Microbial degradation of trifluralin and atrazine residues in soil

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Certificate of authorship

I hereby declare that this submission is my own work and to the best of my knowledge and belief, understand that it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged.

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Paper 2

Chowdhury, I. F., Rohan, M., Stodart, B. J., Chen, C., Wu, H., & Doran, G. S. (2021). Persistence of atrazine and trifluralin in a clay loam soil undergoing different temperature and moisture conditions. *Environ. Pollut.*, 276, 116687. doi.org/10.1016/j.envpol.2021.116687.

References belong to both publications are included within respective chapters but not included in the main reference section.

Author contribution

The candidate contributed to the above-mentioned publications in peer reviewed journals as follows.

Research paper published in Agronomy

The candidate carried out the experiments followed by data collection and statistical analysis, prepared tables and graphs and first draft of the full-length research article. Necessary corrections and revisions suggested by supervisors and the reviewers were also done by the candidate.

Research paper published in Environmental Pollution:

The candidate carried out the experiments followed by data collection and initial statistical analysis, prepared tables and graphs and first draft of the full-length research article. Necessary corrections and revisions suggested by supervisors and the reviewers were also done by the candidate.

"I declare that the author contribution statements stated above are accurate, and I give permission to include the research papers in this thesis."

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Abstract

Pre-emergent herbicides play a crucial role in farming systems by providing early weed control for better crop establishment. However, their persistence in soil for extended period may lead to soil and groundwater contamination. Long term persistence of herbicide residues near the surface zone may affect rotational crops. Several factors namely, soil conditions, chemical structure of herbicides as well as application method and frequency regulate their breakdown in the soil. A recent Grains Research and Development Corporation (GRDC) survey around Australia detected residues of 23 pesticides persisting in soil; with atrazine and trifluralin being frequently detected in New South Wales (NSW) soils. As soil microorganisms are known to be highly adaptable in extreme environmental conditions, certain microorganisms can be used to degrade a range of pesticides in soil.

The first objective of this study was to determine critical concentration levels of trifluralin and atrazine in soil at which crops are susceptible. The second objective was to determine the effect of temperature and moisture conditions on the persistence of atrazine and trifluralin residues in a clay loam soil. The third objective was to determine the changes in microbial community structure and functions associated with various concentrations of trifluralin and atrazine application in clay loam soil.

To fulfil the first objective, critical concentrations of trifluralin and atrazine causing phytotoxic effects to crops were investigated using a bioassay technique with five test crop species including cereal and legume species. Sandy soil was used in this purpose to minimize the interference of organic matter binding herbicide particles so that the actual phytotoxic effects of trifluralin and atrazine can be investigated. Shoot and root parameters of the tested crop species were fitted in logistic equations against herbicide concentrations to calculate effective doses (ED_{50}) for 50% growth inhibition. Both herbicides affected shoot and root parameters of all the test crop species significantly. Trifluralin delayed crop emergence at lower concentrations (0.075 mg/kg) while it completely inhibited the growth of test crops at higher concentrations (2.40 mg/kg). However, atrazine had no significant effect on the crop emergence but drastically reduced overall crop performance. Legumes were comparatively more sensitive than cereals to both herbicides, while lucerne was the most sensitive crop species to both herbicides, with ED_{50} ranging from 0.01 to 0.07 mg/kg soil for trifluralin,

and from 0.004 to 0.01 mg/kg for atrazine. Based on the crop sensitivity, lucerne can be used in soil bioassay techniques to quickly determine trifluralin and atrazine residues in soil before sowing so that a suitable rotational crop can be chosen to minimize the negative impacts of herbicide on crops.

The environmental impact on the dissipation of trifluralin and atrazine in clay loam soil was further investigated in laboratory incubation experiments, under various temperature and moisture conditions. A stochastic gamma model was used to predict the dissipation of both herbicides from the clay loam soil by incorporating environmental factors as covariates to determine the half-life and the days to complete dissipation. Temperature played the crucial role on atrazine persistence while the combined effect of temperature and moisture was critical on trifluralin persistence in the clay loam soil. Rapid loss of atrazine was observed under 30 °C than 10 and 20 °C; with a half-life of 7.50 days and 326.23 days to reach complete dissipation. Trifluralin dissipation was maximum under 30 °C with a moisture content of 70% field capacity (FC); with an estimated half-life of 5.80 days and 182.01 days to reach complete dissipation. Half-life of both herbicides tended to double with every 10 °C drop of temperatures over the range tested. Gamma distribution model estimated that both trifluralin and atrazine could persist in the clay loam soil for several years at ≤20 °C temperature. Moreover, high temperature and moisture conditions reduced their persistence in the clay loam soil which was likely due to the changes in soil microbial community compositions.

The changes in soil microbial community compositions and their functions were evaluated under various concentrations of atrazine and trifluralin in soil using DNA extraction and 16s RNA sequencing. In addition, soil microbial respiration was also investigated under different concentrations of both herbicides. Both herbicides stimulated soil microbial respiration as maximum CO_2-C evolved was recorded under RD5x of the atrazine and RD2x of the trifluralin. The relative bacterial abundance was highest under these concentrations due to promotion of dominant bacterial population. The relative abundance of phylum *Firmicutes* clearly elevated with increasing atrazine concentrations at 20 and 40 DAT, whereas relative abundance of phylum *Actinobacteria* was maximum under RD2x at 40 DAT. On the other hand, trifluralin promoted relative bacterial abundance at later stages (40 DAT) while no change was observed until 20 DAT. The relative abundance of phylum *Actinobacteria* was maximum under RD5x whereas,

relative abundance of *Firmicutes* was increased under RD2x at 40 DAT. At genus level, atrazine and trifluralin stimulated and supported the growth of genus *Bacillus*. Changes in microbial composition upon exposure to atrazine and trifluralin inhibited some bacterial groups due to toxic effects. Atrazine and trifluralin suppressed relative abundance of genus *Kaistobacter* from *Proteobacteria* phylum with increasing concentrations upon exposure. Atrazine had significant effect on overall bacterial diversity whereas effect of trifluralin was nonsignificant. These results conflicted with BIOLOG results as overall soil microbial diversity functions were suppressed by both herbicides studied. This is most likely due to the limitation of BIOLOG technique primarily depending on the fast-growing microorganisms for the colour development while ignoring catabolically inactive and other non-culturable microorganisms. Considering other environmental, chemical and soil properties, the changes in microbial compositions in soil under higher temperature and moisture conditions could promote herbicide degradation by increasing the abundance of some bacterial groups, even though some other groups were inhibited. However, lower temperature and moisture conditions could increase persistence of trifluralin and atrazine in clay loam soil long enough possibly due to reduced microbial activity. Farmers should remain cautious about the carryover potential of trifluralin and atrazine before selecting crops in rotation. Soil should be tested for persistence of suspected herbicides through bioassay or laboratory detection techniques, especially under prolonged drought conditions.

List of abbreviations

$	Dollar
%	Percentage
°C	Degree Celsius
µg	Microgram
2,4-D	2,4-dichlorophenoxyacetic acid
a.i.	Active ingredient
AGRF	Australian Genome Research Facility
AGRTP	Australian Government Research Training Program
AIC	Akaike information criterion
APVMA	Australian Pesticides and Veterinary Medicines Authority
AWCD	Average well colour development
$BaCl_2$	Barium chloride
C	Carbon
cm	Centimetre
CO_2	Carbon dioxide
d	Days
DAS	Days after sowing
DAT	Days after treatment
DDT	Dichloro-diphenyl-trichloroethane
Df	Degrees of freedom
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic Acid
DOM	Dissolved Organic Matter
DPI	Department of Primary Industries
dSPE	Dispersive solid-phase extraction
FAO	Food and Agriculture Organization
FC	Field capacity
g	Gram
GC-ECD	Gas Chromatography-Electron Capture Detector
GRDC	Grains Research and Development Corporation
h	Hour
H_2O	Dihydrogen monoxide
H_3O^+	Hydronium ion
ha	Hectare
HCH	Hexachlorocyclohexane
HCl	Hydrochloric acid
K	Potassium
kg	Kilogram
L	Litre

LOD	Limit of detection
LOQ	Limit of quantification
mg	Milligram
$MgSO_4$	Magnesium sulfate
min	Minute
mL	Millilitre
mm	Millimetre
N	Nitrogen
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometer
NSW	New South Wales
OD	Optical density
OH^-	Hydroxide ion
OTU	Operational Taxonomic Unit
P	Phosphorus
PCR	Polymerase chain reaction
PSA	Primary-Secondary Amine
RD	Recommended dose
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
s	Second
SD	Standard deviation
SDW	Shoot dry weight
SPAD	Soil–plant analyses development
SPME	Solid Phase Microextraction
USA	United States of America
WG	Water-dispersible granules
µL	Microlitre
µm	Micrometre

Chapter 1. Introduction

1.1 Herbicides as medium of weed control

Weeds are classified as an important biological constraint to food production and one of the major yield reducing factors (FAO, 2018). Weeds compete with crops for available resources, such as nutrient, light, water and space, posing a great threat to sustainable crop production. As weed control is critical in respect to increased crop production, significant approaches should be taken to check both the active weed population and the soil seedbank (Graziani et al., 2012). Weed control includes several techniques to destroy or supress weed populations for minimizing competition in crop field. These techniques attempt to maintain a balance between costs involved for weed control and yield loss. Weed control is generally labour intensive considering the variety of options available to eradicate or destroy alien species from the desired field. Considering all the weed control techniques available, chemical weed control is widely accepted among farming communities due to labour insensitivity (Parish, 1990). Chemicals that are used to control, suppress, or kill plants or to severely interrupt their normal growth processes are called herbicides (Beste, 1983). Herbicides provide quick control and when used appropriately, increase efficiency, reduce horsepower and energy requirements (Zimdahl, 2018). As for example, the introduction of 2,4-D in the sugarcane industry, a single knapsack herbicide sprayer was more effective and efficient than 15 labours weeding with hoe (Smith et al., 2011). Atrazine allowed corn cultivation possible and profitable in some parts of the world where it was not possible before (Heri et al., 2011). In addition, application of atrazine increased four-folds of the land area that farmers could grow and manage in USA (Heri et al., 2011). Undoubtedly, the rapid development and adoption of herbicides significantly contributed to global food production, whereas increasing resistance and non-target toxicity of herbicides has subsequently become a global concern.

1.2 Herbicides in Australian agriculture

The Australian farming system has undergone a massive revolution over the past 25 years with the adoption of conservation tillage, including minimal or zero till, which has reduced cultivation practices for weed control (Congreve & Cameron, 2014). Minimal or

zero till refers to sowing of seeds with minimum soil disturbance, allowing reduced evaporation and increasing yields. This revolution has led the foundation of modern technologies in crop production, reducing cultivation practices and also options available for weed control. However, reduced cultivation practices favours weed population which ultimately increases dependence on chemical weed control (Allmaras et al., 1998). Moreover, Llewellyn et al. (2012) reported that the reduced price of the predominant herbicide, glyphosate, was also responsible for the rapid adoption of zero tillage among 78% farmers in 2008. These factors contributed significantly to the increased adoption of herbicides as a sole medium for weed control (D'Emden et al., 2006). Research revealed that herbicide application saves water over tillage practices, allowing 27 mm of extra water in the soil profile and increasing grain yields by 15-25% (Wylie, 2008). Statistics showed that herbicide usage in Australian farming systems is gradually increasing every year (Figure 1.1).

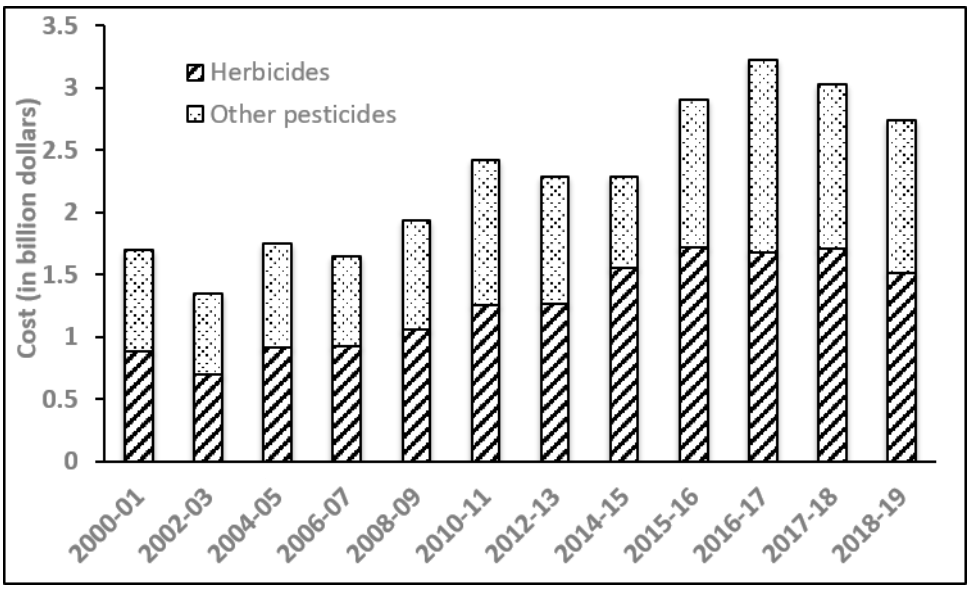

Figure 1.1. Year wise breakdown of herbicide cost in Australian agriculture over total pesticide expenditure, expressed in billion dollars (APVMA, 2021).

At present, Australian farmers spend more than 1 billion dollar per year, which is approximately more than 50% of the annual pesticide expenditure. The increased popularity of herbicides in farming systems has raised concerns about their negative impacts on the environment, human health and agricultural sustainability due to the rapid evolution of herbicide resistance. In Australia, herbicide resistant weed populations are prevalent compared to major grain producing countries (Llewellyn &

Powles, 2001). Adaptation and evolution of herbicide resistance has enabled scientists and growers to radically rethink about existing weed management approach due to the lack of diversity into their weed control programs. Increased use of pre-emergent herbicides could bring diversity in the weed control program. It is estimated that the use of pre-emergent selective herbicides in Australian winter broad acre crops has risen from less than 1 million ha to approximately 7 million ha in a decade (D'Emden et al., 2006).

1.3 Herbicide persistence and impact of herbicide residues on agriculture

Herbicides are applied to soil to control unwanted vegetation that can interfere with the growth and development of commercial crops. Now, persistence is considered the period of time an herbicide remains active in the soil. In general, herbicide persistence is expected until the end of crop harvest but not beyond that. However, existing soil conditions, herbicide chemical structure as well as application method determine the persistence of herbicides in the soil (Eleftherohorinos, 1987; Webster & Shaw, 1996). Long term persistence of herbicides may lead to soil and ground water contamination (Juhler et al., 2001), affect biodiversity and decrease soil heterotrophic bacteria (including denitrifying bacteria) and fungi (Song et al., 2013). Some herbicides can remain in soil for weeks, months or even years. This is advantageous in regards of long term weed control. Herbicides applied in minimal or zero till systems tend to leave a greater concentration of herbicide near the surface zone at the end of the cropping season (Curran, 2016). This portion of herbicide that remains in the soil after use is referred to herbicide residues. Residues could occur in higher concentrations than expected which may affect subsequent crops (Yu et al., 2015). Their persistence can affect sensitive crop species by the residual activity of the herbicide in subsequent years, limiting planting options for farmers.

However, herbicide residues and their persistence in soil are not fully understood in Australia due to lack of proper monitoring. To better understand and manage herbicide residues in the soil, Grains Research and Development Corporation (GRDC) co-invested in a five-year project to conduct a National field Survey of herbicide residues. The soil survey of different crop fields from around Australia prior to sowing in 2015 and 2016 detected residues of 23 chemicals, with trifluralin residues being detected in over

50% of the fields surveyed (Rose et al., 2019). The maximum trifluralin concentration was 5345 µg/kg in 2016. The high detection frequency was partially attributed to its common usage across different cropping systems, which reflects well-known persistency over two years of survey data. On the other hand, atrazine was also found in higher concentrations in New South Wales and South-Australian crop fields (Van Zwieten et al., 2016), with maximum residue concentration of 25 µg/kg detected in 2016. To minimize the negative impact of herbicide residues, currently recommended strategies include routine rotation of fallow and pre-emergent herbicides, reliable record keeping to identify potential residue issues and to plan for tolerant crops or crop cultivars in rotations especially after dry seasons.

1.4 Microbial degradation of herbicide residues in agricultural soil

The increased use of herbicide compounds in agricultural fields leads to the accumulation of herbicide residues in the environment which deserves attention and requires appropriate management strategies. Development of an efficient and sustainable remediation technique is essential for safe crop production and environmental cleanliness. There are several pollutant treatment methods currently practiced, namely recycling, pyrolysis, and contaminated land filling (Wang et al., 2005). However, these methods are neither efficient in reducing the level of pollutants in soil nor sustainable due to high cost associated (Jain et al., 2005). In contrast, biodegradation or bioremediation by microorganisms, is the most cost-effective method available compared to others (Gavrilescu, 2005). Microorganisms are known to have the unique ability to degrade various organic pollutants and capacity to adapt to unfavourable environments (El Fantroussi & Agathos, 2005). A small fraction of the soil biota (certain bacteria and fungi) can quickly develop the ability to degrade certain pesticides when continuously applied to the soil (Parte et al., 2017). Fungi possess oxidative enzymes that are capable of breaking many phenolic ring structures which is less prominent in bacteria but appears to be highly consolidated into a few genera (e.g., *Burkholderia*, some *Actinomycetes*, etc.). Thus, bacteria themselves do not rapidly develop the ability to do so, although the microbial community can adapt in order to enhance the abundances of these degraders in the advent of persistent pesticide application. Often, these pesticides serve as energy and carbon source to soil microorganisms (Aislabie & Lloyd-Jones, 1995). The capacity of a microbial population to degrade pollutants within

an environmental matrix (e.g., soil, sediment, sludge or wastewater) can be improved either by stimulation of the indigenous microorganisms by adding nutrients or by the introduction of specific microorganisms to the local population. Local environmental conditions also play a critical role in this process even degraders are already present in the contaminated site. In some cases, the existing microbial population might not display the appropriate metabolic potential for degradation of the target pollutant (Dangi et al., 2019). The possible reason could be genes present in microorganisms unable to express during biodegradation (Bhatt et al., 2021). Often, a very specific combination of microorganisms (a consortium) and pathways are required to degrade a complex molecule or a mixture of compounds including polyaromatic hydrocarbons, halogenated organics (both aliphatic and aromatic), polychlorinated biphenyls, organophosphorus or triazine pesticides and herbicides (El Fantroussi & Agathos, 2005). In such cases, microbial degradation might be the most efficient and sustainable solution for successful removal of the pollutants from soil.

Chapter 2. Review of literature

2.1 Introduction

Herbicides are chemical compounds that are toxic to weed plants. Modern agriculture relies heavily on herbicides for the control of weeds to maximize yield in crops. It is estimated that herbicide usage in the Australian grains industry increased by more than 30% from 2002 to 2018, from a value of $700M to $1.8 billion (Australian Pesticides and Veterinary Medicines Authority [APVMA], 2019). The increased popularity of herbicides in farming systems has not only raised concerns about their negative impacts on the environment, human health and agricultural sustainability due to the rapid evolution of herbicide resistance, but also raised questions about their fate in soil. Due to excessive use of herbicides, there is great concern about contamination which can lead to soil and water pollution (Juhler et al., 2001), reduced biodiversity and depression in soil heterotrophic bacteria (including denitrifying bacteria) and fungi (Song et al., 2013).

However, understanding the fate of herbicides in soil is a prerequisite for the precise assessment of its behaviour and potential environmental risk (Gianelli et al., 2014). This chapter illustrates a brief understanding about the transport and degradation processes of herbicides upon application to soil. This review encompasses microbial degradation, mechanisms and factors responsible for the degradation of two commonly used herbicides i.e., trifluralin and atrazine in soil, followed by microorganisms associated with degradation and recent advancement in microbial degradation of herbicides.

2.2 Fate of herbicides in soil

Herbicide behaviour in soil is a complex process that varies according to soil type, soil pH, soil moisture, aeration, organic matter and temperature (Sinha et al., 2012). The presence of herbicides in surface and groundwater is of great importance considering the potential impact on human health and the environment (Papadakis et al., 2015). Herbicides can move vertically to contaminate groundwater via leaching or laterally to intrude into surface water. Residual herbicides may also be toxic to sensitive plant and animal species or enter the food chain due to accumulation in grains and crops (Chowdhury et al., 2020; Singh et al., 2014). Soil has several scavenging techniques

comprising chemical, physical and biological processes to minimise the adverse effects of herbicides (Gao et al., 2013; Gu et al., 2012; Jiang et al., 2017; Wang et al., 2014). After application, a major fraction of the applied herbicides may be absorbed by or sorbed to soil particles or decomposed through biotic or abiotic means.

Biotic degradation, or biodegradation, may be defined as degradation of complex herbicide molecules into simple, often more water soluble and less toxic inorganic molecules by enzymatic degradation due to the activity of beneficial microorganisms (Porto et al., 2011; Wood, 2008). Abiotic degradation refers to chemical transformations involved in removal of the herbicides such as photodegradation and hydrolysis. Other processes include volatilization from soil and plants, surface runoff, plant uptake and leaching due to gravitational force.

2.2.1 Volatilisation

Volatilisation is the loss of applied pesticides from plant, soil and water surface in vapour form. Loss due to volatilisation can be as high as 90% of the amount applied for some highly volatile compounds (Taylor & Spencer, 1990), whereas others have been considered as relatively low due to inherent low vapour pressure (Helling, 2005). Many pesticides can be transported far from their initial site of application and may subject to leaching and runoff (Taylor & Spencer, 1990). The consistent presence of atrazine in rainwater, despite of low vapour pressure indicates loss due to volatilisation and vapour drift (Miller et al., 2000). Physiochemical properties of the compound coupled with existing climatic conditions determine the extent of volatilisation (Kookana et al., 1998). Herbicide volatilisation is higher in sandy soils compared to others. Gerritse et al. (1991) calculated the loss of organochlorines through volatilisation in laboratory experiments and found that approximately 90-98% loss occurred from sandy soil within a week; whereas 9-63% loss from a silt loam soil. Volatilisation loss was reported higher in hot and dry weather when compared to cooler weather conditions. Finlayson and Silburn (1996) observed that half of the endosulfan applied to dry soil was lost through volatilisation in dry hot weather. Whereas volatilisation did not occur when the pesticide was applied under cooler conditions. Method of pesticide application also plays a critical role in determining the volatilisation loss. For example, soil-applied pesticides are less susceptible to volatilisation as compared to the foliar-applied ones, and similarly soil

incorporated pesticides are less prone to volatilisation losses than the surface-applied ones (Taylor & Spencer, 1990). The extent of volatilisation loss is maximum during application and immediate after application. Trifluralin is highly volatile, however, soil incorporation immediately after application strongly abated trifluralin volatilisation loss (Bedos et al., 2006).

2.2.2 Leaching

Herbicide movement in soils followed by rainfall or irrigation is often beneficial when root uptake is necessary for weed control. Leaching reduces losses by volatilization and photodegradation, depending on the herbicide chemical properties. Thus, limited leaching may increase soil persistence. Deeper migration reduces the residual herbicide in the upper vadose zone (extends from the top of the ground surface to the water table), and so could lessen persistence in crop production. Such leached chemicals no longer contribute to weed control and may contaminate groundwater or surface water via lateral discharge. Since microbial activity is much lower in the subsurface horizons and in groundwater compared to the vadose zone, herbicide persistence generally is much longer once it moves below the vadose zone. It is believed that low temperature and absence of degrading microorganisms are responsible for the increased persistence of atrazine under vadose zone (Radosevich et al., 1996). The persistence of atrazine in groundwater is quite long (Klint et al., 1993), undoubtedly it is the most commonly detected pesticides in water samples in Australia (Schult, 2012) and USA (Rosecrans & Musgrove, 2020).

Leaching potential has long been predicted based on herbicide and soil characteristics by using various laboratory methods such as adsorption, soil leaching column, and soil thin-layer chromatography tests. Rapid degradation greatly reduces the potential loss by leaching. For example, the herbicide florasulam has very high potential mobility; 68-92% leached through a soil column with the rate of degradation (DT), often expressed as DT_{50} and DT_{90} values, were recorded 2-10 and 16-34 days, respectively. It was judged unlikely to contaminate groundwater (Vencill, 2002). Most herbicide leaching occurs during mass flow of water through the soil matrix, ensuring ample exposure of chemical to soil and soil biota surfaces.

2.2.3 Abiotic degradation

Photodegradation

Photodegradation is one of the primary abiotic degradation processes that occurs only in the presence of light. Photodegradation can be often termed as photolysis which may be affected by various environmental factors including soil temperature, moisture, soil type, pH and humic substances (Wang et al., 2014). Verhoeven (1996) defined photodegradation as "the photochemical transformation of a molecule into lower molecular weight fragments, usually in an oxidation process". Photodegradation of herbicides involves breakdown of organic matter through some organic reactions with the absorption of light occurring generally in surface soils, surface water and in the atmosphere (King et al., 2012). Hydroxylation or decarboxylation (direct photodegradation) and indirect photodegradation by the production of reactive radical species are the major types of organic reactions taking place during photolysis (Fantke & Juraske, 2013). Reactive radical species produced by those oxidation process can degrade persistent compounds in soil (Lutze et al., 2015).

Direct photodegradation may be defined as the chemical transformation of a molecule by fragmentation, to and from electron transfer or intramolecular transformation after absorbing radiation (Schwarzenbach et al., 2003). Conversely, photosensitisers absorb radiation and transfer energy to the herbicides from their excited state during indirect photodegradation, which is followed by several processes as for direct photodegradation.

Several factors have been identified to influence herbicide photodegradation of surface and groundwater including chemical properties, land topography, soil characteristics, weather and agricultural operations (Konstantinou et al., 2001) listed in Table 2.1.

Table 2.1. Factors responsible for exposure to solar radiation and photodegradation (King et al., 2012).

Factor	Potential influence	References
Ozone	Possible slight increase with stratospheric ozone thinning. Potential decreases with high tropospheric ozone.	(Smith et al., 2010)
Latitude	Generally negative relationship. High latitudes susceptible during summer months due to ozone thinning.	(Brandt et al., 2010; Moody et al., 2001; Pancotto et al., 2003)
Season	In grasslands, highest rates during summer in grasslands if seasonally dry, but rates may be higher in spring in areas with summer monsoons. In temperate deciduous forests, highest directly before defoliation in spring or after senescence in autumn. In tropical deciduous forests, highest during dry season.	(Brandt et al., 2010; Henry et al., 2008; Rutledge et al., 2010)
Elevation	Most likely positive relationship due to higher proportion of short-wave radiation and higher total irradiance at high elevations. May be negative relationship in areas where cloud, canopy, or snow cover increases with elevation to the point where litter is shaded.	(Blumthaler et al., 1997)
Cloud cover	Most likely negative relationship. Modest cloud cover can increase diffuse radiation and potentially increase rates on mostly sunny days	(Madronich et al., 1998)
Leaf area index	Generally negative relationship, but especially so with broadleaf architecture.	(Rozema et al., 1999)
Canopy architecture	Higher rates with vertically distributed structure (e.g., grasslands) than horizontally distributed structure (e.g., broadleaf forests).	(Rozema et al., 1999)
Landscape patchiness	Higher rates in open areas versus under shrubs or trees.	(Köchy & Wilson, 1997)
Evenness	Rates per unit mass potentially greater with increased evenness.	(Mlambo & Mwenje, 2010; Throop & Archer, 2007)
Soil reflectivity	Sandy soils may increase albedo and lead to increased rates in adjacent litter.	(Rozema et al., 1999)
Snow	No photodegradation when buried. Potential increase in photodegradation in standing dead if surrounded by snow due to albedo.	No reference found
Soil cover/burial	Decreased rates with increasing soil burial.	(Barnes et al., 2012; Brandt et al., 2010; Throop & Archer, 2007)

Photodegradation of the soil surface is quite different from the aquatic system because soil contains a minimum fraction (<5%) of organic matter and a major fraction (>95%) of minerals. Organic and mineral content are the most important parameters affecting herbicide degradation on the soil surface. Mineral particles occupy the major share of the soil particles in which mainly crystalline and non-crystalline amorphous minerals dominate with an array of hydroxyl groups (Parlar, 1990). Various soil clay minerals containing iron associated with the production of reactive radicals, for example, the hydroxyl radicals which may influence the photodegradation of herbicides in soil (Katagi, 2004; Mantzos et al., 2017; Sleiman et al., 2017). In photodegradation of herbicides, light penetration is limited to a layer of 0.1 to 0.5 mm of soil (Hebert & Miller, 1990; Miller et al., 1989). The degradation rate of metazachlor and quizalofop-p-ethyl herbicides were quick under sunlight irradiation compared to dark conditions (Mantzos et al., 2017). Frank et al. (2002) reported significant differences in half-lives of chemicals in various soil depths. Katagi (2004) found the evaluated depth for direct and indirect photolysis are 0.23 and 0.28 mm, respectively for lab conditions whereas 0.32 and 0.62 mm in the field conditions for most of the herbicides. Ismail et al. (2015) observed the reduction of deltamethrin half-life was higher in presence of light compared to dark. Zhang et al. (2010) found a positive trend between soil depth and half-life of pyrene increasing from 19.80 to 37.46 d as soil depth was increased from 1 to 4 mm. Temperature may have very little or no effect on the herbicide photodegradation. According to Rering et al. (2016), temperature did not significantly influence the photochemical degradation of imazosulfuron.

Hydrolysis

Hydrolysis is the chemical breakdown of a molecule with addition of H_2O, H_3O^+, and OH^- i.e., water molecules. It is one of the major abiotic degradation processes taking place under certain circumstances, such as within groundwater or due to low microbial activity in soil (Wolfe et al., 1990). Hydrolysis rate in soil may be different than in water as soil organic matter content (Stevenson, 1994), clay content (Yaron, 1978), pH (Muller et al., 2007) and temperature (Getzin, 1981) were found to influence hydrolysis of herbicides. Hydrolytic reactions are mainly pH dependant and can be mediated either chemically or biologically (Kookana et al., 1998). Karpuzcu et al. (2013) found the average rate of chlorpyrifos hydrolysis was 0.02 µmol/g/day at pH 7.2 and 30 °C. On the other hand, an

increasing rate of hydrolysis has been observed under acidic conditions for azimsulfuron (Boschin et al., 2007), metsulfuron-methyl and most of the sulfonylurea herbicides (Morrica et al., 2001). Hydrolysis of dimethyl disulphide was faster in neutral or mid-alkaline compared to acidic solutions under constant temperature conditions (Han et al., 2017). However, some exceptions exist regarding the dependency of hydrolysis of some pesticides on soil pH. Shabtai and Mishael (2017); Zhang and Pehkonen (1999) reported that rapid hydrolysis of diazinon in both acidic and alkaline conditions followed half-lives of 0.5, 171 and 6 days at a pH concentration of 3.1, 7.3 and 10.4. It is best to study the hydrolysis of herbicides in a pH range which exists in the field soil, aquifers and environment to know the fate of these chemicals.

Soil organic matter, clay content and type have strong influence on the hydrolysis of herbicides. Liao et al. (2017) reported that abiotic degradation of methyl parathion was significantly related to the natural organic matter and solution pH. Another study reported that higher concentrations of dissolved organic matter (DOM) resulted in significant reduction on the rate of chlorpyrifos hydrolysis (Adams et al., 2016). The influence of clay mineral content in degradation of herbicides are also investigated by several researchers (Shabeer et al., 2014). Baglieri et al. (2013) stated that the catalytic activity of clay minerals was mainly responsible for the adsorption of triclopyr, whereas the rate of the reaction depends on the type of clay, exchangeable cation and the state of hydration. Moreover, hydrolysis allows us to understand the possibility of surface and underground water contamination through the indiscriminate use of herbicides. Basically, when these chemicals leached into the deeper layer due to gravitational force where microbial activity is limited then abiotic degradation is the main process that ultimately determine the fate of herbicides. Among the environmental factors, pH and temperature are most important factors that influence hydrolysis of herbicides. Several studies indicated that pH and temperature regulate the rate of hydrolysis for most of the sulfonylurea herbicides, i.e., pyrazosulfuron ethyl (Singh & Singh, 2013; Zheng et al., 2008), halosulfuron methyl (Grey et al., 2018; Zheng et al., 2008), prosulfuron, primisulfuron methyl, rimsulfuron, and thifensulfuron methyl (Dinelli et al., 1997), rimsulfuron, sulfosulfuron, nicosulfuron, prosulfuron, ethametsulfuron-methyl and metsulfuron-methyl (de Lafontaine et al., 2014), sulfosulfuron, nicosulfuron and rimsulfuron (Cessna et al., 2015).

Oxidation and reduction

Variations of oxidation number in a molecule are referred as oxidation and reduction; where increase represents oxidation and decrease represents reduction. Alternatively, loss or gain of electrons in a molecule can be denoted as oxidation and reduction reactions. Pesticides can only be oxidized or reduced in the soil upon presence of a chemical with adequate redox potential.

Oxidative mechanisms in soils are governed by both oxidative enzymes (Dec & Bollag, 2000) and abiotic catalysts such as metal oxides. However, manganese oxides and hydroxides are major contributors due to their reactivity and frequency in soils (Li et al., 2003). MnOOH and MnO_2 are capable to oxidize a variety of organic contaminants such as phenol (Lin et al., 2009), aniline (Laha & Luthy, 1990) or triazine (Shin & Cheney, 2004).

2.2.4 Biotic degradation

Microbial degradation

Microorganism is a broad term that includes bacteria, fungi, archaea, protists, and viruses, typically representing only 0.1% of the total volume of soil. However, they are involved in some major remediation processes that recycle the waste and pollutants in the environment (Torstensson, 1988). Microorganisms are present in soil regardless of the textural classes and types but in different densities. For example, bacteria may be present in between 10^2 to 10^6 per gram soil (Delgado-Baquerizo et al., 2018) and fungal hyphae may also exist as some many thousands of metres per gram soil. Thus, the total biomass of microorganisms in soil could be several tonnes per hectare (Torstensson, 1988). Microorganisms were reported to play a vital role in waste decomposition (Schneider et al., 2010), regulation of plant growth (Hayat et al., 2010), nutrient cycling (Van Der Heijden et al., 2008) and degradation of various dangerous contaminants and pesticides (Pino & Peñuela, 2011; Zhao et al., 2009). Recent studies suggested the need for rapid exploration of novel microorganisms, their diversity, and innovative ecological functions for the development of bioremediation strategies (Graham et al., 2016; Hua et al., 2015; Martiny et al., 2015; Prosser et al., 2007).

The microbial distribution in soil is regulated by several biotic and abiotic factors. Modification in environmental conditions may alter the equilibrium distribution of the

microbial population. This may be the possible reason for the differences in adaptability of microbial populations in different geographical locations (Verma et al., 2014). For instance, the abundance, composition, diversity, and enzymatic activity of microorganisms present in the rhizosphere can be expressed as a subset of overall soil microbial community, which is influenced by the localised physiochemical properties of the soil (Marschner et al., 2004), that may be different from the bulk soil (Foster, 1986). This is reflected where plant root exudates have been reported to shape the composition and abundance of the rhizosphere microbial community (Wu et al., 2017).

Microorganisms involved in microbial degradation

The removal of pollutants from the soil by various activities of microorganisms is often referred to by several terms, bioremediation, biodegradation, biomineralization, bioaccumulation, biotransformation or co-metabolism (Finley et al., 2010; Park et al., 2003; Shakoori et al., 2000). In agricultural context, the over-reliance of chemical compounds leads to the accumulation of toxic compounds in environment which needs to be removed by any means. In this regard, a special group of microorganisms are reported to do this job by enzymatic transformation into non-toxic compounds, are of special importance (Wang et al., 2005; Wood, 2008). Plants, animals and fungi (Eukaryota) typically remediate pollutants and contaminants through accidental metabolism by broad-spectrum enzymes. Some bacterial extracellular enzymes are able to decompose ring-based compounds into simple compounds for transport across the cellular membrane for metabolism (Fenner et al., 2013). The differences in degradation are due to the sensitivity of chemical products among the eukaryota. For example, the application of organophosphate ester hampers the nervous system of insects but has no effect on microbes. One hypothesis would be that it could be used as a source of carbon and phosphorous if proper metabolizing enzyme accommodates in that microorganism (Fenner et al., 2013). Bacteria predominates the microbial community regardless of the soil depth as they can utilize alternative electron acceptors in such oxygen deficit conditions (Boopathy, 2000). Moreover, bacteria have the greatest capability to produce new metabolic pathways by the evolution of new enzymes for rapid metabolism (Copley, 2009). Bacteria are able to transfer clusters of genes evolved in a bacterium to other organisms by cell-to-cell contact, which is known as horizontal gene transfer

(Emamalipour et al., 2020). This approach allows the development of a protection system against toxic pollutants due to the continuous exposure to various environmental stress and also to take advantage of a broader variety of carbon compounds (Nayak et al., 2018; Parsek et al., 1995; Verma et al., 2014). This is more common among bacteria but also possible between other organisms, where bacteria serve as donor while fungi, plants, and animals serve as recipients (Garcia-Vallvé et al., 2000; Rancurel et al., 2017). There is significant evidence of the generation of new bioremediation pathways within the microbial community by the transmission of genes responsible for biodegradation (Zhao et al., 2017). Nguyen et al. (2018) reported the intra-field evolution and inter-field exchange of 2,4-D catabolic plasmids and genes within a restrained local environment. Bacterial strains engaged in bioremediation processes have been isolated from different locations of the world. These include strains from *Bacillus* (Anwar et al., 2009; Eissa et al., 2014; Liu et al., 2012; Zhu et al., 2010), *Pseudomonas* (Lakshmi et al., 2008; Singh et al., 2003), *Arthobacter* (Aislabie et al., 2005; Evy et al., 2012; Patil et al., 1970), *Ralstonia* (Hay & Focht, 2000), *Rhodococcus* (Park et al., 2003; Zaitsev et al., 1995), *Alcaligenes* (Padmanabhan et al., 2003; Yang et al., 2005), *Nocardiopsis* (Pravin et al., 2012) *Micrococcus* and *Lactobacillus* (Azizi, 2011) and *Acetobacter* (Shakoori et al., 2000). These organisms are highly adaptive in nature and have the capability to degrade a wide range of toxic compounds with the evolution of mutants potentially leading to new metabolic pathways (Suenaga et al., 2001). Laemmli et al. (2000) identified a 2,4-D degrading gene cluster, *tfdII* located on plasmid pJP4 of *Ralstonia eutropha*. Various species within *Pseudomonas*, *Arthrobacter*, *Alcaligenes*, *Cytophaga*, *Actinobacter*, *Moraxella* and *Klebsiella* have been reported to have such types of plasmids (Sayler et al., 1990).

Although microbial degradation of pesticides greatly focused on bacteria, various fungal strains belonging to different genera including *Aspergillus* (Mohamed et al., 2011; Sene et al., 2010; Yu et al., 2011), *Trichoderma* (Sene et al., 2010), *Penicillium* (Peng et al., 2012), *Fusarium* (Sene et al., 2010), *Streptomyces* (Mohamed et al., 2011), *Phanerochaete* (Chirnside et al., 2011), *Rhizopus* (Sene et al., 2010), *Trametes* (Bastos & Magan, 2009), *Lentinus* (Nwachukwu & Osuji, 2007) and *Mortierella* (Badawi et al., 2009) have also been reported to degrade a wide range of pesticides. Fungi mediated bioremediation has been reported to be appropriate due to their extended mycelial networks, low specificity to catabolic enzymes and independency towards utilizing

organic compounds as growth substrate (Chen et al., 2012; Harms et al., 2011). Fungal degradation of pesticides is also regulated by a number of environmental factors including soil moisture (Bastos & Magan, 2009), temperature (Yang et al., 2011; Yu et al., 2011), pH (Yu et al., 2011), aeration (Hussain et al., 2007) and composition of the medium (Kataoka et al., 2010). Identification and characterization of pesticide degrading fungal strains is a prerequisite for the better understanding of fungal bioremediation. Literature is rich in isolation and characterization of a variety of pesticide degrading fungal strains from pesticide contaminated sites worldwide (Derbalah & Belal, 2008; Marco-Urrea et al., 2009; Quintero et al., 2007; Yang et al., 2011; Yin & Lian, 2012). Therefore, fungal strains have been identified capable of degrading various pesticides including alachlor (Chirnside et al., 2011), pendimethalin (Yu et al., 2011), bensulfuron-methyl (Yu et al., 2011), atrazine (Sene et al., 2010), chlorophenol (Zouari et al., 2002), simazine (Fragoeiro & Magan, 2008), trifluralin (Fragoeiro & Magan, 2008), metsulfuron-methyl (He et al., 2006), chlorsulfuron (Boschin et al., 2003), isoproturon (Badawi et al., 2009), diuron (Badawi et al., 2009), linuron (Badawi et al., 2009), glyphosate (Arfarita et al., 2011), metolachlor (Munoz et al., 2011), lindane (Quintero et al., 2008), methyl-parathion (Marinho et al., 2011), endosulfan (Hussain et al., 2007), dichloro diphenyl trichloro ethane (DDT) (Thomas & Gohil, 2011), heptachlor (Xiao et al., 2011), acetamiprid (Wang et al., 2012) and dieldrin (Fragoeiro & Magan, 2008).

Apart from the isolation and characterization of fungal strains capable of degrading a wide range of pollutants, still there are limitations constraining their wider application. Research suggests that fungi mediated degradation is a slow process and often complete removal of the pollutants is not possible (Sasek & Cajthaml, 2005). This might be due to the time required for the adaptation of the fungal strain in a contaminated environment (Kulshreshtha et al., 2014). Moreover, variations in climatic conditions also play a dominating role in this context. Metabolic process and mechanisms governed by biodegradation processes need to be addressed under variable environmental conditions which ultimately contribute to fungal biodegradation in site specific conditions. The changes in environmental conditions will affect the physiology of the fungal species ultimately affecting the degradability of the pesticides. Another important aspect regarding fungal biodegradation is that incomplete degradation of the pollutants may lead to the possibility of increased metabolite toxicity compared to the parent pollutant compound (Boopathy, 2000). Accidental

accumulation of those metabolites in the environmental components may have serious consequences (Badawi et al., 2009; Xiao et al., 2011).

Mechanisms involved in microbial degradation

Soil microbes are an indispensable part of the ecosystem, maintaining biogeochemical cycles through novel transformations in the biosphere (Van Der Heijden et al., 2008; Whitman et al., 1998). As a part of the transformation process, various organic and inorganic compounds deposited in soil are converted to simple compounds through a variety of metabolic pathways adopted by specific microorganism or groups of microorganisms. Under aerobic conditions, herbicides are primarily converted to CO_2 due to oxidation, but other chemicals may also form. Microorganisms require energy for the various metabolic activities they perform within the soil, and they mainly rely on the organic compounds as a source of energy. The question remains about how microorganisms develop their ability to degrade herbicide compounds. Since microbes catabolize herbicide compounds for assimilation as energy source, their interaction with the herbicidal compounds is significant (Table 2.2). Catabolic metabolism is mainly dependant on the suitable chemical structure of herbicidal compounds to be utilised as an energy source by the microorganisms. In this regard, selection of the degrading microorganism is the determining factor whether the herbicide compound will be degraded or not.

Table 2.2. Mechanisms involved in herbicide degradation (Torstensson, 1988).

Degradation mechanism	Outcome
1. Direct decomposition of herbicides through adaptation where herbicide compounds serve as energy sources (catabolism).	Repeated application of same herbicide results in faster degradation. May also arise some serious consequences like persistence of some specific herbicides ex. Phenoxy acids, EPTC (S-Ethyl dipropylthiocarbamate), TCA (Trichloroacetic acid), dalapon.
2. Accidental transformation through peripheral metabolic process (co-metabolism).	All herbicides may be degraded by this mechanism.
3. General activities by microorganisms such as, modification of pH, production of different free radicals and other reactive compounds.	Degradation of herbicides due to the influence of microorganisms on biological and non-biological reactions.

In the case of incidental transformation, herbicide degradation rate depends on the availability of other carbon sources which implies that metabolism rate can be altered by the amount of herbicides or carbon sources. This type of metabolism is more common where the amount of herbicide is comparatively lower than the other available carbon sources. Horvath and Alexander (1970) reported an approach of degradation of stable and non-degrading chemicals by increasing the concentration of the primary substrate for the degradation of chlorinated pesticides. Under the above circumstances, the consequence of adaptation and co-metabolism ultimately determines the microbial degradation of herbicides. So, these two phenomena are of primary interest in determining the mechanisms behind microbial activities in soil. These two phenomena are further described below.

Adaptation

Microbial degradation of herbicide depends on the frequency of herbicide application in soil. Repeated application of the same herbicide in the same field results in increased microbial degradation, suggesting adaptation as a result of selection (Arbeli & Fuentes, 2007; Fang et al., 2015; Lancaster et al., 2010). As a result of the enhanced degradation of herbicides in soil, soil applied herbicides are losing their efficacy (Zablotowicz et al., 2006) and microorganisms are hereby accounted for undermining the effectivity of herbicide compounds. There is some controversy regarding the rapid degradation of

several herbicides by soil microorganisms. This rapid breakdown of herbicide has been reported to be disadvantageous by several researchers whereas others have described it as an uncommon phenomenon having little impact on agriculture (Fox, 1983).

There is a range of opinion available to describe how microorganisms build up their capacity to degrade a certain herbicide. According to Kaufman et al. (1983), a specific signal derived from the applied herbicide or other chemicals is responsible for the microbial adaptation to specific herbicides. Some chemicals may act as a motivator in enzyme secretion which further degrades other chemicals. The phenylurea hydrolase-encoding genes *puhA* and *puhB* were identified in the linuron-degrading actinomycetes *Arthrobacter globiformis* D47 (Turnbull et al., 2001) and *Mycobacterium brisbanense* JK1 (Khurana et al., 2009), respectively. Again, it is not mandatory for the herbicide compound to be substrates for the metabolism process governed by enzyme secretion. Traditional culture-based laboratory investigations mainly concentrated on the monoculture of substrates, but the complete degradation of herbicides is faster and more efficient in microbial consortia rather than single microorganism (Kumar et al., 2021). In regard to this, continuous investigations not only revealed the involvement of microbial communities in the remediation of toxicants in the soil but also broaden the possibility to study the interaction between different microbial species (Torstensson, 1988). These innovative studies further laid the foundation of exploring the adaptation mechanism behind herbicide selectivity of the microorganisms in stress conditions. Herbicide degradation usually shows an initial lag phase where no degradation occurs, followed by a sharp decrease in the concentration (Figure 2.1). The period between herbicide application and initiation of biodegradation is termed as the acclimation period where basically no significant degradation is observed. Zhao et al. (2018) observed a prolonged lag phase followed by higher concentrations of atrazine application, however repeated application of atrazine resulted in faster degradation with decreased half-life (Fang et al., 2015).

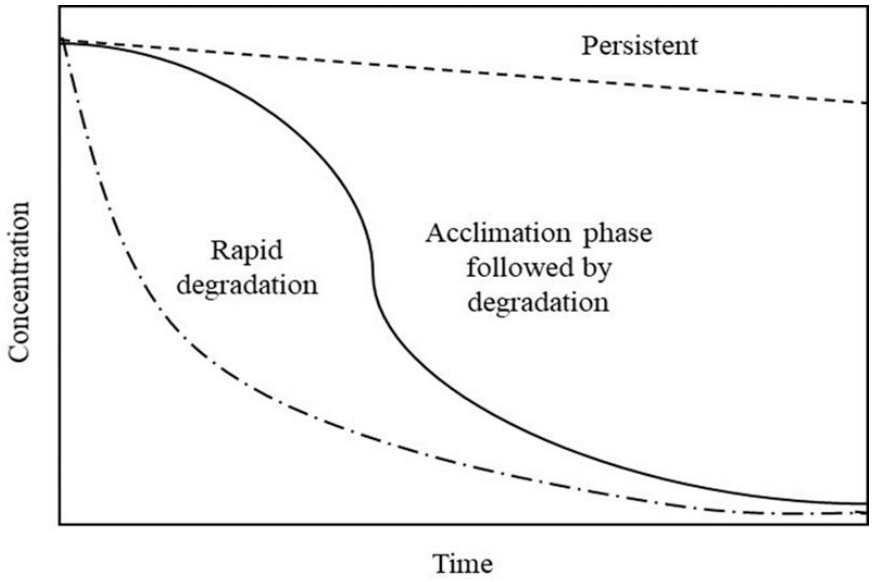

Figure 2.1. Degradation of herbicides in soil over period of time (Boettcher et al., 2001).

This may be due to the multiplication of herbicide degrading organisms during the first application to such a level that increased the degradation rate of herbicide at later applications. Other proposals identified genetic alterations taking place within the microorganism for enzyme synthesis as the main reason for the initial time lapse (Torstensson, 1988). Alterations are mainly due to changes in chromosomal or extra-chromosomal DNA sequences. A specific type of extra-chromosomal DNA, commonly known as plasmid, has been identified to be responsible for the degradation of herbicides (Laemmli et al., 2000). These special types of DNA are smaller than bacterial chromosome and have been reported to bear specific genetic information for biodegradation of herbicides which may be absent in chromosomal genes (Verma et al., 2014). Plasmids are capable of intercellular movement in some microbial communities and provide a pathway for the transfer of the biodegrading genes to other members of the bacterial community (De Souza et al., 1998). Manipulation and transportation of these genes from one organism to other members of the microbial community in such conditions has opened a new horizon in the context of bioremediation.

Co-metabolism

Co-metabolism is an accidental degradation of an herbicide by an enzyme or co-factor associated with the degradation of another compound (USEPA, 2000). The energy derived at this process is neither sufficient to support microbial growth nor activate relevant enzymes involved in the degradation process (Tran et al., 2013). Co-metabolism

is mainly dependant on the substrate metabolism of other compounds. This type of biodegradation is highly sophisticated as only the microorganisms capable to degrade the concerned contaminant are accelerated (Hazen, 2010). This process can be accelerated at low very concentrations particularly to an undetectable limit i.e., parts per trillion, which is the most important advantage (Hazen, 2010). For example, methanotrophs (prokaryotes that metabolize methane for their sole carbon and energy source) have been reported to produce an enzyme called methane monooxygenase, which is capable of oxidizing over 300 compounds (Hazen, 2010). In contrast, some evidence suggest that metabolites produced from this specific type of metabolic pathway may act as inhibitors of microbial degradation (Powell et al., 2011; Rui et al., 2004; Semprini et al., 2005; Sipkema et al., 2000). Microbial co-metabolism may be the effective approach to remove various types of toxic pollutants from soil (Tran et al., 2013; Zhao et al., 2016). According to Torstensson (1988), majority of the herbicides used in agriculture may be degraded by co-metabolism.

Research suggests that no lag phase has been observed in co-metabolic pathways (Moreira et al., 2012; Zhang et al., 2013). Moreover, adaptation is absent in co-metabolism which makes it prominent that repeated application of herbicides did not affect co-metabolic degradation at all. Synthetic chemicals which are not degraded by individual microbial species may be mineralized further via the co-metabolic transformations governed by the combined activity of several microbial species. Co-metabolism of herbicides generally occurs in slow rate due to the lower populations of co-metabolizing microorganisms which will not increase in respect to the chemicals applied (Janke & Fritsche, 1985). Even, a single microorganism can co-metabolize a pollutant completely (Chen & Aitken, 1999; Juhasz & Naidu, 2000; Ye et al., 1995). These co-metabolizing microorganisms can be considered as a good option for the development bioremediation strategies.

2.3 Factors affecting microbial degradation

2.3.1 Temperature

Temperature plays a major role in the ecological distribution of microorganisms interlinked with the metabolic activities and degradation of herbicides in soil (Robador et al., 2016). Davidson and Janssens (2006) demonstrated the rapid increase of soil

microbial respiration with temperature rise. The increased microbial activity could accelerate degradation of pollutants such as herbicides, and the rate of degradation was faster in warmer regions than in the cooler parts due to increased microbial activity with less seasonal variation throughout the year (Racke et al., 1999). In laboratory conditions, generally the effect of temperature variations on herbicide degradation is of minimum attention and 25 °C has been used as a standard temperature (Racke et al., 1999). Wang et al. (2011) and Wang and Xie (2012) showed that the optimum temperature range for atrazine degradation was 20-40 °C. Low temperature induces accumulation of toxic pollutants in the environment (Ma et al., 2011). James et al. (1999) reported that triasulfuron may persist in soils at low temperature. In contrary, Levy et al. (2007) blamed the dry and hot weather in summer of 2003 in Germany for the accumulation of isoproturon in soil due to drastic changes in soil microbial community structure and function. Rapid degradation of clopyralid was observed by Tomco et al. (2016), possibly due to high temperature (14.4 and 16.9 °C) in Alaskan soils. Temperature regulates enzymatic activities required for various biochemical processes in soil, yet very little information is available related to sensitivity of enzymatic activities to varying temperatures in the environment (Trasar-Cepeda et al., 2007). According to Wallenstein et al. (2010), enzyme activation mainly depends on the physical and chemical interactions with soil clay, minerals and organic matter. Studies related to temperature effect on specific enzyme activation for the degradation of herbicide compounds found that higher or lower than the optimum temperature will slow down the degradation process. The optimum temperature for herbicide degradation may vary between chemicals but will generally be in the range of 20-30 °C (Jordan, 1990). Dong and Sun (2016) reported that atrazine residue concentration decreased with increasing temperature, increasing degradation rate and half-life by 3-4 times from shifting temperature 5 °C to 35 °C. Degradation of florasulam was strongly influenced by temperature with half-life ranging from 1.0-8.5 days at 20-25 °C to 6.5-85 days at 5 °C (Krieger et al., 2000).

2.3.2 Soil moisture

Soil microorganisms require moisture for their growth and metabolism. There is a direct relationship between soil microbial activity and moisture content; a decrease in moisture content reduces microbial activity, and rewetting causes a large and rapid

increase in activity (Speight & El-Gendy, 2018). Therefore, degradation process is slow in dry soils and generally increases with increasing moisture content (Dong & Sun, 2016). This may be due to the low microbial activity prevailing under extreme dry conditions (Miles et al., 1984). Since moisture content has significant impact on soil microbial activities, herbicide degradation would be expected to be faster in wet soils. Generally, moisture contents between 50-80% field capacity (FC) levels are considered optimal for microbial activity (Morgan & Atlas, 1989). Atrazine degradation was reported to be 3-4 times higher when soil moisture content was increased from 5% to 20% (Dong & Sun, 2016).

Whereas extreme soil moisture conditions are considered unfavourable for microbial growth and metabolism process, fungal and bacterial oxidative enzymes for degradation are inhibited at low O_2 levels in saturated soils. Excess moisture can accelerate anaerobic transformation of herbicides by reducing the oxygen level, which could hamper the transformation of herbicides (Schroll et al., 2006). Alternatively, soil moisture may not necessarily have any significant effect on the transformation of some herbicides. For example, the half-life of rimsulfuron was reported to be 22.5 and 24.5 days under aerobic and anaerobic conditions, respectively (Schneiders et al., 1993). Some herbicides are reported to be accumulated under anaerobic conditions, e.g., clopyralid (Corredor et al., 2006; Zhao et al., 2011) whereas others breakdown rapidly, e.g., atrazine (Pal et al., 2006).

2.3.3 Soil pH

Soil pH has substantial effects on the reactivity, activity and persistence of applied herbicides in soil, specifically at extreme pH conditions such as less than 4.5 or higher than 7.5 (Monaco et al., 2002). The basic principle is that herbicide degradation is dependent on the charge of the herbicide molecules and herbicides bearing a positive charge will have a strong affinity to the negatively charged soil clay particles. Whereas herbicides bearing a negative charge will be repelled by soil colloids and exposed to transformation (Monaco et al., 2002; Ross & Lembi, 1999). Again, soil pH has a major influence not only on the growth and activity of microorganisms but specifically on the growth of microbes responsible for herbicide transformation (Raeder et al., 2015). Optimization of pH in soil is a difficult task depending on the soil type as variation in the soil pH is comparatively less than in water. In addition, enzymes have an operational pH

range and changes to pH cause inhibition due to denaturation. As most microbial species survive in the pH range of 4.5-7.5 (Msimbira & Smith, 2020), optimization of pH is critical in soil experiments in regards of biodegradation of herbicides.

Tariq et al. (2003) observed highest degradation of HCH isomers (α and γ) in soil slurry with an initial pH of 9.0. Accelerated biodegradation of endosulfan was reported through optimization of pH to 8.0 (Arshad et al., 2008). Literature suggests that optimum condition for biodegradation of pesticides varies with compounds and organisms, but the degradation rate was slow at acidic pH compared to alkaline and neutral pH conditions because acidic pH increases stability of various chemical groups (Reid et al., 2000). Another possible reason may be the reduced activity of bacteria or enzyme involved in the biodegradation process under low pH (Roberts, 1998).

2.3.4 Soil organic matter

Although microorganisms represent only 1 to 8% of the soil organic carbon (Roder et al., 1988), they are responsible for the maintenance of C, N and P cycles and other physio-chemical activities in soil through decomposition, mineralisation and immobilization processes (Sarathchandra et al., 1988). Increase in mineralisation of the herbicides may contribute to the reduction of organic matter content of soil. Low organic matter content of soil may result in slower microbial degradation of trifluralin with high adsorption capacities and as a result less trifluralin available for degradation in soil (Tiryaki et al., 2004). To boost microbial activity in soil, organic matter content in soil should be replenished (Perucci et al., 2000). According to Burns (1975), at least 1.0% of organic matter should present in soil to ensure the activity of indigenous microorganisms that can involve in the transformation of toxic compounds in soil. To increase the organic matter content in soil, application of various organic amendments such as sawdust, municipal waste compost and synthetic biological waste are frequently practiced in different countries (Palma et al., 2002; Said-Pullicino et al., 2004; Vorkamp et al., 2002). Organic amendments e.g., sawdust have higher C:N ratios than compost, resulting in increased microbial activity as microorganisms require carbon and nitrogen as a nutrient for growth and reproduction (Zhang et al., 2021). Addition of organic amendments in the soil has recently gained increasing interests (Scotti et al., 2015), which facilitates development and functioning of terrestrial ecosystems (Izquierdo &

Bedmar, 2008). However, this could lead to a change in the fate and behaviour of herbicides applied in the same soil (Barriuso et al., 1997; García-Jaramillo et al., 2016).

Organic amendment addition will either slow down the microbial degradation process through adsorption (Doyle et al., 1978) or accelerate the remediation process by increasing the microbial metabolic activity (Hance, 1973). Although, herbicide sorption is reported to increase with the addition of organic amendments in soil, dissolved organic matter (DOM) content is also enriched which gradually influences sorption and movement of herbicides in soil (Cox et al., 2001). Marín-Benito et al. (2018) agrees with this statement as they investigated the application of green compost to study the fate of triasulfuron in soil and concluded that soil amendment with green compost not only increased half-life (DT_{50}) in soil due to rapid adsorption by soil particles but also accelerated persistence of triasulfuron by blocking leaching into the soil.

2.3.5 Herbicide structural properties and concentration

Physical and chemical properties of herbicide mainly determine its possibility of biodegradation in the environment. Addition of polar groups such as, OH, COOH, and NH_2 on the phenyl ring makes the herbicidal compound more susceptible to microbial activity (Chowdhury et al., 2008). On the other hand, Cork and Krueger (1991) revealed substituents like halogen and alkyl groups make compounds resistant to microbial degradation. In addition, water solubility and adsorptivity of the herbicide compound are important factors under consideration in this context. Solubility and adsorptivity are inversely related in many herbicide compounds. Herbicides which are likely soluble in water are more prompt to microbial degradation than those which are generally insoluble in water. Chlorinated hydrocarbons such as DDT, pentalene and dieldrin are insoluble in water, sorbed tightly to soil particles and thus are relatively unavailable for microbial degradation (Chowdhury et al., 2008). Again, there is some exception with glyphosate and paraquat which are highly water soluble but adsorbed to soil particles tightly (Williams et al., 2014). Several physical, chemical and structural parameters that determine the possibility of degradation are listed in Table 2.3.

Table 2.3. Effect of physical, chemical and structural properties on the degradability of herbicides (Boettcher et al., 2001).

Properties	**Degradation**	
	Rapid	**Slow**
Solubility in water	Soluble	Insoluble
Size	Relatively small	Relatively large
Functional group substitutions	Few	More
Rapid reduction	In oxidized environment	In reduced environment
Rapid oxidation	In reduced environment	In oxidized environment
Origin	Biologically	Either man made or synthetic
Aliphatics	Up to 10 C-chains, straight chains. Aromatic compounds with one or two nuclei	High molecular weight alkanes, branched chains, polyaromatic hydrocarbons
Substitutions on organic molecules	Alcohols, aldehydes, acids, esters, amides, amino acids	Alkanes, olefins, ethers, ketones, dicarboxylic acids, nitriles, amines, chloroalkanes
Substitution position	p-position, o- or p- di-substituted phenols	m- or o- position, m- di-substituted phenols

2.3.6 Dissolved organic matter (DOM)

Dissolved organic matter (DOM) is the fundamental portion of organic matter having the ability to dissolve in field conditions, and which plays a major role in transportation of pollutants in soil (Kalbitz et al., 2000). Photosynthesis is the primary driver of DOM production in soil which includes organic litter and humus substances accumulated through pedogenesis (Guggenberger et al., 1994; McDowell & Likens, 1988). Soil microbial communities are the substantial agent contributing to the formation of DOM. Guggenberger et al. (1994) investigated DOM structure and fractionation and revealed that DOM may be predominately of microbial origin. Solubility and mobility of various organic compounds and metals are enhanced by DOM (Blaser, 1994; Marschner, 1999; Piccolo, 1994; Zsolnay, 1996) followed by accelerated biodegradation of organic compounds (Raulund-Rasmussen et al., 1998). Previous studies have shown that even a small fraction of DOM can significantly influence the dissipation of various organic compounds, especially DDT and some polychlorobiphenyls (PCBs) (Caron et al., 1985; Hassett & Anderson, 1979; Wershaw et al., 1969). Whereas contradictory results were also reported on the behaviour of the fate of cationic pesticides in soil and water which may be due to the differences in experimental conditions (Barriuso et al., 1992; Klaus et

al., 1998; Pennington et al., 1991; Seol & Lee, 2000). Most studies focussed on the behavioural pattern of herbicides in water bodies while very little is known about their interaction in soil in presence of DOM (Said-Pullicino et al., 2004; Spark & Swift, 2002). Adsorption and desorption behaviour of atrazine, dimefuron and carbetamide herbicides was influenced by the nature of DOM as per observations of Barriuso et al. (1992). They identified a positive relation between soil adsorption capacity and soil organic carbon content which led them to conclude physio-chemical properties of DOM i.e., pH, organic carbon content and conductivity had a strong influence on the sorption behaviour of herbicides. Pre-treatment with DOM solution increased soil adsorption of less soluble atrazine and dimefuron. This increased adsorption may be due to the increased soil carbon content that contributed adsorption of some organic compounds from DOM solution. On the other hand, Pennington et al. (1991) observed that DOM extracted from different soil samples had no significant interaction with the tested herbicides i.e., alachlor, bromacil and metribuzin which may be due to the variation in physio-chemical characteristics of herbicide compounds.

2.4 Microbial degradation of herbicides in Australian soils

Although majority of herbicide degradation studies in Australian soils remain unpublished, very few information is available on the degradation of herbicides in Australian soils under laboratory and field conditions. Bos et al. (1995) reviewed degradation behaviour of triazine, sulfonylureas and dinitroanilines in Australian soils. The degradation of atrazine (Bowmer, 1973; Haigh & Ferris, 1991), sulfonylureas (Blacklow & Pheloung, 1992) and dinitroanilines (Jolley & Johnstone, 1994) have been reported to follow first-order kinetics in Australian soils. However, exceptions to this have been reported. Walker (1990) observed rapid initial degradation of atrazine followed by a slow first order kinetics on a sandy loam soil in Western Australia. A decrease in soil pH from 6.5 to 4.5 resulted in rapid declination of the second phase degradation of atrazine and simazine. Microbial degradation is the primary pathway of atrazine breakdown in high pH soils whereas hydrolysis is more rapid than microbial degradation in neutral to acid soils (Congreve & Cameron, 2014). Biodegradation is a complex process related to chemical, physical and environmental conditions and thus expected to be different across agro-ecological zones. For example, the half-life of atrazine was reported to be more than 100 weeks in two irrigated soils in Australia which

was 13-20 times longer than those reported in Romania and USA (Bowmer, 1991). Overall, the half-life of various herbicides in Australian soils was determined to be significantly different from a USA database (Wauchope et al., 1992).

There is little information about trifluralin degradation in Australian soils. Trifluralin has been extensively used in Australian agriculture since 1970 but majority of the degradation studies mainly emphasizing environmental conditions on the degradation of trifluralin in soil. No biodegradation studies related to trifluralin degradation in Australian soil has been reported so far. But the native microorganisms might have immense possibilities to degrade trifluralin which is remained unexplored. The successful identification of trifluralin degrading microorganisms and specific enzymes governed metabolic pathways will open new horizon in microbial degradation in Australian context.

2.5 Approaches used to study microbial degradation

Recent improvement in microbiology allows us to use various molecular and proteomic approaches to investigate specific microbial catabolic pathways for the biodegradation of herbicides (Ghosal et al., 2016). As mentioned earlier, some bacteria are capable to produce extracellular enzymes to metabolize complex organic and inorganic compounds in order to obtain energy and carbon as a part of their assimilation process. Catabolic genes play an important role in shaping the genetic foundation of herbicide biodegradation, with subsequent identification of these genes permitting application of molecular technology to investigate their function (Widada et al., 2002). Microbial genes which are known to degrade herbicides are listed in Table 2.4. Generally, those catabolic genes are situated on chromosomes, however several have been located to plasmids.

Table 2.4. Isolated bacterial and fungal genes with host organisms (Ortiz-Hernández et al., 2013; Singh & Walker, 2006).

Gene name	Host	Enzyme
Bacterial gene		
opdA	*Agrobacterium radiobacter*	Organophosphorus hydrolase
opd	*Pseudomonas diminuta*	Organophosphorus hydrolase
adpB	*Nocardia* sp.	Aryldialkylphosphatase
Phn	*Bacillus cereus*	Phosphonatase
ophB	*Burkholderia* sp. JBA3	Organophosphorus hydrolase
Imh	*Arthrobacter* sp. scl-2	ND
Mpd	*Ochrobactrum* sp. Yw28 *and Rhizobium radiobacter*	ND
opdE	*Enterobacter* sp.	Organophosphorus hydrolase
opaA	*Alteromonas* spp.	Organophosphorus acid anhydrolase
pepA	*Escherichia coli*	Aminopeptidase A
hocA	*Pseudomonas montelli*	ND
pehA	*Burkholderia caryophilli*	Phosphonate monoesterase
ophC2	*Stenotrophomonas* sp. SMSP-1	ND
OpdB	*Lactobacillus brevis*	Organophosphorus hydrolase
Oph	*Arthrobacter* sp.	ND
Mph	*Arthrobacter* sp. L1	Methyl parathion hydrolase
MphB	*Burkholderia cepacia*	Methyl parathion hydrolase
Fungal gene		
A-opd	*Aspargillus niger*	ND
P-opd	*Penicillium lilacinum*	ND

ND= not determined

Researchers are placing more emphasis on the sequencing of whole genomes from a wide range of microbial populations in the soil to investigate novel genes and degradative elements responsible for the degradation of pesticides. This has provided new insights into the identification of herbicide degrading genes from both culturable and non-culturable microorganisms and provided an increased understanding of innovative metabolic pathways under various environmental conditions, which is essential for the successful implementation of bioremediation techniques. Li et al.

(2007) identified several microbial enzymes, such as organophosphorus hydrolase isolated from bacteria, capable of hydrolysing organophosphate pesticides and utilizing this as a source of carbon. The identified gene (*opd* gene, homologue to *mpd* gene) is highly preserved within plasmid containing 996 nucleotides and is responsible for organophosphorus hydrolases (OPH). Hydroxylation of methyl-parathion was accelerated through the secretion of a specific enzyme methyl-parathion hydrolase. Cui et al. (2001) isolated the gene responsible for hydroxylation of methyl-parathion from the bacterial strains of *Achrobacter, Ochrobactrum, Pseudaminobacter* and *Achrobacter* by comparing the same gene belonging to *Pleisomonas* sp. To date more than 300 genes have been isolated from various culturable bacterial strains worldwide engaged in biodegradation of aromatic compounds (Bhatt et al., 2019). More and more emphasis has been given to DNA and RNA quantification to identify the number of potential biodegrading genes. It is believed that a positive correlation may exist between the relative abundance of biodegrading genes and their ability to degrade contaminants in the environment. Quantitative studies related to DNA and RNA can significantly promote biodegradation of herbicides by identifying the bulk of genes associated in bioremediation. This can allow the manipulation of the environment to promote the increase in the numbers of organisms involved in biodegradation. Repeated application of atrazine resulted in the increase of the microbial population responsible for herbicide degradation (Fang et al., 2015; Yale et al., 2017). Similar results were observed in case of herbicide MCPA (2-methyl-4- chlorophenoxyacetic acid) (Bælum et al., 2008) and glyphosate (Lancaster et al., 2010). Perhaps potential research using DNA and RNA approaches in the identification of biodegrading genes might result in novel understanding related to the management of biodegradation which could lead to regulation of extent and rate of biodegradation (Lovley, 2003).

The absence of degrading microorganism could make the scenario difficult; favouring the herbicide compound persist in soil longer than usual. However, recent research approaches pointed out the possibility of developing genetically modified microorganisms for herbicide degradation (Coelho et al., 2015; Hussain et al., 2018; Verma & Jaiswal, 2016). These genetically modified super microorganisms may degrade the herbicide faster than the usual. Although adaptability of the microorganisms in the contaminated site may be an issue which could lead this strategy ill-fated. Moreover,

the potential risk associated with genetically modified microorganisms to open environment raised common safety concerns and legislative issues (Hussain et al., 2018).

2.6 Research goals

Despite the widespread use of herbicides and consequently their undesirable presence in the environment, microbial degradation pathways of herbicides and their genetic bases remain poorly understood. Researchers identified potential microorganisms for atrazine and trifluralin degradation in soil, but further evaluation of these suspected microbes was not undertaken.

In Australia, herbicides applied in minimal or zero till systems tend to maintain a greater concentration of herbicide near the surface zone at the end of the cropping season, which may result in higher residual concentrations, affecting crops subsequently sown. Currently, there is no option available to combat this problem other than using crop rotation to avoid incompatible crop-herbicide combinations which includes routine rotation of fallow and pre-emergent herbicides, reliable record keeping helping identify potential residue issues, and use of tolerant crops or crop cultivars in rotations after dry seasons.

Environmental fate of trifluralin and atrazine has been extensively investigated in other countries; however, little is known about their persistence in Australian soil. Persistence of both herbicides in soil could potentially affect sensitive crops in rotation, investigation on critical concentrations at which level causing damage to sensitive following crops could help farmers in selecting crop rotation strategies. In addition, environmental factors are known to have a crucial effect on the persistence of herbicides in soil. Again, persistence of herbicides in soil is directly related with the presence or absence of degrading microorganisms in soil, the shift in microbial community structure and diversity upon herbicide exposure could generate valuable information about potential groups of microorganisms benefitted by herbicide application. This study could potentially contribute to the development of bioremediation strategies associated with trifluralin and atrazine degradation in the soil, thereby minimising the negative impacts of herbicides on the environment and the following crops.

2.6.1 Aims of this study

The aims of this study are as follows:

1. To determine critical concentration levels of trifluralin and atrazine in soil affecting growth and development of selected cereal and legume crops (Chapter 3)
2. To determine the effect of temperature and moisture content on persistence of trifluralin and atrazine in clay loam soil (Chapter 4)
3. To determine changes in soil microbial community structure and functions associated with the application of various concentrations of atrazine and trifluralin in clay loam soil (Chapter 5).

Chapter 3. Chapter based on published paper

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Trifluralin and Atrazine Sensitivity to Selected Cereal and Legume Crops

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Abstract: Soil-applied herbicides can persist in sufficient concentrations to affect the growth of crops in rotations. The sensitivity of wheat, barley, oat, lucerne and lentil to trifluralin and atrazine residues were investigated with three glasshouse experiments in 2018 and 2019. Each bioassay crop species was tested against different concentrations of trifluralin and atrazine in sandy soil using a full factorial design. Shoot and root parameters of the tested crop species were fitted in logistic equations against herbicide concentrations to calculate effective doses for 50% growth inhibition (ED_{50}). Results revealed that both shoot and root parameters of all the test crop species were significantly affected by trifluralin and atrazine. Trifluralin delayed crop emergence at the lower concentrations examined, while higher concentrations prevented emergence entirely. Low concentrations of atrazine did not affect emergence but significantly reduced plant height, soil–plant analyses development (SPAD) index, shoot dry weight, root length, root dry weight and number of nodules of all the crop

species. At high concentration, atrazine resulted in plant death. Legumes were found to be more sensitive than cereals when exposed to both trifluralin and atrazine treatments, with lucerne being the most sensitive to both herbicides, ED_{50} ranging from 0.01 to 0.07 mg/kg soil for trifluralin; and from 0.004 to 0.01 mg/kg for atrazine. Barley was the most tolerant species observed in terms of the two herbicides tested. Lucerne can be used to develop a simple but reliable bioassay technique to estimate herbicide residues in the soil so that a sound crop rotation strategy can be implemented.

Keywords: trifluralin; atrazine; herbicide sensitivity; bioassay; cereals; legumes

3.1. Introduction

Farming systems in Australia have undergone a substantial revolution over the past 25 years with the adoption of conservation tillage. The trend towards minimum or zero till has reduced cultivation practices for weed management [1] and driven the increased adoption of herbicides as the primary mechanism for weed control [2,3]. This is a global issue, with herbicides commonly implemented to control weeds that are a persistent risk to crop production [4]. Herbicides account for approximately 60% of total pesticide expenditure across Australian farming systems, costing growers approximately $1.80 billion in 2017–2018 [5]. Herbicide adoption in farming systems has not only raised concerns about their negative impacts on the environment, human and animal health, and agricultural sustainability with the evolution of herbicide resistance, but also raised concerns about their ultimate fates in soil [6–8].

In systems employing conservation tillage, herbicide applications tend to leave a greater concentration of herbicide near the soil surface [9]. The presence of persistent herbicides in this concentrated zone may affect subsequent sensitive crops and compromise overall crop performance [10], thus limiting planting options for farmers. Precise assessment of the herbicide residues to gauge persistence and degradation patterns in soil is crucial for ensuring minimum risk in farming systems practicing crop rotation [11]. A survey of Australian cropping soils undertaken prior to sowing in 2016 detected residues of 23 herbicides, with trifluralin detected in more than 30% of the fields surveyed, with a maximum residue concentration of 5345 µg/kg compared to 590 µg/kg detected in 2015 [12]. The advancement of conservation tillage farming systems associated with relatively higher herbicide application rates may have contributed to the

high detection frequency of trifluralin. In addition, higher concentrations of atrazine residues were also detected in cropping paddocks of New South Wales and South Australia, which was possibly due to the higher persistence of s-triazine herbicides in alkaline soils [13].

Trifluralin, developed in the 1970s, belongs to the dinitroaniline chemical group. It is a popular pre-emergent soil-incorporated herbicide used to control annual grass and broadleaf weeds in field crops [14]. Trifluralin interferes with mitosis and inhibits microtubule assembly by restricting polymerisation of tubulin [15–17], a structural protein of plant cells, subsequently inhibiting growth and resulting in plant death [18]. Being one of the most common pre-emergent herbicides used in conservation tillage systems [19,20], the role of trifluralin in the adoption of no-till farming systems has been acknowledged by several researchers [21–23]. Trifluralin is highly volatile due to high vapor pressure and is generally incorporated into soil after application to reduce losses caused by volatilisation and photodegradation [24]. The level of persistence of trifluralin in soil is regulated by various factors, including soil moisture, temperature, soil type, and duration before incorporation [25–27].

Another common herbicide, atrazine, is often used alone or in combination with other herbicides to control grass and broadleaf weeds in field crops [28,29]. Introduced into the market almost 50 years ago, atrazine now ranks as the second most heavily used pesticide in the world [30]. Atrazine interferes with the photosystem II (PSII) process by affecting adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) production, ultimately reducing efficiency of the CO_2 fixation process [31]. Atrazine is also responsible for the rapid accumulation of reactive oxygen species (ROS) by limiting electron transport system in chloroplasts, causing membrane injury [32,33]. Researchers suggest that atrazine is highly persistent in soil and can be detected even after 22 years of application [34], which indicates a high potential for the contamination of agricultural fields due to its low adsorption, moderate aqueous solubility, and long half-life.

Herbicide recommendation is fundamentally crop-specific. The sensitivity of different crop species to a particular herbicide is related to differences in crop morphology, physiology and phenology [35]. Some herbicides can remain in soil for weeks, months or even years. This is advantageous in regard to long term weed control. However, persistence can affect sensitive crop species by the residual activity of the

herbicide in subsequent years [36]. Therefore, to minimise the negative impacts of herbicide residues in the soil on rotational crops, it is highly recommended to study the residual effects of herbicides on crops utilised in rotational systems [37]. As a result, this study was undertaken to investigate the potential impact of trifluralin and atrazine residues on the establishment and growth of selected cereals and legumes (wheat, barley, oat, lucerne and lentil). The development of a reliable and simple bioassay technique to identify sensitive crop species may allow informed decisions to be made prior to implementing a crop rotation strategy.

3.2. Materials and Methods

3.2.1. Test crop species, herbicides and soil type

Three experiments were conducted under glasshouse conditions at Charles Sturt University, Wagga Wagga, New South Wales in 2018 and 2019. Five crops were chosen based on reported sensitivity to both trifluralin and atrazine [38], being wheat (*Triticum aestivum* cv. Corack), barley (*Hordeum vulgare* cv. Hindmarsh), oat (*Avena sativa* cv. Savannah), lucerne (*Medicago sativa* cv. Stamina) and lentil (*Lens culinaris* cv. Hurricane XT). Seeds of the test crop species were obtained from NSW Department of Primary Industries (DPI), Wagga Wagga, NSW and were tested for germination prior to use. Two commercial herbicides Triflur X (a.i. 480 g/L trifluralin) and Farmozine 900 WG (a.i. 900 g/kg atrazine) were used in this study. Non-autoclaved sandy soils were used to investigate the phytotoxicity associated with each herbicide because autoclaving the soil may alter the toxicity symptoms exhibited by the test crop in natural conditions due to the removal of degrading microorganisms [39]. The soil was determined to have a pH of 6.80 (water), organic matter <0.30%, organic carbon <0.20%, total N, P, and K was 4.00, 7.00 and 4.80 mg/kg, respectively. Prior to use the soil was sieved to 2 mm.

3.2.2. Preparation and application of herbicides at different concentrations

Based on a preliminary study [38], multiple small scale trials were carried out using a broad concentration range for both herbicides to narrow down to a suitable operational range for this experiment. Thus, a concentration series of 0, 0.075, 0.15, 0.30, 0.60, 1.20 and 2.40 mg a.i./kg dry soil was prepared for trifluralin and a series of 0, 0.15, 0.30, 0.90, 1.50, 2.10 and 3.60 mg a.i./kg dry soil was prepared for atrazine. The experiment was

repeated over time, using the above concentration series. Since legume species did not survive the minimum concentration of atrazine (0.15 mg/kg soil) used in first and second experiments (2018), a separate lower concentration series of 0, 0.006, 0.017, 0.05 and 0.15 mg/kg dry soil was prepared for the third trial in 2019.

Each herbicide concentrate was diluted with deionised water at required concentrations and applied by a hand sprayer onto the 1.0 kg dry soil equivalent while the soil was continuously mixed with a cement mixer to approximately 50% water holding capacity, according to Hasanuzzaman, et al. [40]. Soils for control treatments were prepared by applying deionised water only.

3.2.3. Planting and growing test crops

A previously published protocol on crop sensitivity to residual herbicide [41] was used in this study with modifications. Plastic pots (80 × 145 mm) were filled with each herbicide treated soil representing the concentration series. For each crop, five seeds were planted at a depth of 5 cm across the herbicide concentration series. For trifluralin, seven concentrations were tested against five crop species including control, with four replications in a factorial design. For atrazine, seven concentrations were tested against five crop species in first two trials and five lower concentrations were tested for the two legume species during the third trial only. Pots were placed on a bench and blocked by replicates. Pots were hand watered on daily basis with a hand sprayer to maintain moist conditions and allow seedling emergence but avoid overwatering and leaching of herbicides. Pots were maintained in a temperature-controlled glasshouse (30 ± 2 °C) under natural sunlight throughout the 4-week experimental period. Soon after emergence, seedlings were counted and thinned to 3 plants per pot.

3.2.4. Measurements

The total number of seedlings emerged was counted for each of the test crop species. Plant height and leaf chlorophyll content were measured at 28 days after sowing (DAS). Leaf chlorophyll content was calculated as a soil–plant analyses development (SPAD) index with the Minolta SPAD-502 (Konica Minolta Sensing). Measurements were carried out from the leaf lamina of the second uppermost leaves at three different points (tip, middle and base). After the chlorophyll measurements, aboveground parts were harvested by cutting the shoots approximately 2 mm above the soil surface. Shoots were

labelled and bagged accordingly. Shoot dry weight (SDW) was determined after drying the samples at 70 °C for 48 h. Root samples were extracted by gently washing away the soil with tap water and then transferred to a Perspex tray containing deionised water. Samples were imaged at 600 dpi using a flatbed scanner (Epson Expression 11000XL). The scanned images were further analysed by WinRHIZO (Regent Instruments Inc., Quebec, Canada) to determine root length (RL, cm), mean root diameter (RD, mm) and specific root length. Thereafter, root samples were dehydrated at 70 °C for 48 h to determine the root dry weight (RDW). In case of legume species (lucerne and lentil), the total number of nodules were counted under magnification, using a Nikon SMZ25 motorised stereo zoom microscope.

3.2.5. Statistical analysis

All the recorded data were transformed as a percent of control for each of the parameters in order to compare between different treatments of each herbicide tested in three successive trials (except mean root diameter). As there was no significant difference found between first two trials, hence the data were pooled. Statistical analysis was performed using software R operated in RStudio 3.5.3 [42] with a range of R packages including *drc* [43], *ggplot2* [44] and *cowplot* [45] for explanatory data analysis. Data normality and distribution were validated by Q-Q plot and Shapiro-Wilk test of normality. The best fitted model was selected based on the Akaike information criterion (AIC) value. A non-linear two parameter log-logistic model (equation 1) was fitted for the emergence of test crop species (wheat, barley, oat, lucerne and lentil). Other shoot and root parameters of the test crop species were fitted in Weibull four parameter model (equation 2) except for trifluralin concentrations on root diameter and root dry weight of the test crop species and atrazine concentrations on the shoot dry weight of wheat, barley and oat best fitted on a four parameter log-logistic model (equation 3) [46]:

$$f(x) = 1/\{1 + exp(b(log(x) - log(e)))\} \quad (1)$$

$$f(x) = c + (d - c)\exp(-exp\left(b(log(x) - log(e))\right)) \quad (2)$$

$$f(x) = c + (d - c)/(1 + \exp\left(b(log(x) - log(e))\right)) \quad (3)$$

where d = the upper limit corresponding to the mean response of the control treatment, c = the lower limit corresponding to the mean response at the maximum herbicide dose levels, x = the herbicide dose level, e = the effective herbicide dose levels required for the 50% growth inhibition (ED_{50}) and b = the slope of the curve around the inflection point e (ED_{50}).

3.3. Results and Discussion

3.3.1. Effect of trifluralin on the test crop species

The development of trifluralin toxicity was monitored for each crop species from emergence to 28 DAS for each of the concentration series. From this evaluation, it was determined that trifluralin concentrations in soil had a significant effect on the emergence of the test crop species ($P < 0.001$). Emergence of the test species was delayed at the lowest concentration of 0.075 mg/kg. Trifluralin at the highest concentration of 2.40 mg/kg dry soil completely suppressed the emergence for all five test species (Figure 3.1A). Wheat and oat only emerged up to the trifluralin concentration of 0.30 mg/kg soil, while barley, lucerne and lentil had 27.98, 19.58 and 30.82% emergence at the concentration of 0.30 mg/kg, respectively. Almeida and Rodrigues [47] reported that trifluralin inhibits seed germination by interfering with cell division of meristematic tissues. It is due to adsorption primarily by the hypocotyl, followed by seedling radicles during germination [48,49].

Toxicity symptoms associated with trifluralin residues were identified as stunted growth with twisted leaves, swollen hypocotyl and thickened primary root with no secondary roots. Similar types of toxicity symptoms were reported by Senseman [49], Deuber [50]. Trifluralin toxicity induced significant damage in respect to plant height with increasing levels of trifluralin concentration in the soil (Figure 3.1B). Approximately 50% inhibition was recorded in all the crop species under the lowest concentration of trifluralin (0.075 mg/kg soil), with the exception of barley (ED_{50} 0.19 mg/kg). The ED_{50} value of lucerne (0.03 mg/kg) was significantly lower ($P < 0.001$) than the other crop

species as no further elongation was observed after emergence (Table 3.1). Trifluralin interruption on cell mitosis has been acknowledged in literature [51] which resulted in inhibition of root and shoot cell division [52]. Khalil, et al. [53] identified shoot length as the most sensitive parameter to assess trifluralin activity due to sensitivity of the coleoptile node documented in green foxtail [54,55]. Residual activity of trifluralin has been reported to reduce at least 44% plant height in sesame [56] and 80% in rice [57] as compared to control.

In terms of leaf chlorophyll content, a significant reduction ($P < 0.001$) in SPAD index were recorded in all plants treated with trifluralin, with the presence of twisted and yellowing leaves. Increasing trifluralin soil concentrations from 0.075 to 0.30 mg/kg soil resulted in the gradual reduction of SPAD index (Figure 3.1C). Lucerne had the lowest SPAD index at 0.075 mg/kg soil trifluralin concentration compared to others (ED_{50} 0.07 mg/kg) (Table 3.1).

Shoot dry weight was considerably reduced with the increase of trifluralin concentrations in soil, causing as high as 60–70% reduction at the lowest concentration (0.075 mg/kg) compared to control, while plant death occurred at the highest levels (Figure 3.1D). Lucerne was the most sensitive to trifluralin concentrations compared to other crops as the ED_{50} value was significantly lower ($P < 0.001$) than others (0.01 mg/kg soil) (Table 3.1). Chaudhari, et al. [58] reported considerable shoot dry weight reduction (up to 89%) of turnip when exposed to trifluralin at concentrations of approximately 1.70 mg/kg soil. Nosratti, et al. [59] identified the toxicity symptoms of trifluralin including reduction in plant height, chlorophyll content and shoot dry weight.

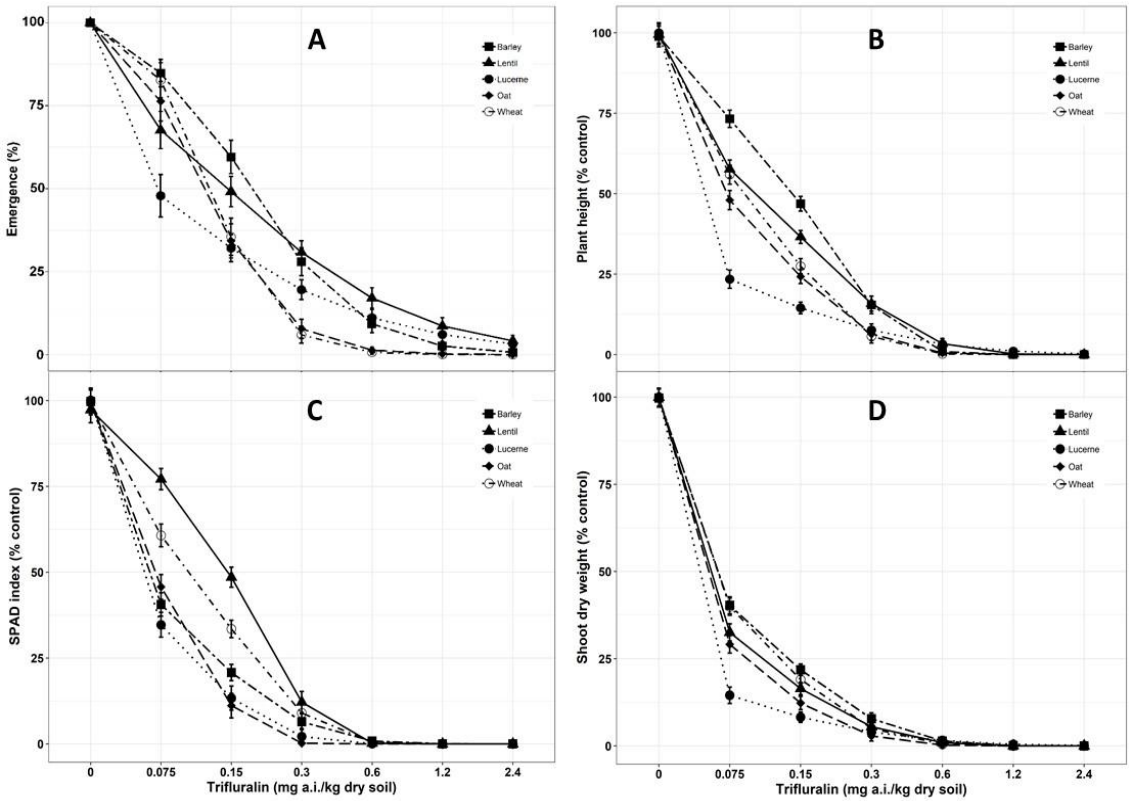

Figure 3.1. Dose-response effects of trifluralin on shoot parameters (**A**. Emergence; **B**. Plant height; **C**. SPAD index; **D**. Shoot dry weight) of barley, lentil, lucerne, oat and wheat at 28 days after sowing (DAS). Lines denote predicted responses according to the equations reported in the materials and methods section. Symbols shown on the graphs are the original means of each parameter (% control) against each dose of trifluralin concentration. Mean value was pooled from eight observational units.

For the root parameters measured, root length, root dry weight and nodulation were highly affected by the presence of trifluralin in the soil. Root length of all the species was reduced by 60–80% even at minimum concentrations of trifluralin in soil (Figure 3.2A). The affected roots were characterised by a thickening of hypocotyls, swollen primary root and absence of lateral root and root hairs, which are similar to results previously reported [49,50]. Oat was the most sensitive crop in regards to root length (ED_{50} 0.01 mg/kg) in both of the trials whereas lucerne performed better (ED_{50} 0.09 mg/kg) (Table 3.1). Wheat, oat and lucerne roots died at 0.30 mg/kg concentration, while lentil roots exhibited their maximum mean root diameter (2.14 mm) at the trifluralin concentration of 0.30 mg/kg soil, which is four times that of the control (Figure 3.2B). However, trifluralin even at the lowest concentration of 0.075 mg/kg soil significantly reduced ($P < 0.001$) root dry weight of all the crop species, with reductions varying from 95% in oat to 69% in lucerne compared to the untreated control (Figure 3.2C). Root development was greatly hampered due to trifluralin toxicity as root dry weight is known to be the most sensitive and precise measures for trifluralin toxicity [60,61]. The ED_{50} for oat in terms of root dry weight was 0.01 mg/kg soil (Table 3.1), significantly lower than the other crop species ($P < 0.001$). Trifluralin interfered with the nodulation process of legume species even at the lowest application rate and the nodulation was completely inhibited at 0.30 mg/kg soil concentration (Figure 3.2D). Variable levels of plant injury and growth reduction due to trifluralin have been reported due to the differences in soil type, temperature, soil moisture and duration of incorporation [25,26,58,62]. Soil organic matter tends to reduce the bioavailability of trifluralin in soil via sorption [63], which is correlated with an increase in soil organic matter [64]. Soils containing high organic matter were reported to adsorb herbicide compounds more likely compared to those with low organic matter [65]. Thus, trifluralin injury in sandy soils was found to be prominent and logical in this experiment as the organic matter content was negligible.

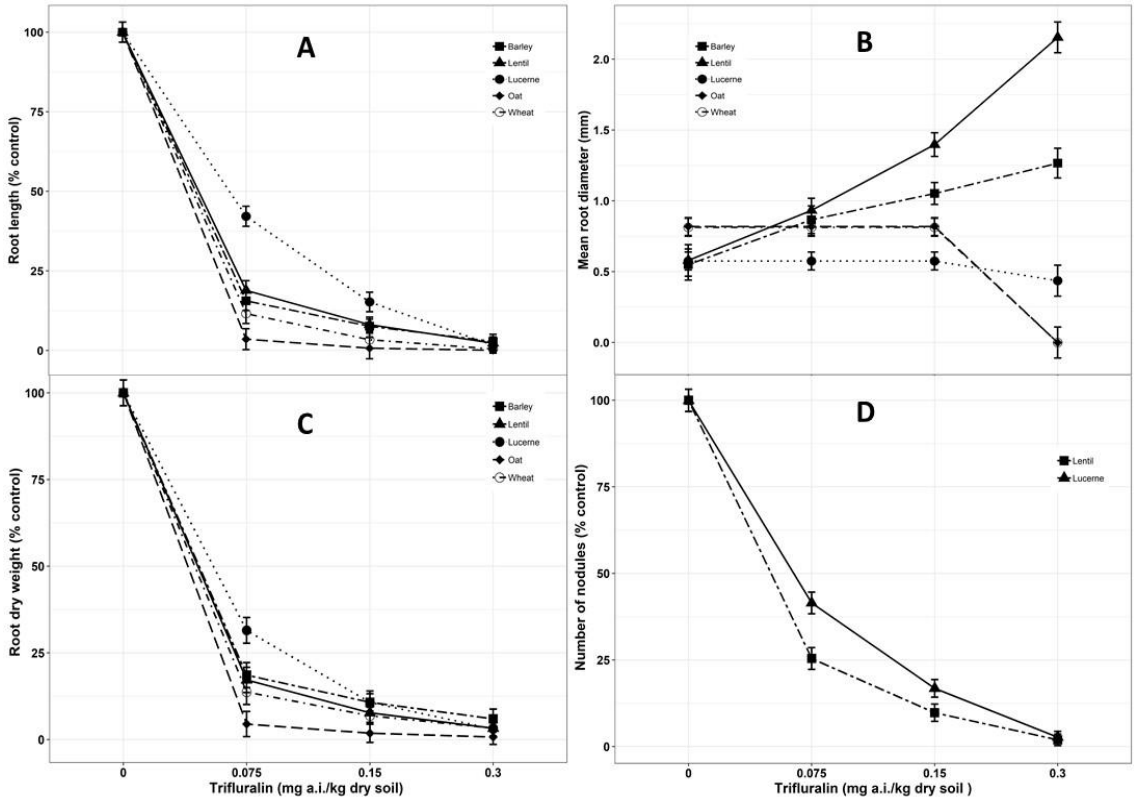

Figure 3.2. Effect of various levels of trifluralin concentrations on root parameters (**A.** Root length; **B.** Mean root diameter; **C.** Root dry weight; **D.** Number of nodules) of barley, lentil, lucerne, oat and wheat at 28 days after sowing (DAS). Lines denote predicted responses according to the equations reported in the materials and methods section. Symbols shown on the graphs are the original means of each parameter (% control) against each dose of trifluralin concentration. Mean value was pooled from eight observational units.

Table 3.1. Estimated regression parameters of the two parameter log-logistic model, four parameter Weibull model (Weibull1) and four parameter log-logistic model for the shoot and root parameters of wheat, barley, oat, lucerne and lentil against various levels of trifluralin concentrations as per equations 1, 2 and 3 mentioned in materials and methods section.

	Crops	***ED_{50} (mg/kg dry soil)***	***d***	***b***
Germination	Wheat	0.12 (0.01)	100 (NA)	3.12 (0.48)
	Barley	0.18 (0.02)	100 (NA)	1.92 (0.25)
	Oat	0.12 (0.01)	100 (NA)	2.63 (0.40)
	Lucerne	0.07 (0.02)	100 (NA)	0.96 (0.18)
	Lentil	0.15 (0.02)	100 (NA)	1.12 (0.17)
Plant height	Wheat	0.12 (0.01)	99.73 (3.17)	1.15 (0.14)
	Barley	0.19 (0.01)	98.84 (3.20)	1.32 (0.15)
	Oat	0.10 (0.01)	99.62 (3.18)	0.95 (0.12)
	Lucerne	0.03 (0.01)	99.97 (3.16)	0.41 (0.09)
	Lentil	0.15 (0.01)	99.48 (3.18)	0.88 (0.10)
SPAD index	Wheat	0.14 (0.01)	99.50 (3.67)	1.14 (0.14)
	Barley	0.09 (0.01)	99.77 (3.66)	0.80 (0.13)
	Oat	0.09 (0.01)	100.00 (3.65)	1.49 (0.26)
	Lucerne	0.07 (0.01)	100.04 (3.65)	0.93 (0.24)
	Lentil	0.19 (0.01)	97.39 (3.82)	1.58 (0.22)
Shoot dry weight	Wheat	0.08 (0.01)	99.83 (2.60)	0.85 (0.10)
	Barley	0.09 (0.01)	99.74 (2.61)	0.75 (0.08)
	Oat	0.06 (0.01)	100.96 (2.60)	0.76 (0.13)
	Lucerne	0.01 (0.01)	100.99 (2.60)	0.37 (0.10)
	Lentil	0.06 (0.01)	97.96 (2.60)	0.69 (0.10)
Root length	Wheat	0.02 (0.02)	100.00 (3.16)	0.65 (0.38)
	Barley	0.02 (0.01)	100.00 (3.16)	0.47 (0.19)
	Oat	0.01 (0.05)	100.00 (3.16)	0.57 (1.48)
	Lucerne	0.09 (0.01)	100.04 (3.16)	1.12 (0.20)
	Lentil	0.03 (0.01)	100.00 (3.16)	0.59 (0.20)
Root dry weight	Wheat	0.01 (0.01)	100.00 (3.72)	1.10 (0.59)
	Barley	0.02 (0.10)	100.00 (3.72)	0.92 (0.43)
	Oat	0.01 (0.03)	100.00 (3.72)	1.33 (2.26)
	Lucerne	0.05 (0.01)	100.01 (3.72)	1.93 (0.53)
	Lentil	0.02 (0.02)	100.00 (3.72)	1.31 (0.61)
Number of nodules	Lucerne	0.09 (0.01)	99.92 (3.22)	1.02 (0.16)
	Lentil	0.05 (0.01)	99.98 (3.21)	0.76 (0.19)

NA= Not applicable; *b*, relative slope of curve around ED_{50} values; *d*, upper limit corresponding to the mean response of the control treatment; *ED_{50}*, the effective herbicide dose levels required for the 50% growth inhibition. SE values are presented in parentheses.

3.3.2. Effect of atrazine on the test crop species

Although pre-emergence application of atrazine primarily targets germination of weed seeds, no significant effect on the emergence of the crop species was observed in all experiments. However, atrazine caused considerable damage to all tested crop species in the current study, regardless of the application rate. Initial toxicity symptoms appeared after two weeks of growth and became more prominent on the tips and edges of the mature leaves, manifested by chlorosis later spreading both upwards and downwards ultimately affecting height, chlorophyll content and shoot dry weight of all the species. Plants died over time due to the inhibition of photosynthesis. Shoots were more affected compared to roots, regardless of the crop species, even though atrazine is reported to concentrate in roots compared to shoots [66]. The plant heights of the three cereal crops were reduced significantly ($P < 0.001$) with the increased concentrations of atrazine (Figure 3.3A). In the third experiment, lucerne survived at the lowest atrazine concentration (0.006 mg/kg soil) and was identified as the most sensitive species based on plant height (Figure 3.3B). At the atrazine concentration 0.15 mg/kg, both lucerne and lentil did not survive, but the cereals had a plant height that was approximately 65% of the control. This study revealed that cereal crops (wheat, barley and oat) were relatively more tolerant to atrazine concentrations than the legume species (lucerne and lentil). Barley was the most tolerant species (ED_{50} 1.21 mg/kg) as it managed to survive under the highest concentration of atrazine tested (3.60 mg/kg soil) while all other crop species died at this concentration (Table 3.2). Zhang, et al. [67] reported 67.10% reduction in shoot length of rice when exposed to 0.40 mg/L of atrazine compared to control.

Chlorophyll is regarded as a sensitive biomarker for plant growth [68]. Figure 3.3C revealed that the SPAD index of all the crop species followed a decreasing pattern with increasing concentration of atrazine and all the plants but barley died due to chlorosis at the highest concentrations in extreme conditions. Lucerne and lentil were the most sensitive species in terms of the SPAD index. Lentil performed better than lucerne as it did not survive the atrazine concentration of 0.017 mg/kg soil, whereas 30% reduction in SPAD index was observed in case of lentil (Figure 3.3D). Huiyun, et al. [69] reported that chlorophyll content inhibition was positively correlated with dosage of atrazine. Barley was the only species that survived the maximum atrazine concentrations (3.60

mg/kg soil) with an ED_{50} value of 1.46 mg/kg, significantly higher ($P < 0.001$) than the other crop species, although the SPAD index decreased to one third of the control (Table 3.2). Chlorophyll content acts as an indicator of the growth and photosynthetic ability of the plant [70]. A decrease in chlorophyll content with the increase of atrazine concentrations in soil indicates the negative effects of atrazine on the growth of plants [69]. Chlorophyll content of rice exposed to 0.80 mg/L atrazine was reduced by 60% compared to the untreated control [67].

A reduction in shoot dry weight was observed with the increase of atrazine residue concentration in soil (Figure 3.3E,F). Approximately 55% reduction of shoot dry weight has been recorded in wheat, barley and oat when exposed to the lowest atrazine concentration (0.15 mg/kg soil), whereas legume species were more sensitive than cereals, having 100% reduction in shoot dry weight at the same atrazine concentration (0.15 mg/kg soil). Higher application rate of atrazine resulted in higher reduction in shoot dry weight which can be related to the higher exposure and absorption of the atrazine residues in soil accompanying root growth. Lucerne had ED_{50} values for shoot dry weight 0.01 mg/kg, which was significantly lower ($P < 0.001$) than other crop species (Table 3.2). Reduction in plant height and SPAD index due to exposure of different levels of atrazine might have contributed to the reduction in the shoot dry weight of all the crop species. Atrazine plays an essential role on seedling growth, inhibiting rice shoot dry weight to an extent of 39% [66] and 48.90% [67]. Phytotoxicity associated with atrazine to wheat, corn, mustard, turnip, pearl-millet and carrot has also been reported by Burhan, et al [71].

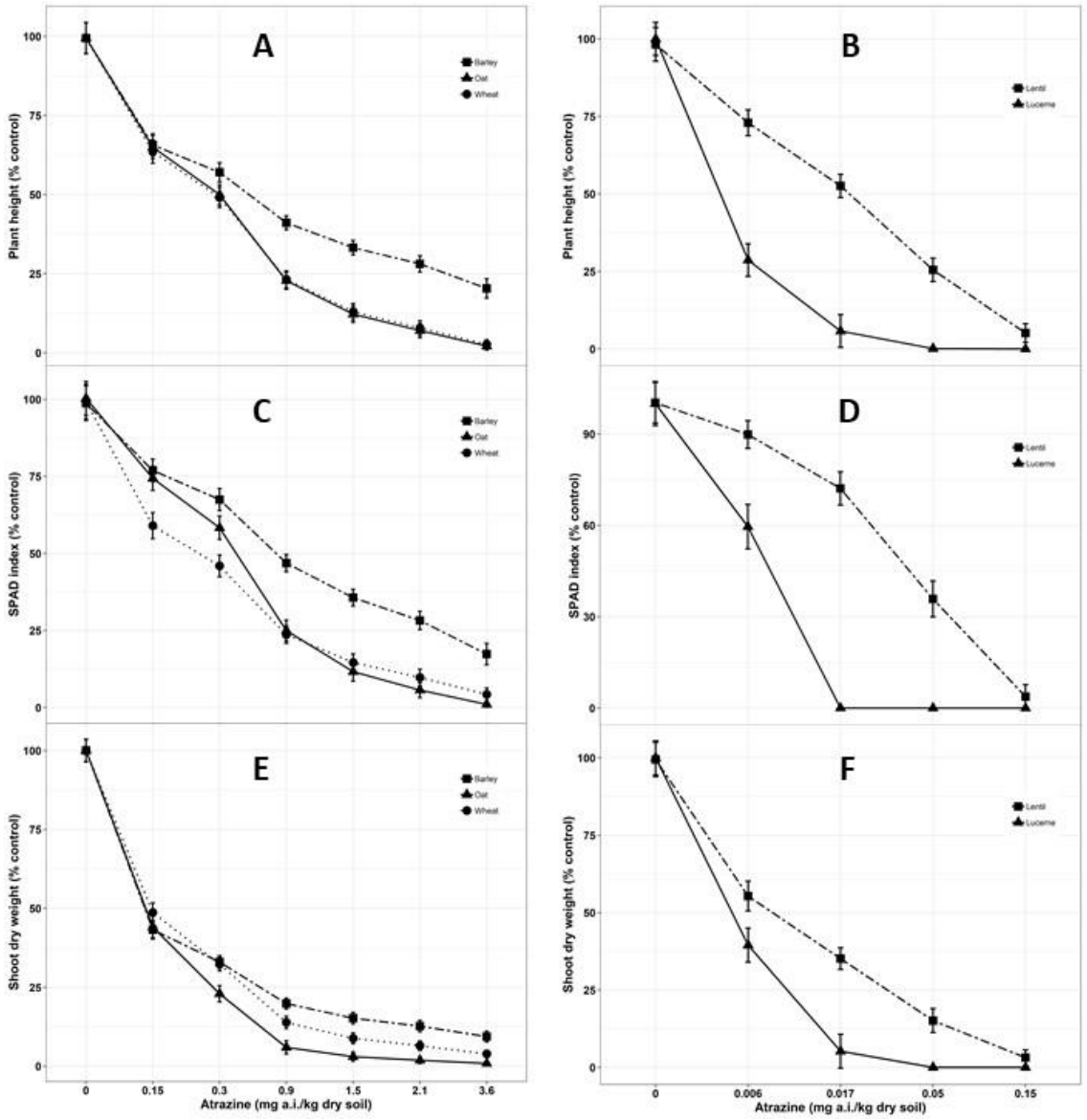

Figure 3.3. Dose-response assay of atrazine concentrations on plant height (**A**. barley, wheat and oat; **B**. lentil and lucerne), SPAD index (**C**. barley, wheat and oat; **D**. lentil and lucerne) and shoot dry weight (**E**. barley, wheat and oat; **F**. lentil and lucerne) at 28 days after sowing (DAS). Lines denote predicted responses according to the equations reported in the materials and methods section. Symbols shown on the graphs are the original means of each parameters (% control) against each doses of atrazine concentration. Mean value was pooled from eight observational units for wheat, barley and oat; whereas four observational units for lentil and lucerne.

Atrazine residues had adverse effects on the root length, root dry weight and nodulation, depending on the concentrations in the soil. It did not affect the mean root diameter and specific root length. Atrazine at the lowest concentration of 0.15 mg/kg soil caused 45–65% reduction in root length in wheat, barley and oat (Figure 3.4A); while lentil and lucerne had 50 and 78% reduction at 0.006 mg/kg soil atrazine concentration

(Figure 3.4B). Lentil experienced greater reduction in root length than lucerne, with the ED_{50} values of 0.003 mg/kg soil significantly lower ($P < 0.001$) compared to others. Figure 3.4C,D depicted similar results regarding root dry weight. Wheat, barley and oat exhibited similar type of sensitivity towards different atrazine levels with 60–75% reductions in root dry weight at 0.15 mg/kg soil atrazine concentration.

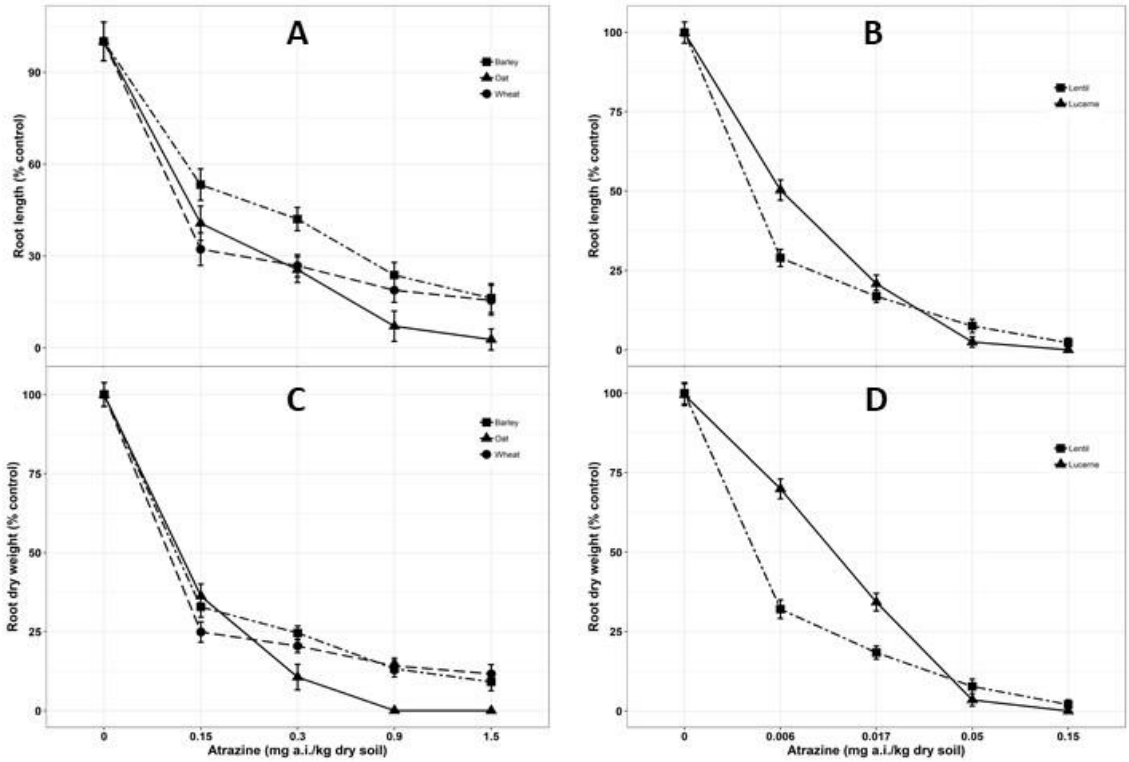

Figure 3.4. Effect of different levels of atrazine concentrations on root length (**A**. barley, wheat and oat; **B**. lentil and lucerne) and root dry weight (**C**. barley, wheat and oat; **D**. lentil and lucerne) at 28 days after sowing (DAS). Lines denote predicted responses according to the equations reported in the materials and methods section. Symbols shown on the graphs are the original means of each parameter (% control) against each dose of atrazine concentration. Mean value was pooled from eight observational units for wheat, barley and oat; whereas four observational units for lentil and lucerne.

Lucerne and lentil were more sensitive and had approximately 65–75% reductions at 0.017 mg/kg concentration. Oat recorded the highest ED_{50} value 0.15 mg/kg soil and lentil the least, 0.004 mg/kg soil (Table 3.2). Reduction of root dry weight due to various levels of atrazine exposure have been acknowledged in the literature [66,67]. Atrazine in soil had a significant effect ($P < 0.001$) on nodulation in legume species. During the first and second trial, no nodule was seen under minimum atrazine concentration (0.15 mg/kg soil) studied and all the plants died after emergence, whereas in the third trial, 62 and 73% inhibition of nodulation was observed in lucerne and lentil under the

minimum residue concentration (0.006 mg/kg soil) when compared to the untreated control (Figure 3.5). No nodules were formed when legumes were exposed to atrazine concentrations more than 0.017 mg/kg of soil. Root is the primary organ by which plants absorb water, nutrient and pollutants as well [72]. Moreover, the majority of the absorbed substances are accumulated in the roots, although some of them are transported to other parts [73]. Therefore, it is apparent that accumulated atrazine in roots caused a reduction in root length, root dry weight and nodulation by damaging cell membranes through oxidative stress, as atrazine can produce reactive oxygen species [32,33].

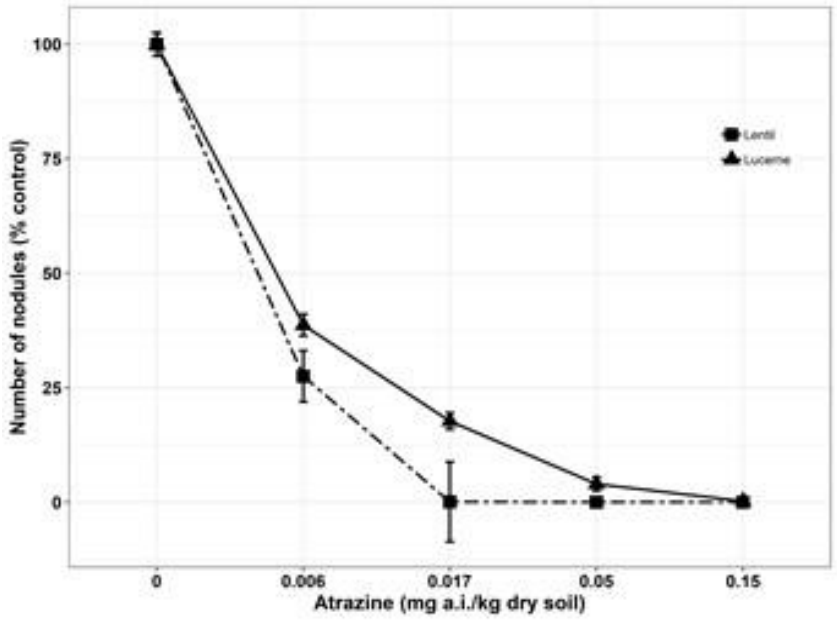

Figure 3.5. Effect of different levels of atrazine concentrations on number of nodules of lucerne and lentil at 28 days after sowing (DAS). Lines denote predicted responses according to the equations reported in the materials and methods section. Symbols shown on the graphs are the original means of each parameter (% control) against each dose of atrazine concentration. Mean value was pooled from four observational units for lentil and lucerne.

Table 3.2. Estimated regression parameters of the four parameter Weibull model (Weibull1) and four parameter log-logistic model for the shoot and root parameters of wheat, barley, oat, lucerne and lentil against various levels of atrazine concentrations as per equations 2 and 3 mentioned in materials and methods section.

	Crops	***ED_{50} (mg/kg dry soil)***	***d***	***b***
Plant height	Wheat	0.51 (0.07)	99.45 (4.91)	0.66 (0.08)
	Barley	1.21 (0.23)	99.45 (4.93)	0.42 (0.07)
	Oat	0.51 (0.07)	99.53 (4.91)	0.69 (0.09)
	Lucerne	0.004 (0.001)	100.00 (5.32)	0.79 (0.34)
	Lentil	0.03 (0.01)	98.22 (5.43)	0.71 (0.12)
SPAD index	Wheat	0.48 (0.09)	99.25 (5.60)	0.57 (0.08)
	Barley	1.46 (0.24)	99.76 (5.64)	0.61 (0.10)
	Oat	0.61 (0.08)	100.33 (5.47)	0.86 (0.11)
	Lucerne	0.01 (0.001)	100.01 (7.21)	2.63 (1.99)
	Lentil	0.05 (0.01)	100.27 (6.66)	1.06 (0.24)
Shoot dry weight	Wheat	0.14 (0.02)	99.94 (3.57)	0.99 (0.12)
	Barley	0.10 (0.03)	100.06 (3.57)	0.63 (0.08)
	Oat	0.13 (0.02)	100.04 (3.57)	1.40 (0.24)
	Lucerne	0.01 (0.001)	100.00 (5.50)	1.11 (0.38)
	Lentil	0.02 (0.003)	99.44 (5.55)	0.55 (0.11)
Root length	Wheat	0.08 (0.08)	100.03 (6.32)	0.22 (0.11)
	Barley	0.41 (0.10)	100.13 (6.31)	0.46 (0.11)
	Oat	0.18 (0.04)	100.09 (6.31)	0.60 (0.19)
	Lucerne	0.01 (0.001)	99.89 (3.34)	0.79 (0.11)
	Lentil	0.003 (0.001)	99.93 (3.32)	0.35 (0.06)
Root dry weight	Wheat	0.03 (0.03)	100.02 (3.78)	0.19 (0.07)
	Barley	0.11 (0.04)	100.09 (3.78)	0.33 (0.08)
	Oat	0.15 (0.01)	100.01 (3.78)	1.14 (0.30)
	Lucerne	0.02 (0.001)	99.46 (3.56)	1.06 (0.13)
	Lentil	0.004 (0.001)	99.96 (3.41)	0.38 (0.07)
Number of nodules	Lucerne	0.01 (0.001)	99.88 (2.52)	0.58 (0.07)
	Lentil	0.01 (0.01)	100.00 (2.58)	1.68 (19.27)

b, relative slope of curve around ED_{50} values; *d*, upper limit corresponding to the mean response of the control treatment; *ED_{50}*, the effective herbicide dose levels required for the 50% growth inhibition. SE values are presented in parentheses.

3.4. Conclusion

The described bioassay technique provides an indication of herbicide residues remaining in soil, especially with those herbicides having persistence potential. Phytotoxicity associated with pre-emergence herbicide depends on the extent of herbicides bounded by the organic matter of soil. To investigate the actual phytotoxicity, interference of organic matter has been minimised by using sandy soils in this study. Our

study revealed that trifluralin and atrazine in the soil can affect the emergence and growth of wheat, barley, oat, lucerne and lentil with the legume species being more sensitive than the cereals. This study revealed that lucerne was the most sensitive crop species compared to others and barley was the most tolerant towards trifluralin and atrazine in the soil. Hence, farmers should be careful about the carry-over issues of trifluralin and atrazine prior to selecting legumes in crop rotation. Lucerne can be used in soil bioassays to quickly determine the levels of herbicide residues in the soil so that suitable crops can be chosen prior to sowing.

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Chapter 4. Chapter based on published paper

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Persistence of atrazine and trifluralin in a clay loam soil undergoing different temperature and moisture conditions

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Abstract

Dissipation kinetics of atrazine and trifluralin in a clay loam soil were investigated in a laboratory incubation experiment under different temperature and moisture conditions. Soil was spiked with diluted atrazine and trifluralin concentrations at 4.50 and 4.25 mg/kg soil, respectively, the moisture content adjusted to 40, 70 and 100% of field capacity (FC) and then incubated in three climatic chambers at 10, 20 and 30 °C. For each of the herbicides, soil samples were collected at 0, 7, 21, 42, 70 and 105 days and analysed by Gas Chromatography- Electron capture detector (GC-ECD). A stochastic gamma model was used to model the dissipation of herbicides from the clay loam soil by incorporating environmental factors as covariates to determine half-life and days to complete dissipation. Results showed that temperature played the greater role on atrazine persistence than soil moisture; while interaction effect of temperature and moisture were significant on persistence of trifluralin over time. Atrazine dissipated more rapidly at 30 °C compared to 10 and 20 °C, with a half-life of 7.50 days and 326.23

days to reach complete dissipation. Rapid loss of trifluralin was observed at 70% moisture content when incubated at 30 °C, with a half-life of 5.80 days and 182.01 days to complete dissipation. It was observed that half-life of both herbicides tended to double with every 10 °C decrease of temperature over the range tested. The model indicated that both atrazine and trifluralin have the potential to persist in clay loam soil for several years at temperature ≤ 20 °C; which could potentially affect following crops in rotation.

Capsule: Application of gamma distribution model indicated that half-life of atrazine and trifluralin tended to double with every 10 °C drop in temperature; with a potential to persist in clay loam soil for couple of years at ≤ 20 °C.

Key wards

Half-life; Complete dissipation; Residues; Persistence

4.1. Introduction

Australian farming systems, with the adoption of conservation tillage facilitated increased reliance on herbicides as primary modes of weed control (Lewis et al., 2016). Statistics showed that Australian growers and farmers spend approximately $1.51 billion in 2018-19; which is more than 50% of the total annual pesticide expenditure (APVMA, 2020). Greater concentrations of herbicide near the soil surface has been reported in conservation tillage system (Curran, 2016), with increased concern about their ultimate fate in soil. Herbicides undergo various biotic and abiotic transformations upon application to soil. It is reported that site-specific agro-climatic conditions coupled with the amount and method of application regulates the level of herbicide persistence in soil (Srivastava et al., 2017). The majority of applied herbicide is degraded either by biotic (microbial degradation) or abiotic degradation (photodegradation and chemical degradation) pathways, which are affected by the immediate environmental conditions, herbicide chemical structure and its affinity to a particular transformation process (Fenner et al., 2013). Recent surveys conducted throughout Australian field soils detected residues of 23 herbicides, trifluralin with the maximum residue concentration of 5345 µg/kg compared to 590 µg/kg detected in 2015 (Rose et al., 2019). In addition, atrazine residues were also detected in higher concentrations of New South Wales and

South Australian field soils (Rose et al., 2016). Our previous studies reported that the presence of trifluralin and atrazine residues in soil may affect following sensitive crops and compromise overall crop performance (Chowdhury et al., 2020). Herbicide persistence is directly related to the fate and dissipation behaviour of the specific herbicidal compound where chemical, environmental and soil properties play an important role in determining their ultimate fate. Therefore, understanding the fate of herbicides in soil is a prerequisite for the accurate assessment of their behaviour and potential environmental risk (Gianelli et al., 2014).

Temperature and moisture are environmental parameters that mostly interlinked with herbicide dissipation in soil because they are known to have a critical effect on the ecological distribution and metabolic activities of microorganisms in soil (Issa & Wood, 1995; Robador et al., 2016). A rapid increase in microbial activity has been observed with an increase in soil temperature (Davidson & Janssens, 2006) because microbial metabolic activities are dependent on specific enzyme activation, which is controlled by temperature (Trasar-Cepeda et al., 2007). Laboratory studies focusing on the effect of temperature on herbicide dissipation tends to range between 4 and 45 °C (Gong et al., 2016; Ma et al., 2017; Moreno et al., 2007; Nousiainen et al., 2014; Triantafyllidis et al., 2010). Similarly, adequate moisture is a pre-requisite for soil microbial activity affecting type and population density of herbicide degrading microorganisms (Issa & Wood, 1999; Wood et al., 1996). The effect of soil moisture on the dissipation of herbicides in soil has been studied by different researchers (Camargo et al., 2013; Castillo & Torstensson, 2007; Dong & Sun, 2016; Issa & Wood, 2005; Martinez et al., 2008). However, the majority of studies focussed on either temperature or soil moisture in isolation, without considering their interaction. Both temperature and soil moisture were reported to have a major influence on accelerating persistence of herbicides in soil by causing drastic changes in soil microbial community structure and functions (Levy et al., 2007; Weber et al., 1993).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5- triazine) is a s-triazine herbicide, used extensively to control annual grass and broadleaf weeds for over 50 years (Scott et al., 2009). Atrazine is mobile in soil and does not get adsorbed by the soil particle and therefore has been frequently detected in surface and groundwater (Liu, 2014). Major degradation pathways of atrazine includes either biotic degradation governed by microorganisms (Meyer et al., 2009), or abiotic degradation such as

chemical and photochemical reactions (Azenha et al., 2003; Baranda et al., 2012). Environmental conditions such as temperature (Dinelli et al., 2000), soil moisture content (Kruger et al., 1993), pH (Qu et al., 2020), oxygen content of the surrounding matrix (Ghosh & Philip, 2004) and soil type (Miller et al., 1997) have been reported to affect persistence of atrazine in soil and natural environmental conditions.

Trifluralin (2,6-dinitro-*N,N*-dipropyl-4-trifluoromethylaniline) is a pre-emergent, soil applied herbicide used to control annual grass and broadleaf weeds. Trifluralin can adsorb onto the organic matter in soil; preventing absorption by the plants (Fernandes et al., 2013). Leaching and soil lateral movement are restricted and therefore trifluralin tends to remain in the soil-incorporation zone (Tiryaki et al., 1997). Trifluralin degradation in soil occurs through photodegradation, chemical processes and microbial activities. However, persistence of trifluralin has been reported to be affected by soil and environmental conditions (Fernandes et al., 2013).

First-order kinetics model is generally used to determine dissipation rates and half-lives of organic pesticides like atrazine and trifluralin in soil and water (Castillo & Torstensson, 2007; Díez & Barrado, 2010; Mamy et al., 2005; Navarro et al., 2004; Prado et al., 2014; Rice et al., 2002; Taylor-Lovell et al., 2002). However, there is some disagreement regarding calculation of pesticide half-lives using first-order kinetics model. For example, herbicide concentration is always a positive value but the log transformed concentration may be negative as well. Moreover, the effect of environmental factors are ignored in the model. However, these limitations can be minimized using two parameter gamma distribution model which is widely used for analysing life-time data (Rohan et al., 2015). Since the herbicide concentration in soil is expected to decline over time, the study can be classified as a survival time of herbicides. Moreover, environmental factors (temperature, moisture, pH, organic matter etc.) can be incorporated into the model, making it easier to fit the model to the data.

Therefore, the following study reports the significance of temperature and soil moisture on the persistence of atrazine and trifluralin in clay loam soil. The aims of this study were to determine the effects of temperature and soil moisture on atrazine and trifluralin dissipation in clay loam soil; and to calculate half-life and days to complete dissipations of atrazine and trifluralin in clay loam soil under different temperature and soil moisture conditions using gamma distribution model.

4.2. Materials and methods

4.2.1 Chemicals and Soil

Analytical grade atrazine and trifluralin were purchased from Sapphire Bioscience (New South Wales, Australia) and Trajan Scientific and Medical (Victoria, Australia), respectively. Solvents for Gas-Chromatography (GC) analysis and soil extraction were of analytical grade and supplied by Sigma Chemical Co. (New South Wales, Australia). Herbicide-free soil (0–20 cm) was collected from a crop field in Collingullie (35.0886° S, 147.1289° E), located 26 kilometres north-west of Wagga Wagga, New South Wales, Australia. Soil collection was carried out randomly from different points of the crop field. Soon after collection, the soil was homogenized, ground, sieved to 2 mm and immediately stored at 4 °C until use. Prior using, soil was air dried for 48 hours and moisture condition was adjusted as required. The absence of atrazine and trifluralin in the soil was confirmed by extraction and analysis. The soil was classified as a clay loam soil with 28% sand, 34% silt and 38% clay, pH - 6.10, organic carbon - 2%; organic matter - 3.44%; N - 91 mg/kg; P - 51 mg/Kg and K - 760 mg/Kg.

4.2.2 Experiment set up

Persistence of atrazine and trifluralin under different temperature and moisture conditions was investigated separately in clay loam soil. Two grams (2 g) of soil (air dry weight basis) was weighed into sterile screw top centrifuge tubes. The herbicides were diluted separately using sterile ultrapure water and applied, in solution, to provide final concentrations of 4.50 and 4.25 mg/kg soil of atrazine and trifluralin, respectively, evenly distributed in the soil. Moisture content of the soil samples was adjusted to 40, 70, and 100% of field capacity (FC) (Hasanuzzaman et al., 2017) using ultrapure water when required. A control consisting of ultrapure water applied to soil was used to ensure no cross contamination occurred during incubation. The concentrations of both herbicides were set based on the highest recommended rates for different crops grown in NSW, Australia, and the residue concentrations of trifluralin and atrazine detected in NSW soil samples based on the survey by Rose et al. (2016).

Capped centrifuge tubes containing soil samples of different moisture content were incubated under aerobic conditions in separate climatic chambers at temperatures of

10, 20 and 30 °C, in darkness. Caps were removed briefly each week to maintain aerobic conditions, while weighing and adjusting moisture content as required. Centrifuge tubes were collected randomly from each of the climatic chambers at 6 different sampling dates 0, 7, 21, 42, 70 and 105 days after incubation. For each of the herbicides, three moisture levels x three temperature levels x four replications were collected randomly at each sampling date. In case of 0 d samples, moisture content of each tube was adjusted with diluted herbicide concentrations at room temperature and extracted hereafter. Thus, 0 d sample concentration values were pooled by calculating mean concentrations for a given moisture content but same for various temperature levels. Centrifuge tubes were stored at -20 °C until extraction and analysis.

4.2.3 Soil extraction

The QuECheRS method (Quick, Easy, Cheap, Efficient, Rugged and Safe) described by (Anastassiades et al., 2003) was used to extract herbicides for analysis. Tubes were spiked with 50 μL of 1.0 μg/ml pendimethalin as an internal standard prior to extraction. For atrazine samples, 5 mL of acetonitrile was added to each centrifuge tube containing soil sample. Tubes were shaken vigorously for 1 min using a vortex mixer at maximum speed and then centrifuged 5 min at 2100 x *g*. The supernatant was recovered, and the soil sample was extracted a second time with 5 mL of acetonitrile. Supernatants were combined in a clean 15 mL centrifuge tube. A mixture of 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate dehydrate and 0.5 g di-sodium hydrogen citrate sesquihydrate was added to remove water with vortex mixing. The extract was centrifuged again for 5 min at 2100 x *g* and the supernatant was transferred to a 15 mL centrifuge tube containing 150 mg PSA (Primary-Secondary Amine) sorbent and 900 mg $MgSO_4$ [(25 mg PSA sorbent and 150 mg anhydrous $MgSO_4$)/ml extract] for dispersive solid-phase extraction (dSPE) clean-up. After vortexing for 30 seconds, the extract was centrifuged for 5 min at 2100 x *g* and supernatant acetonitrile was transferred to 20 mL scintillation vials. Then the extracts were evaporated to dryness under nitrogen. The residues were resuspended in 500 μL of methanol for GC-ECD analysis.

Trifluralin samples were extracted using the atrazine method described above, but with a single 10 mL acetonitrile extraction instead of 2 x 5 mL, and without the use of the

dSPE clean-up step. After concentration, residues were resuspended in 500 µL of methanol for analysis by GC-ECD.

4.2.4 SPME (Solid phase micro extraction) method to measure volatilisation of trifluralin from clay loam soil

Volatilization of trifluralin from clay loam soil was measured using centrifuge tubes with a small hole drilled in the lids for insertion of the SPME fibre. Two grams (2 g) of clay loam soil was measured in a small plastic container and spiked with diluted trifluralin concentrations as mentioned earlier. A piece of aluminium foil was bended round and inserted into the centrifuge tube to prevent the contact of the trifluralin treated soil with the wall of centrifuge tube. Soil was inserted into the inclined centrifuge tube avoiding the contact of the wall which may interfere SPME measurements. Then, SPME fibre was inserted through the lid and incubated at 10, 20 and 30 °C for 15 minutes. After incubation, the SPME fibre was subjected to GC-ECD with manual injection to analyse the amount of trifluralin trapped into the head space. The rest amount of trifluralin remaining in the soil sample was extracted using the same method mentioned above.

4.2.5 Chemical analysis

Atrazine and trifluralin residues were subjected to GC-ECD, simultaneously applying a 6 point calibration under the following conditions: capillary column (30.0 m × 250 µm id × 0.25 µm nominal film thickness, Phenomen 7HG-G010-11, ZB-5ms); carrier gas (nitrogen) 1 mL/min, constant make up (nitrogen) 30 mL/min. Operating conditions: Column temperature ranging 60 to 320 °C; Oven conditions: initial time 1 min at 50 °C; ramp (I): 15 °C/min, to 250 °C hold for 1 min, ramp (II): 50 °C/min, to 300 °C hold for 2 min, total run time: 31 min; detector temperature: 280 °C, injector temperature: 250 °C (splitless), injection volume: 1µL.

4.2.6 Validation parameters

Limit of detection (LOD) and Limit of quantification (LOQ) are two critical measures for the validation of an analytical method ensuring that the analyte is present in the sample. For atrazine, LODs and LOQs were 0.60 and 2.00 µg/kg. On the other hand, LODs and LOQs for trifluralin was 0.015 and 0.05 µg/kg.

The efficiency and accuracy of the method was evaluated and verified by the estimation of each herbicide recoveries from control soil samples. The recovery percentages of atrazine and trifluralin were 86.67±9.38% and 80.41±6.25%, respectively.

4.2.7 Statistical analysis

All statistical analysis was carried out in R 3.6.1 (Team, 2019). The mean values of atrazine and trifluralin concentrations with standard deviations (SD) over the days under various moisture and temperature levels were tabulated.

The aim of the study is to investigate the rate of herbicides dissipation from the clay loam soil with various temperature and moisture levels over the time. In addition, we wish to determine the half-life of herbicides and the number days to complete dissipation of both herbicides from clay loam soil. The gamma model (Rohan et al., 2015) was used to investigate dissipation of the herbicides as it allows us to use environmental factors, such as temperature and moisture content as covariates. In this study, gamma model with inverse link was used to investigate the relationship between changes in herbicide concentrations from the clay loam soil, and effect of temperature, moisture, number of days and their interactions on herbicide dissipation. The best model for the data was determined by the likelihood ratio test. The best model was then used to determine the half-life of herbicides and the days to complete dissipations of herbicides from clay loam soil. To gain these measures, the model equations were re-arranged. For example, If $\frac{1}{\hat{y}} = \hat{\alpha}_0 + \hat{\alpha}_1 \times Day$, the re-arranged equation was $Day = \frac{1}{\hat{\alpha}_1}\left(\frac{1}{\hat{y}} - \hat{\alpha}_0\right)$, where, $\hat{\alpha}_0 \; and \; \hat{\alpha}_1$ are the estimates of the model parameter. For half-life and days to complete dissipation of herbicides on the clay loam soil, $\hat{y} = \frac{\text{mean of original herbicide}}{2}$ and $\hat{y} = 0.1$ respectively.

4.3. Results

Result from soil test showed that the soil sample used for the experiment did not contain the mentioned herbicides, plus we presented soil properties in details.

4.3.1 Loss of atrazine from clay loam soil

The mean values of atrazine concentration (mg/kg soil) with standard deviations (SD) over the time (days) against temperature and moisture are given in Table 4.1.

Table 4.1. Mean values for atrazine concentrations (mg/kg soil) with SD over experimental period under various temperature and moisture levels.

Moisture	Temperature (°C)	0 day	7 days	21 days	42 days	70 days	105 days
40%	**10**	3.60	1.83 (0.06)	1.43 (0.48)	2.41 (0.03)	1.28 (0.18)	0.32 (0.10)
70%	**10**	3.77	2.02 (0.10)	2.05 (0.15)	2.45 (0.21)	1.43 (0.33)	0.30 (0.01)
100%	**10**	4.02	1.94 (0.21)	2.13 (0.05)	2.56 (0.06)	1.38 (0.20)	0.29 (0.03)
40%	**20**	3.60	1.69 (0.09)	1.50 (0.21)	1.84 (0.33)	1.01 (0.32)	0.22 (0.04)
70%	**20**	3.77	1.59 (0.40)	1.69 (0.02)	1.88 (0.15)	0.97 (0.17)	0.22 (0.04)
100%	**20**	4.02	1.26 (0.32)	1.72 (0.08)	1.67 (0.34)	0.60 (0.08)	0.18 (0.13)
40%	**30**	3.60	1.15 (0.09)	1.05 (0.04)	0.90 (0.03)	0.50 (0.02)	0.17 (0.03)
70%	**30**	3.77	1.25 (0.22)	1.08 (0.06)	1.05 (0.29)	0.56 (0.11)	0.10 (0.01)
100%	**30**	4.02	1.21 (0.07)	1.19 (0.14)	0.72 (0.16)	0.47 (0.18)	0.19 (0.02)

0 d samples were not incubated in climatic chambers, but maintained with required moisture levels and extracted immediately after mixing the herbicide in the soil. 0 d sample values represent mean concentration of herbicides in clay loam soil for various moisture content levels in room temperature.

Overall, the concentration of atrazine in the clay loam soil declined over the 105 day incubation period (Table 4.1), indicating less variation within replicates. However, atrazine concentrations were found to be higher at 42 d than 21 d samples at 10 and 20 °C temperature levels; such variation might be due to an experimental error observed under low temperature conditions (10 and 20 °C).

To evaluate the impact of time (day), temperature and moisture on atrazine dissipation in clay loam soil, the full model (main effects with their interactions) was

initially considered. The likelihood ratio test results for the full model are given in Table 4.2. Atrazine dissipation was independent of moisture but dependent on temperature as effect of moisture was non-significant but temperature was found significant on atrazine dissipation (Table 4.2).

Table 4.2. Likelihood results for the full model fitted to atrazine persistence.

	Df	Deviance	Residual Df	Residual Deviance	Pr(>Chi)
NULL			215	147.01	
DAY	1	86.48	214	60.52	<0.01
Moisture	2	0.13	212	60.40	0.71
Temperature	2	4.71	210	55.69	<0.01
DAY x Moisture	2	0.27	208	55.42	0.48
DAY x Temperature	2	13.57	206	41.84	<0.01
Moisture x Temperature	4	0.15	202	41.69	0.93
DAY x Moisture x Temperature	4	0.45	198	41.24	0.65

Three-way interactions of day, moisture and temperature was not significant to explain the variations of atrazine (p-value = 0.65, Table 4.2). Similarly, the two-way interactions of moisture with temperature (p-value = 0.93, Table 4.2) and day (p-value = 0.48, Table 4.2) were not significant. The main effect moisture was also not significant (p-value = 0.71, Table 4.2). However, the main effect of temperature and its interaction with day of incubation were significantly important to explain the loss of atrazine from clay loam soil (Table 4.2). The predicted model for the data is given below.

$$\frac{1}{\hat{y}_i} = \hat{\beta}_0 + \hat{\beta}_1 Day + \hat{\beta}_{1,i} Temp_i + \hat{\beta}_{2,i} Temp_i \times Day$$

$$i = temperature\ levels\ i.e.\ 20\ or\ 30\ ^{\circ}\mathrm{C}$$

Where, $\hat{y}$ is the predicted trend of atrazine and $\hat{\beta}_0, \hat{\beta}_1, \hat{\beta}_{1,20}, \hat{\beta}_{1,30}, \hat{\beta}_{2,20}$ and $\hat{\beta}_{2,30}$ are estimates of the model parameters. The estimated value of the parameters and their standard errors were given in Appendix A and predicted of atrazine loss (thick line) with observed values were presented in Figure 4.1.

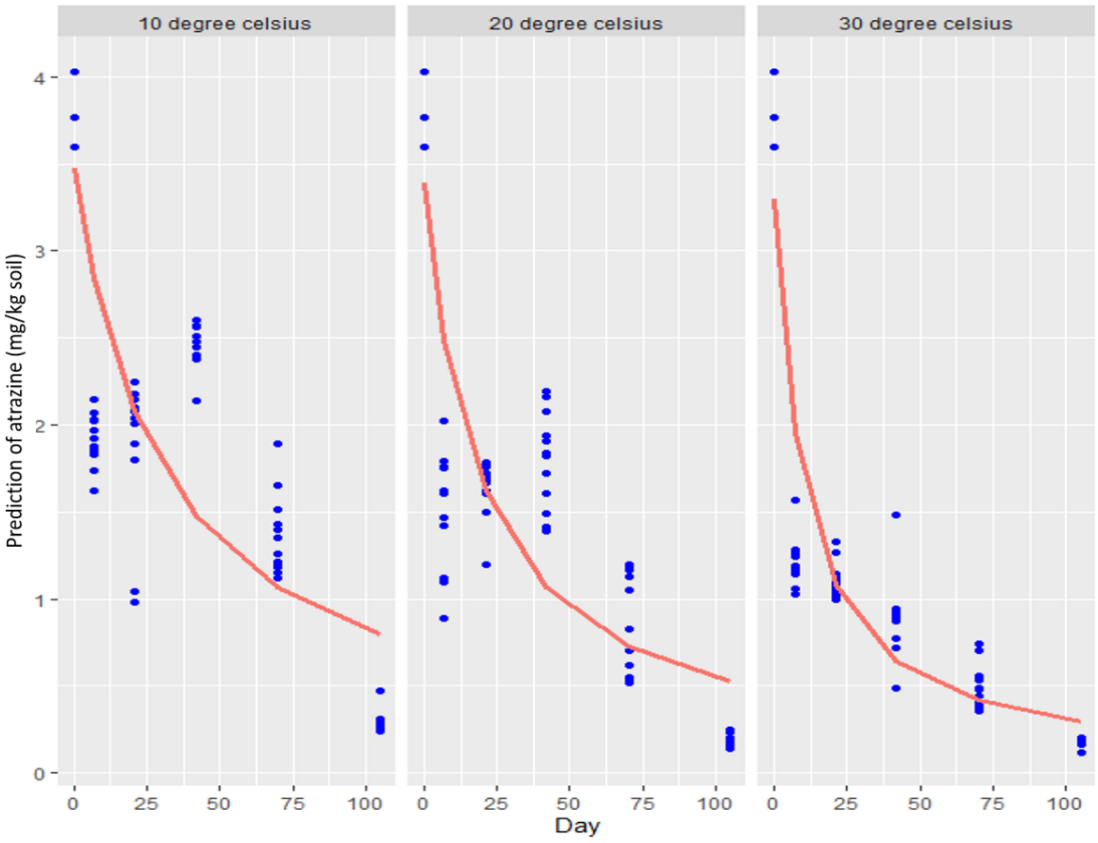

Figure 4.1. Atrazine concentration (mg/kg soil) in clay loam soil as a function of time (days) with gamma distribution curve fitted to the data for 10, 20 and 30 °C temperatures.

The equations for the prediction lines, drawn on the graph are given for three temperatures.

For 10 °C, $\frac{1}{\hat{y}_{10}} = 0.2877 + 0.0093 \times Day$; for 20 °C, $\frac{1}{\hat{y}_{20}} = 0.2949 + 0.0154 \times Day$ and for 30 °C, $\frac{1}{\hat{y}_{30}} = 0.3036 + 0.0297 \times Day$. From the above equations, we found that the trend of atrazine concentration declined over the time, because they are in inverse form. On average, the rate of loss of atrazine from the clay loam soil was 0.90% per day at 10 °C; compared to 1.50% per day at 20 °C and 2.97% per day at 30 °C temperature (Appendix B). The loss of atrazine from the clay loam soil at 30 °C was quicker than 10 °C by 2.00% per day and 20 °C by 1.40% per day. All these results including changing patterns with various temperatures are shown in Figure 4.1.

The estimated days to reach half-life and undetectable levels of atrazine on the clay loam soil for a given temperature are given in Table 4.3.

Table 4.3. Estimated half-life and days to complete dissipations of atrazine in clay loam soil under various temperature levels according to gamma distribution model.

	10 °C	**20 °C**	**30 °C**
Days to reach half-life	25.84	15.07	7.50
Days to complete dissipation	1049.87	631.07	326.23

Half-life and days to complete dissipations of atrazine were highly influenced by temperature conditions. Half-life of atrazine tended to double with every 10 °C decrease of temperature (Table 4.3). Our model suggests that, the complete removal of atrazine from clay loam soil will take 326 days at 30 °C; whereas under low temperature conditions (20 and 10 °C), the persistence of atrazine in clay loam soil will extend for several years (631 and 1049 days, respectively). The model indicated that the complete loss of atrazine from clay loam soil could take several years after application, depending on the prevailing temperature conditions.

4.3.2 Loss of trifluralin from clay loam soil

While degradation is the main route of loss of a pesticide from soil, volatilisation may also occur if the pesticide is resident in the surface layer of soil. Trifluralin loss via volatilisation was determined by measuring the herbicide concentration in the headspace above clay loam soil using SPME. We observed that trifluralin was detected in the centrifuge tube head space under 10, 20 and 30 °C. However, the amount of trifluralin (2.01-2.03 ng) in the headspace was very negligible (0.02%) under different temperature levels studied compared to extracted trifluralin in clay loam soil. The lower amount of trifluralin trapped into the headspace might be resulted due to immediate incorporation of trifluralin after application.

The mean trifluralin concentrations with SD values over the time (days) against temperature and moisture were given in Table 4.4. Overall, trifluralin concentrations in clay loam soil decreased over time.

Table 4.4. Mean values for trifluralin concentrations (mg/kg soil) with SD over the experimental period under various temperature and moisture levels.

Moisture	Temperature (°C)	0 day	7 days	21 days	42 days	70 days	105 days
40%	**10**	3.70	2.89 (0.33)	1.87 (0.35)	1.28 (0.07)	1.15 (0.17)	0.73 (0.02)
70%	**10**	3.70	3.03 (0.42)	1.92 (0.32)	1.34 (0.19)	0.97 (0.11)	0.53 (0.03)
100%	**10**	3.55	3.43 (0.20)	1.95 (0.31)	1.14 (0.16)	1.16 (0.21)	0.61 (0.15)
40%	**20**	3.70	2.58 (0.23)	1.56 (0.36)	0.64 (0.13)	0.45 (0.05)	0.28 (0.07)
70%	**20**	3.70	3.09 (0.46)	1.48 (0.65)	0.65 (0.15)	0.47 (0.03)	0.34 (0.09)
100%	**20**	3.55	3.44 (0.07)	1.75 (0.15)	0.81 (0.11)	0.69 (0.12)	0.68 (0.05)
40%	**30**	3.70	1.73 (0.39)	1.39 (0.19)	0.36 (0.07)	0.18 (0.04)	0.15 (0.01)
70%	**30**	3.70	1.74 (0.18)	1.26 (0.16)	0.25 (0.07)	0.17 (0.03)	0.17 (0.02)
100%	**30**	3.55	1.95 (0.46)	1.54 (0.43)	0.38 (0.01)	0.43 (0.06)	0.38 (0.14)

0 d samples were not incubated in climatic chambers, but maintained with required moisture levels and extracted immediately after mixing the herbicide in the soil. 0 d sample values represent mean concentration of herbicides in clay loam soil for various moisture content levels in room temperature.

For a given temperature, the loss of trifluralin for various moisture levels were approximately similar but some variations were noticed at 7 d samples (Table 4.4). However, trifluralin dissipation from the clay loam soil varied with temperature for a given moisture level (Table 4.4).

To evaluate the impact of time (day), temperature and moisture on trifluralin dissipation in clay loam soil, the full model (main effects with their interactions) was initially considered. The likelihood ratio test results for the full model was given in Table 4.5.

Table 4.5. Likelihood results for the full model fitted to trifluralin persistence.

	Df	Deviance	Residual Df	Residual deviance	Pr(>Chi)
NULL			215	168.23	
DAY	1	123.54	214	44.69	<0.01
Moisture	2	0.23	212	44.46	0.23
Temp	2	4.10	210	40.35	<0.01
DAY x Moisture	2	1.84	208	38.51	<0.01
DAY x Temperature	2	20.53	206	17.98	<0.01
Moisture x Temperature	4	0.30	202	17.68	0.42
DAY x Moisture x Temperature	4	3.36	198	14.32	<0.01

Results showed that the main effect of moisture was not significant to explain loss of trifluralin in clay loam soil (p-value = 0.23, Table 4.5). Similarly, two-way interactions of moisture and temperature was also not significant (p-value = 0.42, Table 4.5). The likelihood ratio test suggest that the three-way interactions of moisture, temperature and day were significant to explain dissipation of trifluralin from clay loam soil. Hence, the full model is considered as a best model, given below.

$$\frac{1}{\hat{y}_{ij}} = \hat{\beta}_0 + \hat{\beta}_1 Day + \hat{\beta}_{1,i} Temp_i + \hat{\beta}_{1,j} Moist_j + \hat{\beta}_{2,i} Temp_i \times Day + \hat{\beta}_{2,j} Moist_j \times Day + \hat{\beta}_{2,ij} Temp_i \times Moist_j + \hat{\beta}_{3,ij} Temp_i \times Moist_j \times Day$$

$$i = temperature\ levels\ i.e. 20\ or\ 30\ °C$$

$$j = moisture\ contents\ i.e. 70\ or\ 100\%\ FC$$

where $\hat{y}_{ij}$ is the estimate for various temperature and moisture levels in a given day and altogether 18 $\hat{\beta}$'s were estimated and given them in Appendix C. Based on the three temperature levels and three moisture levels, we are able to derive nine equations from the above equation. The nine model predictions with observed data are presented in nine panels in Figure 4.2. Overall, fast initial loss followed by slower dissipation of trifluralin was observed under all the treatment combinations. For a given moisture content, the rate of loss of trifluralin from clay loam soil increased as temperature increased (30 °C >20 °C >10 °C) over time. However, the rate of loss of trifluralin from clay loam soil was highest at 5.37% per day at 30 °C temperature with 70% soil moisture (Appendix D), as shown in Figure 4.2.

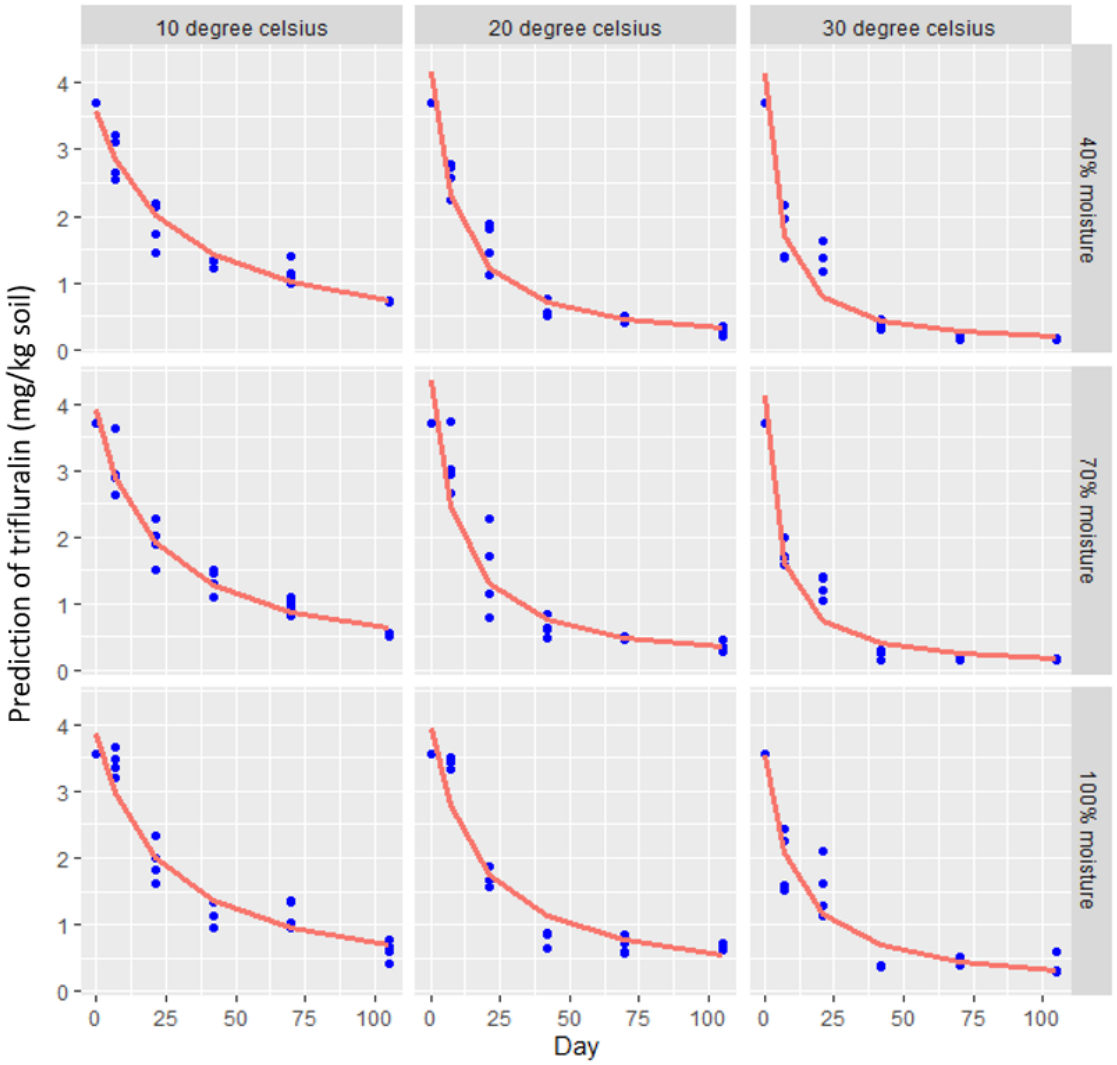

Figure 4.2. Trifluralin concentration (mg/kg soil) in clay loam soil as a function of time (days), moisture levels (40, 70 and 100%) with gamma distribution curve fitted to the data for 10, 20 and 30 °C temperatures.

Similar to atrazine, same equations were used to determine the half-life and days to reach complete dissipation of trifluralin from clay loam soil (Table 4.6).

Table 4.6. Estimated half-life and days to complete dissipations of trifluralin in clay loam soil under various temperature and moisture levels according to gamma distribution model.

	Moisture level (% FC)					
Temperature	**Half-life (days)**			**Days to complete dissipations**		
(°C)	**40%**	**70%**	**100%**	**40%**	**70%**	**100%**
30	6.05	5.8	10.02	197.79	182.01	347.34
20	10.94	12.24	20.43	356.24	384.22	642.73
10	25.52	22.44	26.74	951.51	767.45	853.69

The half-life and days to complete dissipation values of trifluralin under various temperature and moisture conditions varied widely in clay loam soil (Table 4.6). Changes in half-life and days to complete dissipation values for each temperature levels are mainly linked to the influence of soil moisture, especially at high temperatures. At low temperature (10 °C), half-life and days to complete dissipation values of trifluralin were almost identical across all the moisture levels studied. Variations in values were observed under different moisture treatment levels as the temperature increased. According to our results, trifluralin dissipation was approximately twice as fast at higher temperatures (20 and 30 °C) for clay loam soils under 40 and 70% FC, compared to 100% FC (Table 4.6). The model predicts that trifluralin has the potential to persist in clay loam soil for several years (up to 951 days) under low temperature conditions (10 °C). Trifluralin may persist in clay loam soil for long enough (347 days) even at higher temperature (30 °C) under saturated conditions (100% FC).

4.4 Discussion

4.4.1 Atrazine persistence in clay loam soil under various temperature and moisture conditions

Atrazine is considered to be highly persistent in the environment due to its resistance to abiotic hydrolysis (Liu, 2014). Environmental factors are known to have a major influence on atrazine persistence in soil. This study investigated the combined effect of soil moisture and temperature on the degradation of atrazine in a clay loam soil. Results revealed that, atrazine degradation under various moisture levels were not significantly different for a given temperature condition. Martinez et al. (2008) reported that there

was no significant relationship between different moisture levels on herbicide degradation in soil which agrees with the results in the current study. Fastest degradation of atrazine was observed at 30°C while slowest at 10 °C. This may be attributed to increased microbial activity due to increased temperature, resulting in accelerated pesticide degradation (Tariq et al., 2006). An increase in the soil temperature is likely to reduce the adsorption of atrazine to soil because adsorption is an exothermic process, providing greater access to atrazine by soil microbes due to elevated soil solution concentrations (Dao & Lavy, 1978). The results from the current study showed that the half-life of atrazine tends to double with every 10°C decrease over the range tested, which agrees with Mukherjee et al. (2018) who investigated the degradation over the 10-45 °C. Other studies confirm that a 10 °C increase in temperature can cause a decrease in pesticide half-lives in soil by as much as 60% (Bloomfield et al., 2006; Das, 2014).

Atrazine residues were detected in clay loam soil in the current study at 105 days under all three temperature conditions. Application of gamma distribution model for the calculation of the number of days to reach complete dissipation of atrazine in clay loam soil allowed us to investigate the lifespan of atrazine in clay loam soil under different temperature conditions. Gamma model shows that atrazine has the potential to persist in clay loam soil for several years, depending on the soil temperature. Liu (2014) reported that a colder environment can make atrazine more persistent in soil due to almost no microbial activity, as evidenced by detection of atrazine in German soil 18 years after being banned in Germany (LAWA, 2003). Jablonowski et al. (2009) and Jablonowski et al. (2010) investigated long-term aging and bio accessibility study to explore the long-term persistence of atrazine and its metabolites in soil and observed that 25% of the applied atrazine remained in soil after 22 years. Atrazine persistence in soil is not yet completely understood but could be explained due to the binding of herbicide compound to the soil matrix in unavailable forms or lack of metabolic capability (Jablonowski et al., 2011). Although repeated application of atrazine in soil can lead to accelerated degradation due to the emergence of microbial populations (Fang et al., 2015; Krutz et al., 2010; Martinazzo et al., 2010), but a certain fraction of atrazine and its metabolites remain undegraded in soil while still extractable (Jablonowski et al., 2010).

4.4.2 Trifluralin persistence in clay loam soil under various temperature and moisture conditions

Trifluralin is regarded as a moderately persistent herbicide (Koskinen et al., 1986). The main routes of trifluralin loss from soil include volatilization, photodegradation at surface zone, and microbial and chemical degradation within the soil incorporation zone. The results in the current study showed that trifluralin was lost rapidly from clay loam soil during the initial phase, and then more slowly in the next phase over a period of 105 days which is in agreement with Coleman et al. (2020). Due to its high vapour pressure and Henry's constant, trifluralin volatilization losses were reported as high as 60% (Nash, 1983). However, soil incorporation was carried out soon after application in this current study to minimize volatilization loss as soil incorporation has been reported to abate strongly trifluralin volatilization (Bedos et al., 2006). Moreover, volatilization decreases with the increase of organic carbon content due to an increase in soil adsorption. Trifluralin strongly adsorbs by the soil organic matter due to its hydrophobic nature, which decreases its bioavailability to soil microbes (Mamy & Barriuso, 2007; Mamy et al., 2005). The clay content and organic carbon of the soil used in the current study were 38% and 2.0%, respectively, which might have favoured trifluralin adsorption in clay loam soil. Long term persistence of trifluralin in clay soil was also reported by Pritchard and Stobbe (1980).

The gamma distribution model suggested the three-way interactions of moisture, temperature and time (day) to explain the variations in dissipation of trifluralin from clay loam soil. Literature acknowledged that trifluralin behaviour in soil with low moisture lead to major uncertainty due to greater variability in results (Jolley & Johnstone, 1994). The current study demonstrated that soil moisture between 40 and 100% FC had little effect on trifluralin degradation at 10 °C, which agrees with results reported by Solbakken et al. (1982) under greenhouse conditions. Addition of water to air dry soil may affect trifluralin adsorption by increasing solubility followed by enhanced degradation (Jolley & Johnstone, 1994). We observed enhanced trifluralin degradation in clay loam soil with increasing temperature and moisture conditions over time. Higher temperature can stimulate rapid desorption of organic pollutants from soil (Ghosh et al., 2001); resulting enhanced biodegradation by increasing microbial metabolic activities in higher moisture conditions. Because, even at higher temperature (30 °C), trifluralin

degradation was not significant compared to when no moisture was added to the soil (Jolley & Johnstone, 1994). Thus, trifluralin dissipation in clay loam soil may be addressed as a function of soil temperature and moisture. As temperature is known to contribute pesticide behaviour in the environment by affecting pesticide degradation rate, water−air partition coefficient, and water−soil partition coefficient (Paraiba & Spadotto, 2002).

We observed that trifluralin has the potential to persist in clay loam soil for years and a possible concern for the farming community in Australia. Again, trifluralin persistence is highly dependent on site-specific agro-climatic conditions. Many studies in USA reported that persistence of trifluralin in soil may be more than a year (Antonious, 2012; Luchini et al., 2000). Research studies observed that 50% of the initially applied trifluralin was still present in soil after 3 years of application in Latin America (Belden et al., 2003). Karasali et al. (2017) reported that trifluralin residues were still detectable even after 4 years of ban in European Union. These results are in agreement with our findings under certain circumstances. For example, gamma distribution model revealed that under low temperature conditions (10 and 20 °C), trifluralin may persist in clay loam soil for 2-3 years regardless of the soil moisture conditions. Again, the agro-climatic conditions of USA, Latin America and Europe are not comparable with Australian context. Perhaps, this study investigated the potential of trifluralin persistence in clay loam soil under various temperature and moisture conditions; such relationships can be combined with site-specific climatic data (such as temperature and rainfall) to predict trifluralin residues in clay loam soil.

4.5 Conclusion

Variation in soil moisture and temperature can have a dramatic impact on the dissipation of atrazine and trifluralin, making this study essential in understanding and predicting their fates in clay loam soil. Application of gamma distribution model offered more flexibility since environmental factors (temperature and moisture) were incorporated to examine their effects on atrazine and trifluralin dissipation in clay loam soil. Gamma distribution model suggested that persistence of atrazine and trifluralin in clay loam soil could last for a few years, depending on temperature and soil moisture conditions. Their residues in clay loam soil may interfere with following crops in rotation, limiting selection options for the farmers. Moreover, predominant use of trifluralin in

winter crops; atrazine in canola, sorghum as well in fallows might lead to accumulation of these two herbicides in the soil incorporation zone at elevated levels causing serious environmental consequences in future.

Conflicts of interest

The authors declare no conflicts of interest.

Author statement

Imtiaz Faruk Chowdhury: Conceptualization, Methodology, Investigation, Validation, Writing- Original Draft, Funding acquisition. **Maheswaran Rohan:** Software, Formal analysis, Data curation, Visualization, Writing- Review & Editing. **Benjamin J. Stodart:** Validation, Supervision, Project administration, Writing - Review & Editing. **Chengrong Chen:** Supervision, Writing- Review & Editing. **Hanwen Wu:** Validation, Supervision, Project administration, Writing- Review & Editing. **Gregory S. Doran:** Conceptualization, Methodology, Validation, Supervision, Resources, Project administration, Writing- Review & Editing.

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Appendix A. Estimated value of the parameters for atrazine dissipation under various temperature levels over time.

	Estimate	Standard error	t value	Pr(>|t|)
(Intercept)	0.321761	0.029754	10.814	< 2e-16 ***
DAY	0.008566	0.001067	8.029	6.94e-14 ***
Temp_20 °C	-0.038969	0.041278	-0.944	0.346226
Temp_30 °C	-0.045815	0.042466	-1.079	0.281881
DAYxTemp_20 °C	0.007098	0.00181	3.922	0.000119 ***
DAYxTemp_30 °C	0.021937	0.002572	8.528	2.95e-15 ***

Appendix B. Rate of loss of atrazine (% per day) under different temperature conditions.

	Temperature (°C)		
	10	**20**	**30**
Rate of loss of atrazine (% per day)	0.90	1.50	2.97

Appendix C. Estimated value of the parameters for trifluralin dissipation under various temperature and moisture levels over time.

	Estimate	Standard error	t value	Pr(>|t|)
(Intercept)	0.2792	0.0304	9.1787	0.0000***
DAY	0.0102	0.0013	8.1167	0.0000***
Moisture_70%	-0.0230	0.0420	-0.5473	0.5848
Moisture_100%	-0.0212	0.0419	-0.5073	0.6125
Temp_20 °C	-0.0389	0.0427	-0.9106	0.3636
Temp_30 °C	-0.0376	0.0440	-0.8545	0.3938
DAY x Moisture_70%	0.0025	0.0019	1.3194	0.1886
DAY x Moisture_100%	0.0012	0.0018	0.6588	0.5108
DAY x Temp_20 °C	0.0172	0.0027	6.4089	0.0000***
DAY x Temp_30 °C	0.0391	0.0040	9.7974	0.0000***
Moisture_70% x Temp _20 °C	0.0127	0.0591	0.2153	0.8298
Moisture_100% x Temp_20 °C	0.0342	0.0593	0.5772	0.5644
Moisture_70% x Temp_30 °C	0.0242	0.0617	0.3924	0.6952
Moisture_ 100% x Temp_30 °C	0.0624	0.0631	0.9899	0.3234
DAY x Moisture_70% x Temp _20 °C	-0.0044	0.0037	-1.1874	0.2365
DAY x Moisture_100% x Temp_20 °C	-0.0134	0.0034	-3.9866	0.0001***
DAY x Moisture_70% x Temp_30 °C	0.0019	0.0059	0.3171	0.7515
DAY x Moisture_ 100% x Temp_30 °C	-0.0226	0.0049	-4.6129	0.0000***

Appendix D. Rate of loss of trifluralin (% per day) under various temperature and moisture conditions.

Moisture level (% FC)	Temperature (°C)		
	10	**20**	**30**
40	1.02	2.74	4.93
70	1.27	2.54	5.37
100	1.14	1.52	2.80

Chapter 5. Application of atrazine and trifluralin alters microbial community structure and functions in a clay loam soil

5.1 Introduction

Herbicides are phytotoxic chemicals extensively used for destroying various weeds or inhibiting their growth (Gupta, 2017). Due to their persistence and toxicity, several herbicides are often identified in soil, sediments, surface and ground water (Vryzas et al., 2009; Zaya et al., 2011). Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a triazine-based herbicide, which has been used extensively for the control of broadleaf weeds in farmlands worldwide. Atrazine is regarded as highly persistent due to its resistance to abiotic hydrolysis (Liu, 2014). Environmental fate of atrazine in soil is mainly governed by microbial degradation (Huang et al., 2017). Atrazine degrading strains of bacteria were first isolated in 1995 (Huang et al., 2017). Since then, various atrazine degrading species from a number of microbial genera have been identified from soil, such as, *Acidovorax*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Enterobacter*, *Erwinia*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhizobium*, *Rhodobacter*, and *Sinorhizobium* from phylum *Proteobacteria,* and *Arthrobacter, Micrococcus, Nocardioides* and *Rhodococcus* from phylum *Actinobacteria* (Devers et al., 2007; Sajjaphan et al., 2010; Zhang et al., 2012). Recent studies reported that atrazine application induced significant changes in bacterial community structure and functional performance (Chen et al., 2015; Xu et al., 2019); even under lower concentrations (1 mg/L) (Muturi et al., 2017).

On the other hand, trifluralin (2,6-dinitro-N, N-dipropyl-4-trifluoromethylaniline) is a pre-emergence herbicide used to control annual and some dicot weeds in cotton, soybean, wheat and oilseed crops (Anthony & Hussey, 1999). Trifluralin is relatively non-mobile and hence, risk of surface and ground water contamination is minimal (Wallace, 2014). Trifluralin is strongly adsorbed by the soil organic matter due to its hydrophobic nature, which decreases its bioavailability to soil microbes for degradation (Mamy et al., 2005). A number of trifluralin-degrading bacterial isolates were previously identified in soil, and belonged to the genera of *Pseudomonas, Klebsiella, Herbaspirillum* and *Bacillus* (Bellinaso et al., 2003; Carter & Camper, 1975). Majority of studies related to trifluralin degradation in soil only focussed on the identification of potential degraders. However,

little is known about the impact on the soil microbial community structure and composition when exposed to trifluralin.

Pesticides applied directly to either soil or plant foliage may affect the ability of microbial communities colonising soil habitats due to their higher responsiveness to such exposure (Katsoula et al., 2020). However, available data on pesticide application on soil ecology is relatively conflicting particularly for herbicides. Several studies have reported the sensitivity of microorganisms to herbicide application (Allegrini et al., 2015; Barriuso et al., 2010; Karas et al., 2018). In contrast, repeated applications of triazine herbicides did not cause any long-term changes in the abundance of soil microorganisms (Prudnikova et al., 2021). Moreover, Singh and Singh (2016) reported that herbicides such as, trifluralin, dichloral urea, and 2, 4-D did not seriously affect soil microbial abundance, due to the complexity of the soil microbial pool and higher degrees of adaptation rates to various pollutants. The information of herbicidal effects on soil microorganisms is lacking in Australia.

Our previous study reported that both atrazine and trifluralin can cause significant damages to a range of crop species, depending on the herbicide concentration in the soil (Chowdhury et al., 2020). Herbicide concentration in soil primarily depends on herbicide use pattern and persistence behaviour. Atrazine and trifluralin have been reported to persist in soil for 2-3 years depending on environmental conditions (Chowdhury et al., 2021). Repeated use of these herbicides between years, together with drought conditions often experienced in Australian farming systems, could prolong their persistence in soil and maintain herbicide concentrations higher enough to cause injuries to the follow crops. However, the persistence of atrazine and trifluralin may not only affect following crops in rotation but also pose a threat to soil ecology and the environment (Ju et al., 2016). Therefore, a deeper insight is necessary to better understand their impact on the structure and composition of soil microbial communities. Better understanding of the changes in microbial communities will help to identify those microbial groups which are preferentially multiplied in responses to herbicide application. These groups of soil microbes can then be further investigated for their potential to degrade these persistent herbicides.

The objectives of this study were: (1) to investigate the changes in soil microbial respiration, soil microbial community composition and functional diversity under various concentrations of atrazine and trifluralin; and (2) The changing profiles of soil

microbes in responses to herbicide applications would direct future research in identifying novel microbes which could use the herbicides as carbon sources, thereby accelerating the degradation of herbicides and minimising their adverse impact on rotational crops.

5.2 Materials and methods

5.2.1 Chemicals and soil

Analytical grade atrazine and trifluralin was purchased from Sapphire Bioscience (New South Wales, Australia) and Trajan Scientific and Medical (Victoria, Australia), respectively. Solvents for Gas-Chromatography (GC) analysis and soil extraction were of analytical grade and supplied by Sigma Chemical Co. (NSW, Australia).

Soil (0–20 cm) with no atrazine and trifluralin application history was collected from a wheat crop field in Collingullie (35.0886° S, 147.1289° E), located 26 kilometres north-west of Wagga Wagga, New South Wales, Australia. The field soil was randomly collected from the crop field. Soon after collection, the soil sample was homogenized, air dried for 48 hours, grounded, sieved to 2 mm and immediately stored at 4 °C until use. Soil was solvent extracted and analysed by GC-MS according to a previously developed method (Chowdhury et al., 2021) to confirm the absence of atrazine and trifluralin in the soil. The soil was classified as a clay loam soil with 28% sand, 34% silt and 38% clay, pH - 6.10, Organic carbon - 2%; Organic matter - 3.44%; N - 91 mg/kg; P - 51 mg/Kg and K - 760 mg/Kg.

5.2.2 Soil treatment for microbial community structure

To investigate soil microbial community structure and functions upon atrazine and trifluralin application, separate experiments were conducted using sterilised and non-sterilised soil. Most soil sterilisation methods have limitations, and we recognise that some changes to the soil may have occurred, autoclaving was the method of choice due to cost and availability considerations. Soon after autoclaving, the sterility of the soil was verified by plating the soil solution to check for microbial growth. Autoclaving was done only one-time prior experimental setup. Fifty grams of both autoclaved and nonautoclaved soil (dry weight equivalent) was weighed into a plastic bowl. Analytical grade herbicides were diluted with sterile ultrapure water and applied separately, in solution, to provide final concentrations of 4.50, 9.0 and 22.50 mg/kg for atrazine and

4.25, 8.50 and 21.25 mg/kg for trifluralin, corresponding to the recommended dose (RD1x), twice (RD2x) and five times (RD5x) of the recommended dose of herbicides. The moisture content of the soil samples was adjusted to 70% of field capacity (FC) (Hasanuzzaman et al., 2017) using sterile ultrapure water, as required, and the soil was mixed thoroughly for the uniform distribution of the herbicides. Sterile ultrapure water was added for controls and all the treatments were replicated three times. The soils were transferred to 250 ml plastic, screw cap jars and incubated in a climatic chamber at 30 °C as rapid loss of both herbicides were observed at 30 °C (Chowdhury et al., 2021). Caps were removed once each week to maintain aerobic conditions, while weighing and adjusting moisture content as required. For the determination of soil microbial community structure and functional diversity, 15 g of soil was collected at 0, 20 and 40 days after treatment (DAT).

5.2.3 Soil microbial respiration

Soil microbial respiration was measured in nonautoclaved soil samples using the method described by Bartha and Pramer (1965) in a separate experiment. Fifty grams of herbicide mixed soil with 70% field capacity (FC) was transferred in a 1-L sealed glass jar and a 100 ml beaker containing 20 ml of 0.1 M NaOH was placed on a metal stand. The glass jar containing the soil and the beaker was sealed tightly and incubated at 30 °C. The same procedure was followed for the blanks without soil samples. All the jars were taken out at 1, 7 and 21 days after treatment for titration. Soon after removal, 1 ml of $BaCl_2$ was added to the beaker then the residual NaOH was titrated with 0.05 M HCl to the phenolphthalein end point. The amount of CO_2 evolved was calculated from the differences in normality between NaOH blanks and samples.

5.2.4 BIOLOG assay for soil microbial overall activity

Determination of overall soil microbial activity was performed using BIOLOG ECO microplate™ according to the manufacturer's protocol. Five grams (5 g) of nonautoclaved soil sample (dry weight equivalent) was mixed with 50 ml of sterile ultrapure water containing in a 250 ml plastic jar and shaken for 1 hr at 25 ± 1 °C on an orbital shaker. Soil was allowed to settle for 30 min and 1 ml of the upper soil suspension was transferred to a 15 ml glass bottle and serially diluted to 10^{-3} dilution with sterile ultrapure water. For each treatment, 96 wells in the BIOLOG ECO microplate™ (Biolog

Inc. Hayward, CA, USA) was inoculated with 150 µl dilutions using an 8-channel pipette in triplicate. The BIOLOG ECO microplate was placed in a shaking incubator at 25 ± 1 °C after inoculation, and colour development in the wells was measured at 4, 24, 48, 72, 96, 120, 144, and 168 hr, respectively, using a plate reader at a wavelength of 590 nm. The average well colour development (AWCD) was calculated according to Fang et al. (2014) determined as follows:

$$AWCD = \Sigma\, ODi/31$$

where OD_i is the optical density value at a wavelength of 590 nm from each well after subtracting the value from blank.

5.2.5 DNA extraction

The same soil samples used for the microbial community structure, were used for the DNA extraction purpose. Soil sample of 0.25 g (dry weight equivalent) was weighed from each treatment used for total bacterial DNA extraction using the DNeasy PowerSoil Kit (QIAGEN, Chadstone, Victoria, Australia) according to the manufacturer's protocol. The purity and concentration of the DNA extracts were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Scoresby, Victoria, Australia) and each sample diluted to approximately 10 ng DNA μl^{-1} prior to sequencing.

5.2.5 Microbial diversity profiling

Sequencing conditions

PCR amplification and sequencing were performed at the Australian Genome Research Facility (AGRF, Sydney, NSW, Australia). Amplicons for 16S was generated using the primers and conditions listed in Table 5.1. Thermocycling was completed with an Applied Biosystem 384 Veriti and using AmpliTaq Gold 360 (Life Technologies, Australia). Illumina indexing of the amplicons was achieved in a second PCR utilising TaKaRa Taq DNA Polymerase (Clontech). Indexed amplicon libraries were quantified by fluorometry (Promega Quantifluor) and normalised. An eqimolar pool created and adjusted to 5 nM for sequencing on an Illumina MiSeq (San Diego, CA, USA) with a V3, 600 cycle kit (2 x 300 base pairs paired-end).

Assembly and OTU clustering

Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) (Zhang et al., 2014a). Primers were removed, with trimmed sequences being processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010), USEARCH (version 7.1.1090) (Edgar, 2010; Edgar et al., 2011) and UPARSE (Edgar, 2013) software. Within USEARCH, sequences were quality filtered, full length duplicate sequences removed, and reads sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtering using "rdp_gold" database as the reference. To obtain the number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Taxonomy was assigned by QIIME using the Greengenes database (DeSantis et al., 2006). Unless mentioned, default settings were used for all procedures.

Table 5.1. Bacterial diversity profiling primers and sequencing conditions (Yu et al., 2005).

Target	No. of cycles	Initial	Denaturing	Annealing	Extension	Final
16s V3-V4	29	95°C 7 min	94°C 30 sec	50°C 60 sec	72°C 60 sec	72°C 7 min
Forward primer	341F	CCTAYGGGRBGCASCAG				
Reverse primer	806R	GGACTACNNGGGTATCTAAT				

5.2.6 Statistical analysis

Data corresponding to soil respiration and BIOLOG assay were analysed by calculating mean and standard deviation using Microsoft Excel 2016 (Microsoft Corp., USA) with three replicates for each treatment. The statistical significance of different treatments was tested with Tukeys HSD test with 1% levels of significance. The taxonomic diversity of bacterial communities was analysed within the Marker Data Profiling module of MicrobiomeAnalyst (Dhariwal et al., 2017) which implements R version 3.6.1. The relative abundance data of bacterial communities were normalized by data transformation using centered log ratio, as suggested by Gloor et al. (2017). Alpha and Beta diversity analysis was performed using the phyloseq package (McMurdie & Holmes, 2013). Further, Ordination-based Principle Coordinate Analysis (PCoA) were performed

using the Bray - Curtis index and the statistical significance of the clustering pattern in ordination plots were evaluated using Permutational ANOVA (PERMANOVA) ($p<0.05$).

5.3 Results

5.3.1 Diversity profiling of the total dataset

Data revealed that the total number of read counts for atrazine was 1267270, with average counts per sample was 52802. On the other hand, the total number of read counts for trifluralin was 1343482, with average counts per sample was 55978. The number of unclassified sequences for atrazine and trifluralin were 570 and 706, respectively. Diversity profiling of the total dataset corresponding to 16s V3-V4 sequences of soil DNA samples, recorded a total of 11,550 operational taxonomic units (OTUs). OTUs were assigned to 31 phyla, 79 classes, 117 orders, 180 families, and 307 genera. 159 OTUs were identified at species level. The most abundant phyla were the *Actinobacteria*, accounting for 27.49% of all recorded OTUs, and then *Proteobacteria*, and *Firmicutes* accounting for 27.04 and 10.82% OTUs.

5.3.2 Atrazine

Effect of atrazine application on soil microbial respiration

Impact of atrazine on soil microbial respiration was dependent upon atrazine concentration and incubation time (Figure 5.1). Soil microbial respiration was increased with increasing atrazine concentrations at 1, 7 and 21 DAT. Various atrazine concentrations were statistically significant ($p<0.01$) on soil microbial respiration (evolved CO_2-C) and consistently followed the decreasing order as RD5x > RD2x > RD1x > Control. The evolved CO_2-C was the highest with RD5x (11871 µg/g soil) and the lowest with the control soil (4905 µg/g soil) at 21 DAT.

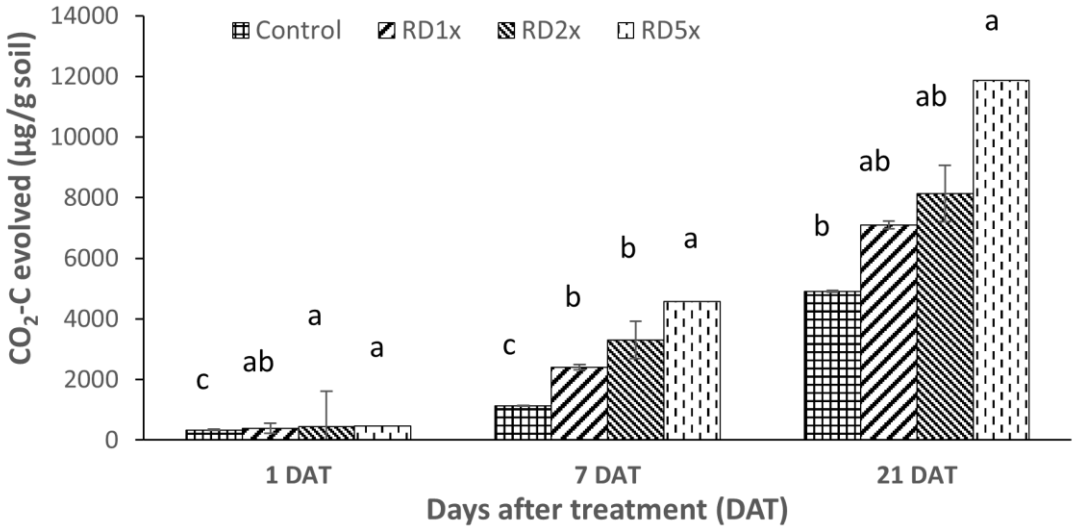

Figure 5.1. The effect of various atrazine concentrations (control, RD1x, RD2x and RD5x) on soil microbial respiration at 1, 7 and 21 days after treatment (DAT) in nonautoclaved soil. Mean values (n = 3) ± SD. Values labelled with various lower case letters are significantly different at $p<0.01$. The recommended dose (RD1x) of atrazine used in this study was set at 4.50 mg/kg soil.

Response of bacterial diversity and structure upon atrazine application

Estimation of the alpha diversity of the bacterial community revealed significant differences between autoclaved and nonautoclaved soil samples (*F value* = -13.06, $P \leq 0.01$) in terms of richness (Chao1). Chao1 index represents the number of species (richness) in a community. Results revealed that bacterial diversity was higher in nonautoclaved soil samples compared to autoclaved soil samples (Figure 5.2A). Interestingly, the bacterial diversity of autoclaved soil samples increased from 0 DAT to 40 DAT, possibly due to some enzyme resistance to autoclaving (Carter et al., 2007), but as the main objective of autoclaving was to reduce the population numbers rather than eliminate them, the minor population increase was considered acceptable. On average for the autoclaved treatments, the control soils had the highest bacterial diversity compared with various atrazine treated soils; whereas RD5x recorded with the lowest bacterial diversity throughout the sampling dates (Figure 5.2B).

In nonautoclaved soil samples, the bacterial diversity in terms of OTU richness followed a decreasing trend from 0 to 40 DAT but the differences were not significant (Figure 5.2B).

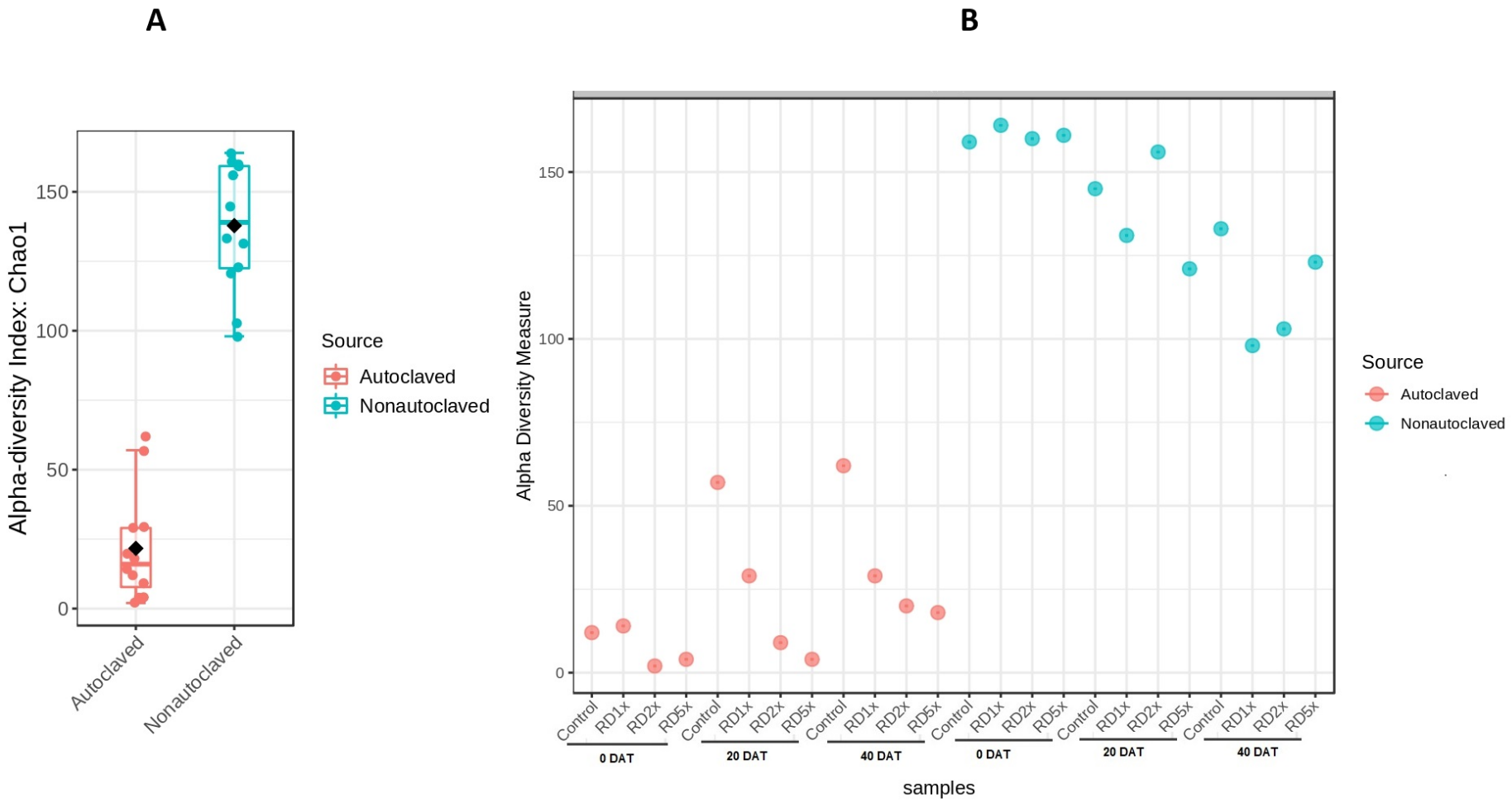

Figure 5.2. Alpha diversity analysis of bacterial communities in autoclaved and nonautoclaved soil samples treated with control, RD1x, RD2x and RD5x of the recommended dose (RD) of atrazine at 0, 20 and 40 days of treatment (DAT). Abundance of OTUs were determined following the sequencing of the V3–V4 region of 16s rRNA genes. The recommended dose (RD1x) of atrazine used in this study was set at 4.50 mg/kg soil.

The PCoA plot represented every treatment as a dot, which is coloured according to their sampling date and labelled as treatment. Firstly, this two-dimensional PCoA plot showed 58% of the total variance between the treatments. On both first and second axis, various atrazine concentrations investigated at 0, 20 and 40 DAT can be easily differentiable. Finally, results showed that the intra-variability between the atrazine concentrations at 0 DAT is much lower than the intra-variability between the atrazine concentrations at 20 and 40 DAT (Figure 5.3). So, the ordination of Bray-Curtis index by PCoA revealed that significant difference between three sampling dates and differences among various atrazine concentrations, especially at 20 and 40 DAT (*P<0.05*).

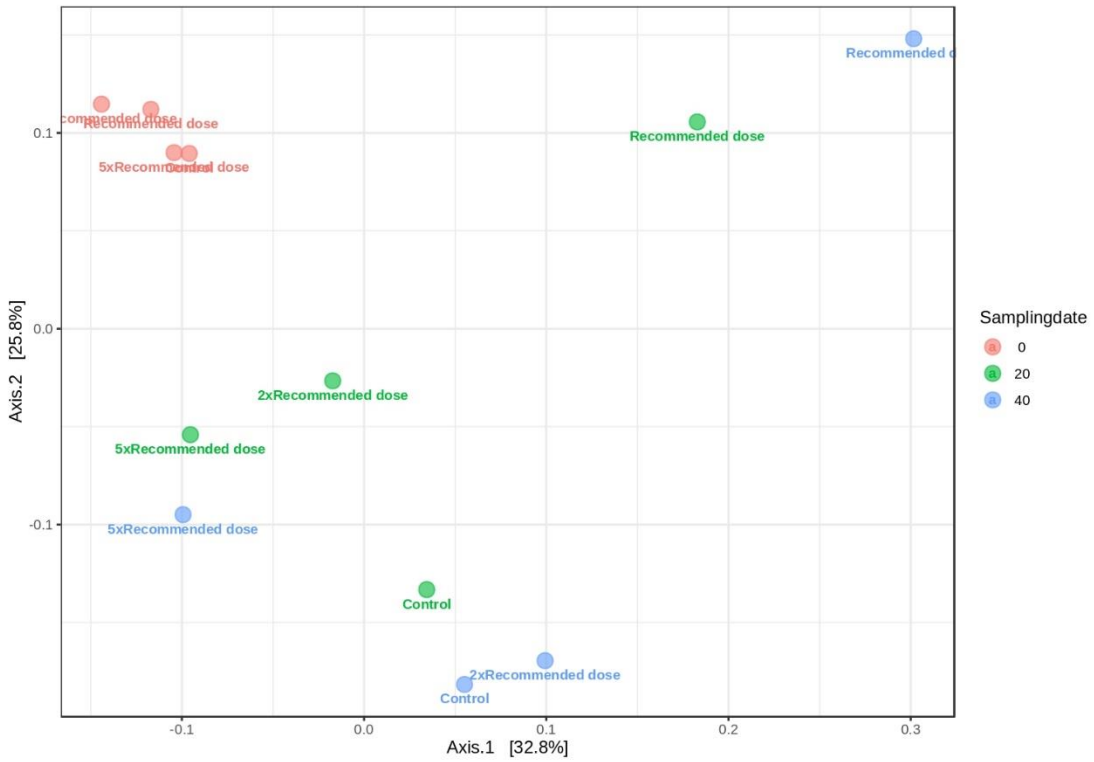

Figure 5.3. Beta diversity analysis of bacterial communities in nonautoclaved soil samples treated with control, RD1x, RD2x and RD5x of the recommended dose (RD) of atrazine at 0, 20 and 40 days of treatment (DAT). Principal coordinate analysis (PCoA) of Bray-Curtis index (r^2=0.28537) was statistically significant using [PERMANOVA] F-value: 1.797; p-value < *0.05*.

Effect of atrazine application on relative bacterial abundance

A total of 15 phyla, and 118 genera were detected in all the atrazine treated nonautoclaved soil samples. No differences in relative abundance were observed after immediate application of atrazine (0 DAT). However, major differences at the phylum and genus levels were observed at 20 and 40 DAT under different atrazine concentrations. The highest relative bacterial abundance was observed under RD5x, whereas RD1x recorded the lowest at 20 and 40 DAT.

The results showed that majority of the sequences belongs to phyla *Actinobacteria, Firmicutes, Proteobacteria, Acidobacteria, Gemmatimonadetes, Bacteroidetes* and *Chloroflexi*, making up 99.55% of the total sequences. However, their response to various concentrations of atrazine varied throughout the incubation period. The relative abundance of phylum *Firmicutes* increased with increasing atrazine concentrations over time (20 and 40 DAT); with exceptions under RD1x at 40 DAT (Figure 5.4A). *Firmicutes* was the most dominant phylum (89 and 61% of the total sequences) under RD5x at 20 and 40 DAT. The relative abundance of phylum *Actinobacteria* was highest under RD2x of atrazine concentrations at 40 DAT. Moreover, relative abundance of *Actinobacteria* was decreased possibly due to dominance of phyla *Proteobacteria* and *Firmicutes* under RD1x and RD5x of atrazine concentrations, respectively. On the other hand, relative abundance of phyla *Proteobacteria, Acidobacteria* and *Gemmatimonadetes* were suppressed under increasing atrazine concentrations at 20 and 40 DAT.

At the genus level (Figure 5.4B), relative abundance of *Bacillus* was increased with increasing concentrations of atrazine at 20 and 40 DAT. Relative abundance of *Bacillus* was as high as 84 and 52% of the total sequences under RD5x of recommended dose of atrazine at 20 and 40 DAT, respectively. The recommended dose (RD1x) of atrazine stimulated relative abundance of *Rhodococcus* at 20 and 40 DAT. However, the relative abundance of *Kaistobacter* was strongly inhibited by all atrazine concentrations compared with untreated control at 20 and 40 DAT.

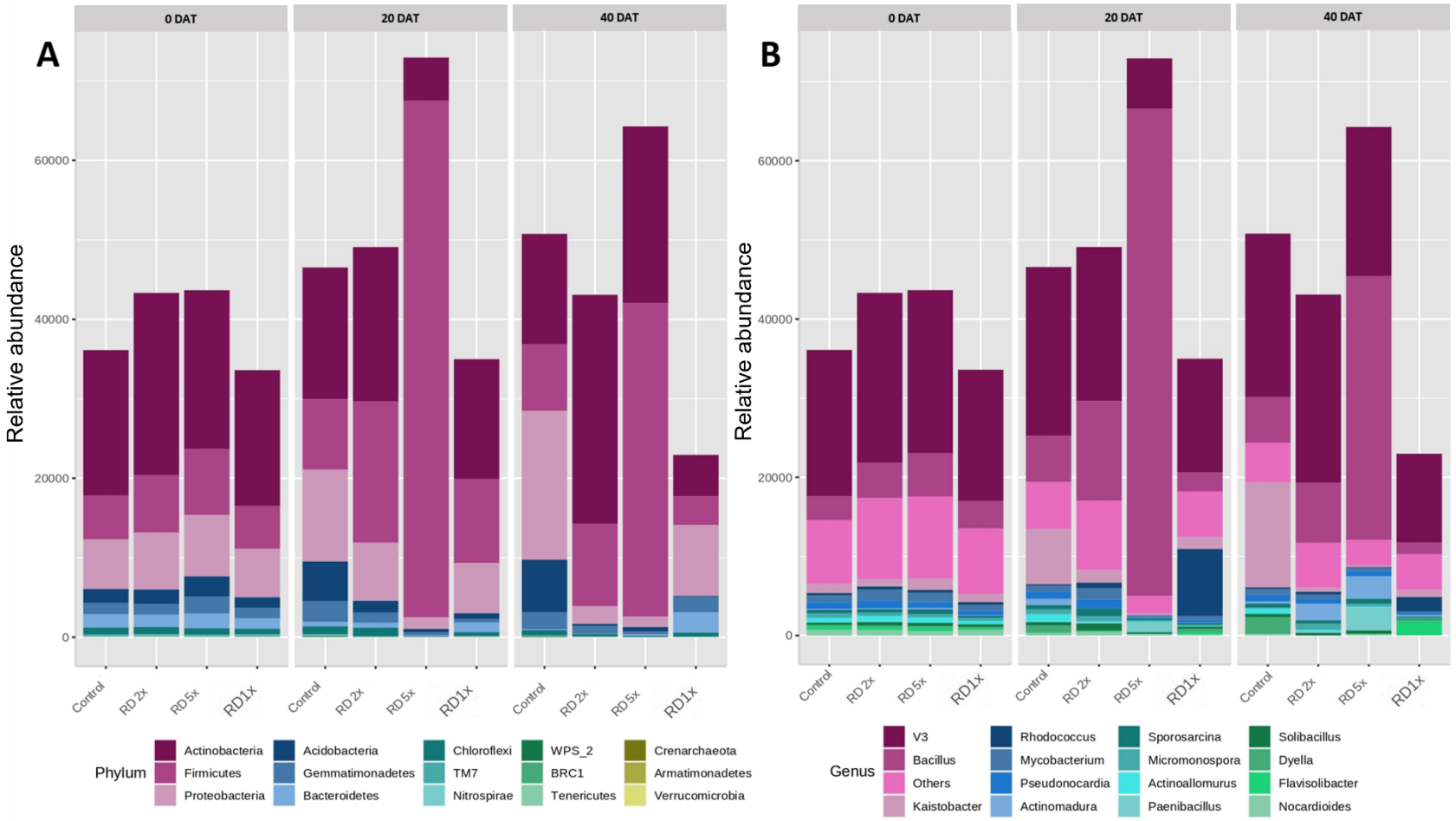

Figure 5.4. The relative abundance of the bacterial phyla (A) and genera (B) under control, RD1x, RD2x and RD5x of the recommended dose (RD) of atrazine at 0, 20 and 40 days of treatment (DAT) in nonautoclaved soil. The 16 predominant bacterial genera with relative abundance are shown at the genus level, and the rest are merged into the others. The recommended dose (RD1x) of atrazine used in this study was set at 4.50 mg/kg soil.

Classical univariate analysis revealed that altogether 7 OTU's were significantly affected by atrazine concentrations applied ($P \leq 0.01$) (Table 5.2).

Table 5.2. Classical univariate statistical comparisons of OTU's at different sampling dates upon exposure to atrazine concentrations.

OTU ID	P values	FDR	Statistics	Phylum
OTU764	1.38E-09	1.26E-06	414.25	*Actinobacteria*
OTU561	7.74E-08	3.53E-05	166.71	*Gemmatimonadetes*
OTU1032	1.18E-07	3.58E-05	151.41	*Actinobacteria*
OTU934	2.60E-06	0.0005933	73.888	*Proteobacteria*
OTU1330	1.71E-05	0.0031262	47.061	*Bacteroidetes*
OTU596	4.42E-05	0.00656	37.28	*Actinobacteria*
OTU595	5.04E-05	0.00656	36.081	*Proteobacteria*

Taxonomic data confirmed that the highest 3 OTU's belong to *Actinobacteria*, followed by 2 OTU's from *Proteobacteria* and 1 each from *Gemmatimonadetes* and *Bacteroidetes* phyla.

Soil microbial carbon utilization upon exposure to various atrazine concentrations

BIOLOG ECO plate assess the oxidative capacity of soil microbial communities representing functional diversity, which can be measured by AWCD (Fang et al., 2014; Wu et al., 2014). Application of atrazine decreased overall activities of soil microbial communities throughout the study duration (Figure 5.5). On day 0 immediately after atrazine application, the AWCD value for all atrazine-treated soils was lower than those in the control soil at the incubation time over 72 hrs. Complete inhibition was observed with atrazine at RD2x and RD5x at 0 DAT during the entire incubation period. It was noted that AWCD value of RD1x was higher than the control soil during the initial period from 4 to 72 hrs after incubation.

At 20 and 40 DAT, the AWCD value for all atrazine-treated soils gradually increased after incubation, except for declining AWCD values from 144 to 168 hrs at 40 DAT. The AWCD values were consistently lower in atrazine-treated soils than the control soil, indicating that atrazine suppressed the overall activities of soil microbial communities.

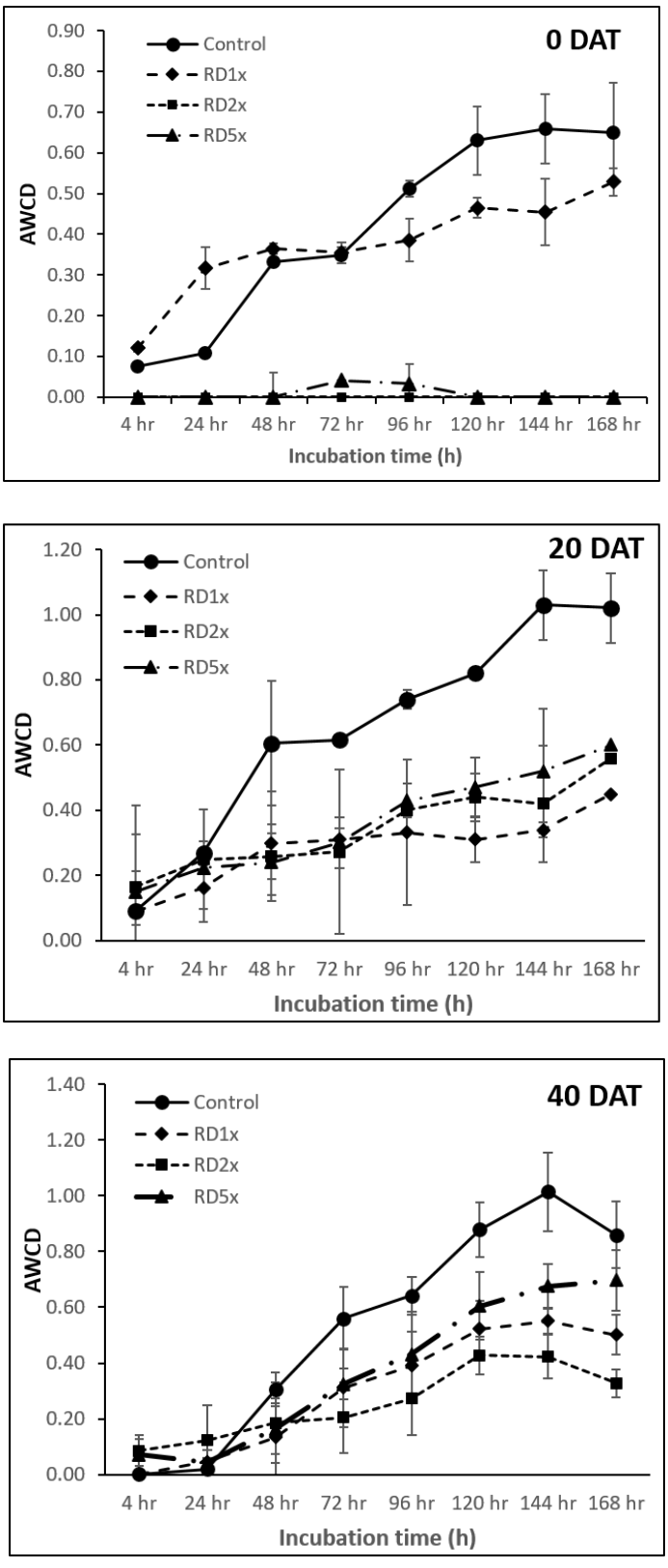

Figure 5.5. AWCD of soil samples under control, RD1x, RD2x and RD5x of the recommended dose (RD) of atrazine at 0, 20 and 40 days after treatment (DAT) in nonautoclaved soil. Mean values (*n* = 3) ± SD. AWCD: Average well colour development. The recommended dose (RD1x) of atrazine used in this study was set at 4.50 mg/kg soil.

5.3.3 Trifluralin

Effect of trifluralin application on soil microbial respiration

Results showed that soil microbial respiration varied significantly under various trifluralin concentrations over time (Figure 5.6). At 1 DAT, no significant difference was observed in terms of evolved CO_2-C with trifluralin treated soil samples and control. However, soil microbial respiration (evolved CO_2-C) increased significantly over time ($p<0.01$) and the highest evolved CO_2-C was recorded with RD2x treatments at 7 and 21 DAT as compared to other treatments (3840 and 9960 µg/g soil, respectively). The RD5x treatment recorded the second highest evolved CO_2-C value which is statistically similar with RD2x.

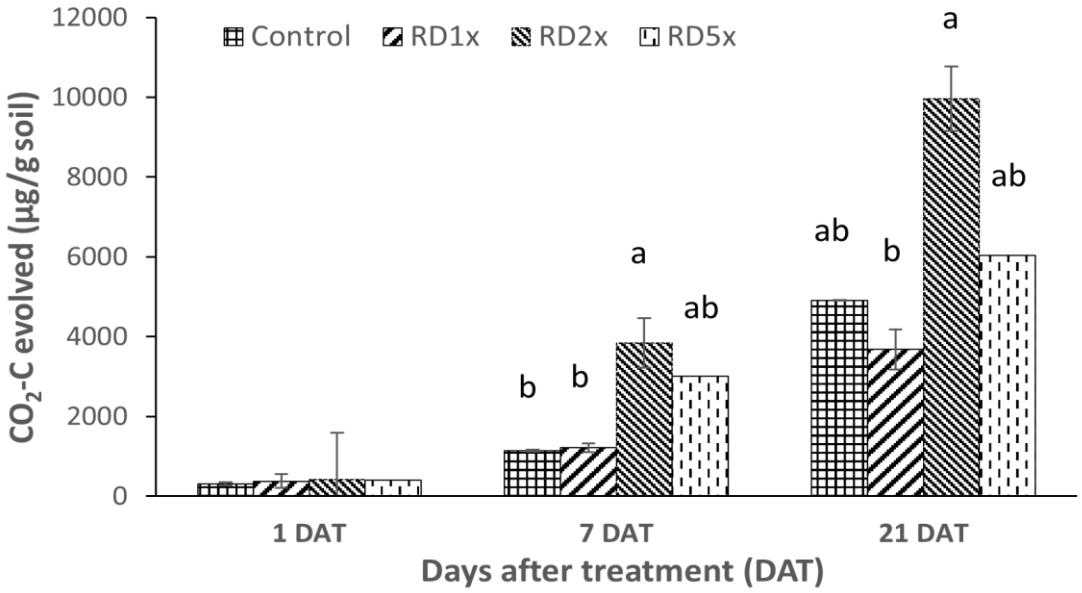

Figure 5.6. Effect of various trifluralin concentrations (control, RD1x, RD2x and RD5x) at 1, 7 and 21 days after treatment (DAT) in nonautoclaved soil. Mean values (n = 3) ± SD. Values labelled with various lower case letters are significantly different at $p<0.01$. The recommended dose (RD1x) of trifluralin used in this study was set at 4.25 mg/kg soil.

Response of bacterial diversity and structure upon trifluralin application

Alpha diversity analysis indicated that bacterial diversity differed significantly between the autoclaved and nonautoclaved soil samples when exposed to trifluralin (F *value* = -16.34, $P \leq 0.01$). In the autoclaved soils, bacterial diversity in the control treatments

increased over time from 0 to 40 DAT but no changes in bacterial diversity was observed under different trifluralin concentrations (Figure 5.7A).

In nonautoclaved soils, the decrease in bacterial diversity for various trifluralin concentrations was not significant (Figure 5.7B). However, the diversity of control and RD1x appeared to decrease throughout the experiment with maximum decrease observed with RD1x from 0 to 40 DAT. On average no changes in bacterial diversity were observed with increasing trifluralin concentrations (RD2x and RD5x) at 20 and 40 DAT.

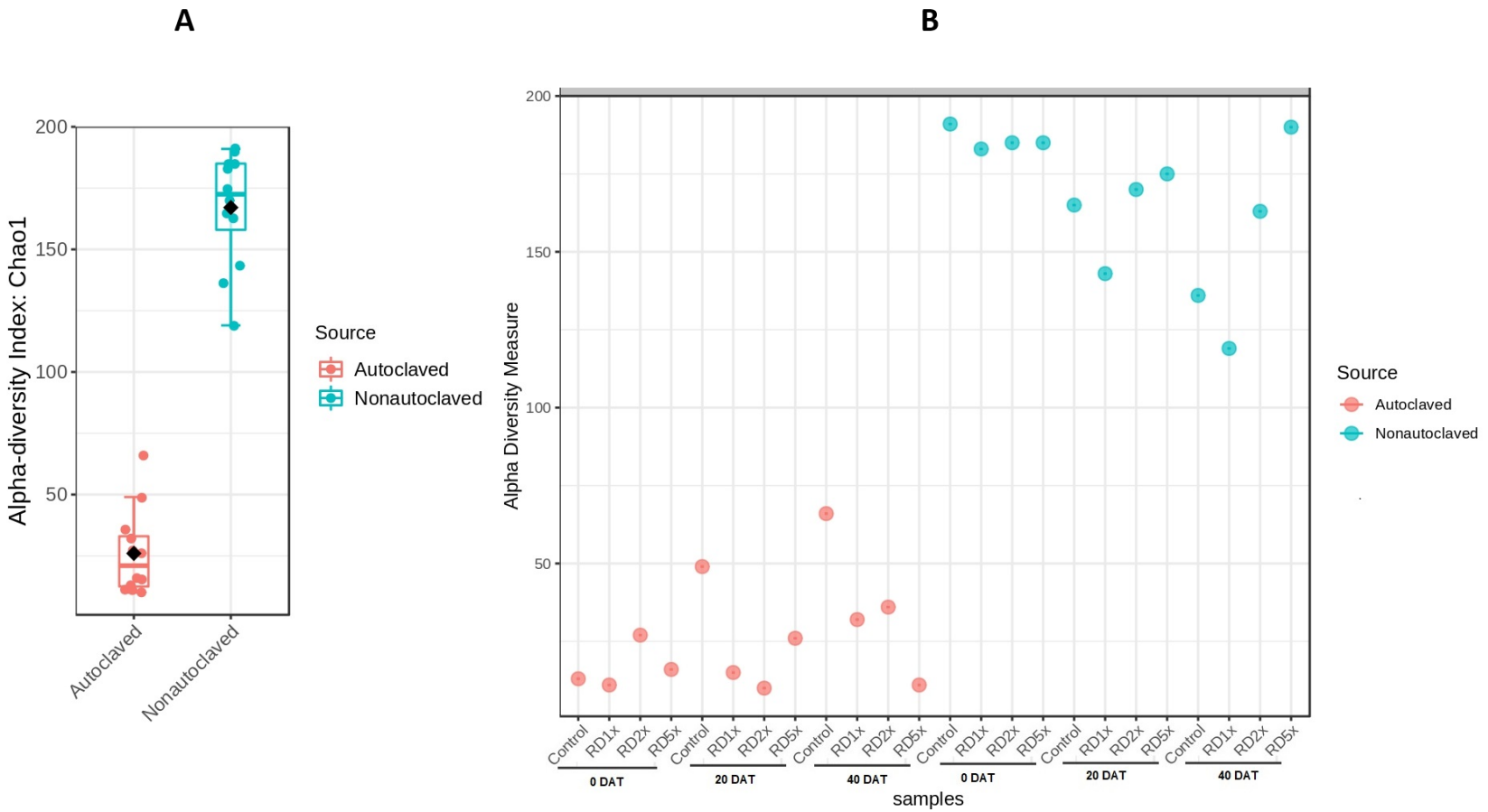

Figure 5.7. Alpha diversity analysis of bacterial communities in autoclaved and nonautoclaved soil samples treated with control, RD1x, RD2x and RD5x of the recommended dose (RD) of trifluralin at 0, 20 and 40 days of treatment (DAT). Abundance of OTUs were determined following the sequencing of the V3–V4 region of 16s rRNA genes. The recommended dose (RD1x) of trifluralin used in this study was set at 4.25 mg/kg soil.

The PCoA plot represented every treatment as a dot, which is coloured according to their sampling date and labelled as treatment. Firstly, this two-dimensional PCoA plot showed 64% of the total variance between the treatments. Various trifluralin concentrations investigated at 0, 20 and 40 DAT could not be clearly differentiable neither on the first nor on the second axis. Finally, results showed that the intra-variability between the trifluralin concentrations at 0, 20 and 40 DAT is similar to the inter-variability between the trifluralin concentrations (Figure 5.8). So, the ordination of Bray-Curtis index by PCoA revealed nonsignificant difference between three sampling dates and among various trifluralin concentrations.

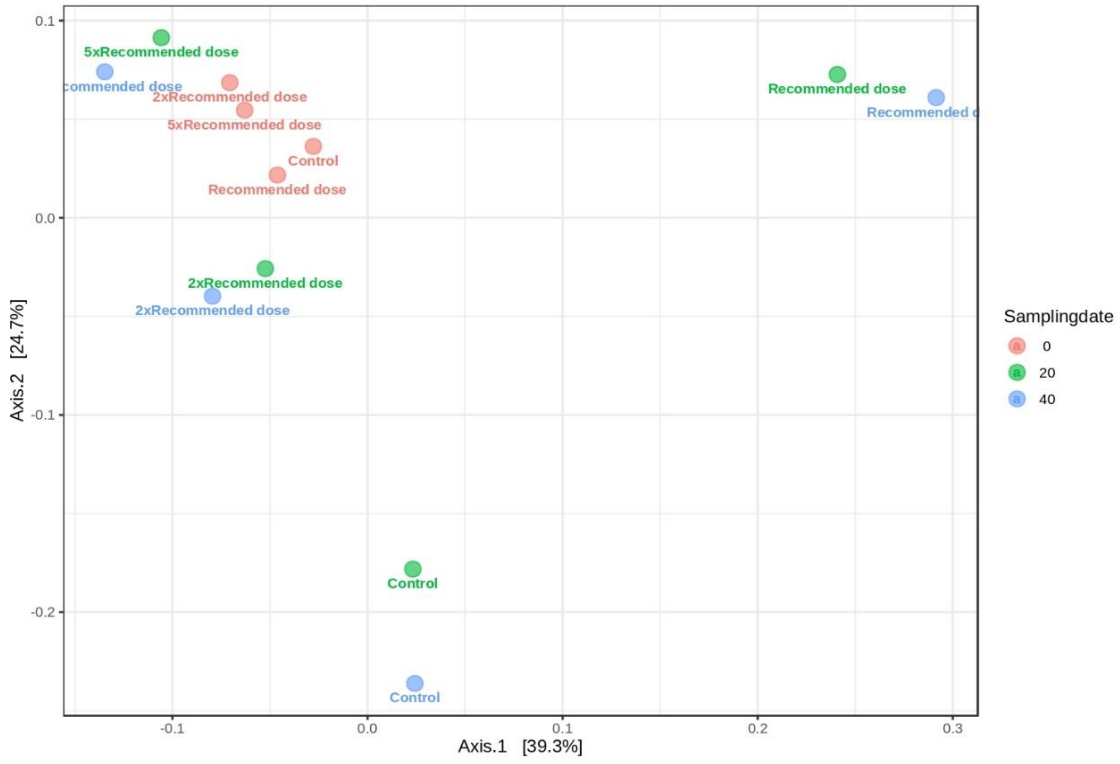

Figure 5.8. Beta diversity analysis of bacterial communities in nonautoclaved soil samples treated with control, RD1x, RD2x and RD5x of the recommended dose (RD) of trifluralin at 0, 20 and 40 days of treatment (DAT). Principal coordinate analysis (PCoA) of Bray-Curtis index (r^2=0.15342) using [PERMANOVA] F-value: 0.81549; p-value < *0.565*.

Effect of various trifluralin concentrations on relative bacterial abundance

Trifluralin treated nonautoclaved soil samples recorded a total of 17 phyla and 129 genera. Relative bacterial abundance was not affected by trifluralin concentrations at 0 and 20 DAT. However, at 40 DAT, highest relative bacterial abundance was observed under RD2x followed by RD5x and the lowest was observed under RD1x.

Results showed that majority of the detected sequences belongs to phyla *Actinobacteria, Proteobacteria, Firmicutes, Acidobacteria, Bacteroidetes, Gemmatimonadetes* and *Chloroflexi*, making up 99.43% of the total sequences. At 40 DAT, *Actinobacteria* was the most abundant phylum, consisting of 49% and 47% of total sequences under RD2x and RD5x of the recommended dose of trifluralin followed by *Firmicutes* (Figure 5.9A). Relative abundance of phylum *Firmicutes* was increased under RD2x of trifluralin concentrations at 40 DAT. Phylum *Proteobacteria* showed higher tolerance to various levels of trifluralin concentrations as its relative abundance was consistent throughout the experimental period.

Very limited taxonomic information was available at genus level (Figure 5.9B). According to the results, relative abundance of *Bacillus* was increased under RD2x, following RD2x > RD5x > RD1x at 20 and 40 DAT. The relative abundance of genus *Rhodococcus* was increased with RD1x and decreased with increasing concentrations at 20 and 40 DAT.

Relative abundance of genus *Kaistobacter* was higher in control treatments over the sampling period (0, 20 and 40 DAT) but strongly inhibited by trifluralin exposure regardless of concentrations applied throughout the experimental period.

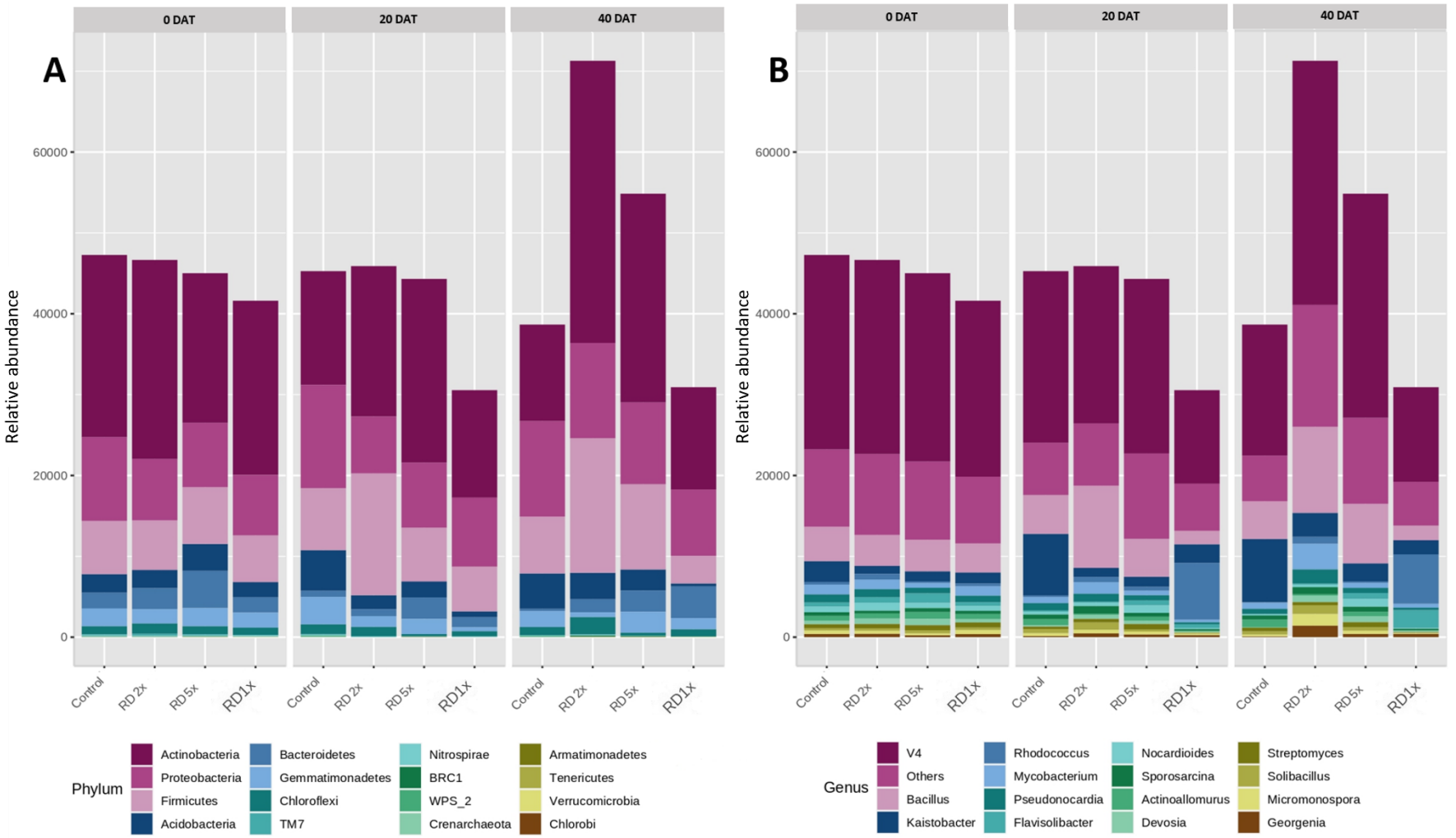

Figure 5.9. The relative abundance of the bacterial phyla (A) and genera (B) under control, RD1x, RD2x and RD5x of the recommended dose (RD) of trifluralin at 0, 20 and 40 days of treatment (DAT). The 16 predominant bacterial genera with relative abundance are shown at the genus level, and the rest are merged into the others. The recommended dose (RD1x) of trifluralin used in this study was set at 4.25 mg/kg soil.

Classical univariate analysis revealed that different trifluralin concentrations significantly affected a total of 5 OTU's at different sampling period ($P \leq 0.01$) (Table 5.3).

Table 5.3. Classical univariate statistical comparisons of OTU's at different sampling dates upon exposure to trifluralin concentrations.

OTU ID	P values	FDR	Statistics	Phylum
OTU363	1.78E-09	1.15E-06	391.56	*Actinobacteria*
OTU359	1.86E-09	1.15E-06	387.72	*Actinobacteria*
OTU937	4.28E-08	1.77E-05	190.75	*Actinobacteria*
OTU1271	5.16E-06	0.0016	62.813	*Bacteroidetes*
OTU1430	2.06E-05	0.00512	44.988	*Acidobacteria*

The number of significant OTU's were determined by p-values adjusted using FDR method. Taxonomic data confirmed that out of 5 OTU's, 3 belong to *Actinobacteria*, followed by 1 of each from *Acidobacteria* and *Bacteroidetes* phyla.

Soil microbial carbon utilization under various trifluralin concentrations

Figure 5.10 represents the dynamic change of the AWCD values in soil upon exposure to control, RD1x, RD2x and RD5x of the trifluralin recommended dose. As the incubation time progresses, the AWCD at 0 DAT followed a general increasing trend except the RD2x treatment. AWCD values at RD5x and RD1x being consistently higher than those in the control soil during the entire incubation period. The RD2x treatment did not behave in a logical manner having no activity until 48 hr of incubation, with a surprising increase at 72 and 92 hr and then again, no activity hereafter.

The general increasing trend of AWCD values was also evidenced at 20 and 40 DAT. Trifluralin treated soils generally had lower AWCD values as compared with the untreated control and the differences widened as the incubation time increased, indicating the suppression of the overall soil microbial activities in trifluralin treated soils.

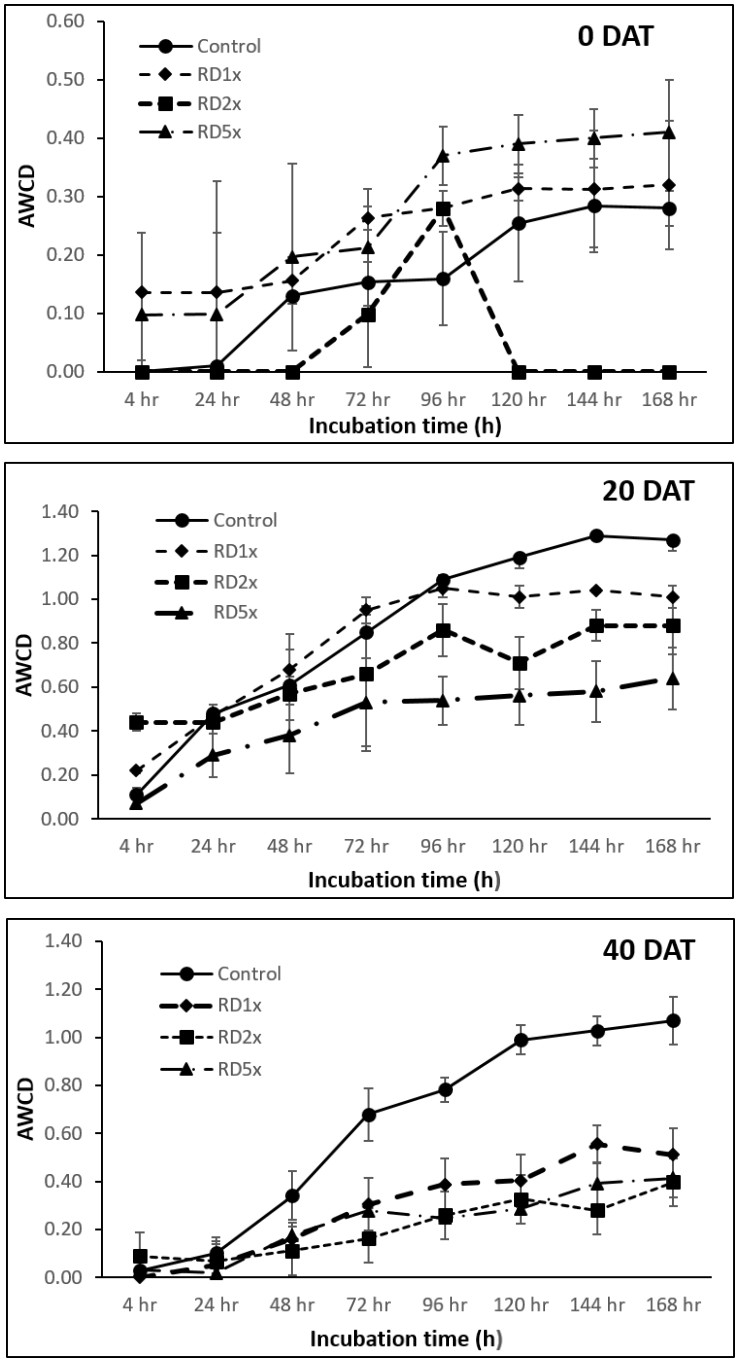

Figure 5.10. AWCD of soil samples under control, RD1x, RD2x and RD5x of the recommended dose (RD) of trifluralin at 0, 20 and 40 days after treatment (DAT) in nonautoclaved soil. Mean values (n = 3) ± SD. AWCD: Average well colour development. The recommended dose (RD1x) of trifluralin used in this study was set at 4.25 mg/kg soil.

5.4 Discussion

This study investigated the dynamic changes in soil microbial respiration, and the shift in bacterial community structure and functions upon application of atrazine and trifluralin at recommended dose (RD1x), twice (RD2x) and five times (RD5x) of the recommended dose separately.

5.4.1 Herbicide effects on microbial respiration

Soil microbial respiration leads to rapid production of CO_2 under aerobic conditions (Cerhanova et al., 2006). It is believed that pesticides degraded microbiologically would result in increased CO_2 production (Bartha et al., 1967). This experiment revealed that soil microbial respiration was stimulated with increasing atrazine concentrations; highest evolved CO_2-C with RD5x treatment examined up to 21 DAT. Radivojević et al. (2008) observed that soil microbial respiration is dependent on atrazine concentrations applied which increased until 60 DAT. This higher respiratory activity is considered to be due to greater microbial activity (Anderson & Domsch, 2010) by microorganisms capable of utilizing pesticides as a source of carbon and energy (Jones & Ananyeva, 2001). Like atrazine, trifluralin applied at RD2x concentrations recorded highest evolved CO_2-C followed by RD5x up to 21 DAT. This is possibly due to an inhibitory effect of trifluralin applied at higher concentrations (RD5x). According to Sağlıker (2009), trifluralin reduced microbial respiration when applied at a concentration greater than 16 mg/kg soil.

5.4.2 Herbicide effects on bacterial diversity and structure

Analysis of the alpha diversity revealed significant variations in the community structures among autoclaved and nonautoclaved soil samples. Similarly, the ordination of Bray-Curtis index by PCoA observed significant variations among three sampling dates and various atrazine concentrations, especially at 20 and 40 DAT. The community structures at 20 and 40 DAT were more complex and diverse than at 0 DAT in nonautoclaved soil, indicating a temporary shift in bacterial community and structure due to atrazine exposure (Wang et al., 2015). Possibly, changes in relative abundance of higher abundant bacterial groups in atrazine treated and control soils might have altered community compositions by supressing low abundance bacterial groups. Low abundant

bacterial groups are known to play an important role in major biogeochemical cycles (Bell et al., 2005) and serve as a seed bank for species richness (Lennon & Jones, 2011).

There is very little information available on overall bacterial diversity and function upon exposure to trifluralin. Alpha diversity analysis observed significant variations on bacterial diversity between autoclaved and nonautoclaved soil samples upon exposure to trifluralin, but beta diversity analysis revealed that trifluralin did not affect the bacterial diversity regardless of concentrations and sampling dates. This result contradicts Du et al. (2018), who reported that trifluralin application significantly increased bacterial abundance, but disturbed bacterial diversity and community structure. However, even if there is not a change in bacterial diversity, there can still be a significant difference in composition. Changes in the composition and structure of microbial communities followed by pesticide application may be due to toxic effects of pesticides on some microbial groups, and proliferation of tolerant species due to reduced competition for space and available nutrients (Johnsen et al., 2001). These variations in response by different bacterial groups to trifluralin exposure might have altered the bacterial community structure.

5.4.3 Herbicide effects on relative bacterial abundance

A total of 15 phyla across 118 genera were detected in soils treated with various concentrations of atrazine. Efficient atrazine degradation has been observed by several bacterial strains belonging to *Acinetobacter*, *Agrobacterium*, *Pseudomonas*, *Shewanella* and *Ensifer* from phylum *Proteobacteria; Arthrobacter, Citricoccus, Nocardioides* and *Rhodococcus* from phylum *Actinobacteria* and *Bacillus* from *Firmicutes* (Devers et al., 2007; Piutti et al., 2003; Zhang et al., 2012). It is evident that majority of the atrazine degrading bacteria belongs to phyla *Firmicutes* and *Actinobacteria* which supports the results of the present study. Maximum relative abundances of *Firmicutes* (under RD5x) and *Actinobacteria* (under RD2x) in this study occurred at 20 and 40 DAT. Again, population dynamics, distribution and activity of soil microorganisms are governed by various environmental factors, such as carbon and energy sources, mineral nutrients, growth factors, ionic composition, available water, temperature, pressure, air composition, electromagnetic radiation, pH, oxidation-reduction potential, surfaces, spatial relationships, genetics of the microorganisms and interaction between

microorganisms (Nannipieri et al., 2003). At genus level, it is obvious that higher doses of atrazine (RD5x) stimulated and supported the growth of *Bacillus.* As some bacteria from *Bacillus* were reported to be higher tolerance against atrazine and potentially could tolerate at least 1000 mg/L atrazine (Zhu et al., 2021). The relative abundance of *Rhodococcus* was increased under RD1x and decreased with increasing concentrations at 20 and 40 DAT. This could be due to the toxic effects resulted from increased concentrations applied. However, higher doses of atrazine (RD5x) supressed relative abundance of *Actinobacteria* at 20 DAT but recovered at 40 DAT which indicates a potential positive co-occurrence with *Firmicutes*, either maintaining a balance in the bacterial community or indirectly supporting atrazine degradation by promoting the growth of *Firmicutes*. This argument can be supported by the findings of Piutti et al. (2002) who observed that *Methylophilus* improved the atrazine degradation rate when co-inoculated with *Arthrobacter*. Presence or absence of some bacterial strains could alter the proportion of some organic components (such as the C:N ratio) in the environment, further affecting the degradation of triazine herbicides. Cook and Huetter (1981) reported a complete degradation of deethylsimazine, a triazine metabolite with the presence of *Rhodococcus corallinus* and *Pseudomonas* sp. strain NRRL B-12228.

This study shows that atrazine supressed the relative abundance of phyla *Proteobacteria, Acidobacteria* and *Gemmatimonadetes* with increasing concentration and incubation time. At the genus level, the relative abundance of *Kaistobacter* was inhibited by various atrazine concentrations at 20 and 40 DAT. The suppression or inhibition of particular microorganisms to specific pollutants might be the result of the toxic effects. Moreover, no co-occurrence of them was observed with known atrazine degrading bacteria. Bardhan (2010) stated that *Kaistobacter* has not been reported as atrazine degraders.

On the other hand, a total of 17 phyla and 129 genera were detected in trifluralin treated soil samples. Relative abundance data revealed no differences were observed between 0 and 20 DAT under various trifluralin concentrations. This is most likely due to possible lag period which allows the adaptation required for bacterial cells exploiting new environmental conditions (Madigan et al., 1997). However, relative abundance of *Actinobacteria* was the highest under RD2x and RD5x at 40 DAT, followed by *Firmicutes* and *Proteobacteria*. At genus level, the relative abundance of *Bacillus* was increased

under RD2x, whereas *Rhodococcus* was increased with RD1x at 40 DAT. Some microorganisms are capable of utilizing pesticides as an energy source and may benefit from pesticide exposure (Russell et al., 2011). Data revealed that trifluralin application significantly decreased the relative abundance of *Acidobacteria* at the phylum level and *Kaistobacter* at genus level. The possible reason could be trifluralin interference on bacterial growth or/and competition among species resulting in extinction (Barabás et al., 2016). Relative abundance of *Proteobacteria* was not impacted at all by various trifluralin concentrations throughout the experimental period, suggesting that they are more tolerant to trifluralin and may utilize trifluralin as a source of carbon and energy, as identified for other herbicides (Cheng et al., 2017; Dong et al., 2015). However, response of different species of microorganisms to pollutants are different (Carter & Camper, 1975), as a result of competition.

5.4.4 Herbicide effects on microbial carbon utilization

Soil microbial diversity functions revealed that AWCD values were significantly affected by the atrazine application. Only RD1x of the recommended dose of atrazine recorded higher AWCD values up to 72 hr of incubation compared to the control at 0 DAT; indicating presence of potential atrazine degrading bacteria in soil. However, at later stages (20 and 40 DAT), AWCD values of atrazine treated soils were consistently lower than the control soil; indicating complete suppression of soil microbial metabolic functions. The AWCD value was initially suppressed by higher atrazine concentrations due to the inhibition of dominant bacteria sensitive to high concentrations of atrazine (Fang et al., 2015). At the later stages (20 and 40 DAT), the AWCD values gradually recovered regardless of the atrazine dose, as a result of possible resistant or degrading microbial populations due to long-term selective pressure of atrazine (Ros et al., 2006). Interestingly, AWCD values under various trifluralin concentrations, indicated an initial stimulation followed by inhibition pattern throughout the experimental period (0 to 40 DAT). The initial stimulation of soil microbial diversity functions indicates the presence of resistant bacterial communities in soil regardless of the concentrations used (Hang et al., 2001; Rainey & Travisano, 1998). Some of them are capable to tolerate extreme concentrations when exposed to (Zhu et al., 2021). However, the reductions in AWCD

values at higher doses of trifluralin (RD2x and RD5x) at 20 and 40 DAT, possibly due to direct toxicity of trifluralin.

Interestingly, BIOLOG results did not comply with the relative abundance of both herbicides in this study. Atrazine and trifluralin doses (RD1x, RD2x and RD5x) obviously suppressed AWCD values at 20 and 40 DAT. But the relative abundance of herbicides reportedly increased with time and doses applied. This is most likely due to the limitation of BIOLOG technique ignoring the activity of catabolically inactive microorganisms in dormant state as well as non-culturable microorganisms (Zhang et al., 2014b). Microorganisms present in the soil regardless of their type and functions, contribute to the total microbial biomass and Phospholipid fatty acids (PLFA) levels. However, mainly fast growing, probably initially non dormant bacteria able to contribute to the colour development in the wells, are involved in this technique (Baath et al., 1998).

5.5 Conclusion

Application of atrazine and trifluralin stimulated the relative abundance but did not affect bacterial diversity. *Bacillus* was the most abundant genus with increased atrazine and trifluralin concentrations over time, while both herbicides suppressed the relative abundance of genus *Kaistobacter*. Herbicide interaction to relative bacterial abundance was rate dependant where both herbicides increased the relative abundance of phyla *Actinobacteria* and *Firmicutes* at different incubations periods. However, the relative abundance data did not comply with soil microbial carbon utilization pattern studied in this experiment. The outcome of this research will contribute to the development of new bioremediation strategy of trifluralin and atrazine in soil.

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Chapter 6. General discussion

Herbicide persistence is the active lifetime of an herbicide in soil which is generally expressed as half-life (Curran, 2016). The half-life is often used as an indicator of herbicide degradability. Degradation may be catalysed by light, or occur via biotic or abiotic processes, and is dependent on various environmental factors. Extensive herbicide use in farming systems may deteriorate overall soil quality, compromise crop performance, affect microbial population and function, and contaminate surface and groundwater. Recent research has identified several herbicides persisting in Australian cropping fields, particularly in low rainfall areas (Rose et al., 2019). Potential risks associated with herbicide persistence include yield loss, reduced farm profitability, and their use may impose limitations crop selection in rotations. Trifluralin and atrazine are the two commonly used herbicides in various farming systems in Australia and were frequently detected in soils with potential persistent activity (Rose et al., 2019). Trifluralin inhibits microtubule assembly by restricting polymerization of tubulin to interrupt cell mitosis (Fernandes et al., 2013); resulting inhibition of shoot and root cell division (Shaner, 2014). However, the extent of injury and growth reduction is variable to plant species due to variations in soil type, temperature, soil moisture and duration of trifluralin incorporation (Horowitz et al., 1974; Kennedy & Talbert, 1977). Similarly, atrazine residues adversely affected the overall performance of the test crop species with increasing concentrations in soil. Atrazine interferes with the photosystem II (PSII) process by affecting adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) production. Atrazine is also responsible for chloroplast membrane injury by accumulating reactive oxygen species. Farmers need to follow label recommendation on the plantback periods for sensitive crops in rotation, especially when environmental conditions are not suitable for herbicide breakdown in soil. Microorganisms are capable of degrading a wide range of pesticides in soils through various enzymatic metabolic pathways (Porto et al., 2011). Moreover, this is cheap, less hazardous, environmentally safe, economic and socially acceptable (You & Liu, 2004). However, degradation is primarily dependant on the microbial composition of the soil (diversity and structure) and existing soil conditions i.e., soil type, temperature, humidity, pH, additional energy sources, etc. (Walker, 1987). Therefore, this study was

carried out to determine the potential damages of trifluralin and atrazine to various cereal and legume crops and to better understand the persistence of trifluralin and atrazine residues in Australian soil under various environmental conditions, and the initial determination of the impact of trifluralin and atrazine on soil microbial composition due to their application.

The initial aim is to investigate the toxic effects of trifluralin and atrazine on the sensitive crop species (Chapter 3). Bioassay techniques are generally used to determine phytotoxic effects of herbicide concentrations present in the soil using sensitive plants as indicators (Hager & Sprague, 2002). This technique indicates critical concentrations of herbicides present in the soil high enough to affect crop growth, yield and quality. To achieve this, a total of five crop species including three cereals and two legume species, were selected based on their sensitivity to trifluralin and atrazine. These included wheat (*Triticum aestivum*. cv. Corack), barley (*Hordeum vulgare* cv. Hindmarsh), oat (*Avena sativa* cv. Savannah), lucerne (*Medicago sativa* cv. Stamina) and lentil (*Lens culinaris* cv. Hurricane XT). The impact of trifluralin and atrazine on the crop species were separately investigated using various concentrations under glasshouse conditions in 2018 and the trials were repeated in 2019. Toxicity symptoms associated with trifluralin were identified as stunted growth with twisted leaves, swollen hypocotyl and thickened primary root with no secondary roots. Similar types of toxicity symptoms were reported by others (Deuber, 1992; Senseman, 2007). Damage induced by trifluralin was observed with increasing levels of trifluralin concentration; about 50% inhibition was recorded in all the crop species under the lowest concentration of trifluralin (0.075 mg/kg soil) used in the soil. Similarly, atrazine residues adversely affected the overall performance of the crop species with increasing concentrations in soil. Legumes were more sensitive to atrazine concentrations in soil compared to cereals, with approximately 65-75% reductions in growth recorded at 0.017 mg/kg atrazine concentration. Atrazine toxicity symptoms include inhibition of photosynthesis; chlorosis affecting height, chlorophyll content and shoot dry weight of all the species. Burhan and Shaukat (2000) reported atrazine similar phytotoxicity to wheat, corn, mustard, turnip, pearl-millet and carrot.

Lucerne was the most sensitive crop species and barley was the most tolerant crop species against trifluralin and atrazine. Lucerne can therefore be used to quickly determine whether trifluralin and atrazine residues are below critical thresholds in soil.

Extremely small concentrations of residues may be damaging to sensitive crops, and thus farmers should be cautious selecting crops in rotation, especially in low rainfall areas. Mangels (1991) reported that drought conditions before planting of rotational crops may cause herbicides to persist longer than usual and consequently injure crops in the rotation.

The length of period from herbicide application to the complete loss of the herbicide product reflects the carryover potential of herbicide. Once applied, it is expected that the active component of the herbicide will be lost from the soil via a range of processes. However, presence of herbicides in the soil in effective concentrations may injure crops. Chapter 3 reported that atrazine and trifluralin residues in soil significantly affected root and shoots of both cereal and legume crop species 21 DAT. Atrazine and trifluralin residues persisted in soil for long enough to compromise overall crop performance. Herbicide persistence in soil is mainly regulated by site-specific agro-climatic conditions. Environmental factors regulate microbial communities and their functions in soil; especially temperature and moisture are known to play a critical role (Cavicchioli et al., 2019). Optimal conditions could support certain microbial communities to degrade herbicide residues in an efficient manner, reducing carryover effects and ensuring safe crop production in rotations. Therefore, persistence of atrazine and trifluralin was investigated in laboratory incubation experiments under different temperature and moisture conditions (Chapter 4). A two-parameter gamma distribution model was used to calculate half-life and days to complete dissipation of herbicides over first-order kinetics model. First-order kinetics models were reported to have some issues in calculating pesticide half-life, which can be minimized using gamma distribution model (Rohan et al., 2015). Results showed that temperature played a greater role on atrazine dissipation over soil moisture, while interaction effects of temperature and moisture were significant on trifluralin dissipation from soil over time. Both herbicides dissipated more rapidly at 30 °C compared to 10 and 20 °C; possibly due to rapid desorption of pollutants observed at higher temperature (Ghosh et al., 2001). Later, moisture content may support trifluralin degradation by increasing microbial metabolic activities at higher temperature (30 °C) (Lichtenstein & Schulz, 1964). Application of the gamma model predicted that atrazine could persist in clay loam soil for 2-3 years depending on soil temperature and moisture conditions. Low temperatures can prolong

atrazine persistence in the soil (Liu, 2014). Jablonowski et al. (2011) explained persistence of atrazine as binding to the soil matrix due to lack of microbial activity or in unavailable forms. Although persistence of trifluralin has been reported in various parts of the world, its environmental fate has not been fully explored so far (Coleman et al., 2020). Results of this study revealed that persistence of trifluralin could last for 2-3 years in clay loam soil under low temperature conditions (10 and 20 °C) regardless of the soil moisture conditions. It is evident that trifluralin has the potential to persist in clay loam soil and may be a possible concern for Australian farming community. These results are in agreement with a study conducted in Australia where Johnstone et al. (1998) observed prolonged persistence of trifluralin in drier locations. This prolonged persistence could be due to low microbial activity under low moisture conditions in soil (Getzin, 1968). Obviously, low temperature and moisture conditions contributed to prolonged persistence of atrazine and trifluralin in soil. The bioassay experiment revealed that after 21 DAT, crop damage was extensive even under minimum concentrations studied for both herbicides. Later, the gamma model showed that higher temperature and moisture conditions reduced atrazine and trifluralin persistence in soil. Increasing the temperature and moisture conditions could have reduced the concentration of residual herbicides to a level where it may not be damaging crop species by accelerating their degradation in soil.

Microorganisms (mainly bacteria and fungi) in soil utilize pesticides as a source of carbon and energy and transform them to CO_2 (Fenner et al., 2013; Siddique et al., 2003). Both temperature and moisture are the main environmental factors that influence microbial growth and development in soil. Higher temperature and moisture conditions could alter microbial community structure and functions by supporting growth of dominant microbial groups. Appropriate identification of dominant microbial groups could contribute to the development of new biodegradation strategies. These aspects were explored in soil containing atrazine and trifluralin residues separately via analysis of the microbiome utilising diversity profiling of the 16s rRNA region (Chapter 5). In addition, soil microbial respiration and activity was also investigated under the same conditions to support sequencing results. This study revealed that atrazine and trifluralin had a stimulatory effect on soil microbial respiration (evolved CO_2-C). Soil microbial respiration was highest under the 5 times recommended dose (RD5x)

treatment of atrazine; while in case of trifluralin, RD2x of the recommended dose recorded the maximum evolved CO_2-C compared to other treatments. The increased respiration could be supported by increased microbial activity, as proposed by (Anderson & Domsch, 2010). The increased microbial activity resulted in possible degradation of herbicides which increased CO_2 production, and the magnitude of the increase extended until carbon of the pesticide molecule oxidized completely (Bartha et al., 1967). Soil microbial respiration results support the relative bacterial abundance data generated from sequencing. RD5x of atrazine and RD2x of trifluralin resulted in the highest relative abundance by promoting dominant bacterial groups. However, both atrazine and trifluralin application suppressed some other bacterial groups as well. Changes in the microbial diversity and structure upon exposure to both herbicides could be due to their toxic effects on some microbial groups and proliferation of tolerant species due to reduced competition for space and available nutrients (Johnsen et al., 2001). Results showed that the relative abundance of phyla *Firmicutes* clearly elevated with increasing atrazine concentrations at 20 and 40 DAT; while the relative abundance of phylum *Actinobacteria* was stable until 20 DAT but increased under RD2x of atrazine concentrations at 40 DAT. On the other hand, relative abundance under different trifluralin concentrations was stable until 20 DAT and phylum *Actinobacteria* was the most abundant at 40 DAT followed by *Firmicutes*. At genus level, atrazine and trifluralin stimulated and supported the growth of *Bacillus* genera. Some microorganisms are capable of utilizing pesticides as an energy source and may benefit from pesticide exposure (Russell et al., 2011). Zhu et al. (2021) reported that some bacteria from *Bacillus* genera showed higher tolerance against atrazine; surviving at least 1000 mg/L atrazine. Matsumura (1982) reported that when the pesticide is toxic to the microorganisms, it may stimulate the growth of resistant strains which can either metabolically or otherwise detoxify it or withstand the toxic-action type metabolism. However, atrazine and trifluralin suppressed the abundance of genus *Kaistobacter* from phylum *Proteobacteria* regardless of concentrations applied over the sampling period. Possibly, microorganisms respond differently to various chemicals (Carter & Camper, 1975) and the toxic interference of various pesticides could result in extinction of certain microbial groups (Barabás et al., 2016).

Interestingly, atrazine had significant effect on overall bacterial diversity but trifluralin did not; according to the ordination of Bray-Curtis index by PCoA. Astaykina et al. (2020) reported increased or stable bacterial diversity followed by greater concentrations of pollutants as certain bacterial species may tolerate higher concentrations due to presence of high hydrolytic activity. Trifluralin application increased the relative abundance through the promotion of members within specific phylum but did not affect bacterial diversity. This might be due to the presence of tolerant bacterial species and changes in their abundance at later stages due to existing competition in the community (MacLean & Gudelj, 2006) as a result of the selection pressure by the herbicide (Ros et al., 2006). Another explanation could be that herbicides were degraded extracellularly and then the by-products of the initial degradation were further broken down by other microbial taxa, resulting in a lag-phase (Bhattacharya et al., 2017).

Overall soil microbial diversity functions were highly supressed by both atrazine and trifluralin application rates used in this study. However, BIOLOG results did not comply with the relative abundance results of this study. Bacterial abundance results reported an increase in relative abundance with time and application rates. This is most likely due to the limitation of BIOLOG technique ignoring the activity of catabolically inactive microorganisms present in the dormant state as well as other non-culturable microorganisms (Zhang et al., 2014). Moreover, BIOLOG technique is mainly dependent on the fast-growing microorganisms for the colour development underestimating the rest (Cycon et al., 2013). Therefore, it is an indicative measure rather than the relative functional expression of the microbial community.

This experiment investigated the potential relation of herbicide application and their possible breakdown in the clay loam soil through increasing the relative abundance of dominant bacterial population. Application of gamma model revealed rapid loss of atrazine was observed when incubated at incubated at 30 °C; while trifluralin loss was highest under 30 °C with moisture content adjusted to 70% FC. Later, incubation of both herbicides under 30 °C with 70% FC moisture content promoted the growth of some bacterial population by increasing their relative abundance in clay loam soil which later degraded both herbicides with increased soil microbial respiration. This study revealed that trifluralin and atrazine treated soils incubated under 30 °C with 70% FC moisture

content promoted the relative abundance of two phyla *Actinobacteria* and *Firmicutes*, and one genus *Bacillus* in clay loam soil. The involvement of these potential bacterial groups enabled possibilities of future research investigating their complete role in trifluralin and atrazine degradation for the development of new bioremediation strategy.

The outcome of this research indicates that persistence of trifluralin and atrazine in soil could interfere the growth and development of sensitive crop species. Based on this study results, some general recommendations could be practiced to minimize the negative impacts of target herbicide residues:

- Including herbicide resistant crops in the crop rotation.
- Avoiding the repeated use of same herbicides which favours herbicide residue accumulation over time.
- Herbicide persistence is greatly affected by site-specific environmental conditions. Farmers should remain cautious during extreme weather events, particularly under prolonged drought conditions where microbial degradation of herbicides are limited due to dry soil conditions. It is recommended that the suspected soil should be tested for persistence of target herbicides through either bioassays or laboratory detection techniques prior sowing.

6.1 Future work

As a result of this research study, the following recommendations are made for future work.

- Further investigation should be carried out to determine sensitivity of trifluralin and atrazine to other crop species using various soil types. Additional varietal screening is necessary before including lucerne in plant-based bioassay techniques for determination of toxicity associated with herbicide threshold concentrations in soil.
- As herbicides are strongly influenced by biotic and abiotic factors, more research should be conducted investigating the fate of trifluralin and atrazine under other key factors such as, soil type, organic matter and pH. These factors directly affect herbicide bioavailability in soil and can generate new insights to understand their fate in soil.

- This study investigates the fate of trifluralin and atrazine in clay loam with no previous application history. However, the effect of repeated application of herbicides may lead to accelerated degradation of the herbicides with clear dominance of dominant bacterial populations. Therefore, detailed investigations should be carried out investigating the impact of repeated application of trifluralin and atrazine on soil microbial community structure and functions associated with.
- As described in this study, two phyla namely *Actinobacteria* and *Firmicutes* and one genus, *Bacillus* was identified from Australian soils having potential to degrade trifluralin and atrazine residues in clay loam soil. Further investigations should focus on successful isolation, identification and characterization of bacterial species from genus *Bacillus*. In addition, for the development of a successful bioremediation strategy, field-based research should be conducted for the verification and the efficacy of *Bacillus* genus.

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