose can possibly reduce AgvE amendment efficiency, exerting a combined toxic effect to soil microorganisms. Alternatively, the higher Roundup doses in combination with AgvE promoted microorganism proliferation, and the loss of the amino group reduced the ability of the analytical protocol used to detect GlyP and AMPA.

Second experiment: GlyP degradation in soil over a three-day period

The analysis of GlyP and AMPA over a three-day period showed that GlyP concentration measured on day one across the treatments 7.5 kg GlyP, 15 kg GlyP and 7.5 kg/AgvE was statistically the same with a range from 0.73 to 0.98 µg GlyP/g soil. The GlyP concentration for the treatment 15 kg/AgvE on Day 1 was significantly ($p<0.05$) lower (0.68 µg GlyP/g soil) than both of the treatments without AgvE (Figure 5.1). The treatments GlyP alone (no AgvE added) on Day 1 showed an increasing concentration of AMPA ($p<0.05$) proportional to the Roundup concentration applied. In contrast, both treatments with AgvE amendment were statistically the same. (Figure 5.1, Day 1).

On Day 2, the GlyP concentration reduced for all treatments with and without AgvE (ranging from 0.47 at 7.5 kg GlyP, to 0.86 µg GlyP/g at 15/AgvE) when compared to Day 1. Similarly, the AMPA concentration dropped on Day 2 (ranging from 1.08 at 7.5kg GlyP, to 1.57 µg AMPA/g at 15 kg GlyP). These results suggest a reduction in GlyP degradation. The average AMPA levels for all treatments dropped to 1.33 µg/g on Day 2, compared to Day 1 with an average of 1.59 µg/g (Table 5.3). On Day 2 the average AMPA concentration was inversely proportional to dissolved total carbon (TC); highest TC of 137.7 mg TC/L ($p<0.05$) was observed on Day 2 relative to that on Days 1 and 3 (Table 5.3).

In contrast, the highest ($p<0.05$) average GlyP degradation concentration of 2.58 µg AMPA/g soil and a reduced TC of 105.7 mg C/L was observed on Day 3 (Table 5.3). The low TC concentration on this day demonstrated a high utilisation of carbon due to

microbial proliferation, which enhanced GlyP degradation. In contrast, the high TC found on Day 2 was the result of an initial fast turnover of organic matter as a result of GlyP degradation on Day 1, followed by low microbial activity reducing the subsequent requirement for carbon. The reduced microbial activity on Day 2 was reflected in the parameters analysed, such as greater TC and low AMPA concentration. With a similar effect, the total dissolved nitrogen (TN) concentration was high on Day 2 compared with Days 1 and 3. Nitrogen is in high demand by soil microorganisms; hence, its high availability on Day 2 was evidence of low microbial activity (Hessen et al., 2004).

The analysis of GlyP's metabolites and soil organic matter mineralisation can help to elucidate the effect of the herbicide on the growth kinetics of soil microorganisms. The trend observed between AMPA, TC and TN across the three days implied cyclic microbial growth, with an initial proliferation phase, followed by a declining phase. (Kovarova-Kovar & Egli,1998). An assumption is that a proliferation phase would subsequently follow.

On Day 3 the highest average concentration of GlyP (1.9 µg/g) and AMPA (2.58 µg/g) was recorded in the soil when the data is compared to the previous days (Table 5.3). This demonstrates gradual GlyP desorption from the soil surface and continuous GlyP degradation. By Day 3 the AgvE amendment improved GlyP degradation in the 7.5 kg/AgvE treatment, with a higher ($p<0.05$) AMPA concentration of 2.89 µg AMPA/g soil, compared to 2.26 µg AMPA/g soil in the treatment 7.5 kg (Figure 5.1). This result demonstrates an enhancement of GlyP degradation exerted by the AgvE constituents.

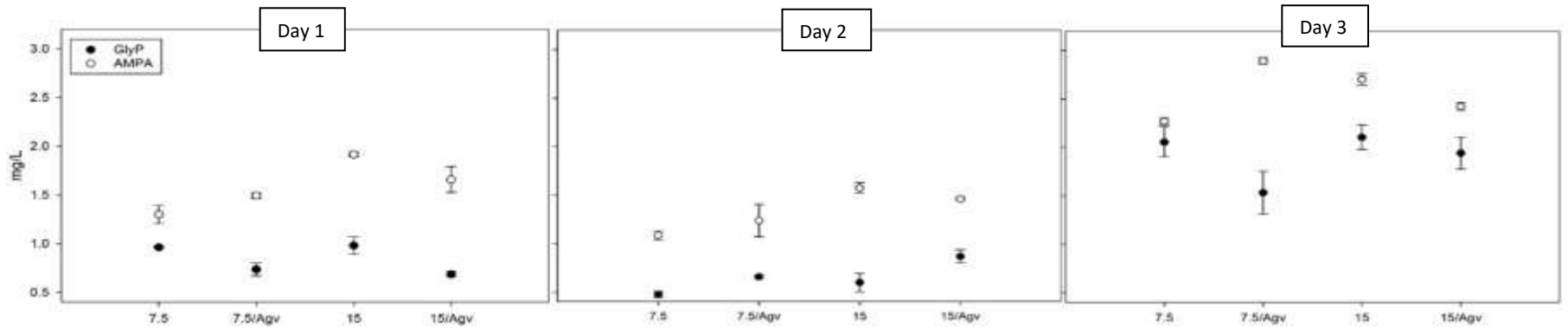

Figure 5.1 Glyphosate and AMPA concentration (mg/L) extracted from soil during a three-day period after Roundup treatments. Mean (n=4) Bars SE.

Table 5.3. Average GlyP, AMPA (µg/g soil), total dissolved carbon (TC); and total dissolved nitrogen (TN) (mg/L) concentration for all Roundup doses, extracted from soil over a three-day period of evaluation.

Day	Glyp		AMPA		TC		TN	
1	0.84	b	1.59	b	115.5	b	10.89	b
2	0.64	b	1.33	c	137.7	a	13.34	a
3	1.9	a	2.58	a	105.7	b	12.07	ab
MSD	0.23		0.18		11.51		1.42	

*Mean values (n=24) in the same column followed by different letter are statistically significant according to Tukey's test (α<0.05). MSD, minimum significant difference.

5.3.2 Metabolic responses of white clover to GlyP doses and AgvE amendment

A) Glyphosate accumulation in white clover shoot biomass

First experiment: Immediate response of white clover plants to Roundup doses and AgvE amendment:

GlyP and AMPA extraction and detection in white clover shoots was performed only in plants from the first experiment. A GlyP concentration of 0.245 µg/g biomass was detected in white clover shoots treated with 90 kg GlyP. A total concentration of 155.52 µg GlyP/g soil was applied as this treatment and assuming a GlyP adsorption rate equivalent to 87.12% for the Pallic soil (value calculated according to Chapter 4.3.2), about 68.48 µg of GlyP was potentially available in soil solution (5 cm depth) for plant uptake. Theoretically, a GlyP concentration of 16.56 µg/g soil was potentially bioavailable in the soil solution for the 60 kg GlyP treatment; however, there was no GlyP detected in the shoots of white clover plants growing on this treatment. This could be explained through immediate microbial activity on the GlyP molecule after application to the soil which reduced the bioavailability of the herbicide (Haney et al., 2002). The GlyP adsorption capacity of the Pallic soil might reduce tGlyP bioavailability in treatments with lower applied GlyP concentration.

Taking into consideration that GlyP and AMPA have a similar charge in soil solution to phosphate, potentially plant-available GlyP was investigated through extraction with $NaHCO_3$ (0.5M), which is an extractant used routinely for evaluating plant-available phosphate in soil samples. The concentration of potentially plant-available GlyP measured in the soil was statistically similar ($p>0.05$) for the treatments of 90 kg (1.13 µg/g soil) and 60 kg (1.24 µg/g soil) (Table 5.4). This plant-available GlyP concentration, measured after Roundup application, did not, therefore, explain the plant uptake of GlyP from only the 90 kg treatment.

GlyP was not detected in the white clover biomass for the treatment 90 kg/AgvE. Glyphosate degradation in the soil was quantified at around 0.13 µg AMPA/g higher in the 90 kg/AgvE treatment than the 90 kg treatment. Similarly, the GlyP concentration detected in the 90 kg/AgvE treatment was 0.18 µg GlyP/g lower than in the 90 kg GlyP (Table 5.4). These results indicate an effect of AgvE amendment on the reduction of GlyP bioavailability for plant uptake.

Table 5.4. Glyphosate and AMPA concentration (mg/L) extracted from soil with $NaHCO_3$ (plant-available) 12 hours after the application of high Roundup doses from 2x to 12x times the maximum recommended dose of 7.5 a.i. kg/ha, alone and combined with AgvE amendment.

Treatment	$NaHCO_3$-extractable			
	Glyphosate		AMPA	
90	1.13	a	0.22	b
90/Agv	0.95	abc	0.35	ab
60	1.24	a	0.38	ab
60/Agv	1	ab	0.41	a
45	0.61	bcd	0.34	ab
45/Agv	0.53	cd	0.42	a
15	0.42	d	0.19	b
15/Agv	0.53	cd	0.19	b
MSD	0.443		0.192	

Tong et al. (2017) evaluated GlyP doses of 5, 50, 200 and 2000 mg/L fed through hydroponic solution over 14 days to tea plants, and found GlyP phytotoxicity with GlyP doses of 200 and 2000 mg/L, but no toxic effect at 5 and 50 GlyP mg/L doses. At the 5 mg/L GlyP dose most of the GlyP uptake detected by the authors was in the tea plant's roots with a concentration of 113.54 mg GlyP/kg biomass at the beginning of the experiment, while translocation to mature leaves was 0.35 mg GlyP/kg biomass. The authors observed a maximum accumulation after 5 days, with concentrations of 294.87 and 14.49 mg GlyP/kg biomass, for roots and mature leaves respectively.

In the current experiment, the GlyP dose of 155.52 µg GlyP/g soil was inferior to the 200 mg/L used by Tong et al. (2017). In addition, in this experiment the GlyP dose was applied directly to the soil, which created the opportunity for immediate GlyP adsorption onto soil surfaces and degradation of the herbicide through microbial activity, which would reduce GlyP plant uptake compared to a hydroponic system where the GlyP molecule is readily-available for plant uptake. In the current experiment, GlyP plant uptake was evaluation on Day 1 only, with a mean concentration of 0.245 µg GlyP/g dry biomass of white clover shoot biomass, which

was in between the concentration range observed on Day 1 (0.35 mg GlyP/kg biomass) for mature tea leaves in the study of Tong et al. (2017).

The results of the present experiment suggest that the translocation of GlyP to white clover shoots is negligible at the recommended commercial rates of Roundup, or even higher, when it is applied to the soil surface. However, in this experiment the uptake of GlyP by white clover roots was not measured. Similarly, detection of tertiary GlyP's metabolites such as phosphonoformaldehyde was out of the scope of this research. This compound may be recalcitrant due to its C-P bonding, cannot be decomposed by a great diversity of soil microorganisms, and represents a potential risk for plant uptake.

B) Gallic acid evaluation in white clover shoot

First experiment: Immediate accumulation of gallic acid by white clover plants treated with Roundup and AgvE amendment

From the three standards of phenolic acids analysed, only gallic acid (GAL) was detected in the plant samples. The GAL concentration of the white clover shoot biomass increased after the application of the Roundup and AgvE amendment treatments. The GAL concentration for the lowest and highest treatments of GlyP (15 kg and 90 kg), with and without AgvE was statistically the same, ranging between 0.4 to 0.38 GAL mg/g, in comparison to the average concentration in the control plants (0.31 mg GAL/g biomass; Figure 5.2). The treatment 60 kg/AgvE amendment had the highest ($p<0.05$) average concentration (0.43 mg GAL/g) compared to the control, but the concentration reduced at the highest dose of 90 kg GlyP. The GAL concentration of the AgvE amendment alone was 0.37 mg GAL/g, significantly higher than the control (Figure 5.2).

These results demonstrated that the Roundup treatments from 15 to 60 kg alone and in combination with AgvE amendment increased GAL in the white clover shoot. This may be due to oxidative damage exerted on the plant. Plants over synthesises phenolic compounds such as GAL in order to reduce oxidative damage caused by abiotic or biotic stress (Nicholson, 1992; Sirin & Aslim, 2018). The cause of this

synthesis may not be GlyP; additives in Roundup can have greater toxicity to living organisms than the GlyP molecule (Brausch, Beall & Smith, 2007).

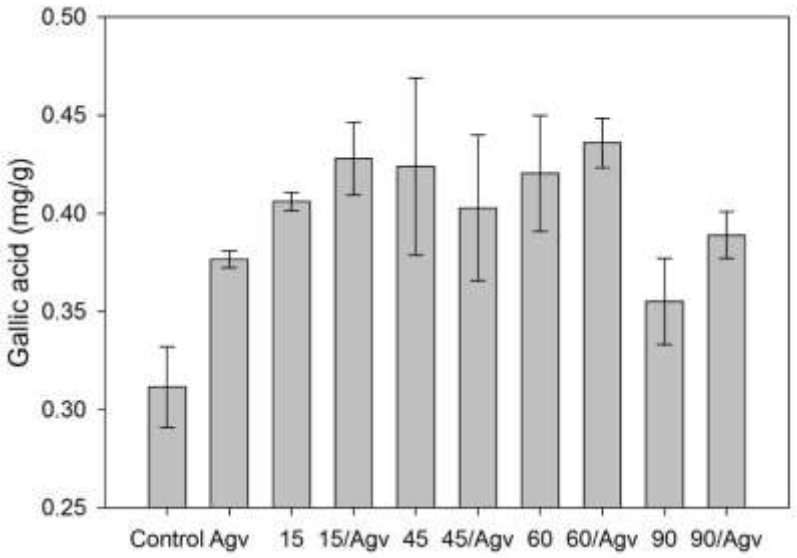

Figure 5.2. Gallic acid concentration (mg/L) extracted from white clover shoots, 12 hrs after the application of high Roundup doses from 2x to 12 x times the maximum recommended dose of 7.5 a.i. kg/ha, alone and combined with AgvE amendment. Mean (n=4) bars SE.

Alternatively, If GlyP root uptake occurred (not analysed in this experiment), GAL accumulation might indicate the potential disruption of the shikimate pathway. GAL is synthesised before the action of the enzyme EPSP in the shikimate pathway, and this enzyme is the main target of GlyP. The functionality of the EPSP enzyme can be compromised by GlyP uptake, and EPSP disruption can be expressed as accumulation of GAL in plant tissues (Zabalza et al., 2017).

However, it is not clear if the GAL accumulation responses observed in the 15 to 60 kg treatments were the effect of the GlyP root uptake or oxidative stress caused by the Roundup additives in combination with AgvE. The AgvE amendment treatment also increased GAL accumulation indicating that both Roundup and AgvE constituents enhanced GAL accumulation in the white clover shoot biomass.

For the treatment 90 kg GlyP, where GlyP uptake was detected in white clover shoot biomass, GlyP distribution through the plant could be related to the reduction in GAL concentration effected by this treatment. This could suggest that GlyP affected white clover metabolism beyond the EPSP enzyme activity, which infers a disruption of the

shikimate pathway. GlyP uptake possibly interfered with the enzymes involved in the synthesis of GAL precursors, which reduced its production. GlyP has been shown to reduce the efficiency of other enzymes apart of EPSP, such as the nitrogenase enzyme of the nitrogen-fixing bacteria (Fan et al., 2017). Glyphosate has also been shown to inactivate superoxide dismutase and glutathione peroxidase in fish (Modesto & Martinez, 2010). Glyphosate's chelating properties could even disrupt ion transport or other metabolic processes not necessarily involved with enzymatic activity (Nowack, 2003), which might reduce GAL synthesis. Donnini et al. (2016) observed a significant reduction of anthocyanins in grapevine berry skin due to the residual effect of a GlyP application of 2.4 kg/ha of Roundup Bioflow targeting weeds.

The AgvE amendment in the treatment 90 kg/AgvE increased the GAL concentration compared to Roundup dose 90 kg alone. However, the AgvE amendments did not influence metabolic activity for the Roundup doses from 15 to 60 kg, indicating the effect of AgvE was relevant only in extreme conditions such as in the 90 kg treatment.

Second experiment: evaluation of gallic acid concentration in white clover shoot biomass over a three-day period

The treatments 15 kg/AgvE and 7.5 kg/AgvE a day after application yielded a higher ($p<0.05$) GAL concentration of 0.406 and 0.376 mg/g, respectively in comparison to the control. The control and AgvE treatment were statistically similar to the treatments of 7.5 kg GlyP and 15 kg GlyP, with a GAL concentration ranging from 0.311 to 0.353 mg/g (Figure 5.3). These results demonstrated a combined effect between AgvE and Roundup enhanced GAL accumulation in white clover shoots.

At the second day an opposite effect was observed relative to the first day. The GAL concentration dropped in all treatments and ranged from 0.37 to 0.35 mg GAL/g. The AgvE only treatment and the control had a higher GAL concentration of 0.43 mg/g and 0.38 mg GAL/g, respectively on Day 2. These results suggested that the AgvE amendment treatment could cause oxidative stress in white clover plants. It is possible that GAL was taken up by plants in the AgvE treatment, as another possible reason for this result. The GAL concentration of the AgvE aqueous extract was analysed by HPLC and the average GAL concentration was 0.112 mg GAL/ g agave leaf powder (Chapter 3.7.1). Rizwan et al. (2012) reported a range of 0.1054 to 0.3935 mg/g

concentration of total phenolic compounds for *Agave attenuata*. Kroyer (2004) reported a total phenolic concentration in red clover *Trifolium pratense* of 153 mg/g. In the current experiment, about 0.018 mg GAL/pot was potentially available for plant uptake and in combination with the white clover's synthesis of phenols; this combined effect might have increased GAL accumulation.

GAL accumulation from the AgvE amendment treatment occurred over Days 1 and 2, whereas the GAL concentration for the 7.5 and 15 kg GlyP treatments reduced on Day 2 and remained stable on Day 3 relative to the control. Overall, potential stress caused by both the GlyP doses and AgvE amendment was alleviated by Day 3 when no significant metabolic responses were observed.

In both experiments, the AgvE amendment treatment increased the GAL concentration compared to the control. AgvE constituents potentially exerted an oxidative stress to white clover plants; studies have demonstrated that phenolic acids can interfere with indole-3-acetic acid metabolism in maize stems (Lee, 1980). Phenolic compounds such as GAL are over synthesised by plants under biotic or abiotic stress in order to overcome the presence of reactive oxygen species (ROS) (Sirin & Aslim, 2018; Nicholson, 1992). Studies have demonstrated that an exogenous phenolic compound, such as p-coumaric acid 0.25 mM, applied to white clover plants can enhance plant growth and mycorrhizal colonization, but a higher concentration of 1mM p-coumaric acid (1mM of GAL = 170.12 mg GAL/L) will cause growth inhibition (Fries et al., 1997). In the present study the amount of GAL applied from the AgvE amendments was 0.018 mg GAL per pot, which is lower than the GAL accumulated by the white clover plants of around 0.33 mg GAL/g. However, AgvE constituents includes a suite of organic acids, phenolic acids, saponins and other compounds that might have influenced white clover's metabolic response.

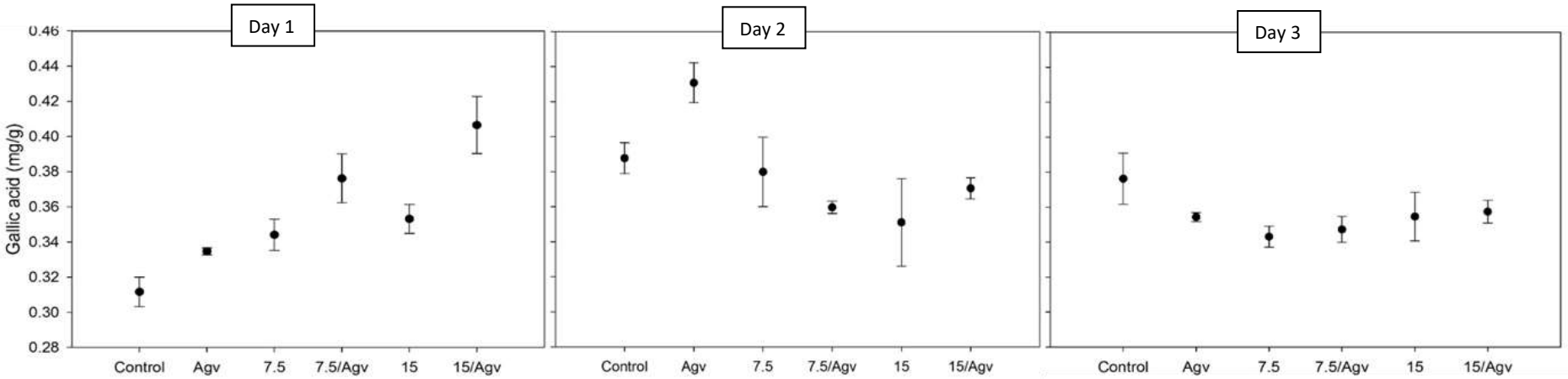

Figure 5.3 Gallic acid concentration (mg/g) extracted from white clover shoot biomass, across three days of evaluation. Mean (n=4) bars SE.

C) Evaluation of the organic acid concentration in white clover shoot biomass.

First experiment: Evaluation of immediate organic acid accumulation in white clover shoots after treatment with Roundup and AgvE amendment

From the six organic acid standards analysed, only tartaric acid (TAR) was detected in the white clover shoot biomass. There was an increase in the concentration of TAR in plants proportional to the concentration of the GlyP treatments with and without AgvE from 15kg to 90 kg. The highest ($p<0.05$) TAR concentration was observed at the 90 kg GlyP/AgvE (3.02 mg/g), and the lowest at the 15 kg GlyP (1.95 mg TAR/g). The control treatment had a TAR concentration of 1.7 mg/g and the AgvE treatment increased TAR to 1.96 mg/g (Figure 5.4). In all the treatments, the TAR concentration was higher with the application of the AgvE amendment compared to the Roundup doses alone. These results show a combined effect of AgvE amendments and Roundup application on TAR accumulation in the white clover shoots.

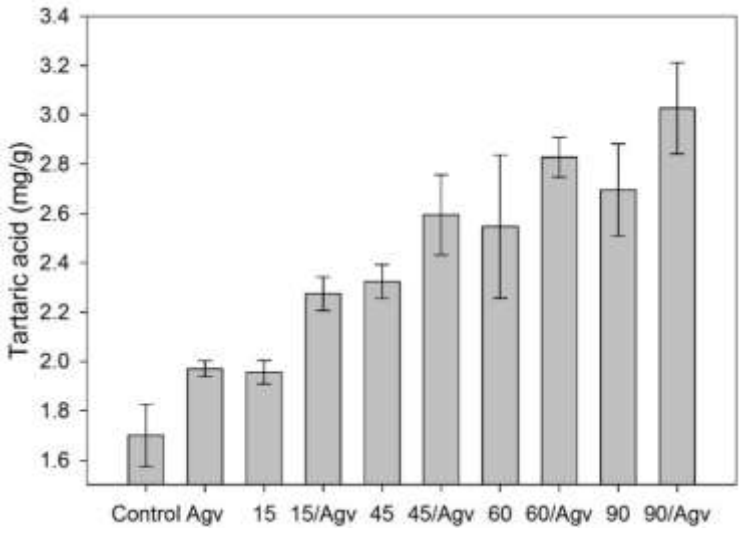

Figure 5.4 Tartaric acid concentration (mg/L) extracted from white clover shoots 12 hrs after the application of high Roundup doses from 2x to 12 x times the maximum recommended dose of 7.5 a.i. kg/ha, alone and combined with AgvE amendment. Mean (n=4) bars SE.

Second experiment: evaluation of the organic acid concentration in white clover shoot biomass over a three-day period

On the first day of evaluation the AgvE treatment had the highest ($a<0.05$) TAR concentration, followed by the treatments 7.5 kg/AgvE and 15 kg/AgvE, with a concentration of 1.86, 1.54 and 1.51 mg TAR/g biomass, respectively. The control had the lowest TAR concentration of 1.17 mg TAR/g. On Day 2, no significant differences were observed across the control and the treatments, with a similar response on Day 3 (Figure 5.5).

The results observed in both experiments demonstrated that the AgvE amendment had a significant effect on the accumulation of TAR in white clover shoots. The following water-extractable organic acids were detected from the Agave powder: oxalic, citric and succinic acid with an average concentration of 4.04, 86.15 and 162.92 mg/g biomass (Table 4.11). These concentrations correspond to a mass applied per pot for both experiments of 0.67, 14.35 and 27.15 mg/pot for oxalic, citric and succinic acid, respectively.

The elevated TAR concentration in the treatments with AgvE amendment suggest that plant uptake of organic acids potentially occurred. The AgvE amendment applied had a significant amount of succinic acid (27.15mg/pot) when compared to the other organic acids detected in the Agave powder. Succinic acid and TAR are carboxylic acids, and they participate as metabolic intermediaries between primary and secondary plant metabolism. Organic acids can balance a plant's metabolic functions under abiotic and biotic stress due to their chelating and antioxidant properties (Lopez-Bucio, 2000). TAR is an active hydrogen donor and acceptor (Ghosh & Adhikari, 2006). TAR and succinic acid are reciprocal derivatives; both molecules only differ because TAR has two more hydroxyl groups than succinic acid. Thus, it can be speculated that succinic acid was metabolised to TAR, as a donor acceptor of radical species for detoxification within the plant due to the oxidative stress caused by the treatments.

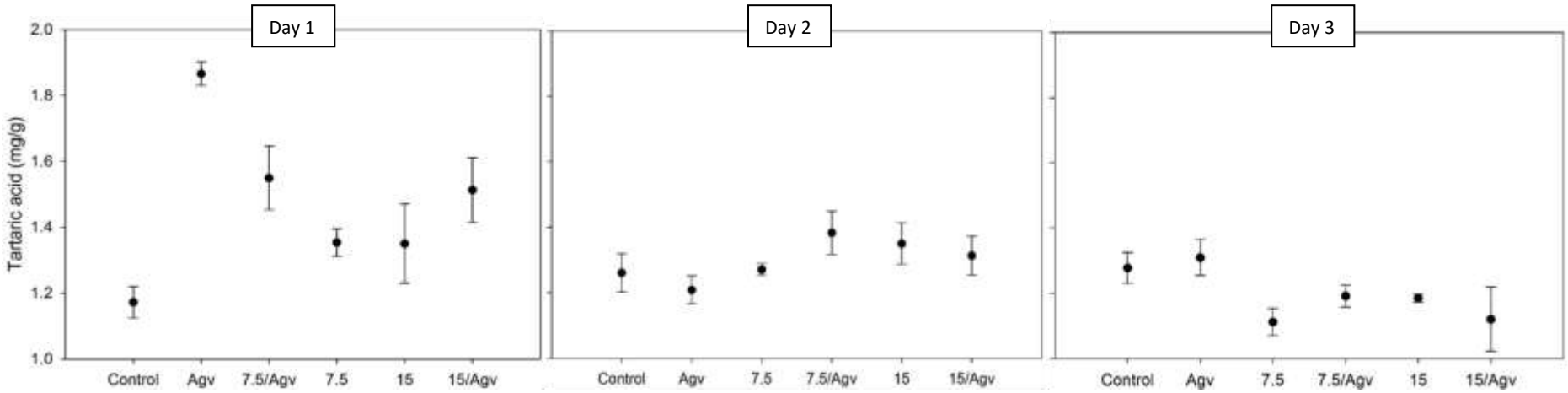

Figure 5.5. Tartaric acid concentration (mg/g) extracted from white clover shoot biomass, across three days of evaluation. Mean (n=4) bars SE

On the other hand, organic acid accumulation might be an indication of oxidative stress exerted by the combined effect of AgvE amendments and Roundup application on the plant. Organic acids play a significant role in stress signalling and detoxification (Lopez-Bucio et al., 2000). In the first experiment, TAR accumulation increased with increasing Roundup dosages regardless of the AgvE amendment. This response demonstrated that GlyP induced a metabolic impairment in the plant's carbon cycle. This effect was similar to the work done by Donnini et al. (2016), and they found greater titratable acidity in the skin of grapevine berries of plants treated with Roundup. The shikimate pathway is supplied by carbohydrates coming from the plant's primary metabolism, either from glycolysis products or from the pentose phosphate pathway, where organic acids are the intermediaries (Matsuki, 1996). The disruption of the EPSP enzyme may have led to accumulation of precursors metabolites before the action of the EPSP enzyme, including carbon compounds such as organic acids (Zabalza et al., 2017).

5.4 Conclusion

AgvE amendment increased the extractable AMPA concentration in soil at the Roundup dose of 15 kg. This observation demonstrates that AgvE promoted the immediate mineralisation of the herbicide. However, AgvE in combination with higher Roundup doses potentially exerted a synergistic toxic effect and reduced GlyP degradation to AMPA. The GlyP accumulation in the white clover shoot biomass was mitigated by the use of AgvE amendment at the 90 kg Roundup dose, however for all other treatments, including treatments more similar to commercial doses, there was no detection of GlyP in plant biomass. GlyP was only detected in the 90 kg Roundup treatment alone where a concentration of 0.245 µg GlyP/g dry biomass was detected in the white clover shoot biomass.

AgvE amendment improved GlyP degreadation in the soil over three days of evaluation at the maximum recommended Roundup dose of 7.5 kg/ha. Roundup doses alone, and in combination with AgvE amendment, enhanced the accumulation of gallic acid and tartaric acid, suggesting metabolic disruption. However, for Roundup

doses of 7.5 and 15 kg/ha metabolic stress was alleviated on Day 3 after treatment application. Overall, the use of AgvE amendments helped to reduce the occurrence of GlyP in the soil and its distribution in white clover shoot biomass. However, further research is required to elucidate the direct effect of AgvE amendments on soil microorganisms, plant metabolism and the occurrence of GlyP's tertiary metabolites.

6. Recommendations and further research

The results of this thesis can assist decision-making around farm management practices which aim to mitigate GlyP and AMPA displacement from the farmland. This thesis has contributed to the understanding of the interaction between soil properties and GlyP. Such understanding is crucial to help define appropriate GlyP rates and application periodicity according to crop and soil type in order to reduce potential GlyP bioavailability.

Phosphate and GlyP have similar sorption behaviour in the soil, and this can increase GlyP desorption because phosphate displaces the herbicide from soil surfaces. Therefore, appropriate measures to reduce phosphorus accumulation in farmland should be taken in order to reduce GlyP mobility, particularly when soils possess a reduced anion retention capacity. In addition, soil physical properties define water infiltration, which plays a significant role in the displacement pathway of the herbicide. Because GlyP is water soluble it can be transported by surface runoff, and in some cases leaching.

The results of this thesis demonstrated that the Pallic soil possessed a low anion retention capacity and high propensity for compaction, which may increase the risk of GlyP solubilisation and surface runoff losses from farmland. In contrast, the Brown soil with a free-drained structure and high chemical reactivity had high GlyP percolation. In this regard, the Brown soil possessed elevated anion retention capacity as a function of the Fe oxy-hydroxide fraction. High Fe reactivity enabled the adsorption, but also the eventual desorption of GlyP from the soil, and this can increase GlyP displacement from the soil. The Allophanic soil had the lowest GlyP displacement risk due to its greater anion retention capacity, which was dependant on the Al oxy-hydroxide fraction that formed more stable bonds with the herbicide than the Fe fraction. Phosphorus accumulation influenced the vertical displacement of GlyP. The results of this thesis demonstrated that phosphorus occupied the soil reaction sites increasing GlyP mobilisation risk.

The accumulation of GlyP and its degradation product in the farmland has boosted the development of GlyP remediation strategies. The results observed in this thesis have demonstrated that Agave amendments possess potential for enhancing the

degradation *in situ* of the herbicide through microbial decomposition. Biochemical profiling of Agave leaf powder helped to elucidate the effect of Agave components on the degradation of GlyP. Preliminary results demonstrated that Agave powder increased the desorption of phosphate from the soil. This can contribute to a reduction of P fertilisation requirements while at the same time promote P desaturation. Therefore, Agave amendment would promote GlyP immobilisation in the soil. Soil induced respiration results suggested that Agave amendments participated in the proliferation of soil microorganisms, which in combination with GlyP rates triggered soil induced respiration, implying greater microbial activity over the herbicide compared to soils without Agave amendments.

The results observed in the glasshouse experiment using one-month old white clover plants demonstrated that Agave amendment increased GlyP degradation to AMPA in the soil when using the maximum recommended Roundup rate of 7.5 kg a.i./ha and the doubled dose. In addition, Agave amendment nullified the detection of GlyP in the white clover shoot biomass for the Roundup dose of 90 kg a.i./ha; which is a promising effect of the Agave amendment for reducing the bioavailability of GlyP. Nevertheless, Agave amendment in combination with high Roundup doses of 45 and 60 kg reduced the detection of AMPA, possibly due to a synergistic toxicity caused to soil microorganisms. Alternatively, the observed reduction of AMPA could respond to N microbial uptake, which enhanced the presence of GlyP's tertiary metabolites.

The scope of this thesis was to analyse GlyP and its main degradation product AMPA, using the HPLC FMOC-Cl method; which relies on the derivatization of the amino group of GlyP and AMPA with the FMOC moiety. Following the nutrient uptake mechanisms of soil microorganisms, the high availability of carbon sources coming from the high Roundup doses and Agave amendment might trigger an increased requirement of nitrogen. If soil microorganisms removed (and utilised) the amino group of GlyP and AMPA, then the remaining metabolite phosphonoformaldehyde would be undectable by the method used in this thesis because of the lack of the amino group. Phosphonoformaldehyde has the phosphonic acid functional group and studies have demonstrated that the phosphonic acid C-P moiety can replace the phosphate group of nucleic acids under controlled conditions. Thus, analysis of this GlyP metabolite in environmental samples should be considered in further research, as most of the

studies currently only targets GlyP and AMPA. The residual effects of the C-P moiety in the soil remain uncertain and this is a major knowledge gap for GlyP studies.

White clover metabolism was disrupted by high Roundup doses alone and combined with Agave amendments. Tartaric acid and gallic acid accumulated in white clover shoot biomass could be a response to oxidative stress caused by the various components of these treatments. However, when using the Roundup doses of 7.5 and 15 kg the transient metabolic condition was alleviated after three days. Alternatively, the accumulation of gallic acid might be due to uptake of GlyP by plants which disrupted the shikimate pathway. However, this assumption remains uncertain because GlyP and AMPA was only analysed in white clover shoots not roots. Further research is needed to clarify the effect of the herbicide and Agave amendments on white clover metabolism. Similarly, the potential remediation effect of Agave amendment rates and application timing on perennial crops should be analysed and these represent a greater risks for GlyP uptake.

Overall, the results of this thesis can help in the development of effective farm management practices that reduce the potential displacement and bioavailability of glyphosate. Similarly, the results demonstrated that Agave has potential use for GlyP remediation. However, further research is required in order to clarify the effect of the herbicide on specific soil microorganisms, plant metabolism of a range of pastoral species and organs of those species, and the occurrence of GlyP's tertiary metabolites.

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APPENDIX

Appendix. Mean square and F values of ANOVA factorial analysis (Soil type and GlyP dose) from the experiment Soil induced respiration on Chapter 4.3.5

Factor*	TOC		TC		IC		TN		SIR	
	MS	F	MS	F	MS	F	MS	F	MS	F
Soil type*	10241.4	550.21	10218.68	552.41	0.01	0.42	8481.68	497.94	3.2	139.84
GlyP dose*	38.08	2.05	39.24	2.12	0.12	2.74	73.44	4.31	0.92	40.4
Model	2078.74	111.68	2075.13	112.18	0.1	2.27	1755.09	103.04	1.43	62.49
Error	18.61		18.49		0.04		17.03		0.022	

Appendix 1. Phosphorus adsorbed (mg P/ g soil) by soils after saturation using a 1,000 mg KH_2PO_4 solution. P desorbed (mg P/ g soil) after shaking one hour the P saturated soils with $CaCl_2$ 10mM. Mean (n=3)±SD.

Soils	P mg/g soil			
	P adsorbed	SD	P desorbed	SD
Pallic	0.710	±0.076	0.040	±0.003
Brown	3.319	±0.047	0.039	±0.002
Allophanic	5.038	±0.036	0.028	±0.001

*P adsorbed= [(total P added)-(residual P measured from saturation solution)]