APPLICATIONS OF SOLID-STATE $^{15}\text{N}$ NMR SPECTROSCOPY TO THE STUDY OF NITROGEN CYCLING IN SUB-TROPICAL FOREST PLANTATIONS

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DECLARATION

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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Figure A.2: Influx and efflux of a) calcium, b) chloride and c) nitrate ions in hydroponic solutions containing slash pine seedling. Uptake values are the average taken for the time period shown above each column and have been corrected for the number of plants present. Positive values represent influx into the roots, while negative values represent efflux out.

Figure A.3: Influx and efflux of a) phosphate and b) sulfate ions in hydroponic solutions containing slash pine seedling. Uptake values are the average taken for the time period shown above each column and have been corrected for the number of plants present. Positive values represent influx into the roots, while negative values represent efflux out.

Figure A.4: Influx and efflux of a) magnesium, b) ammonium and c) potassium ions in hydroponic solutions containing eucalyptus seedling. Uptake values are the average taken for the time period shown above each column and have been corrected for the number of plants present. Positive values represent influx into the roots, while negative values represent efflux out.

Figure A.5: Influx and efflux of a) calcium, b) chloride and c) nitrate ions in hydroponic solutions containing slash pine seedling. Uptake values are the average taken for the time period shown above each column and have been corrected for the number of plants present. Positive values represent influx into the roots, while negative values represent efflux out.

Figure A.6: Influx and efflux of a) phosphate and b) sulfate ions in hydroponic solutions containing slash pine seedling. Uptake values are the average taken for the time period shown above each column and have been corrected for the number of plants present. Positive values represent influx into the roots, while negative values represent efflux out.

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Figure A.15: $^{13}$C NMR spectra of Eucalyptus whole stem samples, taken after harvesting from eucalypts grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure A.16: $^{13}$C NMR spectra of Eucalyptus root samples, taken after harvesting from eucalypts grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure A.17: $^{13}$C NMR spectra of Eucalyptus are stem samples, taken after harvesting from eucalypts grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure A.18: $^{13}$C NMR spectra of Eucalyptus bark samples, taken after harvesting from eucalypts grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure A.19: Hot water soluble anions found in SL, SM, SO, LO, and MO samples over 8 weeks. Graphs show results for a) nitrite, b) nitrate, c) phosphate, d) sulfate, e) fluoride and f) chloride.

Figure A.20: Hot water soluble cations found in SL, SM, SO, LO, and MO samples over 8 weeks. Graphs show results for a) ammonium, b) potassium, c) calcium, d) magnesium and e) sodium.
CONFERENCE PRESENTATIONS

The Australian Institute of Physics (AIP) Bi-annual Conference, Sydney Australia, 2005 (Poster Presentation)

3rd European Symposium on NMR Spectroscopy in Soil, Geo and Environmental Sciences, Friesing, Germany 6th – 9th August 2006 (Oral Presentation)

The International Symposium on Forest Soils and Ecosystem Health, Noosa, Australia August 2007 (Oral Presentation)


Each chapter and the findings presented in this thesis are in the process of being reformatted and submitted for journal publications.
**ABBREVIATIONS**

- **NMR**: Nuclear magnetic resonance
- **CP**: Cross polarisation
- **MAS**: Magic angle spinning
- **DD**: Dipolar dephasing
- **IC**: Ion Chromatography
- **FC**: Field Capacity
- **WUE**: Water use efficiency
- **LA**: Leaf area
- **AN**: Ammonium Nitrate (double $^{15}$N enrichment)
- **AS**: Ammonium Sulfate (double $^{15}$N enrichment)
- **CN**: Calcium Nitrate (double $^{15}$N enrichment)
- **UR**: Urea (double $^{15}$N enrichment)
- ***AN**: Ammonium Nitrate (single $^{15}$N enrichment of the ammonium ion)
- **A*N**: Ammonium Nitrate (single $^{15}$N enrichment of the nitrate ion)
- **SM**: Soil and a Mixture of plant components
- **SL**: Soil and Leaves
- **LO**: Leaves Only
- **MO**: Mixture of plant components Only
- **SO**: Soil Only
- **GS**: Glutamine Synthetase
- **GOGAT**: Glutamine:2 oxoglutarate amidotransferase
- **$\alpha$KG**: $\alpha$–Ketoacid
- **NAD(P)+**: Nicotinamide adenine dinucleotide phosphate
- **NAD(P)H**: Nicotinamide adenine dinucleotide phosphate (oxidised)
- **ATP**: Adenosine triphosphate
- **ADP**: Adenosine diphosphate
## SYMBOLS

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ABSTRACT

The aim of this research project was to use $^{15}$N nuclear magnetic resonance (NMR) spectroscopy to better understand nitrogen (N) cycling processes in forest plantations. In particular, the studies were designed to link to the effects of forest management practices and environmental conditions. $^{15}$N NMR cross polarization/magic angle spinning (CPMAS) and dipolar dephasing (DD)-CPMAS experiments of some simple N-containing compounds found widely in nature were first undertaken. This was done in order to understand how different sample conditions, such as the presence of moisture, sample mixing and dilution, affected the intensity and the observability of the NMR peaks corresponding to N containing functional groups. Our results exhibited changes in NMR signal intensities and various time constants calculated. In the case of variable contact time experiments, use of an equation that predicts both a bi-exponential rise and decay was found to fit the data obtained more accurately. The optimised parameters for $^{15}$N NMR CPMAS were then used along with other analytical techniques such as ion chromatography, total C and N and elemental analyses to study plant nutrient uptake, plant decomposition and the effect of forest ecosystem disturbances, in this case an insect infestation. These analytical data were also correlated with the changes in the $^{13}$C NMR spectra in order to gain a more comprehensive understanding of the chemical transformations in the forest ecosystem. The following section gives a brief account of the key research outcomes related to the N cycling in sub-tropical forest plantations.

1) Effects of fertiliser-N form used on nutrient uptake and assimilation in the plantation tree species, *Eucalyptus Pilularis* and *Pinus Elliotti*

Plants were grown hydroponically with $^{15}$N-enriched ammonium nitrate, ammonium sulfate, calcium nitrate or urea as their only N source. $^{15}$N NMR analysis of different components of the plants revealed that the N composition of the bark from *Pinus Elliotti*, and new leaves and the bare stem of *Eucalyptus Pilularis* were possibly dependent on the fertiliser-N forms used in the hydroponic solution. This is most likely due to differences in the way the plant is assimilating, cycling and storing organic N in the plant. No significant variations in C constituents of these plant components were found using $^{13}$C NMR. While the N composition in other plant components did not differ significantly, total N and C changed in all components based on the fertiliser used. The hydroponic fertilizer solutions were also monitored and it was found that eucalypts in ammonium nitrate fertiliser solution used almost as twice as much water when compared to the other fertiliser-N species, therefore exhibiting a reduction in water use efficiency (WUE). This is most likely due to a host of
reasons including loss of water due to transpiration at night and loss of C due to plant respiration. Ion chromatography (IC) analyses carried out on the hydroponic solutions the plants were placed in, found negative correlations between the amount of nitrate-N taken up by both the eucalyptus and pine plants, and the uptake of phosphate, sulfate and chloride combined. A negative correlation was also found between ammonium-N with that of calcium uptake by the plant. Strong positive correlations were found between phosphates and the combination of magnesium and potassium ions. All correlations could be explained by the plant root’s need to maintain electroneutrality. Frequent sampling of hydroponic solutions for IC analyses found that this electroneutrality could be maintained either through the efflux of nutrients from the plant back into the solution, and/or by lowering the uptake of specific like charges in order to let a similar charged nutrient enter the root. Pine plants grown in urea solutions showed the potential for urea to be taken up intact, however evidence of both microbial and chemical conversion of urea to ammonium and nitrates were observed in all urea solutions. Therefore, it is possible that pine plants utilised both the organic nutrient urea as well as the inorganic N forms. Alternatively, our experiments show that it is unlikely eucalypts were able to take up urea directly, as these plants revealed difficulties in maintaining good overall plant health and survivability.

2) The short-term nutrient dynamics during decomposition of plant components of Eucalyptus Pilularis residues in soils

This experiment was designed to compare samples with chemical compositions made to simulate the affect of windrowing in soil. Two sets of plant residues were prepared, one consisting of $^{15}$N-enriched eucalyptus leaves, stem and roots, mixed with soil collected from a Queensland plantation (abbreviated as SM) and the other $^{15}$N-enriched eucalyptus leaves in soil (abbreviated as SL). These were left to decompose in an incubator for 8 weeks to identify the short-term nutrient dynamics occurring during decomposition. During the 8-week period, the $^{15}$N NMR spectral data for organic-N did not reveal any significant change in the $^{15}$N-chemical environments. However, interesting connections were observed between the total C and N content as well as changes in the amount of inorganic-N and other ions extracted from both decomposing SL and SM substrates in hot water. Rapid changes in the C content of samples were attributed to the losses of CO$_2$ to the atmosphere and transformation of C. These transformations included the loss of carbohydrate-C peak intensities for both the leaves and the mixed samples, along with increase in aliphatic-C due to the microbial resistance of these groups and possible cross-linking. It is however possible that signal distribution changes could impart due to functional groups moving closer to paramagnetic centres. The hot water extracts indicated that soluble C has been removed to a greater extent with advancing decomposition, with the SL samples having more soluble C than the SM
samples. $^{15}$N and $^{13}$C NMR signal intensities of hot water extraction residues were found to decrease most likely due to increasing paramagnetic concentrations. Some $^{15}$N signal intensities were found to increase after KCl extraction. This could partly be explained by the presence of the paramagnetic iron, copper and manganese. It has been previously reported that the removal of these paramagnetic species from samples is not complete and effective. This means that techniques are needed to gain an understanding of their behaviour and how these impact on the NMR signal observability and intensity. An alternate reason for the loss of signal may be due to the removal of mobile organic molecules by hot water, leaving behind a higher concentration of rigid adsorbed inorganic molecules. As the loss of mobile structure could represent a loss of possible sinks for proton spin relaxation, this may decrease the signal intensity of the resulting NMR spectrum. It has been shown for mixtures of glycine and urea that as the concentration of the mobile compound (glycine) decreased the observability of urea in the NMR also decreased. The opposite is true for KCl extractions, which removed the mostly inorganic fraction, therefore concentrating the organic mobile groups and hence signal intensity. Our NMR spectral investigations confirm the complexities associated with paramagnetic species, molecule mobility, presence of moisture and temperature. The impacts of these complexities on qualitative and quantitative $^{13}$C and $^{15}$N NMR spectral characterisation have been observed. It is important therefore to use NMR spectral data in conjunction with other chemical analyses data such as total C and N content and elemental analyses to get a better understanding of the nature of processes taking place during decomposition in soil.

3) The impact of an environmental condition, such as, an insect infestation has on the N cycling in the forest ecosystem:

Insect infestations can limit tree growth and establishment, with the most damaging being chewing insects that defoliate plantation trees. Leaf beetle larvae and moth caterpillars were chosen in this study, as they are both common chewing insects found in Australian plantations. The insects, both larval and adult forms were allowed to feed on $^{15}$N-enriched eucalyptus leaves. These larvae/caterpillar and adult stages as well as the eucalyptus leaves were analysed by $^{15}$N and $^{13}$C NMR spectroscopy in order to study the N and C chemical transformations that take place during insect metabolism. Droppings from all insects, both larval and adult, were also collected for NMR analysis. $^{15}$N NMR showed that while the insects were of similar N composition to that of the eucalyptus leaves, their droppings showed large amounts of aromatic heterocyclic N similar to those present in uric acid and allantoin. $^{13}$C NMR analysis showed the opposite, with the insect droppings being similar to that of the leaves, while the insects themselves were metabolising C, and transforming to a different C profile. These results show that in addition to changes in the forest ecosystem
recovering from defoliation, there are clear potential for changes in the N and C cycles due to insect dead bodies and their droppings during a heavy insect infestation. That is, the insects are not only removing leaves which would eventually fall off and decompose but also adding nutrients to the forest soil. This was confirmed in our study in which the insects were converting leaf chemical constituents into different chemical forms such as high content of aromatic heterocyclic N containing species. These compounds may have a different impact on the microbial communities and the flow of nutrients in the C and N cycles, both in terms of the chemistry and the time required for decomposition in the forest ecosystem.
CHAPTER ONE

INTRODUCTION

1.1. Project Background

Forest industries are faced with the growing global demand for timber and forest based products due to the expansion of construction and manufacturing industries. There is, however, an increasing public awareness of the need for conservation of natural forests. This is coupled with new concerns over global warming and the potential for forest plantations to be used in carbon sequestration and carbon accounting. These demands have all led to a rapid expansion of forest plantations around the world. There is therefore an increased need for the development of effective management strategies and practices, as this is necessary to enable forest plantations to be environmentally sustainable, in turn addressing the forementioned concerns.

A sustainable forest plantations industry is one of the main aims of the Australian Forestry Industry (Australia's Forests at a Glance, 2007). To achieve this, a better understanding of forest nutrient cycles, quality indicators in both plant and soil, and the affect of different management practices have on these properties are required. Development of these management strategies will therefore lead to enhanced forest productivity and environmental benefits.

In this thesis, these management issues and strategies will be discussed with an emphasis on the impact of management practices, particularly on the nitrogen cycle in a forest ecosystem. Soil organic matter (SOM) is the principal source of nitrogen in most forest ecosystems, with nitrogen being an essential nutrient for plant growth and survival. Nitrogen transformation rates and quality in both plant and soil can be influenced by different management techniques. Therefore, to improve forest plantation health and productivity, development of sound strategies for management, and quality indicators at all stages of the nitrogen cycle are needed.
This chapter provides an initial look at the Australian forestry industry, focussing on the Queensland Department of Primary Industries: Forestry (QDPI-Forestry), the management techniques employed and the effects of animals and insects on a forest plantation. The aims and objectives of this thesis are then given with references to subsequent chapters, followed by an introduction to the nitrogen cycle and the processes that take place.

1.2. Plantation Forests

1.2.1 The Australian Forestry Industry

According to the Australian government’s Department of agriculture, fisheries and forestry report Australia’s Forests at a Glance (2007), Australia has the world’s sixth largest total forested area covering 164.4 million hectares, or 21% of Australia’s total land area. This forested area consists of 162.7 million hectares of native forest, and 1.75 million hectares of plantation forest. It is the intention of both the government’s forestry department, and the forestry industry, to increase the area of plantation forests to 3 million hectares by the year 2020, with an average of 70,000 hectares of new plantations currently being planted every year.

Effective management of forest plantations provides not only environmental sustainability, but also helps to provide economical sustainability through increased productivity and efficiency. The Australian forest industry currently makes an important contribution to Australia’s economy and will continue to do so due to increasing demands for timber-based products.

At present, Australia exports wood-based products such as wood chips, paper, panel, and sawn timber mainly to Japan, New Zealand and China, generating an export income of AUD$2.09 billion per year. Australia also imports pulp, paper, panel and sawn wood products from countries such as New Zealand, Indonesia, USA and Finland. Imports in 2006 totalled AUD$4.1 billion (Figure 1.1). The Australian forestry industry’s objective is to achieve import replacement and create export opportunities, of which can be done by increasing Australia’s plantations. By
improving sustainable forest management practices, these export opportunities can be both economically and environmentally sustainable.

Generally, climate and soil type determine where plantations can be positioned, with the majority based in regions where rainfall is greater than 700mm per year. By understanding important nutrient cycles in forest plantation ecosystems and the effect forest management has on these processes, the area in which plantations can be based may be expanded. Different techniques can help improve soil quality or make plants more tolerant to unfavourable environmental conditions.

![Figure 1.1: Australia’s top import countries and export destinations of forest timber products. Import/export data was collected over 2004-05 and 2005-06 respectively (Australia’s Forests at a Glance, 2007).](image)

### 1.2.2 Queensland Department of Primary Industry

This project was carried out in collaboration with Griffith University and the Queensland forestry industry. The Queensland Department of Primary Industries: Forestry (QDPI-Forestry) softwood plantations cover over 186,033 hectares in Australia, making it one of Australia’s largest plantation estates (Australia’s Forests at a Glance, 2007). Almost 90% of these plantations are in southeast Queensland. Further expansion is planned through acquisition of more land. The major softwood tree species grown include *Pinus caribaea* (Caribbean Pine), *Araucaria cunninghamii* (Native Hoop Pine), *Pinus elliotti* (Slash Pine), and hybrids of Slash and Caribbean Pine.
QDPI-Forestry has also established a hardwood program in southeast Queensland, which now consists of 37,496 hectares. This was established to enable a transition from Crown native forest harvesting to hardwood plantation harvesting. Species currently recommended for these plantations by QDPI-Forestry include *Eucalyptus cloeziana* (Gympie Messmate), *Eucalyptus pilularis* (Blackbutt) and *Eucalyptus argophloia* (Western White Gum).

With the transition from native forest harvesting to plantation forest harvesting, there comes an increased need for effective management of soil quality. In mature, native stands, the soil has stabilised over the years and the effects of any past disturbances are therefore considered to now to be negligible. In plantation forests, the soil is disturbed by harvesting approximately every 25 year time period, which in turn causes major disturbances to soil nutrient cycles. The loss of potential soil nutrients and therefore soil quality by the removal of the preceding rotation’s trees can have a detrimental effect on the subsequent rotation of forest trees. Different management regimes are normally used to restore nutrient balances for healthy growth of subsequent rotations. Some of these regimes include the use of fertilisers or the retention of harvest residues by the formation of windrows, which involves residues being pushed into mounds and left to decompose on site. In both instances, the nutrients are returned to the soil, but it is of interest to find out the rates of decomposition of these windrows and availability of the nutrients for plant’s uptake.

The current thesis research has looked at the uptake of nitrogen from different fertilizer sources by both softwood and hardwood plant species and the results are presented in Chapter 3. Chapter 4 has focussed on evaluating the decomposition of plant residues mixed with soil, with an emphasis on the variation of carbon and nitrogen chemical profiles during decomposition. Chemical characterisation techniques such as $^{15}$N and $^{13}$C nuclear magnetic resonance (NMR) spectroscopy and ion chromatography (IC) were used to follow $^{15}$N, $^{13}$C isotopes and other ions in these processes. The resulting nitrogen environments could then be determined in order to help us better understand the nitrogen cycling process under different management practices employed by QDPI-Forestry.
1.2.3 Management Practices

Sustainable management practices of forest plantations are needed to increase the beneficial, and minimize the adverse effects of plantation operations (Lee et al., 2003). Effective site management can enhance forest productivity, provide environmental benefits and reduce the magnitude of any negative impacts. In the forestry industry, there are many practices and techniques imposed during the life cycle of a plantation and any impacts of such activities on the plantation as well as the environment must be taken into consideration. Some of the management practices and their beneficial effects are listed in Table 1.1.

Table 1.1: Examples of forest plantation management practices and techniques used and their beneficial effects on the plantation and environment (*Australia's Forests at a Glance*, 2007; Eldridge, 2007; Lee et al., 2003).

<table>
<thead>
<tr>
<th>Management</th>
<th>Technique Implemented</th>
<th>Beneficial Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed Germination</td>
<td>Gravitational separation of seeds to pick out larger, healthy seeds</td>
<td>Improved and more constant germination rates</td>
</tr>
<tr>
<td>Potting Media</td>
<td>Using more free draining mediums such as coarse bark with sand</td>
<td>Better drainage and therefore root growth</td>
</tr>
<tr>
<td>Plant Containers</td>
<td>Pots designed with interior ridges</td>
<td>Train roots downwards and reduce coiling</td>
</tr>
<tr>
<td>Watering</td>
<td>Managed and reduced watering regimes</td>
<td>Hardened, drought tolerant plants</td>
</tr>
<tr>
<td>Planting</td>
<td>Planting trees in wetter months in favourable conditions</td>
<td>Better plant survival</td>
</tr>
<tr>
<td>Site Preparation</td>
<td>Windrowing/mounding of the previous rotation residues</td>
<td>Retains nutrients in soil for next rotation</td>
</tr>
<tr>
<td>Weed Control</td>
<td>Use of herbicides to reduce the tree need to compete for nutrients</td>
<td>Increased early growth</td>
</tr>
<tr>
<td>Nutrition</td>
<td>Use of site and species specific fertilizers</td>
<td>Increased growth</td>
</tr>
<tr>
<td>Thinning</td>
<td>Removal of trees at young age to optimal trees/hectare rate</td>
<td>Larger individual tree sizes and better plant survival</td>
</tr>
<tr>
<td>Pruning</td>
<td>Removal of live branches less than 2cm diameter</td>
<td>Minimise knot defects</td>
</tr>
<tr>
<td>Insects</td>
<td>Use of genetic material of plantation trees resistant to insect attack</td>
<td>Growth of insect resistant trees</td>
</tr>
<tr>
<td>Trials</td>
<td>Trials looking into wood quality, silviculture, tree survival etc…</td>
<td>Information on how to improve forest production</td>
</tr>
<tr>
<td>Modelling</td>
<td>Allow for information input, and estimations of productivity rates</td>
<td>Can adjust management practices based on information provided</td>
</tr>
<tr>
<td>Surveillance</td>
<td>Helicopter surveillance can be used to identify whether the plantation is healthy</td>
<td>In drought affected stand, thinning may be brought forward to improve health</td>
</tr>
</tbody>
</table>
There is a need for acquiring in-depth knowledge on these processes, which in turn will help make well-informed management decisions. For example, even with the use of genetically modified plant species to withstand harsh environmental conditions, there is always a need for appropriate plantation establishment techniques, site preparation, weed control and nutrition which will collectively contribute towards any gains made in the venture (Lee et al., 2003). Unless there is an understanding of the impact of the soil conditions, nutrient cycle, pests and diseases on tree survival, growth and wood quality, optimum management cannot be achieved and may result in plantation failure.

In this investigation, management practices of *Eucalyptus pilularis* and *Pinus elliotti* and their impact on the nitrogen cycle have been studied. The relevance of these plants in this study is because they are commonly grown in plantations, representing softwood and hardwoods managed by QDPI-Forestry. As nitrogen is a macronutrient essential for plant survival, an understanding of nitrogen cycling processes in forest soils will add value to overall forest plantation management.

### 1.2.4 Animals and Insects

Plants and soil are not the only contributors of material to the nutrient cycle in a forest ecosystem. Animals also contribute by eating and metabolising plant material, then returning these metabolites to the soil via faeces and animal remains. *Australia’s Forests at a Glance* (2007), has identified that 3817 animal species are forest-dependent, including mammals, birds, frogs and reptiles. In order for plantation forests to be ecologically sustainable, it is important to plan carefully the plantation harvesting operations to minimise adverse effects on animal populations (Eldridge, 2007).

Insects are also of concern to the forestry industry, as they can be detrimental to the health of the plantation. An environmental condition such as an insect infestation can limit tree growth and establishment, with the most damaging being chewing insects, which defoliate plantation trees. For example, in 2005 the California pine aphid (*Essigella californica*), damaged 60,500 hectares of plantation trees in NSW (Eldridge, 2007). Defoliation of trees can cause serious environmental damage, which in turn has a dramatic effect on the ecosystem when present in large numbers.
The loss of leaves can cause a decrease in the transpiration and plant growth and therefore an increase in plant mortality (Lovett et al., 2002). This can then affect the nitrogen cycle in a forest ecosystem. Further study is needed to ascertain the chemical changes in soil as a result of these changes on the forest ecosystem, and strategies to manage them in a way that is positive to forest plantation sustainability and productivity.

The changes in nitrogen transformations as a result of insect infestations need to be monitored from eucalyptus foliage, through the metabolism of this plant material by leaf-chewing insects and finally their waste products, which are returned to the soil in a plantation environment. This would then demonstrate any changes in the nitrogen cycle that will impact on levels of soil nutrients available to the plants.

1.3. Aims and Objectives of the Thesis

The aims of the current thesis research are to (1) test, develop and apply solid state $^{15}$N nuclear magnetic resonance (NMR) spectroscopy techniques, to advance our understanding of both the composition and quality of nitrogen in plant material and soil organic matter (SOM), and (2) advance the understanding of nitrogen (N) cycling processes under different forest management practices and environmental conditions in order to improve productivity and sustainability of plantation forest ecosystems. The focus of these two aims will be on plantation forest ecosystems such as those found in sub-tropical Queensland, Australia.

This project, funded by the ARC-Linkage grant scheme, was carried out in collaboration with the QDPI-Forestry, and Griffith University (GU), located in Nathan, Queensland. Initially, the project was to be in conjunction with a PhD student from GU. Samples were to be received from GU, which had already been examined using solid-state $^{13}$C NMR spectroscopy. The objective of this thesis research was to analyse any $^{15}$N present in these samples using solid-state $^{15}$N NMR spectroscopic facility at University of Western Sydney (UWS) located in Parramatta North, New South Wales. The $^{15}$N enrichment of these samples, however, was very low (approximately 0.5% $^{15}$N atom), and acquisition of spectral data within a
reasonable time period failed to provide spectra with adequate signal-to-noise ratio and then to gather useful information on the different types of $^{15}$N functional groups. Since the low levels of $^{15}$N enrichment does create difficulties in the optimisation of the NMR experiments, it was therefore decided that a separate project needed to be set up, involving growth of highly $^{15}$N enriched plants at UWS, which could then be analysed with $^{15}$N NMR spectroscopy.

The aim of determining the significance of solid-state $^{15}$N NMR spectroscopy for analysing forest ecosystems material was still valid for these new experiments. This was carried out using the following research objectives:

1. Optimise solid-state $^{15}$N NMR spectroscopy techniques and characterise $^{15}$N NMR spectra for different nitrogen chemical environments and then apply the techniques to field samples.

2. Study the effects of fertilizer management, with respect to plant uptake and assimilation of different forms of N found in fertilisers by both softwood and hardwood plant species and the effect on the nitrogen partitioning within the plant.

3. Better understand the effects that management practices, such as windrowing, have on soil nitrogen, and its incorporation into soil organic matter.

4. Study the effects of an environmental condition such as an insect infestation may have on the forest N cycle.

These four objectives were achieved by conducting the following experiments in ideal lab conditions:

1. Examination of commercially available $^{15}$N enriched model compounds using solid-state $^{15}$N NMR spectroscopy, in order to optimise $^{15}$N NMR acquisition parameters to reflect the different $^{15}$N chemical environments both qualitatively and quantitatively. (Chapter 2)
2. Analysis of $^{15}$N-enriched *Eucalyptus pilularis* and *Pinus elliotti* plant material grown hydroponically using $^{15}$N NMR techniques, to examine variations in the assimilation of different N forms and the influx and efflux of nutrient ions in solution. (Chapter 3)

3. Use of $^{15}$N NMR, IC and C/N analyser techniques to study the decomposition of $^{15}$N-enriched *Eucalyptus pilularis* residue material mixed with Queensland forest plantation soil in a temperature controlled incubator. (Chapter 4)

4. Examine $^{15}$N enriched insect life-cycle stages and their faeces in order to follow their metabolism and understand what other chemical forms of N, compared to $^{15}$N enriched foliage, may be introduced into a forest ecosystem. (Chapter 5)

Results from these ideal environments could then be related back to real forest plantation conditions.

In order to advance the understanding of nitrogen cycling processes under different forest management practices and environmental conditions, a brief overview of the nitrogen cycle is presented in the subsequent section.

### 1.4. The Nitrogen Cycle

#### 1.4.1 Basic Overview

Nitrogen (N) is an important element required in all biological life, and is therefore of major importance in all terrestrial and aquatic ecosystems. For example, it is involved in the structure of chlorophyll, proteins and nucleic acids such as DNA and RNA. The nitrogen cycle is a biogeochemical cycle describing the chemical changes that take place in nitrogen and N-containing compounds when cycled in our environment. The three main pools of nitrogen on Earth are the atmospheric, oceanic and biosphere, with the atmosphere being the largest (Table 1.2).
Table 1.2: Estimate of nitrogen in the three major pools (Mengel & Pilbeam, 1992), where Pg = 1 x 10^{15} grams.

<table>
<thead>
<tr>
<th>Reservoir</th>
<th>Nitrogen Stock (Pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Atmosphere</td>
<td>3,866,000</td>
</tr>
<tr>
<td>2. Oceanic</td>
<td>6</td>
</tr>
<tr>
<td>3. Biosphere</td>
<td></td>
</tr>
<tr>
<td>Vegetation Biomass</td>
<td>10</td>
</tr>
<tr>
<td>Soil Organic Matter (0-100cm deep)</td>
<td>95 – 140</td>
</tr>
<tr>
<td>Microbial Biomass</td>
<td>2</td>
</tr>
</tbody>
</table>

Plants are autotrophic organisms, meaning that they survive in an inorganic environment, therefore taking up nitrogen in the form of minerals, such as, ammonium (NH₄⁺) and nitrates (NO₃⁻) present in the soil. These inorganic forms are then converted into organic nitrogen forms such as, proteins and amino acids, within the plant. Animals on the other hand are heterotrophic organisms, meaning they live in an organic environment, and depend on organic forms of nitrogen to survive, through ingestion of plant material or other animals. Animals then return nitrogen to the soil via faeces or animal remains. Plants return nitrogen to the soil via litter such as leaves, stems and roots, and also dead plants. Microbes in the soil then convert these organic nitrogen-containing products back to mineral forms, therefore becoming available once again to the plant.

This gives us a basic view of the nitrogen cycle. The main focus of the thesis is on the nitrogen cycle in the biosphere. This includes processes that take place in plants, animals and soil. Figure 1.2 and the following sections will give a more detailed explanation of the major processes relevant to these sections in the nitrogen cycle.

1.4.2 Nitrogen from the Atmosphere

Nitrogen Fixation

Approximately 78% of our atmosphere is nitrogen, with the largest amount present in the form of dinitrogen gas (N₂), followed by small amounts of nitrogen oxides (NO₂) and ammonia (NH₃). Most organisms, however, cannot use dinitrogen as it requires large amounts of energy (approximately 167 kJ/mole) to break the covalent bond connecting the two nitrogen atoms. By breaking this bond, the nitrogen atoms can
join with other atoms to form useful nitrogen containing compounds. Dinitrogen gas can only be broken down in three ways:

- **Biological Fixation:** Certain bacteria and cyanobacteria species can break down dinitrogen and convert into mineral forms of nitrogen, such as ammonium (NH$_4^+$) and nitrates (NO$_3^-$). These microbes can either live free in the soil or in a symbiotic relationship with a plant.

- **Atmospheric Fixation:** Lightning provides enormous amounts of energy that can break down dinitrogen, and convert it into nitrogen oxides (NO$_2$). These oxides dissolve in water to form nitrates (NO$_3^-$), and are carried back to earth via rain.

- **Industrial Fixation:** Under high temperatures and pressure used in industrial processes, dinitrogen can be converted into ammonia gas (NH$_3$), which is returned to the atmosphere or used to make fertilizers such as ammonium nitrate (NH$_4$NO$_3$).

These processes are known as nitrogen fixation, with each one breaking dinitrogen down into mineral nitrogen forms, which can then be used by plants.

### 1.4.3 Soil Nitrogen Processes

Many nitrogen processes take place in the soil, including nitrification, denitrification, mineralisation, immobilisation, volatilisation, adsorption and leaching. Chapter 4 of this thesis will look into the decomposition of harvest residues on plantation soils and will provide an understanding of the net result of all concurrent processes taking place in a lab environment that will help make connections to those processes in the field. A summary of these processes is thus provided here.

**Nitrification**

Ammonium (NH$_4^+$) produced from the nitrogen fixation processes and deposited in the soil, can either be used directly by plants (known as assimilation), or oxidized by nitrifying bacteria. This is done by bacteria of genera *Nitrosomonas* or *Nitrococcus*, which oxidize NH$_4^+$ to form nitrites (NO$_2^-$). They are then further oxidized by bacteria of genera *Nitrobacter* into nitrates (NO$_3^-$). This process requires the presence
of oxygen and is called nitrification. In poorly drained soils, there tends to be a build up of ammonium (NH$_4^+$), as the availability of oxygen is limited, and therefore the ammonium cannot be converted to nitrates. The pH of soil can also affect nitrification in soils.

Figure 1.2: A detailed diagram of the nitrogen cycle.
**Denitrification**

Nitrates not used by the plants, can be returned to the atmosphere through a process known as denitrification. This involves bacteria of genera *Pseudomonas* and *Clostridium* which live in anaerobic conditions (where there is no oxygen present). These bacteria use the nitrates (NO$_3^-$) as their oxygen supply, converting it into dinitrogen, which is released back into the atmosphere.

**Mineralization**

When an insect or animal excretes, or a plant, insect or animal dies leaving its remains, they all contain forms of organic nitrogen that will become part of the soil organic matter (SOM) content during the decomposition process. Therefore SOM can include material such as:

- plant litter such as leaves, stems and roots
- insect and animal manure
- insect and animal remains
- feathers
- microbes including bacteria and fungi (there is roughly 3 billion per gram of soil) and all their remains
- sewage

All of these products will be at various stages of decomposition while in the soil, and contain organic forms of nitrogen which plants are unable to use. Mineralization is the process in which microbial decomposers, such as bacteria and in some cases fungi, aerobically or anaerobically convert this organic nitrogen into mineral nitrogen in the form of ammonium (NH$_4^+$). Therefore, this process is also known as ammonification and the basis is to convert nitrogen to a form that is then available to plants once again.

While it is known that organic N forms such as proteins, amino acids and sugars, and nucleic acids, can be rapidly mineralised by a vast variety of soil microbes (Tate, 1995), it has been found that the mineralisation of organic N is not proportional to the amounts found in soil (Tate, 1995). The location of organic N compounds within various soil components has a larger effect on N mineralisation rates than the quantity of organic N present in the soil and its chemical structure. For example, organic N forms may become bound to clay particles in the soil, dissolved in soil
water or immobilised (see next section) in the microbial biomass. Each of these circumstances will make access to, and therefore the mineralisation of organic N more difficult. N mineralisation is also dependent on soil conditions such as temperature, water content and soil structure. Goovaerts and Chiang (1993), have shown that N mineralisation in soil varies both spacially and temporally, and these variations within less than 1 m$^2$ of soil are mainly due to differences in water content, and therefore the amount of oxidisable carbon found in the soil.

Bolton et al (1993), have also shown that higher amounts of organic N and biological activities are found in soil that is directly influenced by growing plants. This could be due to the fact that more plant litter is dropped on these soils leading to more organic N input. The litter acts as a kind of mulch layer, protecting the soil from moisture loss, and regulating the soil temperature better. This was also shown more recently in a windrowing experiment performed by Blumfield and co-workers (2004), where plantation harvest residues were pushed into long mounds, otherwise known as windrows, which followed site contour lines. The temperature of soil under the windrows was found to be constant, while soil temperatures of soil without windrowning varied between day and night.

Soils in the vicinity of plant root growth could benefit from the roots rhizosphere community. This community of micro-organisms and plant roots produce polysaccharides having a higher affinity towards colloidal minerals and soil organic matter. The soil around these micro-organisms and roots therefore form micro-aggregates, which in turn improves soil aeration, water infiltration and root penetration, all of which leads to better soil conditions and mineralisation rates.

**Immobilization**

Immobilization is the alternative to mineralization. When the microbes take the organic nitrogen in, instead of processing and excreting as ammonium, a portion is absorbed into the microbe itself, becoming part of the microbe’s make-up, and therefore retaining the organic form of N. This nitrogen is unavailable to plants, and immobilized for a period of time out of the nitrogen cycle. The same is true for insects and animals.
**Volatilisation**

Volatilisation is a chemical reaction by which ammonium (NH$_4^+$) in alkaline environments dissociates and forms the ammonia gas (NH$_3$), where it is then released back into the atmosphere.

\[ \text{NH}_4^+ + \text{OH}^- \rightleftharpoons \text{NH}_3 + \text{H}_2\text{O} \]

Therefore the soil pH will determine the extent of volatilisation. For example, urea fertilisers undergo higher volatilisation rates than other fertilisers when first applied to soil as urea fertilisers increase the soil pH. (Sparks, 2004).

**Adsorption and Leaching**

Clay particles in soil have a negative charge. Therefore, any positively charged organic or mineral forms of nitrogen in the soil, including ammonium (NH$_4^+$), can be attracted to and adsorbed onto these clay particles instead of being assimilated by the plant. Nitrates (NO$_3^-$) on the other hand are negatively charged and are therefore repelled by the clay particles allowing them to move more freely though the soil. As a result, nitrates can move down through the soil into groundwater leading to a loss of nitrogen in soil ecosystem that could have been available for plant growth. This process, known as leaching, can lead to problems in streams and other water systems, in particular, the over supply of nutrients in water can contribute to eutrophication that can result in algal blooms, death of aquatic life due to excessive demand for oxygen and therefore, overall contamination of the water body.

1.4.4 **Plant Nitrogen Processes**

Many nitrogen processes also take place within plants themselves. Chapter 3 of this thesis will look at the uptake of nitrogen in the fertiliser solutions by plants, and the effect different nitrogen species found in fertilisers have on the partitioning of nitrogen within the plant based on nitrogen uptake, assimilation and metabolism. Once again, these are all processes that need to be taken into account when interpreting results in Chapter 3, therefore a summary of these processes is provided in the subsequent sections.
Nitrogen in Plants

Nitrogen (N) is found in many plant molecules and structures that are involved in important processes such as photosynthesis, growth and signalling of soil conditions. These molecules can include proteins, chlorophyll and plant hormones such as cytokinin. A nitrogen deficiency therefore leads to symptoms such as yellowing of leaves as well as slow and stunted growth. The roots and leaves of a plant usually contain the highest amounts of N, as these are the points of N uptake, and N deposition respectively. The stem, in particular its woody tissues, is very low in N. However, the stem plays an important role allowing the translocation of N between the roots and shoots of a plant.

Photosynthesis in a plant is strongly affected by N availability, as more than half of a leaf’s N is involved in the photosynthetic process (Lambers et al., 1998) including N found in chlorophyll. Pons and co-workers (1989), showed that the N concentration per unit leaf area was highest in leaves which received more sunlight, and declines towards the bottom of a plant, where there is less sunlight. A strong negative correlation has also been shown between leaf lifespan and both leaf N and leaf photosynthetic rates in conifer and broadleaved plant species (Reich et al., 1995). This may also be due to the fact that as the leaf gets older, it is overshadowed by younger leaves that get more sunlight. Iivonen and co-workers (1999) have also pointed out that the photosynthetic capacity of a plant is related to N concentrations in the plant’s leaves. Also nutrient limitations may affect seedling growth initiation, therefore affecting N content and hence the seedling’s capacity for photosynthesis.

A large proportion of a plant’s N is in the form of soluble amines and amides, therefore making N very mobile within the plant (Flowers & Yeo, 1992). For example, deciduous trees and plants have the ability to mobilize N from the leaves they are about to shed, and store it within its stem, branches and roots until it is needed for regrowth and development of young leaves (Rossato et al., 2001; Tilsner et al., 2005). This process is also known as resorption.
**Nitrogen Uptake**

N ions are taken up in large amounts by plants in order for new growth to occur. Nitrate (NO$_3^-$) and ammonium (NH$_4^+$) ions are the two major forms of N taken up by plants (Mengel & Pilbeam, 1992). It has been shown however, that some plants have the ability to take up organic N forms such as amino acids (Persson & Nasholm, 2002; Warren, 2006; Warren et al., 2000). The inorganic ions found in the soil are taken up by the roots and transported inside the plant. The roots provide a selective barrier for the ions to cross and enter tissues that transport water and ions to the plant shoots. This barrier also prevents solutes from leaching out.

Due to the large amount of N taken up by the plant, it is common for the rhizosphere and surrounding soils to become acidic when NH$_4^+$ is the main N-source, or alkaline when NO$_3^-$ is the main source. This means, the soil pH often influences the ratio of NO$_3^-$ to NH$_4^+$ taken up and affects the root’s ability to maintain electroneutrality. For example, if the plant has an influx of NO$_3^-$, it is likely to be accompanied by an increased influx of cations such as Ca$_2^+$, Mg$_2^+$, K$^+$, or an efflux of other anions such as SO$_4^{2-}$ or a OH$/\text{HCOO}^-$ group in order to maintain electroneutrality. The larger influx and thereby loss of NO$_3^-$, however, tends to increase the soil’s pH. An influx of ammonium will see an increased SO$_4^{2-}$ influx, or an efflux of Ca$_2^+$, Mg$_2^+$, K$^+$ or H$^+$ groups, therefore making the soil more acidic (Mengel & Pilbeam, 1992).

The uptake of N ions and other solutes by plant roots is closely coupled with the flow of water (Flowers & Yeo, 1992). Water is transported one way from the roots, up the plant’s xylem, to other parts of the plant where water has been lost due to transpiration. Therefore any species soluble in water, such as NH$_4^+$ and NO$_3^-$, gets transported along with the water.

**Assimilation**

Assimilation is the process in which the plant, once it has taken up either ammonium or nitrates from the soil via its root, makes it available to be incorporated into organic molecules such as proteins, amino acids and nucleic acids. In order to be incorporated into these organic forms, all inorganic nitrogen must first be converted to ammonium. If the plants assimilate ammonium straight from the soil, no
conversion is needed. When the plant takes up nitrates, these ions need to be converted to ammonium at some point in the plant. This can take place in the root cells, or the nitrates can be taken to the leaves, via the xylem, for assimilation. This conversion from nitrates to ammonium involves two enzymes in the plant:

\[
\begin{align*}
\text{NO}_3^- & \xrightarrow{\text{NR}} \text{NO}_2^- \xrightarrow{\text{NiR}} \text{NH}_4^+ \\
\text{NR} & = \text{the enzyme nitrate reductase } (2\text{H}^+ + \text{NO}_3^- + 2\text{e}^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}) \\
\text{NiR} & = \text{the enzyme nitrite reductase } (8\text{H}^+ + \text{NO}_2^- + 6\text{e}^- \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O})
\end{align*}
\]

Nitrates are usually more abundant in soils than ammonium. Also, despite the fact that nitrates need to be converted to ammonium and therefore take more energy to be assimilated, most plants prefer to assimilate nitrates.

It has been previously shown that growth rates can be increased if both \(\text{NO}_3^-\) and \(\text{NH}_4^+\) are present (Guo et al., 2007), however it is not known exactly why this is the case. Lee and Drew (1989) found that the presence of \(\text{NH}_4^+\) inhibits the influx of \(\text{NO}_3^-\) in barley plants. MacKown et al (1982), found that in maize, \(\text{NO}_3^-\) however, does not inhibit \(\text{NH}_4^+\) uptake. There is also more recent evidence of plants being able to take up organic N forms, such as simple amino acids like glycine, as an alternative to inorganic nitrogen (Persson & Nasholm, 2002; Warren, 2006; Warren et al., 2000). Our research involved another simple organic N form, urea, to examine the uptake and assimilation within the plant. Aarnes and co-workers (1995), have previously shown that the conifer Norway spruce has the ability to utilise urea directly.

The preferential nitrogen mineral uptake by plants of importance to QDPI-Forestry, and its relevance to growth, has been covered in the current thesis research reported in chapter 3. In this project, the uptake of plants grown with different N sources was examined in order to understand how each different N form effects plant growth and chemical structure. Eucalyptus and Pine species grown by QDPI-Forestry were chosen, and were grown hydroponically in the presence of either \(\text{NH}_4^+\) only, \(\text{NO}_3^-\) only, with both \(\text{NH}_4^+\) and \(\text{NO}_3^-\) together or urea as their only N source available. Ion chromatography was then used to analyse hydroponic solutions, while NMR was
used to examine the plant material. Results from both forms of analysis provided a comprehensive picture of the nitrogen cycling taking place within the two species of plant, and their preferences for particular forms of nitrogen.

**Metabolism**

In higher plants, N that has been reduced to $\text{NH}_4^+$ is assimilated and then metabolised via the glutamine synthetase/glutamine: 2-oxoglutarate aminotransferase (GS/GOGAT) pathway.

$$\text{Glutamate} + \text{NH}_4^+ + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + \text{Pi}$$

$$\text{Glutamine} + 2\text{-oxoglutarate} + \text{NAD(P)}H + H^+ \rightarrow 2\text{ Glutamate} + \text{NAD(P)}^+$$

N can then be incorporated into $\alpha$-amino groups found in amino acids such as asparagine, arginine, alanine and lysine (Aarnes et al., 1995; Canovas et al., 2007; Thorpe et al., 1989). Basically once converted to $\text{NH}_4^+$, it is incorporated into amide groups (eg proteins, glutamate, aspartate etc.) and then converted into amino groups (eg. glutamine, arginine etc.) via reactions with ketoacids, enzymes, ATP and ADP.

In contrast to the plant xylem, which is composed of mostly dead cells and used for transport of solutes in an upward direction only, the phloem is composed of living cells and can transport soluble organic compounds in both directions. Therefore the phloem acts as a sink for the N metabolic products from the GS/GOGAT pathway, along with products of photosynthesis, which have not been immediately incorporated into plant material. These can then be transported down the phloem to non-photosynthetic parts where they can be used for new growth, incorporated into proteins such as those for long-term N storage in seeds and vegetative organs (Canovas et al., 2007), or recycled back up the phloem or into the xylem where they can move back up the plant (Pate, 1989; Pate & Arthur, 2000; Singh, 1998). N transferred to storage proteins can be mobilised at a later time when required for plant growth (Canton et al., 2005).
1.4.5 Plant Effects on Soil
Because plants and soil are involved in nutrient cycles, they have the ability to influence on each other, and, in the long term, themselves. For example, if a plant has a high uptake of nutrients, and therefore a high nutrient concentration in its litter material, it could lead to an increase in soil fertility which then in turn benefits the plant. Other effects also need to be considered, such as decomposition and mineralisation rates, etc. As mentioned previously, the thesis research involves investigating of the partitioning of N within different sections of the plant. The affect these different sections, and therefore concentrations of N in the plant material, has on decomposition of eucalyptus plant material on Queensland forestry soil has been examined in Chapter 4.

1.4.6 Links Between the N and C cycle
The N cycle has a driving effect on the C cycle (Hogberg, 2007; Magnani et al., 2007), but as to how much is still under debate. This has recently become an important issue with elevated greenhouse gas levels, including NO₂ and CO₂ gases, and increases in industrial fixation of N into fertilisers. These new N sources now surpass natural biological fixation (Vitousek et al., 1997). It is under debate, however, how much of this extra N is actually being taken up by the plants (Nadelhoffer et al., 1999), and how much may be leached into groundwater systems (Aber et al., 1998).

It had long been known that increased N uptake can increase the plant’s foliar biomass and concentration of the enzyme/protein Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) which is involved in a plant’s fixation of carbon (Townsend et al., 1996). This leads to an increased photosynthesis capacity of the plant, and therefore an increase in the assimilation of CO₂. As more C is assimilated, more is stored, for instance, in the plants woody stem material. This woody material decomposes slower than other parts of the plant and therefore C will be sequestered in the woody stem for a longer period of time. It has also been found that higher levels of N content in plant litter leads to slower decomposition, and therefore a longer time in which C is sequestered. It is also reported that low levels of nitrogen have higher rates of decomposition especially targeting the release of sequestered
nitrogen in soil (Craine et al., 2007). Therefore, current thesis research on decomposition of plant and soil mixtures with different levels of nitrogen could be used to understand the changes in the carbon chemical profile indicative of the level of decomposition using data from the C/N analyser and the solid state $^{13}$C NMR spectroscopy.

The age of a forest also needs to be taken into account when considering C sequestration. As Magnani et al (2007) has pointed out, the ability of a forest to sequester C will be small when plants are young and small, increasing until they are at their optimum primary production age. As a tree ages beyond this point, their ability to sequester C falls, and if a stand is harvested, sequestered C falls to zero.

1.5. Thesis Overview

A more detailed look at the nitrogen cycle, and the affect forest plantation management strategies have on the nitrogen species present, has been further explored in Chapters 3, 4 and 5. Chapter 3 has focussed on the effects of fertilizer management, with respect to plant uptake and assimilation of different forms of N found in fertilisers, and the effect on the nitrogen partitioning in the plant. Chapter 4 then looks at the effect of decomposition of eucalyptus residues and the influence windrowing and the different residues types have on decomposition rates. Finally, the chemical profile of N in insect material has been examined in Chapter 5 in order to see what affect an insect infestation could have on a forest ecosystem and hence the nitrogen cycle. This involved research into the nitrogen forms present in the different growth stages of insects’ themselves, during metabolism of plant material and the deposition of waste that is returned to forest soils.

While Chapter 1 provides an introduction to the forestry industry and the nitrogen cycle, Chapter 2 will provide an introduction to important aspects of using nuclear magnetic resonance (NMR) spectroscopy. This instrument was used as the main form of analysis and also has been used to examine different simple nitrogen containing chemical structures that are involved in the nitrogen cycle, in order to optimise NMR acquisition parameters. These parameters can then be applied to more complex environmental samples such as plant and soil materials.
1.6.References


CHAPTER TWO

ESTABLISHING QUANTITATION IN SOLID STATE $^{15}$N NMR

2.1. Introduction

In chapter 1 the important role that organic N plays in the N cycle for a forest plantation was discussed. Knowledge of the chemical structure of organic N species is important in understanding reactivity and reaction pathways. Smernik & Baldock (2005), have indicated that there is disagreement within the scientific community regarding the nature of organic N in soils. It is possible that a fraction of N is not seen and therefore not accounted for, or that some analytical techniques employed have biases towards certain N species. In this chapter the use of solid-state $^{15}$N NMR spectroscopy for identifying and quantifying both organic and inorganic forms of N is explored, and reasons for any unobservable N or biases that may occur with certain functional groups are discussed.

While other forms of analysis can provide information about the content of N in a sample, solid state NMR can also provide qualitative information on the environments and functional groups N is involved in. Solid state NMR is also non-destructive, therefore samples can be re-run if needed or used in multiple experiments. The non-destructive nature is also of benefit when compared to other forms of analysis, such as acid hydrolysis and pyrolytic techniques, in which the chemical nature of a sample may be altered, possibly leading to artifacts in measurements.

Solid state $^{13}$C NMR has been a powerful tool for analysing the structure of plant and animal materials (Blumfield et al., 2004; Knicker et al., 1996a; Newman & Redgwell, 2002; Schaefer et al., 1987; Smernik et al., 2002) and complex geochemical matrixes such as soils, coals and kerogens for over 30 years (Keeler &
Issues related to quantification have been the subject of considerable debate and have increased understanding in analytical procedures and techniques to process data. However the matter is complex and does not lend itself to simple integration of routine spectra (Kogel-Knabner, 1997; Mao et al., 2000; Smernik & Oades, 2000, 2003), although semi quantitative or relative analyses serve as a useful method to compare changes in spectra. There are, however, difficulties that affect the quantitative measurement of signals recorded using solid-state $^{13}$C and also $^{15}$N NMR spectra. Problems include differences in ideal parameters required for NMR analysis due to the heterogeneous nature of most natural organic matter, the various chemical environments incorporated in samples and the presence of paramagnetics, particularly in soil samples. All of these can affect NMR signal intensities and therefore absolute quantitation of the resulting spectrum.

$^{15}$N solid state NMR spectroscopy has been slower to develop as a tool for analysing these complex samples. Because of the low sensitivity, natural abundance and concentrations of $^{15}$N nuclei, organic samples demand long analysis times, usually in the order of days and sometimes weeks, therefore limiting the application of NMR spectroscopy. The sensitivity of $^{15}$N nuclei, and therefore the intensity of its spectrum, is 1/50th of that compared to a $^{13}$C experiment. Kogel-Knabner (1997) has pointed out that unenriched samples are able to be analysed with $^{15}$N NMR, and a decent spectrum obtained, if the sample contains at least a 1% concentration of N by weight.

A number of studies on the application of solid-state $^{15}$N NMR to the analysis of many different organic materials have recently been carried out. Techniques to increase content and improve visibility of $^{15}$N in plants and soil include the $^{15}$N-enrichment in plants (Bruns-Nagel et al., 2000; Knicker, 2003; Knicker et al., 1996a; Smernik & Baldock, 2005b), and treatment of soils with HF, which removes paramagnetics (Knicker & Hatcher, 2001; Kogel-Knabner, 1997; Smernik & Baldock, 2005a). These techniques therefore reduce experimental times substantially. Recent $^{15}$N NMR studies have been carried out on a large range organic materials, which involve both enriched and unenriched plant materials, (Coker & Schaefer,
1985; Knicker & Lüdemann, 1995a), algae (Knicker, 2000a; Knicker & Hatcher, 2001; Zang et al., 2001), insect materials (Schaefer et al., 1987), organic matter in soils (Clinton et al., 1995; Dick et al., 2004; Schmidt et al., 1997; Smernik & Baldock, 2005a), sediments (Knicker & Hatcher, 1997), coals (Knicker et al., 1995b, 1996b) and kerogen (Kelemen et al., 2002) to name a few.

There has been some controversy over interpretation of NMR spectra for organic materials, especially soils (for a good summary see Smernik and Baldock 2005), in particular, the high concentration of amide linkages observed in soil organic matter, and lack of other N functional groups. These results are in disagreement with those obtained from other methods, and suggest that some nitrogen may not be seen using NMR. For example, Schulten and Schnitzer (1998) suggest that up to 35% of nitrogen found in soil organic matter is heterocyclic. This is backed up by techniques such as X-ray spectroscopy and pyrolysis-gas chromatography-mass spectroscopy (Py-GC-MS) (Leinweber & Schulten, 1998; Patience et al., 1992). All of these techniques, however, are destructive, and alter the chemical structure of the sample at some point, leading to possible artefacts and non-quantitative results as mentioned previously.

Knicker (2002) has previously used 1 and 2 dimensional solid-state double-CPMAS NMR methods, where polarisation is transferred from $^1$H to $^{15}$N nuclei, which is then passed on to $^{13}$C nuclei. By doing this, the spectrum obtained will only show $^{13}$C nuclei, which are very close to $^{15}$N nuclei. Hence this gives more information on the $^{15}$N and $^{13}$C environments and interactions. Analysis undertaken on $^{13}$C and $^{15}$N enriched humic fractions and plant residues after 9.5 months of degradation found coupling between amide-C and N-substituted-alkyl-C functional groups. This therefore confirmed that amide-N was the major form of N present, and was mostly found to be joined to alkyl-C groups. These findings would also suggest that N has been immobilised by microbial populations into peptide structures.

If it is true that some N is not seen using NMR, this could be due to, for example, low protonation, the presence of paramagnetics or overlapping of the amide peak with other N peaks. Nevertheless Smernik and Baldock showed by the application of spin counting techniques that 68-93% of nitrogen was observed by direct polarisation
technique experiments and even less, namely 37-80% using indirect pulse techniques, depending on the sample type. This does not mean results are non quantitative, since agents like paramagnetics species, which may interfere with and hinder the NMR observations, may be swamping observed nitrogen but similarly for all structural groups. Moreover, experiments that quantify nuclei may be limited due to a variety of additives that may interact with the matrixes being measured. This can be chemical or physical due to a change in crystallinity, which is discussed later in this chapter.

In this Chapter a short introduction to solid state NMR is provided initially, focussing mostly on the pulse sequences used. This is followed by a more in-depth literature review on $^{15}$N and $^1$H spin relaxation processes and the use of time constants, which define the nuclei spin decay processes, and various studies which show how these processes help describe N functional groups. A study of $^{15}$N enriched model compounds undertaken for this thesis is then described along with results acquired. In this work we look at some simple N model compounds found widely in nature, including urea, amino acids, ammonium salts and heterocyclic N compounds. These are also materials that may be used as models when studying nitrogen incorporation in plant and animal materials, soils and recent sediments. The effects of mixing these materials on the relative $^{15}$N NMR signal intensities are examined.

### 2.2. Solid-State NMR Spectroscopy

The theory of solid state NMR spectroscopy is not presented here in detail, as detailed in depth explanations are found in many specialized NMR textbooks such as (Abrahams et al., 1998; Fukushima & Roeder, 1981; Wilson, 1987). There are also many journal publications which also provide detailed descriptions of NMR theory and its application to various organic materials (Kogel-Knabner, 1997; Mathers et al., 2000; Preston, 1996, 2001; Smernik & Baldock, 2005a; Smernik & Oades, 2000, 2001, 2003) and inorganic samples (Kim et al., 2004; Metz et al., 1996; Ratcliffe et al., 1983). For the purpose of this thesis, a brief explanation of NMR theories relevant to the studies presented in this thesis are given, particularly focusing on the pulse sequences used, and the nuclei spin relaxation parameters calculated by analyzing data acquired by $^{15}$N NMR spectroscopy.
NMR takes place when the nuclei of a particular atom are exposed to a static magnetic field while electromagnetic energy is applied at a particular frequency. This frequency, known as the Lamour frequency, is defined by the nucleus type and the magnitude of the static field. A physical interaction between the electromagnetic pulse and the nuclei (NMR) will only occur for atoms which possess a nuclear spin with a quantum number greater than zero \((I > 0)\). When nuclei with nuclear spin are placed in a magnetic field, their angular momentum causes them to precess around the direction of the magnetic field, in the same way a spinning top precesses around earth’s gravitational field.

The frequency at which the nuclei precess depends on the magnetic field and the proportionality constant that equates the precession frequency with the constant field strength, known as the gyromagnetic ratio \((\gamma)\). For a given nucleus, as the field intensity increases, the precession or Lamour frequency increases. This results in an increase in detection sensitivity of the NMR spectrometer, depending on the atom’s \(\gamma\) value. For example, Table 2.1 shows that the \(\gamma\) for \(^1\text{H}\), is much higher than that of \(^{15}\text{N}\), meaning \(^1\text{H}\) is more sensitive to NMR detection and therefore gives a stronger signal than \(^{15}\text{N}\), which is the least sensitive nucleus. The ability to acquire a spectrum also depends on the abundance of these nuclei (Table 2.1). Because \(^{15}\text{N}\) has a low \(\gamma\) and also a low natural abundance, it is quite insensitive. Careful optimisation and the application of refined NMR techniques are therefore required to provide optimum signal to noise ratio when recording \(^{15}\text{N}\) spectra by NMR.

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>Spin</th>
<th>(\gamma) (radT(^{-1})sec(^{-1}))</th>
<th>% Natural Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^1\text{H})</td>
<td>(\frac{1}{2})</td>
<td>(2.675 \times 10^{-7})</td>
<td>99.99</td>
</tr>
<tr>
<td>(^{13}\text{C})</td>
<td>(\frac{1}{2})</td>
<td>(6.73 \times 10^{-7})</td>
<td>1.12</td>
</tr>
<tr>
<td>(^{15}\text{N})</td>
<td>(\frac{1}{2})</td>
<td>(-2.71 \times 10^{-7})</td>
<td>0.37</td>
</tr>
<tr>
<td>(^{31}\text{P})</td>
<td>(\frac{1}{2})</td>
<td>(10.83 \times 10^{-7})</td>
<td>100.00</td>
</tr>
<tr>
<td>(^2\text{H})</td>
<td>1</td>
<td>(4.12 \times 10^{-7})</td>
<td>0.02</td>
</tr>
</tbody>
</table>
2.2.1. Chemical Shift

In an NMR spectrum, peaks representing chemically different environments of a particular nucleus are distributed according to their chemical shift value. This chemical shift is primarily influenced by electron shielding associated with different functional groups, which affects the magnetic field the nuclei are subjected to. For example, a chemically distinct carbon atom in solution NMR gives a single NMR line. The chemical shift for this line is related to the electronic shielding about the nucleus providing useful information about the environment for the nucleus being analyzed. Furthermore, for solution NMR the rapid molecular motion or tumbling will cause the 3D variation in the shielding to be averaged and its effect can be ignored.

For solid state NMR, there is little molecular motion and since the shielding is not equal along different directions this produces tensors that broaden the chemical shift peak. Often the shielding is not symmetric and the chemical shift tensor describes the magnitude and direction of this 3D shielding. This results in nuclei in different chemical environments resonating at different frequencies, or chemical shifts, in an NMR spectrum. For example, an amino acid would have increased electron shielding for C bearing a hydroxyl group and hence electron deshielding of any other remaining C will occur (Wawer & Zielinska, 1997, 2001). In a $^{13}$C NMR spectrum, the carbonyl C peak will therefore be further downfield relative to any other C functional groups, due to its increased electronegativity.

The chemical environment surrounding one functional group can also affect its chemical shift. Ratcliffe and co-workers (1983) have previously used $^{15}$N NMR to look at the chemical shift of various ammonium ($\text{NH}_4^+$) salts. It was found that the $\text{NH}_4^+$ peak has a different chemical shift depending on its environment (Table 2.2). It was concluded that this was due to changes in the $\text{NH}_4^+$ ion geometry and anion environment. The shifts could be explained by the different bond lengths and electronegativity of interacting anions.

It was also found that the $\text{NH}_4^+$ associated with oxyanions produced peaks within a chemical shift range of approximately 10ppm wide, which was different from those associated with halides. This may be due to the lattice or environment type present. It
was also found that (NH₄)₂SO₄ and (NH₄)₂HPO₄ had two crystallographically different NH₄⁺ ions, and therefore may show two peaks in an NMR spectrum. Different ammonium salts had different T₁ values, with NH₄I needing a repetition delay of 90 seconds, while the other samples needed 2-5 seconds.

Table 2.2: Observed ¹⁵N chemical shifts in ammonium salt relative to ¹⁵NH₄Cl (Ratcliffe et al., 1983)

<table>
<thead>
<tr>
<th>Salt</th>
<th>Chemical Shift (ppm)</th>
<th>Salt</th>
<th>Chemical Shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹⁵NH₄I</td>
<td>16.5</td>
<td>¹⁵NH₄H₂PO₄</td>
<td>-15.8</td>
</tr>
<tr>
<td>¹⁵NH₄Br</td>
<td>2.1</td>
<td>¹⁵NH₂NO₃</td>
<td>-17.4</td>
</tr>
<tr>
<td>¹⁵NH₄Cl</td>
<td>0.0</td>
<td>(¹⁵NH₄)₂C₆H₄O₆</td>
<td>-19.3</td>
</tr>
<tr>
<td>¹⁵NH₂SCN</td>
<td>-5.1</td>
<td>(¹⁵NH₄)₂Cr₂O₇</td>
<td>-24.1</td>
</tr>
<tr>
<td>(¹⁵NH₄)₂HPO₄</td>
<td>-14.3, -17.0</td>
<td>(¹⁵NH₄)₂Cr₂O₇</td>
<td>-24.1</td>
</tr>
<tr>
<td>(¹⁵NH₄)₂SO₄</td>
<td>-14.7</td>
<td>NH₄SC¹⁵N</td>
<td>169.9</td>
</tr>
<tr>
<td>¹⁵NH₄HCO₃</td>
<td>-15.6</td>
<td>NH₄¹⁵NO₃</td>
<td>336.0</td>
</tr>
</tbody>
</table>

2.2.2. Pulse Sequences

Because of the low natural abundance of ¹⁵N, peak broadening due to chemical shift anisotropy (CSA) and heteronuclear dipolar interactions, various NMR techniques and pulse sequences can be used to overcome these problems, enhance resolution and improve the signal to noise ratio. Techniques include magic angle spinning (MAS), which is used in conjunction with all pulse sequences reported here, cross polarisation (CPMAS), Bloch decays and dipolar dephasing (DD-CPMAS). Each of these pulse sequences also allows a more in-depth exploration of the data recorded by providing the opportunity to acquire certain time constants, of which can be used to help define the environments specific N functional groups are in. An explanation of both the pulse sequences and time constants used in this research (Chapters 2, 3, 4 and 5) and also previous research by others is provided as follows.

2.2.3. Magic Angle Spinning

When analysing a solid-state sample using NMR, peak broadening due to chemical shift anisotropy (CSA) occurs. This is due to a whole range of chemical shifts being observed at the same time, which is caused by restricted molecular motion found in a solid sample. In solution samples, CSA is averaged out naturally due to rapid time-
averaged molecular motion. In solid samples, CSA can be averaged out by spinning the samples at an angle of approximately 54.74° to the magnetic field, known as the “magic angle”. Since peak broadening is in part due to CSA, reduction of CSA by magic angle spinning (MAS), will also reduce the peak broadening. MAS is employed in all NMR pulse sequenced undertaken in this research.

The speed at which a sample rotor is spun is also important. If the CSA is greater than the spinning rate of the rotor, a series of spinning side bands (SSB’s) will occur. It is a common occurrence for sidebands to overlap with sample peaks, making quantitative analysis tricky. The intensity of the SSB’s is usually distributed evenly on either side of the central bandwidth, and can be reduced by increasing the spinning speed. This however, may lead to broadening of all peaks, therefore high spinning speeds are not necessarily a better option. Various NMR pulse sequences (Dixon, 1985) and probe types which allow for higher spinning speeds (Preston, 2001) exist for eliminating SSB’s. It is however possible to produce quantitative spectra by integrating the peak intensities of non-overlapping SSB’s, and adding twice this amount to the central bandwidth it originated from.

2.2.4. Bloch Decays

Bloch Decays, otherwise known as direct polarisation magic angle spinning (DPMAS), is the most straightforward pulse sequence, where a 90° radio frequency (RF) pulse is applied to rotate nuclei, and is tuned to the Larmor frequency of the nuclei to be observed (Figure 2.1). They are then allowed to relax, in turn inducing a current in the NMR spectrometer’s receiver coils. It is this process which allows us to acquire a signal. During this acquisition time, protons in the sample are decoupled using high power decoupling pulse, to eliminate strong heteronuclear dipolar interactions. In other words, the protons are given a long RF pulse which tips them over, and maintains their orientations until the observed nuclei have fully relaxed. This avoids any interaction of protons with the relaxing nuclei of interest (e.g. 15N).

One problem with using 15N and 13C NMR Bloch decay pulse sequences arises due to the very long delay times needed to allow these nuclei to fully relax, therefore causing excessive analysis times. This, however, depends on the chemical
environment associated with the nuclei. For example, the extreme increase in delay times needed for $^{15}$N have been discussed by Smernik and Baldock (2005b). They have shown that in order to see a true representation of nitrate-N in plant material, delay times need to be in the order of 200 – 1000 seconds. The N found in the organic compound urea has been shown to take up to 96 minutes to relax, which would therefore require an extremely long delay time in order to see the nuclei quantitatively in an NMR spectrum.

Another problem, associated with detection limits, occurs due to the very low natural abundance of the $^{15}$N isotopes. Low abundance can be overcome, in part, by running more scans (Kogel-Knabner, 1997). However, to obtain a satisfactory spectrum with a reasonable signal to noise ratio, the large number of scans required leads to excessive analyses times. This can be in the order of days or even weeks, which in turn may then present a problem in maintaining the tuning of the NMR spectrometer. Another way to overcome the problem of low natural abundance is to enrich samples with either the $^{15}$N or $^{13}$C isotopes needed for NMR analysis (Bruns-Nagel et al., 2000; Knicker, 2003; Knicker et al., 1996a; Smernik & Baldock, 2005b). This technique was utilized in the research undertaken for this thesis with the use of $^{15}$N enriched model compound and $^{15}$N isotopically labelled fertilisers to grow $^{15}$N-enriched plants.

The benefit of using a Bloch decay pulse is that it is possible to obtain a true representation of N composition in the sample analysed. Quantitative spectra can only be obtained, however, if the recycle delay time is at least 5 times $T_{1N}$, the time it takes for the N spin-lattice to relax (Taylor, 2004; Wilson, 1987). If this is not achieved, saturation will occur, and hence a quantitative spectrum will not be
observed. It is common to compare Bloch decay spectra with spectra from other techniques such as CPMAS (see next section), to point out which N chemical environments may be underestimated (Smernik & Baldock, 2005a).

2.2.5. Cross Polarisation

The cross polarisation, magic angle spinning (CPMAS) technique involves the transfer of magnetic energy (or polarisation) from the more abundant proton nuclei, to the less abundant $^{15}$N nuclei. This is done by applying a pulse in the $^1$H field and placing the proton in a spin-lock condition with observed nuclei (e.g. $^{15}$N or $^{13}$C). At the beginning of the spin lock the $^{15}$N field is applied with optimised coupling conditions between the two nuclei for a period known as the contact time. During this time, under optimised conditions, the best transfer of energy between the protons and the $^{15}$N nuclei occurs, thereby enhancing the $^{15}$N signal (Figure 2.2). This cross-polarisation can only be achieved for $^{15}$N nuclei within close proximity (up to 4 – 5 bond lengths maximum) of a proton. Therefore, in a sample where $^{15}$N nuclei have no protons attached or in the close vicinity (eg. aromatic N), their signal may be underestimated or at worst not even observed using CPMAS. This may also be the case for $^{15}$N nuclei that are protonated, but are highly mobile (eg. NH$_3$ groups). In this case efficient cross polarisation does not take place due to weak dipolar interactions. Therefore the resulting spectra peak for these groups may be suppressed if longer contact times are not used to allow efficient energy transfer. This is where a Bloch decay spectrum, if time permits, is useful to compare and point out where any spectrum under-estimations have occurred.

Another advantage in using CPMAS is the rapid relaxation of proton nuclei spins to equilibrium, which allows much shorter delay times between pulses. This advantage, coupled with the enhanced $^{15}$N signal due to cross polarisation, leads to shorter experimental times compared to a Bloch decay experiment. For CPMAS experiments, the rate of the free induction decay (FID), which is the decay of signals with respect to time collected, is determined by the time constant $T_1^H$, which represents the proton relaxation time. In cases where relaxation is slow the nuclei must fully relax before the next pulse, and recycle delays should always be at least 5 times $T_1^H$ in order not to saturate the spectra obtained (Taylor, 2004; Wilson, 1987).
At the commencement of contact time, the signal intensity will initially rise due to the transfer of magnetic energy. This can be characterized by the cross polarization time constant $T_{NH}$, where $1/T_{NH}$ is the rate at which energy is transferred per millisecond. The decay of the signal intensity, which may start while cross polarization is still occurring, involves the relaxation of protons under spin-lock conditions, which is characterized by $T_{1\rho H}$ (where $1/T_{1\rho H}$ is the rate of decay per millisecond), and the proton dipolar spin-lattice relaxation, $T_1H$.

**Variable Contact Time Experiments**
An array of time constant parameters, which define the transfer of magnetization and relaxation process, can be obtained using variable contact time (VCT) experiments. This involves conducting a number of CPMAS experiments where the contact time ($t$) has been varied. By plotting the natural log of the peak intensities obtained versus the contact time (Figure 2.13), the parameters $T_{1\rho H}$ and $T_{NH}$ (in the case of $^{15}\text{N}$ NMR) can be obtained. $T_{NH}$, the rate at which magnetisation is transferred from H to N, depends on the internuclear vector between N and any adjacent protons and also dipolar interactions between the two nuclei. Therefore the value for $T_{NH}$ will depend on a number of factors such as:

- The distance between H and N.
- The ratio of N to H’s. For example, high levels of protonation of the N will transfer greater magnetisation, more quickly, than unprotonated or low levels of protonation of the N.
- Molecular motion, as it reduces dipolar interactions needed for magnetisation transfer.
While the rate of increase in magnetisation is defined by $T_{NH}$, the decay of magnetisation is controlled by the relaxation of the proton spin-lattice in the rotating frame. The rate of this relaxation is defined by the parameter $T_{1pH}$. This decrease in magnetisation is due to the fact that polarisation transfer from protons cannot be maintained indefinitely (Hartman-Hahn conditions), so at some point, during or after cross polarisation begins, the competing effect of proton relaxation will dominate. The value for $T_{1pH}$ of a particular functional group will vary depending on:

- Molecular motions experienced, due to reduced dipolar interactions
- Effectiveness of intrinsic spin diffusion, which can average $T_{1pH}$’s present over a range of up to approximately 10nm
- Uniformity of the sample’s structure, eg. crystalline or amorphous (depending on region sizes)
- The presence of paramagnetic species

Because the rate of build up of magnetisation is defined by $T_{NH}$, and the rate of decrease is defined by $T_{1pH}$, the signal intensity ($I_t$) can be determined by the following equation:

$$I_t = I_0 \alpha^t \times [1 - \exp(\alpha t / T_{NH})] \times \exp\left(-t / T_{1pH}\right)$$

Equation 2.1

Where $\alpha = (1 - T_{NH} / T_{1pH})$, $I_t$ is the signal intensity at contact time $t$ and $T_{NH}$ and $T_{1pH}$ are constants. $I_0$ is the signal intensity extrapolated to a zero contact time based on the decay of $\exp(-t / T_{1pH})$ only after longer contact times. That is, $T_{1pH}$ can only be found when $T_{1pH} \gg T_{NH}$. If $T_{NH}$ approaches similar values to $T_{1pH}$, magnetisation transfer can never be completed before substantial relaxation of the proton spin-lattice has occurred. This will then quantitatively affect the spectrum so only a small fraction of the total signal can be measured. The ratio of $T_{NH}$ to $T_{1pH}$ can assist in discovering whether all signal for a species is being observed.
$T_{1p,H}$ is strongly influenced by the presence and proximity of paramagnetics. In some cases paramagnetics may assist in the relaxation process. Thus a range of $T_{1p,H}$’s in a mixture can prohibit quantitative measurements for all components. It is also possible that $T_{1p,H}$’s are so short that nuclei coupled to protons will not be visible. In such cases it is necessary to measure the polarisation rate $T_{NH}$ along with the $T_{1p,H}$ for the various components. It is difficult, however, to assert values for $T_{NH}$’s for all species without analysing model compounds.

While these points are important and have a strong impact in determining the limits for absolute quantification, relative quantification may still be possible provided the environment for the nuclei is consistent.

### 2.2.6. Dipolar Dephasing

Magic angle spinning and high-powered decoupling help to reduce line broadening and therefore produce higher resolution spectra. Dipolar dephasing CPMAS (DD-CPMAS) involves turning the high powered decoupling off for a short period of time $(t)$, which follows cross polarisation and is before acquiring data when running a CPMAS pulse sequence (Figure 2.3). During the middle of the delay time a 180 deg pulse is applied in order to refocus to remove any phase distortions or any other offsets such as chemical shifts and peak splitting (Alemany et al., 1983).

The delay time causes the linewidth of the peak for $^{15}$N nuclei closely associated with protons, to become very broad, or dephased, to a point where they are unobservable.

![DD-CPMAS pulse sequence](image)

**Figure 2.3: DD-CPMAS pulse sequence**
What remains in the spectrum are the peaks of unprotonated $^{15}$N nuclei or $^{15}$N nuclei with high molecular motion. This technique therefore provides further information on internuclear distances and molecular motion. Dipolar dephasing is generally used in $^{13}$C NMR spectroscopy to investigate the dipolar coupling between $^{13}$C-$^1$H (Alemany et al., 1983; Smernik & Oades, 2001; Wilson, 1987).

By plotting the natural log of peak intensity verse the dephasing time, the transverse relaxation $T_{2DD}$, can be calculated. This is done by using either Equation 2.2, which is based on an exponential decay, or Equation 2.3, which is based on gaussian decay (Alemany et al., 1983; Smernik & Oades, 2001).

\[
I_t = I_o \times \exp(-t/T_{2DD}) \quad \text{Equation 2.2}
\]

\[
I_t = I_o \times \exp(-t^2/2T_{2DD}^2) \quad \text{Equation 2.3}
\]

Where in both equations $t$ is the dephasing time, $I_t$ is the signal intensity at time $t$, $I_o$ is the signal intensity at $t = 0$, and $T_{2DD}$ is the transverse time constant. By plotting $\ln(I_t/I_o)$ against $t$ (Figure 2.12), for exponential decay, the reciprocal of the slope ($-1/m$) is equal to $T_{2DD}$. For gaussian decay $T_{2DD}$ can be found by calculating $(-1/2m)^{1/2}$ from a plot of $\ln(I_t/I_o)$ verse $t^2$ (Figure 2.12).

2.2.7. **Spin Counting**

Spin counting is a method in which the integrated signal area, measured for a known standard spectrum, is quantitatively related to the known amount of $^{15}$N contained in the standard. It can then be compared to other samples with known $^{15}$N content, to see if all nitrogen is being seen. This quantitative technique provides an absolute “observability” of N functional groups in a particular sample.. It can therefore be seen if any $^{15}$N in the sample is over- or under-represented in the sample’s spectrum.

Spin counting can be used in conjunction with the Bloch Decay and CPMAS pulse sequences, and also difference spectra, in order to identify the under- or over-representation of any peaks in an NMR spectrum. The only condition required in
using this method, is that all nuclei are fully relaxed during the recycle delay. Therefore, once again, the recycle delay must be at least 5 times the time it takes the slowest nuclei being observed to relax. If this criterion is not met, relative spin counting can still be used, where the difference in observability of a set of similar samples is compared to a standard. Both absolute and relative observability techniques will be used throughout this thesis.

For $^{13}$C and $^{15}$N NMR spin counting has been extensively used by Smernik and co-workers to find the observabilities of various model compounds and species contained in soils (Keeler & Maciel, 2003; Smernik & Baldo, 2005a; Smernik & Oades, 2000, 2003), sewage sludge (Smernik et al., 2004) and $^{15}$N enriched plant materials (Smernik & Baldo, 2005b). For example, Smernik and Baldo (2005b) looked at highly $^{15}$N enriched plant material, including leaf, stem and root samples of *Eucalyptus globulus*. Observability for leaf and stem samples were both 80% using CPMAS, and 85% using Bloch decay pulse sequences. Roots, however, had an observability of 68% for CPMAS, and 89% for the Bloch decay. By making a difference spectrum (where the resulting CPMAS spectrum is subtracted from the Bloch decay spectrum), the large difference in observability of the roots was found to be due to the under-representation of amino and nitrate groups.

**2.3. NMR Analysis and Sample Physical Properties**

An understanding of the relationship between physical properties of samples, studied in this thesis, and data acquired by NMR spectroscopy is important. It is possible by processing NMR data to gain a better understanding in the:

1. Intra and inter molecular dipolar interaction
2. Crystal structure and molecular bonding
3. The effects of molecular group mobility
4. The effects of protonation
To facilitate this study a range of model compounds, with varied characteristics, were used to provide an explanation of the link between NMR analyses and the differences between these properties.

The two main model compounds chosen were urea and α-glycine. Both compounds are organic molecules which are found widely in nature, and contain N in the form of an NH$_2$ group. The environments, associated with the NH$_2$ group, in these compounds are quite different. Independent NMR measurements of α-glycine and urea were then compared to measurements for other N containing samples such as ammonium nitrate, ammonium chloride, ammonium sulfate; and uric acid, and the amino acids lysine, arginine, asparagine, and histidine. To compare NMR measurements for these compounds demands different pulse sequences and time constants, to define the different relaxation processes, are needed in order to identify N in the different environments. The following is a review of the affects physical properties such as crystal structure, intra- and inter-molecular bonding, mobility, protonation and paramagnetics have on dipolar interactions and time constants. In this review, particular reference has been given to glycine and urea.

2.3.1. Dipolar Coupling and Dipolar Interactions

Dipolar coupling is the affect two bonded atoms have on each other. As discussed earlier in this chapter, dipolar coupling is affected by the gyromagnetic ratio (γ) of the nuclei observed. $^{15}$N nuclei have a small γ (Table 2.1), and therefore will have smaller dipolar coupling with other nuclei. This is one reason why $^{15}$N nuclei give a less intense CPMAS spectrum than, for example, $^{13}$C, as the dipolar coupling between $^{15}$N and $^1$H are weaker than that between $^{13}$C and $^1$H. Dipolar couplings can be investigated using DD-CPMAS. For example, $T_{2DD}$ values acquired by $^{13}$C DD-CPMAS show that CH$_2$ and CH groups, which have strong dipolar coupling, will have low $T_{2DD}$’s, followed by higher values for by CH$_3$ groups which exhibit moderate coupling, and finally, non-protonated C giving the highest $T_{2DD}$’s due to weak coupling (Alemany et al., 1983). $T_{2DD}$ values, however, are also affected by molecular mobility, which is discussed in section 2.3.3.
Dipolar interaction, on the other hand, is the effect different functional groups or environments have on a particular coupling or atom. Both intra and inter-dipolar interactions can occur either within a single molecule or with adjacent molecules. These interactions are influenced by three important factors, which are considered here and discussed also in the following sections. Firstly, the environment a particular molecule is in, secondly the mobility of molecular groups and thirdly the bond length between adjacent nuclei. Small bond lengths between $^{15}\text{N}$ and $^1\text{H}$ lead to stronger dipolar interactions, whereas larger bond lengths will give weaker dipolar interactions. More mobile groups will also have weak dipolar interactions between adjacent nuclei, while rigid functional groups will give stronger interactions. The affects of crystal structure, molecular bonding, mobility and protonation on dipolar interactions are discussed respectively in the following sections.

If the environment has an affect on dipolar interactions, then the dipolar interactions influence the relaxation processes and therefore the time constants calculated for a sample. This has been exploited in various research studies by comparing protonated and deuterated versions of an otherwise identical sample. Deuterons ($^2\text{H}$) have a much smaller gyromagnetic ratio than $^1\text{H}$ (Table 2.1), and therefore have weaker dipolar interactions with surrounding nuclei.

For example, a study has recently been undertaken by Yang and Muller (2007), where the urea inclusion compounds, 1,6-dibromohexane ($\text{BrC}_6\text{H}_{12}\text{Br}$), were recrystallised with either urea ($\text{NH}_2\text{CONH}_2$) or a deuterated form of urea ($\text{N}_2^2\text{H}_2\text{CON}^2\text{H}_2$). After undertaking $^{13}\text{C}$ NMR CPMAS experiments it was found that values for $T_{1\rho}\text{C}$ and $T_{1\rho}\text{H}$ increased when $\text{BrC}_6\text{H}_{12}\text{Br}$ was mixed with the deuterated urea (Figure 2.3). This arises due to reduced dipolar interactions with $^2\text{H}$. The fact that there is a change, however, shows that protons are involved in interactions within the urea crystal lattice, otherwise a change in $T_{1\rho}$ values would not have been observed. A reduction in the peak line width of the urea carbonyl group upon deuteration was also observed, which suggests there may be interference between the $^1\text{H}$ decoupling RF field and molecular motions the urea may be experiencing, such as rotation about the C=O and C-N bonds.
Table 2.3: Results from C-1 of BrC₆H₁₂Br¹³C NMR spectrum (Yang & Muller, 2007).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>T_{ip}C</th>
<th>T_{ip}H</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrC₆H₁₂Br/urea</td>
<td>45.6</td>
<td>1.2</td>
</tr>
<tr>
<td>BrC₆H₁₂Br/urea-²H</td>
<td>54</td>
<td>9.0</td>
</tr>
</tbody>
</table>

2.3.2. Crystal Structure and Molecular Bonding

Glycine is the smallest of the amino acids, consisting of an amine NH₂ group in its neutral state (i.e. electrically neutral), and is found in human and animal biological matter, as well as plant and soil materials. This compound also serves as a model for larger biomolecules, such as proteins, which involve the linking of various amino acids. Glycine has been studied extensively, with more recent investigations focusing on its three main polymorphs (α, β and γ), and their growth (Chew et al., 2007), quantitative identification (Cao et al., 2002), crystal structure (Ramachandran et al., 2007) and properties (Boldyreva et al., 2003a, 2003b; Taylor et al., 2006).

Glycine is zwitterionic, meaning it is electrically neutral, but carries a positive and negative charge on different atoms contained in this compound. It is therefore polar, and highly soluble in water. The NH₂ group in glycine, with its polar nature, lends itself to both intra- and inter-molecular bonding with other protons, forming an amino NH₃ group. In a pure glycine sample, conjugation is experienced through a NH…O bond between the amine N and carbonyl C either in one molecule, or between adjacent molecules.

The three polymorphs of glycine differ by how their crystal structures are packed together. When studying glycine it is therefore important to note which polymorphs are present. If the different polymorphs have different structural packing, molecular motion may differ, and relaxation time constants will therefore also be different. For example, the high molecular motion found about the αC-NH₃ bond in α-glycine, allows the NH₃ group to act as a sink for proton spin lattice relaxation (Taylor et al., 2006).

Taylor and co-workers (2004) have compared NMR relaxation parameters between the three main glycine polymorphs. Parameters include relaxation of ¹H, ¹³C and ¹⁵N.
(T₁H, T₁C and T₁N respectively) and relaxation of ₁H, ₁³C and ₁⁵N in the rotating frame (T₁ρH, T₁ρC and T₁ρN respectively). The results obtained (Table 2.4) show that the different crystal structures give significantly different relaxation parameters. Therefore the polymorph that a particular nucleus is incorporated in has a large affect on its behaviour. Mathias and co-workers (1988) have also previously shown that the chemical shift of peaks found in the ₁⁵N CPMAS spectra of solid polyamides differ depending on whether the α or β crystal forms is present.

Table 2.4: Solid-state ₁H, ₁⁵N and ₁³C CPMAS NMR relaxation times for and α, β and γ-glycine measured on a 300MHz spectrometer. Relaxation times for β were measured with a static sample (Taylor, 2004).

<table>
<thead>
<tr>
<th>Glycine Structure</th>
<th>T₁H (sec)</th>
<th>T₁ρH (msec)</th>
<th>T₁C (sec)</th>
<th>T₁ρC (msec)</th>
<th>T₁N (msec)</th>
<th>T₁ρN (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>0.25</td>
<td>44</td>
<td>12 (C=O)</td>
<td>180 (C=O)</td>
<td>240</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11 (NCH₂)</td>
<td>43 (NCH₂)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β</td>
<td>29</td>
<td>0.4</td>
<td>8 (C=O)</td>
<td>12 (C=O)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.3 (NCH₂)</td>
<td>300 (NCH₂)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ</td>
<td>4</td>
<td>4.1</td>
<td>55 (C=O)</td>
<td>270 (C=O)</td>
<td>150</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>22 (NCH₂)</td>
<td>53 (NCH₂)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Crystallinity can also affect the width of a peak, due to lower CSA. Powell and Mathias (1989) used ₁⁵N NMR to show peak width differences between samples of crystalline and amorphous (non-crystalline) samples of nylon 6. The crystalline sample had a half-width (i.e. half peak height width) of 2.4ppm. A broader peak with a half-width of 6.3ppm was located slightly downfield and was associated with the amorphous fraction of nylon 6, with its peak appearing midway between α and β crystal peak shifts. This was confirmed by comparing T₁N (N spin lattice relaxation times) values, which were 416 seconds (based on an exponential decay) for the crystalline sample, and 29.6 and 1.9 seconds (results from a biexponential decay) for non-crystalline samples. The two T₁N’s produced by the amorphous form is the result of different crystal structures, or a lack of crystal structure, being present in the one sample. The shorter T₁N represent structures with more mobility, while the longer T₁N represent rigid structures.

In the α-glycine structure, three interactions are taking place: 1) Van der Waals forces, 2) electrostatic forces (mainly dipole-dipole interaction) and 3) an H bond framework (Perlovich et al., 2001). Each amino H is bonded to an O (Figure 2.4).
Two of these bonds help form ribbons, which then form sheets bound through van der wall forces. The sheets are held together through the 3rd amino H, which is bonded to an O in the sheet above. In α-glycine, lateral H bonds (in ribbons) are stronger than the interlayer H bonds (between sheets).

Urea on the other hand is planar, with its carbonyl O accepting four amide H bonds (Muidinov & Zorkii, 1999; Taylor et al., 2007; Williams & McDermott, 1993). It is therefore also highly soluble in water, due to extensive hydrogen bonding. Urea’s crystal packing is very open, with planar ribbons of urea making long square tunnels.

Figure 2.4: Crystal structure of α-glycine a) structure (Perlovich et al., 2001), b) general view (Taylor 2004 13c). The dotted lines represent protons involved in intermolecular NH…O bonds.

Figure 2.5: Crystal structure of urea: a) general view, b) structure and agglomeration of C(Z) chains. Distances are in angstroms and angles in degrees. The dotted lines represent protons involved in intermolecular NH…O bonds.
with a cross section of 4 x 4 Å (Figure 2.5). It is for this reason that urea is a popular inclusion compound, as the long square tunnels provide a space to include or transport other compounds, thereby possibly changing the appearance or properties of the final compound (Yang & Müller, 2007). Urea is an organic molecule, however, and is widely found in nature. It is commonly found in the urine of mammals, amphibians and some fish. Derivatives of urea, such as uric acid and allantoin, can be found in the excretes of birds, reptiles and insects, as they are less soluble in water and therefore allow these small animals to be more water efficient.

2.3.3. Effects of Mobility

Molecular motion within a functional group reduces the dipolar interactions between the nuclei involved. The time it takes for cross polarisation to occur during CPMAS will therefore increase with increase in motion, leading to high $T_{NH}$ values. The amount of time needed for spin diffusion will also increases, hence longer $T_{1\rho H}$ values are observed. For example, when looking at the mobility of polymers in tomato cell walls using $^{13}\text{C}$ NMR, Fenwick et al (1996), found that $T_{CH}$ values varied depending on the section of tomato and therefore functional groups of the $^{13}\text{C}$ spectra obtained. For methyl C groups longer polarisation transfer times were observed, even though this group is protonated with protons. This is due to the free rotation of the methyl group; therefore increased transfer times are seen. Methylene and methine C groups do not rotate, and are characterised by fast, or short, $T_{CH}$ values. This was also found in research conducted by Alemany and co-worker’s (1983).

$T_1$ values for nuclei, on the other hand, will decrease with mobility, as the mobile group acts as a sink for relaxation of the nuclei involved, therefore relaxation is quicker. This was found by Andrew and co-workers (1976), in a study of $T_1H$ values of the NH$_3$ groups of a group of amino acids. $T_1H$ values for $\alpha$-glycine, serine, aspartic acid, tyrosine, cystine, histidine, and tryptophan were found to be 42, 62, 75, 96, 55, 79 and 129 msec respectively, where glycine has the highest molecular motion. It was therefore found that a highly mobile NH$_3$ group provided an efficient mechanism for the relaxation of protons. This was also found by Taylor et al. (2006) in a study of $\gamma$-glycine. $T_1$ measurements were made for $^{15}\text{N}$, $^{13}\text{C}$ and $^1\text{H}$, and were compared to the same measurements made on $\gamma$-glycine with a deuterated amino
group (ND$_3$). As deuterons have a much weaker dipolar interactions with surrounding nuclei, significantly higher T$_1$H values were observed for the dueterated samples due to the longer amount of time needed for spin-lattice relaxation (Table 2.5). Lower T$_1$N and T$_1$C values for undeuterated samples also suggested that the protons in the mobile NH$_3$ group act as a sink for relaxation, for all γ-glycine nuclei.

Table 2.5: T$_1$ values found for deuterated and undeuterated γ-glycine (Taylor et al., 2006)

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>T$_1$ (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH$_3$</td>
</tr>
<tr>
<td>$^1$H (methyl)</td>
<td>4</td>
</tr>
<tr>
<td>$^{15}$N-amino</td>
<td>150</td>
</tr>
<tr>
<td>$^{13}$C-methylene</td>
<td>22</td>
</tr>
<tr>
<td>-carbonyl</td>
<td>55</td>
</tr>
</tbody>
</table>

Urea, on the other hand, experiences two forms of rotation; 1) whole body flip about the carbonyl bond, and 2) cis-trans isomerisation internally about its C-N bonds (Figure 2.6). The rate that each of these motions takes place has been investigated by Williams and McDermott (1993), using deuterium NMR. T$_1$ values were used to measure the whole body flip and the cis-trans isomerization rotations per second, and both forms of rotation were found to increase with temperature (Table 2.6), indicating that there was an increase in rotations taking place. A reduction in the peak line width of the urea carbonyl group upon deuteration has been observed by Yang and Muller (2007), suggesting there may be interference between the $^1$H decoupling RF field and molecular motions the urea may be experiencing. Therefore, reduced peak widths indicate that more molecular motion is taking place.

T$_{2DD}$ values are also affected by molecular rotations, with highly mobile groups producing longer T$_{2DD}$ values due to weaker dipolar coupling and interactions. DD-CPMAS T$_{2DD}$ values have been studied extensively using $^{13}$C NMR (Alemany et al., 1983; Smernik & Oades, 2001). For example, in a DD-CPMAS study of natural organic materials including, cellulose, hemicelluloses, lignin, protein, chitin, charcoal, palmitic acid and wax, Smernik and Oades (2001), found that the methyl carbon groups in waxes exhibited slower decays, and therefore higher T$_{2DD}$ values, even though the carbon is highly protonated. This is due to high molecular rotations
of the methyl group, which therefore weakens the strength of the $^{13}$C-$^1$H dipolar coupling.

![Molecular Diagram](image.png)

Figure 2.6: Illustration of the two active rotations found in a urea molecule, consisting of a whole body rotation about the carbonyl and a local rotation about the C-N bond (Williams and McDermott 1993).

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Rotation Type</th>
<th>Activation energy (kcal/mol)</th>
<th>NH…O bond distance (Å)</th>
<th>Rotations (s$^{-1}$) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine (NH$_3$)</td>
<td>amino N-H bond</td>
<td>6.8</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Urea (NH$_2$)</td>
<td>cis-trans</td>
<td>18.5</td>
<td>2.33, 2.14</td>
<td>1.5 x $10^4$</td>
</tr>
<tr>
<td>whole body</td>
<td>15.5</td>
<td>2.33, 2.14</td>
<td>8</td>
<td>6050</td>
</tr>
</tbody>
</table>

### 2.3.4. The Effect of Protonation

The effects of protonation have been shown extensively with $^{13}$C NMR spectroscopy. Because $T_{CH}$ relies upon the internuclear vector between N and any adjacent protons (r), non-protonated C will cross polarise more slowly than protonated C. Alemany et al (1983), provides a summary of $T_{CH}$ results previously found for mostly polymer compounds. It was found that methylene/methine cross polarised in 15-50 usec, followed by protonated aromatic C and methyl C which took 45-100 usec and 80-160 usec respectively. Longer $T_{CH}$’s for methyl C are due to the higher amount of rotation present. The longest $T_{CH}$’s were found for quaternary aliphatic C, non-protonated aromatic C and non-protonated carbonyl C, all of which took approximately 300 usec. For analysing carbon in the polymer compounds, $T_{1p}H$ for polymer ranged between 3 to 8 msec. It was also found some carbons with a lot of
mobility may not cross polarise and therefore, will not be observed. For these results though, $T_{1\rho H} >> T_{CH}$ for all types of $C$, so all should cross polarise and relax efficiently to give a quantitative spectrum.

2.3.5. Presence of Paramagnetics
Samples with a significant paramagnetic content can experience changes in NMR spectra, particularly due to signal loss and peak broadening. The presence of paramagnetics causes magnetic field inhomogeneity with NMR analysis, as they inherently generate their own small magnetic fields. Furthermore, metals contain unpaired electrons which strongly affect the nuclear spin of nuclei (e.g. H) in a close proximity of paramagnetic species and hence spin diffusion (Smernik & Oades, 2002). These two processes can therefore lead to reduced proton relaxation times ($T_1H$, and $T_{1\rho H}$) and hence the broadening of NMR peaks. The presence of high paramagnetic contents can lead to a reduction in NMR signals where they may not be observable (Mathers et al 2002).

The extent to which NMR signals may be affected depends on the paramagnetic content (amount and type) and distribution in the sample being analysed. The main paramagnetic compounds found to contribute to signal loss include iron, copper and manganese, with iron producing the greatest adverse effect (Mathers et al 2002; Smernick & Oades, 2002). If any of these paramagnetics are present as free ions in a sample, then they are more likely to affect molecules in the whole sample, thereby causing an even loss of signal across the entire NMR spectrum acquired. However, if they are involved in organic-metal structures or on cation exchange sites, they can selectively diminish specific regions of a NMR spectra depending on the compound they are in close proximity to (Smernik & Oades, 2002).

A method commonly used for improving NMR signal intensity is treatment with hydrofluoric acid (HF) to remove mineral matter such as paramagnetics. When the mineral matter is removed, it also increases the concentration of organic matter and hence the observability of the resulting NMR spectrum. The HF treatments used by various researchers involve treating samples containing paramagnetic concentrations usually between 2 – 10% (Dai, Johnson & Driscoll 2001; Keeler & Maciel 2003;
The samples made to study decomposition in the experiments for this thesis however, have been made with $^{15}$N-enriched plant material, and have a high plant material to soil ratio. For this reason HF treatment has not been undertaken as signal to noise ratios obtained were adequate. The effect of paramagnetics on the samples containing soil and plant residues during the decomposition experiment will be discussed further in Chapter 4.

### 2.3.6. Biexponential Equations

The use of equations that predict exponential rise and decay for experiments such as VCT’s are commonly used for sample analysis (see Equation 2.1). However, in some cases these equations have not represented the data obtained accurately. Therefore the use of an equation which predicts biexponential rise and biexponential decay has been used by previous researchers (Fenwick et al., 1996; Gabrielse et al., 1994; Smernik, 2006; Smith et al., 2005). These equations therefore provide two sets of $T_{NH}$ and $T_{1\rho}$'s in the case of VCT experiments, and can result in a superior fit. The reason for this is due to “fast” and “slow” cross-polarising and relaxing components being present in the one sample. This especially needs to be taken into account when analysing samples containing mixtures of different compounds, such as soil and plant samples.

For example, Powell and Mathias (1989) have previously used a biexponential equation to find nitrogen relaxation rates ($T_1N$) for the non-crystalline region of a nylon 6 sample. From the two $T_1N$’s obtained they concluded that the shorter value was due to extremely mobile N or liquid-like fraction, whereas the longer $T_1N$ value was due to a more restricted N mobility in the fraction. Biexponential equations have been used in experiments throughout this thesis, and are discussed further on page 55 and in the results section of this chapter.
2.4. Experimental Method

2.4.1. Model Compounds

Unenriched compounds were either purchased from Sigma Aldrich or found at the UWS North Parramatta labs (originally purchased from Sigma Aldrich or Labchem). $^{15}$N-enriched compounds, which included glycine (98%), and urea (98%), were purchased from Cambridge Isotope Laboratories (Cambridge UK). All model compounds were first used without any further refinement, i.e. at ambient moisture levels. Samples were then dried at 140°C (with the exception of urea, which was dried at 70 °C), until a constant mass was reached, and then rerun on the NMR spectrometer to observe any differences that may occur. The moisture content of each of the samples is shown in Table 2.7.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Water (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>4.30%</td>
</tr>
<tr>
<td>Urea</td>
<td>2.48%</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.05%</td>
</tr>
<tr>
<td>Asparagine</td>
<td>14.29%</td>
</tr>
<tr>
<td>Histidine</td>
<td>5.46%</td>
</tr>
<tr>
<td>Arginine</td>
<td>18.17%</td>
</tr>
</tbody>
</table>

All samples were all ground to a fine powder using a mortar and pestle before NMR analysis. The molecular structures of all model compounds can be found in Figure 2.7 Figure 2.8. Mixes of two different dried model compounds were made by grinding the two dried samples together with a mortar and pestle until they had fully blended. No recrystalisation took place.

For this investigation it was important to find out the polymorphic form of glycine, which had been acquired from Sigma-Aldrich (as the form had not been specified). To confirm the form present, an XRD analysis was performed (Figure 2.9) and the measured spectrum was compared with XRD spectra of both $\alpha$ and $\gamma$–glycine polymorphs reported by (Ramachandran et al., 2007). It was found that only $\alpha$–glycine was present in the sample used for this study.
2.4.2. $^{15}$N NMR Spectroscopy

Solid-state $^{15}$N NMR Bloch decays, CPMAS and DDCPMAS spectra were all obtained on a Bruker 200MHz spectrometer operating at 20.28MHz for $^{15}$N. Samples were loaded into 4mm zirconium oxide rotors with Kel-F caps, and spun at 7 kHz.
Free induction decays were obtained during a 0.3sec acquisition time for $^{15}$N-enriched glycine, and 0.01 sec for all other samples over a 25 kHz sweep width. Chemical shifts were measured with respect to glycine as an external standard at -347ppm (Knicker & Hatcher, 2001).

All spectra were Fourier transformed with a 30Hz Lorentzian line broadening. Spectra were integrated using MestRec computer software. Absolute intensities were found by integrating peak areas then correcting for the amount of sample present in the rotor during experiments (in mmol for model compounds and grams for eucalyptus material) and the number of scans used. Preliminary experiments showed that a linear relationship existed between the number of scans and signal intensity (Figure 2.10). Error in integrated peak intensities were estimated to be approximately 10% after running, integrating and comparing multiple standard sample spectra, and taking into account background signals and drift in NMR tuning for long experiments.

![Figure 2.10: The linear relationship between the number of scans and peak intensity for $^{15}$N-enriched glycine.](image)

**CPMAS**

For CPMAS experiments, a pulse width of 4.6µs was used with a contact time of 1.5 msec (except for the VCT experiments). The number of scans ranged from 30-1000 for the $^{15}$N-enriched compounds, up to 100,000 for the unenriched compounds.

The delay time chosen for glycine analyses produced a maximum peak intensity, and thus minimised saturation. This delay time of 5 seconds was also found to be well
over 5 times $T_1^H$ reported for the $\alpha$-glycine polymorph. The same delay time of 5 seconds used for glycine was also used for all model compounds. For analysing urea, $T_1^H$ is reported to be in the order of 67 minutes (Taylor et al., 2007), requiring delay times of over 5 hours for one scan and therefore leading to unacceptable experimental times. It was therefore decided for urea analysis, that even though saturation would occur, resulting in less than maximum peak intensities, time constants such as $T_{1\rho}^H$ and $T_{NH}$ could still be obtained because there was sufficient signal. Furthermore, spin counting could still be used to compare relative changes in observability as oppose to absolute observability. For analysing the Eucalyptus sample an optimum delay time was found by undertaking a variable delay time experiment. Changing the delay time from 0.5 to 10 seconds did not change signal intensities, and therefore it was assumed all protons contained had fully relaxed.

**DDCPMAS**

For experiments, which used the DDCPMAS pulse sequence, the NMR operating parameters used were the same as those used for CPMAS experiments, with the addition of a delay dephasing time ($t$). An array of $^{15}$N NMR DD-CPMAS spectra were first obtained using $^{15}$N-enriched glycine and urea over dephasing times ($t$) ranging from 0 - 200 $\mu$sec. It was found that after approximately 45 $\mu$sec, the entire signal from urea-N had disappeared (Figure 2.11). Urea and glycine spectra were

![Figure 2.11: Left: full dipolar dephasing experiment for $^{15}$N-enriched glycine glycine and the corresponding linear line of best fit. Right: Dried $^{15}$N-enriched glycine (-347ppm) and urea (-302ppm) with dipolar dephasing delays of 0 $\mu$sec and 45 $\mu$sec.](image-url)
then obtained for 15 different dephasing times over the range of 0 – 45µsec. It was found that both glycine and urea were best described by exponential decay when a plot of ln(I_t/I_o) versus dephasing time was obtained (Figure 2.12). Because the unenriched samples take 1 – 2 days to run an experiment, it was of interest to be able to acquire only two data points to work out T_{2DD}. This was first tested with glycine and urea by comparing the T_{2DD} values obtained from the 15 point experiment to results for T_{2DD} using only two points, 0 and 45µsec (Table 2.1). It was found that the two experiments were in agreement if a 10% error in integrated signal intensities was assumed for the two point experiments, as discussed earlier in this section.

![Figure 2.12](image.png)

**Figure 2.12:** Signal intensity as a function of dephasing time up to 45µsec for 15-enriched glycine and urea using either t (exponential decay) or t² (gaussian decay).

<table>
<thead>
<tr>
<th>Compound</th>
<th>R²</th>
<th>T_{2DD}</th>
<th>T_{2DD}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>0.9709</td>
<td>17.6 ± 0.5</td>
<td>18.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.9925</td>
<td>114.7 ± 0.9</td>
<td>113.8</td>
</tr>
</tbody>
</table>

**Table 2.8:** Comparison of results for an initial T_{2DD} experiment based on an 15 points over the range of t = 0-45µsec, versus the use of only two points at t equals 0 and 45µsec. T_{2DD} was best described by exponential decay (Equation 2.2). The R² value for the two point analysis is 1.0 due to there being only two points.
**VCT Experiments**

Variable contact time (VCT) experiments were carried out for the $^{15}$N-enriched model compounds and eucalyptus material, as well as unenriched uric acid. VCT experiments comprise of an array of experiments set up with the same parameters described for CPMAS experiments, only the contact time was varied. After tuning, signal intensity was measured as a function of contact time between $^{15}$N and $^1$H. The dependence of the signal on contact time was described using a model based on Equation 2.1, described previously in this chapter. This model curve was fitted using Microsoft Excel solver. The time constants $T_{NH}$ and $T_{1pH}$ were then obtained.

VCT experiments were first carried out on $^{15}$N enriched glycine. It was found that the model calculated using Equation 2.4, which predicts an exponential rise and exponential decay, did not fit the experimental data well. Therefore, a biexponential model, Equation 2.4, which is based on Equation 2.1, was used to observe whether two sets of time constants were present.

\[
I_t = \left[ I_0[1] \alpha_{[1]} \times \left[ 1 - \exp\left( -\frac{t}{T_{NH[1]}} \right) \right] \right] \times \left[ \exp\left( -\frac{t}{T_{1pH[1]}} \right) \right] + \left[ I_0[2] \alpha_{[2]} \times \left[ 1 - \exp\left( -\frac{t}{T_{NH[2]}} \right) \right] \right] \times \left[ \exp\left( -\frac{t}{T_{1pH[2]}} \right) \right]
\]

*Equation 2.4*

where $\alpha_{[1]} = 1 - \left( \frac{T_{NH[1]}}{T_{1pH[1]}} \right)$, $\alpha_{[2]} = 1 - \left( \frac{T_{NH[2]}}{T_{1pH[2]}} \right)$, $I_t$ is the signal intensity at contact time $t$, $T_{NH[1]}$, $T_{NH[2]}$, $T_{1pH[1]}$ and $T_{1pH[2]}$ are time constants using excel solver, and $I_0[1]$ and $I_0[2]$ are the signal intensities of each component extrapolated back to a contact time of zero.

This new equation predicts biexponential rise and biexponential decay, and therefore presents two sets of $T_{NH}$ and $T_{1pH}$’s, however it is possible to use an infinite number of components. The biexponential equation was found to give a good fit here. Biexponential equations have previously been used to find various time constants (Fenwick et al., 1996; Gabrielse et al., 1994; Smernik, 2006; Smith et al., 2005) and have been shown to represent data more accurately. This is due to “fast” and “slow” cross-polarising and relaxing components being present in the one sample. This
model resulted in a superior fit to the experimental data (Figure 2.13). Plots were then made showing the two sets of time constants against one another (Figure 2.14).

![Graph showing experiment data comparison]

Figure 2.13: $^{15}$N NMR CPMAS variable contact time experiment for $^{15}$N-enriched $\alpha$-glycine (-347ppm). Model 1 is based on Equation 2.1 (exponential), while model 2 is based on Equation 2.4 (biexponential).

### 2.5. Results and Discussion

The results reported here represent changes in relaxation processes when the dried model compounds glycine and urea were a) in the presence of moisture, b) mixed together and c) diluted with KCl. The relaxation processes were calculated using data acquired by variable contact time (VCT) experiments.

It was found for all samples that a single exponential model (Equation 2.1) which predicts an exponential rise and exponential decay of the recorded signal did not fit the experimental data well (Figure 2.13). For this data the relaxation processes that occurred were better described by a model that accounts for a biexponential rise and biexponential decay (Equation 2.4). This biexponential model predicted two sets of time constants for the rise and fall of signal, as discussed in section 2.3.6. Plots were then made showing the two sets of time constants against one another, such as those seen for glycine and urea in Figure 2.14.
2.5.1. VCT Results: The Effect of Moisture

VCT experiments were first carried out on $^{15}$N-enriched urea and glycine separately at ambient moisture levels. Samples of $^{15}$N enriched glycine and urea were then dried to a constant mass to remove any moisture that may be present. VCT experiments were then carried out in order to acquire sets of time constants, and were then compared to the ambient moisture samples.

![Graph](image)

Figure 2.14: $^{15}$N NMR CPMAS variable contact time experiment for $^{15}$N-enriched urea (left) and $^{15}$N-enriched glycine (right), at ambient moisture levels, based on Equation 2.4. Graphs also show the two separated components from the biexponential model fit.

On drying, glycine and urea lost 4.3 and 2.5% of their mass respectively, indicating the amount of moisture present. The graphs obtained for both the glycine and urea samples at both dried and ambient moisture levels are shown in Figure 2.15 and Figure 2.16, while the resulting time constants are shown in Table 2.9.

**Glycine**

Results for the glycine at ambient moisture levels displayed one $T_{1\rho}H$ and two $T_{NH}$ values (Figure 2.15, Table 2.9). According to Gabrielse (1994), compounds which exhibit two different components from a biexponential model, in this case two $T_{NH}$ values, indicates that different motions within a sample may be present. Two different $T_{NH}$ values, however, can also represent two different distances between N and H atoms in a sample, therefore giving two rates of cross polarisation. The two $T_{NH}$ values of 3.94 and 0.44 msec found here show that both slow and fast cross polarising amino groups respectively are present in the one sample, even though
glycine has only a single nitrogen in its structure. This could be due to either different crystal packing within the sample, or molecules at the crystal edges acting differently from those within the crystal as they are not as attached to adjacent molecules.

Another possibility which should be considered is that the pressure experienced by the glycine crystals in the rotor may cause distances between glycine molecules, or bond distances within a molecule, to decrease. This may then cause the crystal lattice to deform and may therefore explain why two different $T_{\text{NH}}$'s have been found. Estimations of the pressure experienced in the rotor were calculated to be in the order of mega-pascals (MPa). Dawson and co-workers (2005) have previously looked at the effect of pressure on glycine polymorphs, and found that $\alpha$-glycine was stable at pressures up to 23 GPa. Therefore there should be no structural differences in $\alpha$-glycine due to NMR spinning, and hence no change in glycine $T_{\text{NH}}$ values.

The single $T_{1p}$H of 130msec suggests that the spin lattice diffusion/relaxation of protons in the rotating frame is efficient and does not vary for changes in motions found within the sample. This would suggest that only one crystal form (i.e. $\alpha$-glycine) must be present. However, the presence of moisture may also provide a mechanism for proton diffusion if extensive hydrogen bonding is taking place. The latter can be excluded as the presence of one $T_{1p}$H here, would suggest a single crystal exists, as this is consistent with XRD results reported in section 2.4.1 (Ramachandran et al., 2007) for the glycine used.

By comparing the contribution of signals from the fast and slow components ($I_{0[1]}$ and $I_{0[2]}$ from Equation 2.4), it was found that the slow cross polarising component contributed to 59% of the total signal for glycine containing moisture. It can therefore be said that 59% of glycine-N is cross polarising more slowly than the other 41%. This is most likely due to differences in mobility of the amino group. An alternative reason for the presence of two $T_{\text{NH}}$ values can be due to differences in bond length. This cause would result in slight changes in the chemical shift of the glycine peak or produce peak splitting, which are both usually associated with changes in bond distance (Ratcliffe et al., 1983) or both crystalline and amorphous sections being present (Mathias et al., 1988; Powell & Mathias, 1989). Since peak
shifts and splitting were not observed then these causes can be ruled out as an explanation. Since no new peaks were observed in the spectrum either, then this rules out the possibility that some glycine was not in its zwitterionic form of an NH$_3^+$ amino group and the N is thus highly protonated.

The dipolar dephasing time constant ($T_{2DD}$), was also found for the moist glycine sample. The high $T_{2DD}$ of 113.8msec (Table 2.9) suggests the amino group is highly mobile, as a lack of attached protons does not apply. As the formula applied to dipolar dephasing experiments produces a single $T_{2DD}$ value, this value is thought to be an average, as it has been shown from the VCT results that two different motions of the glycine amino group are present.

For dried glycine similarly, two values for $T_{NH}$, of 2.8 and 0.23msec were found, along with only one value for $T_{1ρH}$ of 99msec. The values for these time constants are significantly different to those found for the moist sample, with the dried samples exhibiting shorter cross polarisation times as a result of faster relaxation of protons under spin-lock conditions. The presence of water is expected to increase mobility of the glycine molecules, thus increasing $T_{NH}$ and $T_{1ρH}$ due to reduced dipolar interactions between molecular glycine. A decrease in the mobility of glycine is also suggested from the dipolar dephasing experiments, as the calculated $T_{2DD}$ values decreased from 113.8msec for moist glycine to 78.0msec when it was dried (Table 2.9).

The fact that there is only one $T_{1ρH}$ still observed with the removal of water, suggests that this is not due to extensive hydrogen bonding with water. While it may be due to one crystal type being present, it is also possible that the high molecular mobility of the amino group is acting as a sink for spin diffusion, therefore providing efficient relaxation on protons in the rotating frame. Efficient spin diffusion of the glycine amino groups has also recently been shown by Taylor and co-workers (2006) and discussed earlier in this chapter in section 2.3.3. If mobility is the dominant control over this spin lattice diffusion, then variation in this high level of mobility will cause a change in the $T_{1ρH}$, still producing a single value because of its dominant control.
The chemical shift peak half widths (i.e. the half height width) were measured for both the dried and moist samples of glycine. The peak half width increased from 6.4 ppm for the moist sample, to 34.7 ppm when dried. This also suggests change in mobility, as the presence of water would allow the sample to become more “liquid-like”, therefore reducing any CSA effects present.

Observability is influenced by the presence of water in samples. The presence of moisture in glycine reduced the signal intensity, measured with a contact time of 5 msec, when compared to the signal intensity for dried glycine (Table 2.9). This effect has been seen by Smernik (2006), when observing the effects of moisture content on soil organic matter. At high moisture levels (up to 22%), spectra were found to decrease slightly in intensity, however, chemical shifts and distribution of the signals were unaffected. The decrease in intensity with moisture levels was found to be mostly due to longer $T_{1pH}$ values as a result of increases molecular mobility. This should also coincide with longer $T_{NH}$ values. It is useful to note that the $T_{1H}$ values were also found to decrease with moisture content for these soil samples. This may also explain why the difference in peak widths observed for ambient and dried glycine samples examined here, as a decrease in $T_{1H}$ would show that there is less CSA, therefore leading to narrower half peak widths.

**Urea**

Urea at ambient moisture levels was found to have a single $T_{1pH}$ of 12.8 msec and two $T_{NH}$ values of 0.12 and 0.034 msec (Figure 2.16, Table 2.9). These values are lower than those of glycine, with the lower $T_{1pH}$ suggesting the crystalline form of urea is more rigid than glycine. Once again this can suggest that there is efficient proton spin-diffusion under spin-lock conditions, possibly due to a single crystal being present or extensive hydrogen bonding taking place with the presence of water.

The two $T_{NH}$ values indicate that there are probably two different mobilities present in the structure. However, the spectrum displays a slight shoulder upfield of the urea peak (Figure 2.11). This hints that there may be two slightly different crystal types present. The $T_{NH}$ values are much smaller than those found for glycine, showing that urea is cross polarising more rapidly. The fact that in their crystalline form, urea has
an NH₂ group while glycine has an NH₃ group would suggest that cross polarisation should be faster for glycine due to higher protonation, however, this was not found here. This is most likely due to higher molecular motions found around the glycine αC-NH₃, which also explain the longer T₁pH and T₂DD values also found for glycine.

Williams and McDermott (1993) have previously compared the activation energies for glycine-NH₃ and urea-NH₂ rotation (Table 2.6, section 2.3.3). While glycine must break three stronger N-H bonds in order to rotate, compared to urea that needs to break only two weaker bonds, the activation energy required to rotate the glycine NH₃ group is smaller. It was suggested that this maybe is due to urea’s C-N bond acting as a partial double bond (C=N), which is more rigid and less able to rotate. Hence the results acquired here for urea also show this crystal structure is more rigid than glycine. This view is further supported by the smaller T₂DD value of 17.6msec found for the moist urea (Table 2.9) compared to the T₂DD value of 113.8msec for the moist glycine. Urea also has strong NH…O bonds between tapes (Table 2.6, section 2.3.3), while glycine has weaker van der waals forces holding its ribbons/sheets together (Williams and McDermott 1993). Therefore weak dipolar interactions would lead to longer Tₐ’s needed, which may be why Tₐ is longer for glycine than it is for urea.

As discussed previously in section 2.3.3, there are two types of motion present within the urea crystal lattice; cis-trans and whole body rotations (Figure 2.6). If a whole urea molecule flips around its carbonyl, then the two NH₂ groups will undergo the same motions as the molecule turns. Only one side of the adjacent urea molecules however, will be affected by this flipping. When the urea flips, it also causes steric interactions between the adjacent urea molecules, causing the bond length between H and N on the flipping urea molecule to be affected and therefore become shorter. On the other hand, if cis-trans rotation occurs, it affects only one NH₂ group, and therefore only one will experience this motion. Therefore both types of motions found in urea crystals could explain why two Tₐ values occur. At ambient temperature however, which is relevant for this research, the cis-trans rotations happen a lot more often than whole body rotations (Table 2.6).
Upon drying the urea sample, relaxation analysis produced two components for $T_{1pH}$ equal to 3.4 and 15.9 msec, and two components for $T_{NH}$ equal to 0.21 and 0.015 msec. The appearance of a second $T_{1pH}$ value, would imply that the single value that was found for the moist sample, was due to efficient diffusion of protons in the rotating frame brought on by inter-dipolar interactions between the urea and water molecules. The two $T_{1pH}$’s found for the dried sample, along with the peak shoulder which is also observed in the dried urea spectrum, suggests that two crystallographically different structures may be present, i.e. the presence of both a more crystalline and a more amorphous structure. This could also be due to different mobilities existing between the two NH$_2$ groups found in urea.

It is useful to compare the single $T_{1pH}$ value, for the moist urea, of 12.8 msec with the two $T_{1pH}$ values for the dried urea, with one value of 15.9 msec being higher and one lower value of 3.4 msec. Comparing the single value for the moist sample with the higher value obtained from the dried sample, suggests that the presence of water assists in spin diffusion or a slight increase in mobility due to the lack of hydrogen bonding with water molecules. The lower $T_{1pH}$ is most likely due to a more crystalline component of the dried sample becoming more rigid with the absence of water.

The two $T_{NH}$ values for dried urea, on the other hand, are higher than those found for the moist sample. One explanation that could be argued indicates that this could be due to bond lengths between urea molecules being longer than those between urea and a water molecule. If this explanation is true there would need to be a chemical shift difference between moist and dried urea sample peaks. This difference in the spectrum was not observed, indicating that differences in bond length were not present. Increased $T_{NH}$ values could also be brought about by increased mobility. The half peak height width changes that occurred due to drying samples were different for glycine and urea samples. For glycine the width increased from 6.4 to 34.7 ppm while for urea the width decreased from 94.9 to 74.5 ppm upon drying. For urea this could be because of a possible decrease in $T_1H$ values.
Recent research by Taylor and co-workers (2007), found that $T_1$ for urea is 67 minutes. Delay times needed for CPMAS experiments therefore need to be at least 5 times this value (over 5 hours for one scan!) in order to acquire quantitative spectra. Because delay times used for the urea $^{15}$N NMR experiments were only 5 seconds, thus saturating the signal, which therefore leads to a strong decrease in observability compared to dried glycine is expected. If there were any changes in $T_1$ upon drying of the urea sample, this would result in differences in the observability of spectra due to slight changes in saturation.. It was found here that upon drying of urea, a significant increase in observability was seen (Table 2.9), especially at a contact time of 1 msec. This would indicate that there has been a decrease in $T_1$, which would also explain the increase in peak half width with drying.

2.5.2. VCT Results: The Effect of Compound Mixing

Mixes of dried urea and glycine were made, in order to see whether these two compounds, which are widely found in nature, have an affect on one another’s time constants and observabilities. The mixtures are expressed as a percentage of the total millimoles present in the mixture, and are shown in Table 2.11. Separate VCT plots were made for each varied mix of glycine and urea enabling time constants to be calculated for the different mixes. These VCT plots for different mixes are displayed for the glycine peak at -347 ppm and the urea peak at -302 ppm in Figure 2.17 and Figure 2.18 respectively. The VCT for the 19% urea was not found, as the signal to noise ratio of the $^{15}$N NMR spectrum for that particular mixture made integration of the signal impossible to obtain.

The biexponential model equation used for these mixtures showed that glycine had one $T_{1\rho}$ and two $T_{NH}$’s for all mixtures. Therefore, even with increasing urea content, glycine exhibited efficient relaxation of protons in the rotating frame. This is consistent with the single $T_{1\rho}$ values, as was found for 100% dried glycine in the previous section. The urea crystals present in all of these mixtures also acted similarly to the 100% dried urea sample discussed earlier, in that it has two $T_{1\rho}$ and two $T_{NH}$’s.
Results for glycine showed that no significant changes for time constants or peak widths were recorded with increasing urea content, and therefore decreasing glycine content (Table 2.11). This is despite the fact that with an increase in urea, there is a decrease in the N : H ratio of the mixtures and an increase in possible NH…O bonds available between the two compounds present. Observabilities also remained roughly unchanged relative to uncertainties accumulated between NMR tuning stability and repeated integrations (approx. ± 10-15%). Averages of $T_{1\rho}H$, $T_{NH}$, %observability and peak half width provided uncertainties of between 2 and 15% based on standard deviation with n equal to 5. This is also seen in Figure 2.17. These results suggest that glycine is either not experiencing any intermolecular bonding or dipolar interactions with the urea molecules present, or any interactions which may be taking place, are not affecting glycine to any extent that enables a change to be observed.

Urea on the other hand, shows significant changes depending on the mixture with glycine. It was observed that as the glycine content increased, the set of $T_{NH}$’s increased accordingly. When $T_{NH}$ is plotted against the % urea present in the mixture, a linear relationship was found (Figure 2.21a). Each of the $T_{1\rho}H$ values, in the set, acted differently with increased glycine content. The first component showed a small increase, while the second component showed larger increases. When plotted against the %urea present, the first component showed a linear trend, while the second component change was best described by fitting an exponential line of best fit (Figure 2.22a).

The percentage of observability for the urea $^{15}$N NMR spectra, generated for different mixtures, show that with increasing glycine content there is a significant increase in observability. This can be seen in Figure 2.18. As discussed previously, this may due to a decrease in saturation of the signal causing a decrease in $T_{1\rho}H$. This may be due to interactions between urea and glycine, leading to the glycine amino group acting as a sink for the relaxation of protons in the urea molecules. This would depend on the distance between adjacent molecules.

The increase in observability of urea upon mixing with increasing amounts of glycine implies that urea is being influenced by molecular interactions with glycine. It is unusual, however, that glycine does not show any affects if this interaction occurs.
An explanation for this anomaly could be that the dilution of the urea molecules causes changes in the time constants, rather than interactions with glycine.

### 2.5.3. VCT Results: The Effect of Dilution

To find out if dilution was affecting TNH and T1pH values obtained for the glycine and urea mixes, mixtures of urea with potassium chloride (KCl) and glycine with KCl were made. KCl was used here as an inert diluent that does not interact with the urea, but acts to separate the urea crystals. VCT graphs for glycine and urea when mixed with KCl can be viewed in Figure 2.19 and Figure 2.20 respectively. The results are compared here with either 100% glycine or 100% urea. All sample mixtures were dried to a constant mass before performing NMR experiments.

Results for glycine mixed with KCl showed no real changes in T_{NH}, half peak width or observability results within uncertainties (Table 2.10). T_{1p}H was found to be slightly lower than the results obtained with the glycine and urea mixtures. This may be due to the very small amount of glycine used in the mixture with KCl (8.9%), as only a tiny amount of 15N-enriched glycine was available. This dilution of glycine may have finally broken down the efficiency of spin diffusion between protons in the rotating frame.

Results for urea (Table 2.10) reveal that both sets of time constants, that is a set for T_{1p}H and T_{NH}, change due to dilution with KCl, with the second components in the sets of T_{NH} and T_{1p}H exhibiting the largest changes. To identify if these changes correlated with those found in the urea and glycine mixtures, T_{NH} and T_{1p}H results for the two different urea and KCl mixtures were plotted with the urea and glycine mixture results against the percentage urea present in the mixture (Figure 2.21b and Figure 2.22b respectively). If graphs show that the line of best fit still fits all the data points presented, then the differences in time constants in the glycine and urea mixtures must be due to dilution (otherwise the KCl mixture data wouldn’t fit). If the urea mixed with KCl data does not fit, then changes in the urea and glycine mixtures must be due to interactions taking place between these two compounds.
In the plot of $T_{NH}$ versus percent urea, $R^2$ values (i.e. correlation coefficients), were found to decrease significantly for the fast relaxing $T_{NH}$ component ($R^2 = 0.906$ down to 0.553). It can therefore be suggested that the changes in the fast cross polarising $T_{NH}$’s should in part be due to the interactions with glycine in the urea and glycine mixtures. When the urea and KCl mixture results were added to the plot of slow cross-polarising $T_{NH}$’s, the $R^2$ values decreased from 0.89 to 0.84. The changes measured by this set of relaxation parameters would imply that the nitrogen is acting differently due to dilution.

In the plots of $T_{1pH}$ versus percent urea, the first component of the $T_{1pH}$ values measured for the KCl mixtures, strongly disagreed with those found in the urea and glycine mixtures, with the $R^2$ values dropping from 0.969 to 0.192 when the KCl mix data was added. It can therefore be said that this component of the urea crystal is acting differently in the glycine mixtures due to interactions with the glycine. The second component of the $T_{1pH}$’s for the KCl mixture strongly agreed with the data from the glycine mixtures, with $R^2$ values changing from 0.992 to 0.987. This puts forward the argument that this component of the urea nitrogen is strongly influenced by dilution, and virtually no interactions with glycine are taking place.

Observability of the urea spectrum upon mixing with KCl was found to increase with increase in KCl content; however, observability was the same for both mixtures when the urea content varied. Therefore the increase in observability cannot be attributed to dilution, and is still most likely due to some interactions, which probably affect the spin diffusion rate through the glycine molecules present in urea and glycine mixtures.
Figure 2.15: $^{15}$N NMR CPMAS variable contact time experiments for $^{15}$N-enriched glycine (-347ppm) at ambient moisture and dried at 140°C until a constant mass was reached. Intensities have been corrected for the amount of N (mmol) present in each sample.

Figure 2.16: $^{15}$N NMR CPMAS variable contact time experiments for $^{15}$N-enriched urea (-302ppm) at ambient moisture and dried at 70°C until a constant mass was reached. Intensities have been corrected for the amount of N (mmol) present in each sample.
Figure 2.17: $^{15}$N NMR CPMAS variable contact time experiments for $^{15}$N-enriched glycine (-347ppm) and glycine when mixed with different amounts of $^{15}$N-enriched urea. Intensities have been corrected for the amount of N (mmol) present in each sample.

Figure 2.18: $^{15}$N NMR CPMAS variable contact time experiments for $^{15}$N-enriched urea (-302ppm) and urea when mixed with different amounts of $^{15}$N-enriched glycine. Intensities have been corrected for the amount of N (mmol) present in each sample.
Figure 2.19: $^{15}$N NMR CPMAS variable contact time experiments for $^{15}$N-enriched glycine (-347ppm) and glycine when mixed with different amounts of KCl. Intensities have been corrected for the amount of N (mmol) present in each sample.

Figure 2.20: $^{15}$N NMR CPMAS variable contact time experiments for $^{15}$N-enriched urea (-302ppm) and urea when mixed with different amounts of KCl. Intensities have been corrected for the amount of N (mmol) present in each sample.
Table 2.9: VCT results for $^{15}$N enriched urea and glycine samples at both ambient moisture levels, and after drying. All dried samples were dried to a constant mass.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peak $^{\frac{1}{2}}$ Width (ppm)</th>
<th>$T_{NH}$ (msec)</th>
<th>$T_{1\rho\rho\rho}$ H (msec)</th>
<th>$\alpha_{[1]}$</th>
<th>$T_{NH}$ (msec)</th>
<th>$T_{1\rho\rho\rho}$ H (msec)</th>
<th>$\alpha_{[2]}$</th>
<th>%I$<em>{0[1]}$ : %I$</em>{0[2]}$</th>
<th>t = 1 msec</th>
<th>t = 5 msec</th>
<th>T$_{2DD}$ (usec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Glycine (Dried)</td>
<td>34.7</td>
<td>2.78</td>
<td>99</td>
<td>0.97</td>
<td>0.23</td>
<td>99</td>
<td>1.00</td>
<td>59 : 41</td>
<td>100.0%</td>
<td>100.0%</td>
<td>78.0</td>
</tr>
<tr>
<td>100% Glycine (Moisture)</td>
<td>6.4</td>
<td>3.94</td>
<td>130</td>
<td>0.97</td>
<td>0.44</td>
<td>130</td>
<td>1.00</td>
<td>69 : 31</td>
<td>102.7%</td>
<td>90.6%</td>
<td>113.8</td>
</tr>
<tr>
<td>100% Urea (Dried)</td>
<td>74.5</td>
<td>0.21</td>
<td>3.4</td>
<td>0.94</td>
<td>0.015</td>
<td>15.9</td>
<td>1.00</td>
<td>72 : 28</td>
<td>1.17%</td>
<td>0.36%</td>
<td>12.0</td>
</tr>
<tr>
<td>100% Urea (Moisture)</td>
<td>94.9</td>
<td>0.12</td>
<td>12.8</td>
<td>0.99</td>
<td>0.034</td>
<td>12.8</td>
<td>1.00</td>
<td>58 : 42</td>
<td>0.31%</td>
<td>0.14%</td>
<td>17.6</td>
</tr>
</tbody>
</table>

^ % Observability is compared to the 100% Dried Glycine sample. Results are corrected for amount of sample in the rotor and millimole of $^{15}$N present for both urea and glycine.

Table 2.10: VCT results for $^{15}$N enriched urea and glycine samples mixed with KCl. All samples have been dried to a constant mass.

<table>
<thead>
<tr>
<th>% Compound 1 in Mix *</th>
<th>Peak $^{\frac{1}{2}}$ Width (ppm)</th>
<th>$T_{NH}$ (msec)</th>
<th>$T_{1\rho\rho\rho}$ H (msec)</th>
<th>$\alpha_{[1]}$</th>
<th>$T_{NH}$ (msec)</th>
<th>$T_{1\rho\rho\rho}$ H (msec)</th>
<th>$\alpha_{[2]}$</th>
<th>%I$<em>{0[1]}$ : %I$</em>{0[2]}$</th>
<th>t = 1 msec</th>
<th>t = 5 msec</th>
<th>T$_{2DD}$ (usec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Glycine</td>
<td>34.7</td>
<td>2.8</td>
<td>99</td>
<td>0.97</td>
<td>0.23</td>
<td>99</td>
<td>1.00</td>
<td>59 : 41</td>
<td>100.0%</td>
<td>100.0%</td>
<td></td>
</tr>
<tr>
<td>8.9% Glycine (+ 91.1% KCl)</td>
<td>34.4</td>
<td>2.4</td>
<td>101</td>
<td>0.98</td>
<td>0.22</td>
<td>101</td>
<td>1.00</td>
<td>64 : 36</td>
<td>111.2%</td>
<td>113.0%</td>
<td></td>
</tr>
<tr>
<td>100% Urea</td>
<td>74.5</td>
<td>0.21</td>
<td>3.4</td>
<td>0.94</td>
<td>0.015</td>
<td>15.9</td>
<td>1.00</td>
<td>72 : 28</td>
<td>1.17%</td>
<td>0.36%</td>
<td></td>
</tr>
<tr>
<td>54.6% Urea (+ 47.4% KCl)</td>
<td>71.7</td>
<td>0.25</td>
<td>6.0</td>
<td>0.96</td>
<td>0.069</td>
<td>2.8</td>
<td>0.98</td>
<td>59 : 41</td>
<td>2.33%</td>
<td>0.55%</td>
<td></td>
</tr>
<tr>
<td>19.5% Urea (+ 80.5% KCl)</td>
<td>67.0</td>
<td>0.39</td>
<td>4.0</td>
<td>0.90</td>
<td>0.077</td>
<td>0.9</td>
<td>0.92</td>
<td>65 : 35</td>
<td>2.31%</td>
<td>0.65%</td>
<td></td>
</tr>
</tbody>
</table>

* % is based on millimoles of model compound present.

^ % observability is compared to the 100% Glycine sample. Results are corrected for amount of sample in the rotor and millimole of $^{15}$N present for both urea and glycine.
Table 2.11: VCT results for two set time parameter results for $^{15}$N enriched urea and glycine samples, and mixtures of both. All samples have been dried to a constant mass.

<table>
<thead>
<tr>
<th>% Compound 1 in Mix * (+ % Compound 2)</th>
<th>Peak $^{1/2}$ Width (ppm)</th>
<th>Possible O…H…N bonds</th>
<th>N:H ratio</th>
<th>$T_{NH}$ (msec)</th>
<th>$T_{1P}$ (msec)</th>
<th>$\alpha_{[1]}$</th>
<th>$T_{NH}$ (msec)</th>
<th>$T_{1P}$ (msec)</th>
<th>$\alpha_{[2]}$</th>
<th>%I$<em>{0[1]}$/ %I$</em>{0[2]}$</th>
<th>t = 1 msec</th>
<th>t = 5 msec</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Glycine</td>
<td>34.7</td>
<td>3.0</td>
<td>5.0</td>
<td>2.8</td>
<td>99</td>
<td>0.97</td>
<td>0.23</td>
<td>99</td>
<td>1.00</td>
<td>59 : 41</td>
<td>100.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>81.0% Glycine (+ 19.0% Urea)</td>
<td>36.0</td>
<td>3.2</td>
<td>4.0</td>
<td>2.0</td>
<td>103</td>
<td>0.96</td>
<td>0.21</td>
<td>103</td>
<td>1.00</td>
<td>60 : 40</td>
<td>104.0%</td>
<td>103.3%</td>
</tr>
<tr>
<td>65.6% Glycine (+ 34.4% Urea)</td>
<td>35.1</td>
<td>3.3</td>
<td>3.3</td>
<td>2.1</td>
<td>111</td>
<td>0.98</td>
<td>0.18</td>
<td>111</td>
<td>1.00</td>
<td>67 : 33</td>
<td>103.1%</td>
<td>111.3%</td>
</tr>
<tr>
<td>44.8% Glycine (+ 55.2% Urea)</td>
<td>35.1</td>
<td>3.6</td>
<td>2.9</td>
<td>2.0</td>
<td>110</td>
<td>0.98</td>
<td>0.22</td>
<td>110</td>
<td>1.00</td>
<td>65 : 35</td>
<td>109.6%</td>
<td>114.9%</td>
</tr>
<tr>
<td>17.6% Glycine (+ 82.4% Urea)</td>
<td>34.8</td>
<td>3.8</td>
<td>2.3</td>
<td>2.3</td>
<td>108</td>
<td>0.98</td>
<td>0.19</td>
<td>108</td>
<td>1.00</td>
<td>69 : 31</td>
<td>99.3%</td>
<td>108.5%</td>
</tr>
<tr>
<td>100% Urea</td>
<td>74.5</td>
<td>4.0</td>
<td>2.0</td>
<td>0.21</td>
<td>3.4</td>
<td>0.94</td>
<td>0.015</td>
<td>15.9</td>
<td>1.00</td>
<td>72 : 28</td>
<td>1.17%</td>
<td>0.36%</td>
</tr>
<tr>
<td>82.4% Urea (+ 17.6% Glycine)</td>
<td>78.5</td>
<td>3.8</td>
<td>2.3</td>
<td>0.24</td>
<td>4.5</td>
<td>0.95</td>
<td>0.020</td>
<td>6.5</td>
<td>1.00</td>
<td>66 : 34</td>
<td>1.32%</td>
<td>0.41%</td>
</tr>
<tr>
<td>55.1% Urea (+ 44.9% Glycine)</td>
<td>77.2</td>
<td>3.6</td>
<td>2.9</td>
<td>0.26</td>
<td>5.1</td>
<td>0.95</td>
<td>0.024</td>
<td>2.9</td>
<td>0.99</td>
<td>68 : 32</td>
<td>1.83%</td>
<td>0.51%</td>
</tr>
<tr>
<td>34.4% Urea (+ 65.6% Glycine)</td>
<td>71.0</td>
<td>3.3</td>
<td>3.3</td>
<td>0.33</td>
<td>6.0</td>
<td>0.94</td>
<td>0.037</td>
<td>1.2</td>
<td>0.97</td>
<td>68 : 32</td>
<td>2.81%</td>
<td>0.79%</td>
</tr>
</tbody>
</table>

* % is based on millimoles of model compound present.

^ % observability is compared to the 100% Glycine sample. Results are corrected for amount of sample in the rotor and millimole of $^{15}$N present for both urea and glycine.
Figure 2.21: Percent urea present versus the resulting two urea $T_{NH}$ values for a) urea + glycine mixes only, b) urea + glycine and urea + KCl mixes

Figure 2.22: Percent urea present versus the resulting two urea $T_{1pH}$ values for a) urea + glycine mixes only, b) urea + glycine and urea + KCl mixes
2.5.4. DDCPMAS

As discussed in section 2.4.2, it was found that the dipolar dephasing curves produced by plotting the natural log of intensity vs. the dephasing delay $t$, was best explained by an exponential decay equation (Equation 2.2). For both $^{15}\text{N}$ enriched urea and glycine samples, an array of 15 data points between 0 and 45 $\mu$sec (Figure 2.12), was compared to a two data point experiment which included delays at 0 and 45 $\mu$sec only (Table 2.8). The reason for running these experiments is due to unenriched organic N samples needing long experimental times; therefore it is not feasible in most cases to obtain more than two data points. Comparisons of the two sets of experiments showed that they were in agreement if the two-point experiment were assumed to have an error of approximately 10% as discussed in section 2.4.2.

The samples of $^{15}\text{N}$-enriched glycine and urea were then compared to unenriched amino acids histidine, lysine, asparagine and arginine, uric acid and ammonium nitrate at both ambient moisture levels, and after being dried (with the exception of uric acid). All samples were run using the two point experiments, with $t = 0$ and 45 $\mu$sec. Comparisons of spectra obtained at 0 and 45 $\mu$sec can be found for dried urea and glycine in Figure 2.11 (right). Spectra for dried histidine, asparagine, arginine, lysine and uric acid are shown in Figure 2.23. Results for $T_{2DD}$ values found are shown in Table 2.12. It was observed that $T_{2DD}$ values for both dried and ambient moisture samples fell into groups, where $T_{2DD}$ for $\text{NH}_4 \approx \text{NH}_2 < \text{NH} \approx \text{N} < \text{NH}_3$ in general for samples at both ambient moisture levels and when dried.

The increased mobility of glycine’s amino $\text{NH}_3$ group in the presence of moisture, as discussed in section 2.5.1, is further confirmed here as a higher $T_{2DD}$ value of 113.8$\mu$sec was observed when the sample was at an ambient moisture level. This is due to the decreased strength of dipolar interactions that comes about because of the increased mobility. Hence when the sample is dried, there is less mobility, therefore stronger dipolar coupling, and therefore a reduced $T_{2DD}$ of 96.8$\mu$sec. An alternate reason for differences in $T_{2DD}$ values of glycine in the presence of water could be that the water protons are interacting with the glycine. However, if this was the case it would be expected that this would cause better cross polarisation, and therefore lower $T_{2DD}$ values, both of which were not found here, with both $T_{\text{NH}}$ and $T_{2DD}$ values for...
increasing with the presence of water (as shown in Table 2.9 and Table 2.12 respectively).

Increased T_{2DD} values were also found in all the amino groups for the amino acids analysed, with the exception of lysine. This exception may be due to the NH_{2} group in lysine experiencing high molecular motions in the presence of moisture, and when dried. The NH_{2} peak in the lysine ^{15}N spectrum is absent most likely due to extremely high mobility, with only the amino peak at −339ppm observed (Figure 2.23). When dried, this NH_{2} group may become slightly more rigid, allowing the NH_{3} amino group to become more mobile, explaining the higher T_{2DD} when dried. With the other amino acids, the amounts T_{2DD} decreased with drying did not show a relationship with the moisture content (Table 2.7), with arginine showing the smallest difference in T_{2DD} values, but the highest moisture content.

The rigidity of urea, as shown by the very fast cross polarisation and T_{1ρH} times compared to glycine (Table 2.9), was further confirmed here with the resulting small T_{2DD} values of 17.6 and 15.3μsec when moist and dried respectively. Large differences between moist and dried T_{2DD} values were not observed for compounds containing NH_{4}, NH_{2}, NH and N groups as compared to the amino groups that did. This reveals that time constants for amino-N are generally influenced more strongly by the presence of water.
Figure 2.23: The resulting $^{15}$N DD-CPMAS NMR spectra produced for uric acid at ambient moisture levels and the dried model amino acids histidine, lysine, arginine and asparagine where $t = 0$ and 45 µsec.
Table 2.12: Results for $T_{2\text{DD}}$ of model compound run as found at ambient moisture levels, compared to the same compounds dried to a constant mass.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Shift (ppm)</th>
<th>$T_{2\text{DD}}$ (µsec)</th>
<th>Ambient</th>
<th>Dried</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp NH$_2$</td>
<td>-265.8</td>
<td>12.4</td>
<td>12.7</td>
<td></td>
</tr>
<tr>
<td>*NH$_4$NO$_3$</td>
<td>-350.0</td>
<td>15.1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Urea NH$_2$</td>
<td>-301.0</td>
<td>17.6</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>Arg NH$_2$</td>
<td>-308.3</td>
<td>19.4</td>
<td>15.4</td>
<td></td>
</tr>
<tr>
<td>*NH$_4$NO$_3$</td>
<td>-358.4</td>
<td>23.3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Arg NH</td>
<td>-300.9</td>
<td>25.3</td>
<td>16.3</td>
<td></td>
</tr>
<tr>
<td>Arg NH$_3$ (amino)</td>
<td>-342.7</td>
<td>26.9</td>
<td>22.6</td>
<td></td>
</tr>
<tr>
<td>Uric Acid Peak 3 ^</td>
<td>-260.0</td>
<td>27.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>His NH</td>
<td>-203.9</td>
<td>28.4</td>
<td>27.8</td>
<td></td>
</tr>
<tr>
<td>Uric Acid Peak 2 ^</td>
<td>-246.0</td>
<td>33.4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>His N</td>
<td>-190.5</td>
<td>39.3</td>
<td>35.4</td>
<td></td>
</tr>
<tr>
<td>Arg NH (double bond)</td>
<td>-291.4</td>
<td>39.8</td>
<td>33.4</td>
<td></td>
</tr>
<tr>
<td>Uric Acid Peak 4 ^</td>
<td>-264.0</td>
<td>57.6</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lys NH$_3$ (amino)</td>
<td>-338.7</td>
<td>58.1</td>
<td>77.4</td>
<td></td>
</tr>
<tr>
<td>Asp NH$_3$ (amino)</td>
<td>-340.2</td>
<td>76.3</td>
<td>62.4</td>
<td></td>
</tr>
<tr>
<td>NH$_4$*NO$_3$</td>
<td>-4.8</td>
<td>78.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Uric Acid Peak 1 ^</td>
<td>-228.0</td>
<td>82.4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>His NH$_3$ (amino)</td>
<td>-333.0</td>
<td>102.7</td>
<td>77.3</td>
<td></td>
</tr>
<tr>
<td>Gly NH$_3$ (amino)</td>
<td>-347.0</td>
<td>113.8</td>
<td>96.8</td>
<td></td>
</tr>
</tbody>
</table>

^ Uric acid contains four heterocyclic NH groups. Specific peak assignments however, have not been made here.
2.6. Conclusions

The objective of this chapter has been to optimise solid-state $^{15}$N NMR spectroscopy techniques and characterise $^{15}$N NMR spectra for different nitrogen chemical environments. This was achieved by undertaking $^{15}$N NMR VCT experiments which allowed observations as to how different N-environments affect various time constants. This work is important as the study of samples containing natural organic matter, which includes soil, plant, animal and insect material to name a few, involves mixes of many different N-species and chemical environments. This chapter has pointed out that N-containing compounds involved in mixtures can have an affect on total NMR signal acquisition as well as nuclei relaxation parameters. This would mean that the both qualitative and quantitative N spectral information will be affected.

In this research, a biexponential model equation was used for $^{15}$N NMR CPMAS VCT experiments, and was found to fit data acquired for N-containing compounds more accurately than an exponential equation. These samples included both pure samples of urea and glycine, or mixtures of the two compounds. It was therefore demonstrated that there are at least two differently relaxing components for both the organic model compounds urea and glycine, as well as mixtures of the two. The two sets of time constants $T_{1pH}$ and $T_{NH}$ obtained for samples, as well as the NMR observability of $^{15}$N spectra attained, were found to be influenced by several factors. These included the ratio of compounds in samples mixtures, the presence of moisture and sample dilution.

The $\alpha$-glycine polymorph was used throughout this research. It was pointed out that before running NMR experiments, it is important to know the polymorphic form present in the sample, as differences in glycine structure could give different time constants. The glycine amino group was found to be highly mobile, with VCT results exhibiting a single high $T_{1pH}$ value while DDCPMAS revealed a high $T_{2DD}$ value. This single $T_{1pH}$ indicates that there is efficient spin diffusion occurring, and it is therefore likely that this amino group is acting as a sink for proton spin lattice relaxation. Mobility of the amino group and possibly the glycine molecule itself is
increased when in the presence of moisture. This was seen by increases in $T_{1\rho H}$, $T_{NH}$ and $T_{2DD}$ when moisture was present, along with a decrease in peak width. No major effects on time constants were observed when mixing with urea or KCl.

Urea on the other hand, was found to be more rigid than glycine, exhibiting faster $T_{NH}$ and $T_{1\rho H}$ values, as well as a low $T_{2DD}$ value. The two $T_{NH}$ components obtained, however, suggest that there are two different mobilities present in the urea sample, possibly due to cis-trans and whole body rotations. Differences were found between urea time constants $T_{1\rho H}$ and $T_{NH}$ when dried and in the presence of water. When dry $T_{1\rho H}$ was found to split into two components, whereas in the presence of moisture only a single $T_{1\rho H}$ was observed. This would suggest that there were some interactions between the urea crystals and water molecules when present. It may be that the water was acting as a sink for spin diffusion, causing only a single $T_{1\rho H}$ to be observed.

Higher observabilities of the $^{15}\text{N}$ NMR signal were found for dried urea samples. Peak widths were found to decrease upon drying, most likely due to lower $T_{1H}$ values. As the recycle time needed for urea CPMAS experiments is in the order of hours, and the delay times used in these experiments were in the order of seconds, signal saturation may have occurred. If $T_{1H}$ values have been lowered however, there should be less saturation occurring. Therefore an increase in NMR observability would be expected, and would explain the results obtained for urea.

Urea time constants were shown to be sensitive to mixing with glycine content, and dilution with KCl. The urea $^{15}\text{N}$ NMR spectra obtained for urea and glycine mixtures showed increases in both $T_{NH}$ components and one of the $T_{1\rho H}$ components. The second $T_{1\rho H}$ component decreased. The ratio of urea and glycine in mixtures also affected the observability of the urea NMR spectrum. It was found that as the glycine content increased, so did the observability. Changes in both time constant and total NMR signal intensity were found to be due in part to interactions with the glycine, and also due to dilution of the urea molecules. Increases in observability with dilution were also shown when urea was mixed with KCl.

$^{15}\text{N}$ NMR DD-CPMAS was undertaken on samples of organic and inorganic N-containing compounds, including glycine, urea, histidine, lysine, asparagine and
arginine, uric acid and ammonium nitrate. These were analysed at both ambient moisture levels and after drying using two dipolar dephasing times of 0 and 45 µsec. Results showed that $T_{2DD}$ values fell into general groups, where $T_{2DD}$ values for $\text{NH}_4 \approx \text{NH}_2 < \text{NH} = N < \text{NH}_3$ for samples at both ambient moisture levels and when dried. Large differences between moist and dried $T_{2DD}$ values for amino groups in particular, backing up VCT experimental results, that they influenced by water content.

In subsequent Chapters, the changes in N chemical environments in plant, soil and insect material will be examined using $^{15}\text{N}$ NMR spectroscopy. It is therefore inherent that these samples will contain many different N-species. This research has exhibited that mixed model compounds can have an effect on each other, and therefore similar effects may occur in natural organic matter. The presence of small amounts of moisture has also been shown to cause large differences in nuclei relaxation parameters. These results therefore need to be taken into account when analyzing samples which may contain mixtures of different N-containing compounds.

2.7. References


CHAPTER THREE

EFFECTS OF NITROGEN FORM ON PLANT NUTRIENT UPTAKE AND PARTITIONING

3.1. Introduction

As discussed in Chapter 1, a sustainable forest plantations industry is one of the main aims of the Australian Forestry Industry (Australia's Forests at a Glance, 2007). Increases in the global demand for wood based products require intensive development of plantation management strategies that will lead to enhanced forest productivity and environmental benefits. To achieve this, a better understanding of forest nutrient cycles, quality indicators in both plant and soil, and the effect different management practices have on these properties are required.

While the application of management practices to weeding, thinning, pruning, planting (Chapter 1, Table 1.1) and tree breeding schemes can increase productivity, plantation forests cannot be successful unless nutrient requirements of the trees are optimised. Soil nutrient levels depend on the location and use of land. Plantation soils are detrimentally affected by disturbances such as harvesting, with the removal of the preceding rotation’s trees and which therefore results in the export of nutrients from the system (Beever et al., 2007; Lewis & Ferguson, 1993). Plantations are therefore reliant on the use of fertilisers, particularly to increase nitrogen levels in soils.

In this Chapter the effect of fertilizer management is studied with respect to plant uptake and assimilation of nitrogen from different sources commonly present in fertilizers. Both softwood and hardwood plant species that are of importance to Queensland Forestry were used. The studies were designed to understand the effect of fertiliser type on the nitrogen uptake, partitioning and relative composition of different nitrogen containing chemical environments within the plant using $^{15}$N NMR spectroscopy. It was also important to investigate whether there is a link between the
fertilizer nitrogen uptake and the overall carbon gain. Although it is known that the influx and efflux of both cationic and anionic nutrients take place while maintaining the electrical neutrality in the plant, it is of interest to investigate whether any links exist between fertilizer nitrogen ions and the uptake of other nutrient ions by the plants. These studies were carried out using C/N data, $^{13}$C NMR spectroscopy and ion chromatography (IC).

While studies into the uptake of nitrogen in different forms and at different concentrations are common, the use of NMR spectroscopy will provide the edge to identify relative qualitative and quantitative changes in nitrogen chemical environment during metabolic processes within the plant. Differences in the form and distribution of N within plant material could therefore have an effect on tree growth and wood quality, as well as plantation soil quality considering that these harvest plant residues are left to decompose and expected to introduce nutrients back to the soil. This aspect is covered in Chapter 4. While Chapter 1 gave a review of N uptake and assimilation in plants, the subsequent section of this chapter will focus on tree species grown by the forestry industry and their recommended fertilizers, the uptake processes with respect to fertilizer species and recent research into N cycling in trees.

### 3.1.1 Fertilisers

**Use in Plantations**

Nitrogen, phosphorus and potassium (N, P, K) are the nutrients required in the greatest amounts for plant growth in general, with N being the most growth limiting. Other macronutrients required in large amounts include sulphur, calcium and magnesium (S, Ca, Mg). Micronutrients, which are needed only in trace amounts, include manganese, zinc, iron, boron, copper and molybdenum (Mn, Zn, Fe, B, Cu, Mo). Many fertiliser companies provide inorganic fertiliser mixtures that reflect the needs of specific crop varieties across most agricultural soils.

Nutrient deficiencies can result in growth disorder, sickness and deformations in trees that may also result in becoming more susceptible to insect attacks, and tree mortality. All of these are potentially damaging to plantation productivity and the quality of wood produced. By supplementing the naturally occurring nutrients in
plantation soils with man-made fertilisers, most of these deficiencies and imbalances can be corrected. In a forest plantation this supplementation is needed, as with each harvest, nutrients that have become stored in the tree’s woody biomass are removed. An example of the amount of N removed upon harvesting of plantation radiata pine has been compiled by Lewis & Ferguson (1993) and shown in Table 3.1. Coupled with this export of nutrients, comes a heavy requirement for nutrients by the next rotation’s seedlings. Consequently these disturbances have a substantial effect on nutrient cycling systems, in particular the N cycle, and therefore require N-containing fertilisers to help balance the nitrogen loss due to removal of trees for wood production. (Blumfield & Xu, 2006; Blumfield et al., 2004; Lewis & Ferguson, 1993; Schreiner & Scagel, 2006; Williams & Woinarski, 1997).

Large amounts of litter-fall from a tree, and therefore potential nutrients returned to the soil, counteract the leaching of nutrients from the system. When large trees are harvested, however, and young trees are planted in the next rotation, the amount of litter-fall is greatly reduced, and hence not enough nutrients to counteract the leaching processes. The lack of nutrients due to leaching can also arise from the time period it takes for the litter-fall to decompose and then to have it available for the uptake by the plants. For these reasons, man-made fertilisers are needed and may likely have to be increased over successive rotations (Laclau et al., 2003).

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Stand Basis</th>
<th>Dry wt. Logged (kg/ha)</th>
<th>N Drain in log (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>Unthinned</td>
<td>ca. 400,000</td>
<td>199</td>
</tr>
<tr>
<td>30</td>
<td>Unthinned</td>
<td>409,000</td>
<td>312</td>
</tr>
<tr>
<td>35</td>
<td>Twice thinned</td>
<td>362,000</td>
<td>230</td>
</tr>
<tr>
<td>40</td>
<td>Four times thinned</td>
<td>270,000</td>
<td>148</td>
</tr>
</tbody>
</table>

The use of fertilisers has also been accompanied with the practice of windrowing in Queensland forest plantations. When a tree is harvested, it is only the stem that is of major use for wood products. Stems are cut to size by machinery on-site, and harvest
residues such as leaves, twigs, branches, bark and roots are left behind. Whereas previously these residues were burnt or removed (McMurtrie & Dewar, 1997), it is now common practice to mound these residues in long rows along contour lines within the plantation (Blumfield et al., 2004; Bubb et al., 1999; Mathers et al., 2000). These mounds are called “windrows”, and allow at least a part of the natural organic matter that was removed from the forest plantation to decompose on-site, thereby returning nutrients to the soil system. Decomposition in Australian soils, however, is usually slow due to limitations in moisture and temperature (Williams & Woinarski, 1997). Large amounts of nutrients are therefore trapped in the decomposing dead plant material, also known as the litter layer, or in the case of a forest plantation, the harvest residue windrows.

The decomposition of eucalyptus material in soil is explored in detail and presented in Chapter 4. It is beneficial, however, to look at N uptake and assimilation in trees in order to see what chemical forms of N in plant residues are eventually introduced back to the forest soils to decompose, and compare that with the forms that may be removed with harvesting of stems. The impact that the chemical form of N present in fertilisers has on any of these parameters therefore been investigated. In this Chapter the affect that N form has on plant uptake and assimilation is studied, as well as partitioning of N within the plant. For example, if most of a tree’s N is deposited in the leaves, then this may be returned to the system via windrowing. However, if the N form supplied by a fertiliser results in the stem becoming more concentrated in N-containing compounds, then this N will be removed when harvesting takes place, therefore leading to greater losses from the soil system.

Use of fertilisers enables better tree growth rates and site capacity. For example, Ward and associates (1985) found that the application of N fertilisers to Eucalyptus saligna, significantly increases the height and trunk diameter of the trees. Applications of some fertiliser elements, such as phosphorous (P), are only needed once during a plantation rotation in order to help the tree make better use of its naturally occurring resources. This application usually occurs early in the stand’s rotation. N-containing fertilisers, however, require repeat applications. Plantation soil quality, stage of growth and nutritional needs of plants will have to be taken into account when applying N-containing fertilisers, as excessive amounts lead to
inefficient use and may have detrimental affects on the environment. Uneven matching of tree requirements to N application results in the leaching of nitrates into groundwater and therefore nearby streams and rivers, or volatilisation of any unused fertiliser N. Examples of losses that may be experienced are provided in subsequent sections of this chapter.

N fertilisers are likely to be most beneficial on poor quality soils, such as sites that contain large amounts of gravel and sand. The N status of plantation trees is usually monitored by visual diagnosis or foliage analysis. Foliage nutrient levels have been used to determine the successful responses of trees to fertilisers (Ward et al., 1985) and have been found to be good indicators of tree quality in Eucalyptus species (Herbert, 1990; Olsen & Bell, 1990). Foliage analysis however, does not take into account the highly dynamic nature of N within the tree itself (Flowers & Yeo, 1992; Patrick et al., 2001). Canovas and co-workers (2007) have recently researched the production of storage proteins in pine trees. It was found that N fertilisation led to higher levels of the amino acid arginine in pinewood and needles. Storage proteins rich in arginine may therefore better reflect the long-term deposition of N, and hence the N status of the tree.

While concentration and application of N-containing fertilisers enhance growth and productivity that leads to wider ring patterns in stem cross-sections, no direct correlations on wood quality have been observed. It is the occurrences of knot and grain-distortion effects, however, that increase or decrease wood quality. Adjusting fertiliser concentrations and application rates to improve these qualities has not been found to be beneficial though. In this research, application of various N fertilizers and their uptake by the plants will be studied. An emphasis has been put on N levels and partitioning of N in tree stems reflective of the extent of growth and N that is exported upon harvesting.

**Nitrogen Form**

Soil minerals produced through mineralisation of organic matter in the soil are present even before the introduction of nitrogen fertilisers. The dominant N containing minerals can differ depending on location, soil type, rates of
mineralisation, tree species present and number of disturbances. For example, it has previously been found that the dominant form of N in hoop pine plantations has changed from ammonium in established plantations, to nitrate in inter-rotation and early establishment periods (Blumfield et al., 2004; Bubb et al., 1998). This change has also been observed in other conifer forest plantations, and also coincides with changes in soil pH and microbial communities (Kronzucker et al., 1997). As pointed out by Blumfield and co-workers, research is needed to gauge seedling responses to different N-containing fertilisers, which can also help provide insights into seedling responses to dominant N forms that occur naturally in a plantation.

Ammonium, nitrates and urea are the most common components used in the man-made N-containing fertilisers. Table 3.2 provides an example of recommended fertilisers and application rates for new hardwood plantations ("Managing hardwood plantations," 2005). Here Starterfos®, which contains mono-ammonium phosphate (MAP), and Nitram®, which contains ammonium nitrate, are used as N-containing fertilisers. Fertilisers are applied in the first 18 months of plantation establishment. Previous research has shown that the health and appearance of a plant can be affected by both the amount of N available and also the form of N used. Examples include experiments on the effects of N fertiliser form on plants such as tobacco (Wang et al., 2003), hoop pine (Blumfield & Xu, 2006), norway spruce (Aarnes et al., 1995), French bean (Guo et al., 2002; Guo et al., 2007), pine (Griffin et al., 1995; Persson & Nasholm, 2002), Eucalyptus (Warren, 2006; Warren et al., 2000).

These N losses can be minimised by changing fertiliser types to suit environmental conditions, such as applying nitrates in dry weather and ammonium in wet weather (McTaggart et al., 1997) and also by matching the N supply and demand (Prasertsak et al., 2001a). For example, the current recommendation by Prasertsak and associates (2001a) for urea application on dairy pastures is to spread just prior to rain, in order to reduce ammonia volatilisation, and spreading nitrates in dry weather, when leaching is less likely to occur.
Table 3.2: Recommended fertilisers and application rates for new hardwood plantation seedlings ("Managing hardwood plantations," 2005). Starterfos® contains mono-ammonium phosphate (MAP), while Nitram® contains ammonium nitrate.

<table>
<thead>
<tr>
<th>Month</th>
<th>Age (months)</th>
<th>Fertiliser</th>
<th>Content N (%)</th>
<th>Rate (kg/ha)</th>
<th>Fertiliser N</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>March</td>
<td>0</td>
<td>Starterfos®</td>
<td>10%</td>
<td>21.9%</td>
<td>275</td>
<td>28</td>
</tr>
<tr>
<td>January</td>
<td>9</td>
<td>Nitram®</td>
<td>34%</td>
<td>0</td>
<td>180</td>
<td>61</td>
</tr>
<tr>
<td>September</td>
<td>18</td>
<td>Nitram®</td>
<td>34%</td>
<td>0</td>
<td>180</td>
<td>61</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>150</strong></td>
<td><strong>60</strong></td>
<td></td>
</tr>
</tbody>
</table>

The fertilisers applied in the thesis research are made up of inorganic salts containing either ammonium only, nitrate only, ammonium combined with nitrate or the organic compound, urea. These fertilisers are similar to those used for eucalyptus and pine tree species grown by the QDPI-Forestry. When the fertilizer is in an organic form, such as urea, the N is unavailable for immediate plant uptake and requires the activity of the microbes to mineralise over time, considered as slow release of nutrients. However, it has been shown that some species of plants have the ability to utilise organic N forms directly, such as N in the form of amino acids (Persson & Nasholm, 2002; Warren, 2006; Warren et al., 2000). Our studies included monitoring of the health and growth parameters of the tree seedlings, as well as their nutrient and water uptake in order to determine preferences for particular fertiliser N-forms, which included ammonium, nitrates and the organic N-form urea.

**Nitrogen Losses**

Information on the magnitude of fertiliser N losses such as those from volatilisation, and N losses due to soil decomposition processes, are essential in order to improve the efficiency of plantation productions (Prasertsak et al., 2001a). The loss of N during decomposition processes are discussed in more detail in Chapter 4. The losses due to processes such as the volatilisation of N from directly applied fertilisers are discussed in the subsequent section.

The fate of urea N loss in banana plantations (Prasertsak et al., 2001b), and dairy pasture soils (Prasertsak 2001a) in tropical north Queensland, Australia, has been previously studied by Prasertsak and co-workers. Urea is mainly used for
fertilisation, as it is cheap and has a good formulation. On the downside, it can have large N losses. The main reasons for this can be:

- The soil and plants (depending on the species) may have a high urease activity, and therefore results in the hydrolysis and conversion of urea to ammonia (ammonia volatilisation). In the case of the dairy pasture, most of the urea applied can be lost within 2 weeks
- Large runoff and erosion N losses due to high rainfall
- High denitrification rates as a result of waterlogging

It has been reported that the first sampling in the dairy pasture carried out 11 days after application, had no urea N present in the soil, with roughly 8% of the applied N ending up as ammonium, and 65% as nitrates. The high nitrate content is expected because of the rain and dew present throughout the experiment. These moist conditions are favourable for the conversion of ammonium to nitrates (Tan, 1994), as are the high temperatures found in tropical Queensland. The rest of the applied N must have therefore been lost from the system through a combination of ammonium volatilisation, denitrification and leaching.

Final measurements of the fate of the applied $^{15}$N are found in Table 3.3. The 20% loss due to ammonium volatilisation falls within the range of what others have found. As ammonium volatilisation was measured, there was no runoff and therefore insignificant leaching and little ammonia was lost from plants, the remaining 20% of applied N lost must be due to denitrification. Denitrification potential was found to increase with high moisture and temperature conditions (Estavillo et al., 1996). It has also been previously found that grassland soils can lose up to 30kg/ha per day of N through denitrification (Jarvis et al., 1995).

The effect of wet or dry soil on the fate of urea has also been studied in the banana plantation experiment. It was found that most of the urea was hydrolysed within 4 days in both wet and dry soil situations. Nitrification rates, however, increased when soil was rained upon, with 20% of N applied being converted to nitrates within 9 days. Even though rain was significant, due to the plantation’s canopy, not enough
Table 3.3: Fate of applied $^{15}$N enriched urea in a sub-tropical Queensland banana plantation and a dairy pasture (Prasertsak et al., 2001b).

<table>
<thead>
<tr>
<th>Fate of applied $^{15}$N</th>
<th>Banana Plantation</th>
<th>Dairy Pasture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.4% (leaves)</td>
<td></td>
<td>38% (shoots)</td>
</tr>
<tr>
<td>3.3% (stem)</td>
<td></td>
<td>4% (stolen + roots)</td>
</tr>
<tr>
<td>2.8% (fruit)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0% (roots)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5% (other)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil (0-110cm)</td>
<td>60%</td>
<td>18%</td>
</tr>
<tr>
<td>Ammonia volatilisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.2% (N lost in dry)</td>
<td></td>
<td>20%</td>
</tr>
<tr>
<td>17.2% (N lost in wet)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaching and Denitrification</td>
<td>Combined 5% loss</td>
<td>20% (from denitrification)</td>
</tr>
</tbody>
</table>

was reaching the soil, and hence washing the urea in. Therefore, some of the urea applied in the experiment would have been lost by ammonium volatilisation.

In experiments by Blumfield & Xu (2006), on the application of different N-fertilisers to hoop pine seedlings, it was found that in the first 4 months most N losses were likely to have occurred from denitrification. No leaching occurred, as all runoff was collected and recycled back into the pots. Losses were less after 4 months of fertiliser application possibly due to the use of N by the plants, the binding of N to soil particles, or N becoming protected by soil microsites. Approximately 30% of fertiliser applied $^{15}$N was not recovered for all fertiliser N-forms and application rates. Therefore N loss was not limited by denitrifying microbe activity, hence the more N that was applied, the more N that was lost (Blumfield & Xu, 2006).

In a study of gas emissions from arable soils fertilised with either ammonium-N or nitrate-N, Liu and associates (2007) found that soils under no tillage conditions at 60% field capacity emitted N$_2$, N$_2$O and CO$_2$ in quantities found in Table 3.4 over a 14-day period. Small amounts of sulphur containing gases may also be emitted (Li et al., 2006). Li et al (2006) had results showing H$_2$SO$_4$ and COS being emitted at rates of 1.2 and 0.8µg m$^{-2}$ h$^{-1}$ respectively, which is equivalent to 0.403 and 0.269 mg m$^{-2}$ day$^{-1}$. It can be concluded from these papers and results, however, that the majority of emitted gases is in the form of CO$_2$. 
Table 3.4: A comparison of gas emissions from arable soils fertilised with N in the form of either ammonium or nitrate (Liu et al., 2006)

<table>
<thead>
<tr>
<th>Fertiliser Used</th>
<th>N₂ + N₂O (mgNkg⁻¹)</th>
<th>N₂O (mgNkg⁻¹)</th>
<th>CO₂ (mgCkg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄⁺</td>
<td>16.3</td>
<td>4.5</td>
<td>450</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>5.9</td>
<td>2.0</td>
<td>267</td>
</tr>
</tbody>
</table>

3.1.2 Acquisition of Water and Nutrients

Plants need osmotically active soluble nutrients (solute), as they supply the driving force for water uptake, which in turn maintains the hydrostatic pressure that stops cell walls from collapsing in non-woody plants (Flowers and Yeo 1992). Nutrient uptake is also necessary for the activation and stability of proteins required for growth. Plants acquire nutrients either by specific ions moving towards plant roots, such as soluble nitrates moving towards roots in groundwater, or by roots growing and moving towards nutrients. This can be aided by fungal associations which can increase the effective radius of the roots (Schmidt et al., 2006; Tate, 1995). Canovas and co-workers (2007) have shown that NH₄⁺ uptake is aided by mycorrhizal fungal associations in pine, whereas NO₃⁻ was unaffected.

The roots surface area of a plant also determines how much access there is to nutrient supply and hence uptake. For example, the very fine roots found in eucalyptus plants (< 0.5mm diameter), greatly increase their surface area and therefore their uptake efficiency (Barrow, 1977; Williams & Woinarski, 1997). In experimental research of root water uptake, uptake rates are frequently expressed per unit root volume, rather than per root dry mass, as volume would give a better indication of the surface area of the root exposed to water/fertiliser solution. For example, two roots may have the same dry mass, but may have different volumes and therefore densities. Root tissue density (RTD) can be influenced by chemical composition of the root material. For example, increased lignification and content of substances with high molecular weights (eg starch) would increase RTD. (Ryser & Lambers, 1995). Differences in RTD can then affect the hydraulic conductivity of the roots and hence water uptake rates (Guo et al. 2007).
Relationships between roots and above ground biomass, in particular shoots, exist and help determine the balance between the uptake of nutrients and water, and the assimilation of carbohydrates. Therefore, more roots would indicate a greater nutrient uptake. However, there is less need for nutrient if there is a small amount of above ground biomass present. The larger the above ground biomass, the greater capacity there is for photosynthesis and assimilation of carbohydrates and hence there is a larger requirement for nutrients and therefore roots (Williams & Woinarski, 1997). The ratio between roots and shoots is known to vary according to environmental conditions. For example, when water supply is low, the roots proliferate to reach ground water, and therefore the root to shoots ratio will be high. When water supply is high, the root to shoot ratio will decrease (Zimmer & Grose, 1958).

Nutrient inorganic ions are taken up by roots, however, before they are transported inside they must first cross a selective barrier in order to enter and be transported via tissues up to shoots. These barriers also prevent solutes leaking out of root tissues. As discussed in Chapter 1 (Section 1.4.4), plant roots also need to maintain electroneutrality. Therefore any influx of ions into roots requires a decrease in the uptake of ions of a similar charge, or an efflux. Once inside the root, the tree xylem acts as a one-way transport system from roots to shoots. It transports large amounts of water to parts of the plant where water has been lost due to transpiration. Anything dissolvable, including any nutrients, will get a free ride up to the plant shoots. The transport of nutrients therefore relies on the need and hence transport of water. Leaf stomata remain opened during transpiration whilst a plant is undergoing photosynthesis.

While the xylem transports inorganic solutes up the plant and relies on transpiration, the phloem transports organic products of photosynthesis back to non-photosynthetic plant parts such as seeds, roots and developing leaves. These parts therefore act as a sink for N. Any compounds not used for new growth, such as amino acids, can then be cycled and hence “stored” in the phloem until they are needed (Rossato et al., 2001; Tilsner et al., 2005). This is also known as phloem loading. The phloem can therefore act as both a source and a sink for organic compounds. For example, in a vineyard study of N remobilisation in “pinot noir” grapevines, it was found that as much as 50% of the N and P required for growth came from stored nutrients within
its own trunk and roots (Schreiner & Scagel, 2006). It was also evident in the work on hoop pine previously mentioned by Blumfield & Xu (2006), that seedling were not showing major changes in total N content when exposed to different N fertilisers, as they were still relying on N stored reserves. Therefore it was suggested that hoop pine seedlings planted in new plantations could rely on their own N reserves for nutrients for up to 12 months.

It has been found more recently however, that certain plant species are able to absorb organic N forms, such as amino acids, directly as a nutrient source, without having to mineralise first into inorganic N-forms by soil microbial communities. Some examples include conifers which can utilise the amino acids arginine and glutamate (Canovas et al., 2007; Persson & Nasholm, 2002) and also urea (Aarnes et al., 1995). Nasholm and coworkers (2001) found that 19-23 % of dual-labelled glycine added to soil around wheat plants was taken up in intact form. A similar behaviour has been observed for Eucalyptus species (Warren, 2006).

In the thesis study, the effect of N fertiliser form used and how it influences the distribution of different chemical groups of \(^{15}\)N and \(^{13}\)C reserves on seedling stems will be analysed using \(^{15}\)N and \(^{13}\)C NMR spectroscopy. This data will provide information on the relative qualitative information on chemical composition of xylem and phloem. IC will be used to monitor the uptake of inorganic N (NH\(_4^+\), NO\(_3^-\) and NO\(_2^-\)) as well as a wide range of other anions and cations. The efflux of any ions will also be identified and their relative distribution as a function of different fertilizers will be investigated.

### 3.1.3 Nitrogen Signalling and Cycling in Plants

While studies have shown that the root to shoot ratio of a plant can change depending on supply of water, some studies have found links between the root to shoot ratio and the N-source supplied to the plant. For example, plants grown in the presence of NO\(_3^-\) have large leaf expansion and inhibited root growth (Stitt & Scheible 1999; Walch-Liu et al 2005). This may, however, be due to elevated plant cytokinin levels with increased NO\(_3^-\) levels within the plant. The presence of NO\(_3^-\) simulates cytokinin, which stimulates leaf cell division and expansion, therefore leading to the
larger leaf areas (Walch-Liu et al. 2005). Stitt & Scheible (1999) also found in their studies that root growth is inhibited when excess, unreduced NO$_3^-$ accumulates in shoots. Therefore there must be a mechanism for communication about both the external and internal N supply between the roots and shoots of a plant (Walch-Liu et al., 2005). It is also possible that the external and internal N status involves a separate mechanism for each. Walch-Liu and co-workers (2005) have suggested that amino acids could act as signal transmitters, as they are cycled around the plant. No correlations have been found however, between N uptake or N source and the flux and composition of amino acids.

In a study on amino-N cycling in wheat, it was found that large amounts of N were cycled through roots as well as root xylem sap and shoots (Cooper et al., 1986; Cooper & Clarkson, 1989). It was suggested that by cycling N, the plant forms a reserve of labile N in the form of amino groups. In times of stress, the plant can draw upon these reserves. It was also pointed out that constant cycling will incur greater energy costs to the plant, with repeated loading/unloading of the phloem and xylem. Cycling of N back down the plant however, may give feedback to roots on the plant’s N status and therefore help control the uptake of N by the roots.

Canovas and co-workers (2007) reasoned that cycling of the amino acid arginine and the amide containing amino acids glutamine and asparagine, is important in the metabolism of N in conifers. If N supplied by soils to the plant is low, then an efficient system for N storage and cycling in the plant is needed. It was also pointed out that N fertilisation increases the arginine content of needles and wood, and may therefore reflect the N status of the plant. Storage proteins are rich in arginine and the amide amino acids. Asparagine is also a key N transporter as it is soluble, more stable than glutamine and has a high N/C ratio. Therefore it accumulates as a mobile N form in sink tissues. Mobile bark storage proteins have been found in the angiosperm Populus (Cooke & Martin, 2005). Here it was found that N accumulated in branches during autumn and disappeared from the bark in spring, most likely due to use in new growth.
3.1.4 Root Exudations

It is interesting to find that root exudations are not limited to inorganic ions only and can include organic molecules as well. Root exudations of amino acids and amides have been previously reported for *Eucalyptus marginata* and *Eucalyptus calophylla* (Bowen, 1969) and *Pinus radiata* (Malajczuk & McComb, 1977) species. Malajczuk & McComb (1977) found that these two species of Eucalyptus exuded up to 20 different amino acids over 42 days of sampling, as well as sugars and organic acids. *E. marginata* was found to exude almost twice as much as *E. calophylla*, however the make up of the root exudates of both plants was basically the same.

The previous studies by Malajczuk and McComb (1977) have reported that amino acids have been collected directly from root exudates of eucalypt species and also from root exudate samples that have been hydrolysed with 6M hydrochloric acid. Upon comparison, the hydrolysed samples showed a considerable increase in amino acid content, suggesting that the samples must have contained soluble proteins and/or peptides. Comparisons of the total amino acid exudates, before and after hydrolysis for both species are shown in Table 3.5. It was concluded that eucalyptus root exudates provide a significant source of nutrients for the life cycle of microorganisms living on or near the root surface. The extent of exudates produced would

<table>
<thead>
<tr>
<th>Amino Acid Exudates</th>
<th><em>E. calophylla</em></th>
<th><em>E. marginata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Hydrolysis</td>
<td>7.42</td>
<td>16.54</td>
</tr>
<tr>
<td>After Hydrolysis</td>
<td>12.60</td>
<td>23.11</td>
</tr>
</tbody>
</table>

Table 3.5: Total amino acids exudates from *E. calophylla* and *E. marginata* after 4 weeks growth, before and after hydrolysis with 6M hydrochloric acid (micromoles per gram oven-dried roots) (Malajczuk & McComb, 1977).

<table>
<thead>
<tr>
<th>Solution Used</th>
<th>Total amido-/amino N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>104.5</td>
</tr>
<tr>
<td>Phosphate – deficient</td>
<td>248.5</td>
</tr>
<tr>
<td>N –deficient</td>
<td>25.1</td>
</tr>
</tbody>
</table>

Table 3.6: Total amide and amino acid exudates from *Pinus radiata* after 4 weeks in nutrient solutions. (Moles x 10⁻⁹ per plant) (Bowen, 1969).
also have an affect on the species selectivity/specificity of micro-organism populations. For example, it was shown that low concentrations of amino acids can attract certain micro-organisms.

In another study, Bowen (1969) found that exudates of *Pinus radiata* were affected by the nutrient solution supplied, with phosphate deficient pines exuding almost two and a half times more than pines with a complete solution after 4 weeks (Table 3.6). This was thought to be due to an increase in free amides and amino acids in these plants. Nitrogen deficient solutions showed roughly 75% less root exudates compared to the complete solution. The root exudates in all cases were found to contain up to 11 different amino acids and amides. Based on these results, it was suggested that phosphate deficient and nitrogen rich plants might have an increased susceptibility to micro-organisms in the plants rhizosphere due to these increases exudations (Bowen, 1969).

### 3.1.5 Tree Species

*Eucalyptus Pilularis* (Blackbutt) and *Pinus Elliotti* (Slash Pine) were chosen for the thesis research as they are both grown by the Queensland Forestry Industry, with *Eucalyptus* and *Pinus* species being hardwoods and softwoods respectively. They also represent two different types of trees, with *Eucalyptus* being an angiosperm (flowering and fruit bearing trees), and *Pinus*, a gymnosperm (seed bearing). Some differences that occur between these two types can be ascribed to differences in the physical structure of the xylem and phloem, and therefore how these species cycle nutrients.

For example, the xylem in an angiosperm is made up of a mixture of vessels and tracheids. Tracheids are single, elongated, lignified cells which act like long upright tubes. Vessels however, are long cells that are connected end to end by perforated plates, and have larger cell diameters than tracheids. While angiosperms xylem contains a mixture of these two types of cells, the xylems of gymnosperms only contain tracheids. The strength and structure of both vessels and tracheids can have an affect on plant health. For example, it has been pointed out by Williams and Woinarski (1997) that the narrower, thick-walled vessels found in the xylem of
Table 3.7: A comparison of two species grown by the QDPI-Forestry, and therefore chosen for this research ("Hardwoods advice - Timber species notes index," 2007).

<table>
<thead>
<tr>
<th>Botanical Name</th>
<th>Eucalyptus pilularis</th>
<th>Pinus elliotti</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>Angiosperm</td>
<td>Gymnosperm</td>
</tr>
<tr>
<td>Family Name</td>
<td>Myrtaceae</td>
<td>Pinaceae</td>
</tr>
<tr>
<td>Local Name</td>
<td>Blackbutt</td>
<td>Slash Pine</td>
</tr>
<tr>
<td>Tree Size (height)</td>
<td>med - large (40-60m)</td>
<td>medium (30-35m)</td>
</tr>
<tr>
<td>Colour: Heartwood</td>
<td>pale brown</td>
<td>reddish brown - yellow</td>
</tr>
<tr>
<td>Sapwood</td>
<td>slightly paler brown</td>
<td>pale yellow - yellow</td>
</tr>
<tr>
<td>Grain</td>
<td>moderately coarse</td>
<td>generally straight</td>
</tr>
<tr>
<td></td>
<td>textured and uniform</td>
<td></td>
</tr>
<tr>
<td>Air Dry Density (at 12% moisture)</td>
<td>930 kg/m³</td>
<td>625 kg/m³</td>
</tr>
<tr>
<td>Strength Groups*</td>
<td>Seasoned: SD2</td>
<td>SD5</td>
</tr>
<tr>
<td></td>
<td>Unseasoned: S2</td>
<td></td>
</tr>
<tr>
<td>Stress Grades^</td>
<td>Seasoned: F11, F14, F17, F22</td>
<td>F4, F5, F7, F8, F11</td>
</tr>
<tr>
<td></td>
<td>Unseasoned: F17, F22, F27, F34</td>
<td>F7, F8, F14, F17</td>
</tr>
<tr>
<td>Durability #</td>
<td>Above ground: Class 1</td>
<td>Class 4</td>
</tr>
<tr>
<td></td>
<td>In ground: Class 2</td>
<td>Class 4</td>
</tr>
<tr>
<td>Hardness Rating</td>
<td>Class 2 (hard)</td>
<td>Class 4 (firm)</td>
</tr>
<tr>
<td>Hard/Softwood</td>
<td>hardwood (angiosperm)</td>
<td>softwood (conifer)</td>
</tr>
<tr>
<td>Vessels</td>
<td>medium to large</td>
<td>absent</td>
</tr>
<tr>
<td>Tracheids</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Sieve tubes/cells</td>
<td>Tubes</td>
<td>Cells</td>
</tr>
<tr>
<td>Parenchyma</td>
<td>not visible without lens</td>
<td>absent</td>
</tr>
<tr>
<td>Resin Canals</td>
<td>absent</td>
<td>numerous</td>
</tr>
</tbody>
</table>

* Timbers are classified into strength groups with seven classes for seasoned timbers (SD1 - SD7) and eight classes of unseasoned timber (S1 - S8), where class 1 in each case is the highest strength.

^ High F-numbers indicate higher stress tolerance

# Timers are classified into classes according to durability, where class 1 in each case has the highest durability and class 4 has the lowest.
Eucalyptus wandoo, may explain why this species is able to tolerate lower water potential and therefore lower hydrostatic pressures than other eucalypt species.

Similar differences are also found in plant phloem structure, with gymnosperm phloem containing sieve cells, which are like xylem tracheids. Angiosperm phloem contains sieve tubes, which are like xylem vessels in that they are connected end to end by sieve plates that have pores. These vessels are interesting in that they contain what is known as p-proteins, which block these pores to stop loss of liquids and therefore nutrients if the plant is cut or wounded. When pores are open, flow and transport of solutes is fast, however, pore diameter can control transport rates.

Differences in physiological parameters of interest to plantation production, such as wood density, strength, colour and durability are given for the species chosen for this research in Table 3.7. Differences between growth rates and how these two species assimilate and metabolise were observed in the research for this thesis using solid-state $^{15}$N and $^{13}$C NMR spectroscopy, as well as ion chromatography (IC). Wikberg and Maunu (2004), have previously used solid-state $^{13}$C CPMAS NMR to study changes in the chemical structure of wood upon thermal modification. During this study differences between the gymnosperms/softwood Picea abies (L.) Karst. (spruce), and the angiosperms/hardwoods Betula pendula (birch), Populus tremula (aspen) and Quercus robur (oak) were investigated. Major differences between untreated softwood and hardwood samples found in the spectra were:

- The main units of lignin present, as softwoods contain guaiacyl (G), while hardwoods contain both guaiacyl and syringyl (S) units.
- The composition and percentage of hemicellulose present.

3.1.6 Effects of Nitrogen Form

Nitrogen and Water Uptake

In a study of the influence of N-form on Phaseolus vulgaris L. (French bean), Guo and co-workers (2007) set up a split-root system in which roots of a single plant were split between two vessels, each with a fertiliser solution containing a specific N-form. Fertiliser N-forms used included $\text{NH}_4^+$, $\text{NO}_3^-$ and water only. These were used
in different combinations, as shown in Table 3.8. The N uptake during both day and night was monitored, and hence the influence each fertiliser type had on the plant.

In Table 3.8 it can be seen that N-uptake was higher in nitrate containing vessels (NN, NO and NA) during the day, however, at night it was highest in ammonium containing vessels (AA, NA and AO). In NA roots, during the day, the NA-nitrate root contributed approx. 80% of the uptake of N. At night the NA-ammonium roots contributed to 79% of the N uptake. It is apparent that ammonium-roots all took up approximately the same amount of N, for all combinations during both night and day periods, with the exception of AO during the day. Nitrate-roots however vary greatly between all time periods and combinations. Table 3.8 also shows that N uptake is slightly greater when nitrates and ammonium are both present.

Table 3.8: Results tabulated from experiments on N uptake of Phaseolus vulgaris L. (French bean) using a split-root system over day and night periods (Guo et al 2007). Values in parentheses are modified results showing the total N uptake during the day, night and over a 24-hour period for each treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vessel</th>
<th>N Uptake (µmol/day)*</th>
<th>N Uptake (µmol/night)*</th>
<th>Total N Uptake (µmol/24hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN</td>
<td>NO₃⁻-N</td>
<td>995</td>
<td>232</td>
<td>2371</td>
</tr>
<tr>
<td></td>
<td>NO₃⁻-N</td>
<td>937</td>
<td>207</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1932)</td>
<td>(439)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>NH₄⁺-N</td>
<td>421</td>
<td>397</td>
<td>1521</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺-N</td>
<td>350</td>
<td>353</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(771)</td>
<td>(750)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>NO₃⁻-N</td>
<td>1794</td>
<td>105</td>
<td>2719</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺-N</td>
<td>420</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2214)</td>
<td>(505)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>NO₃⁻-N</td>
<td>2193</td>
<td>54</td>
<td>2247</td>
</tr>
<tr>
<td></td>
<td>N free</td>
<td>(2193)</td>
<td>(54)</td>
<td></td>
</tr>
<tr>
<td>AO</td>
<td>NH₄⁺-N</td>
<td>538</td>
<td>363</td>
<td>901</td>
</tr>
<tr>
<td></td>
<td>N free</td>
<td>(538)</td>
<td>(363)</td>
<td></td>
</tr>
</tbody>
</table>

*Where day and night consists of 14 and 12 hours respectively

Differences in water uptake (WU) were also found between the different fertiliser groups by Guo and co-workers. Water uptake of NN and NO roots was found to be approximately the same, while NA-nitrate root doubled its WU. The NA-roots however, had increased in root volume. After 24 hours ammonium roots had less
aquaporin mRNA expression than nitrate roots, where aquaporin are integral membrane proteins that act as water channels, selectively allowing water to pass through while discriminating against other ions and solutes. After 2 days, water uptake for ammonium roots was significantly lower than other N-forms. Root tissue densities of *Phaseolus vulgaris* L. were significantly lower when treated with ammonium solution than compared to nitrates. Therefore water uptake may have been affected due to the differences in densities hence influenced by the N-form used. It was suggested that the lower root tissue density under ammonium compared to nitrates, may reflect lower starch levels in the ammonium supplied root tissues, due to the higher amount of energy required to detoxify ammonium in the plant.

**Growth Parameters**

The form in which N is supplied either naturally in soils or in fertilisers can have both benefits and adverse affects on a plants health, depending on the species. For example, supply of fertiliser N in the form of ammonium only, can have adverse affects on leaf water potential, leaf expansion rates due to low cytokinin synthesis in roots (Walch-Liu et al., 2005) and reduced water uptake (Guo et al., 2002). Guo and co-workers (2007) found that *Phaseolus vulgaris* L. (French bean) plants fertilised with ammonium only solutions lead to reduced leaf area, shoot and root dry mass formation, potassium uptake, aquaporin expression and therefore a reduced water uptake rate when compared to plants fertilised with solutions containing nitrates. It was found that leaf area was highly correlated with water uptake.

On the other hand, Blumfield & Xu (2006) found that there was no significant difference in growth parameters (eg. height, stem diameter, biomass dry weight) of *Araucaria cunninghamii* (Hoop pine) seedlings with the application of different N-forms (ammonium sulfate, ammonium nitrate, potassium nitrate and urea) and application rates (0, 150 and 300 mg N kg\(^{-1}\) dried soil). This lack of growth response, however, was suggested to be due to the plants still using internally reserved supplies accumulated before the experiment, as they were not grown from seed. For this reason, the plant may not have exhibited any influence of fertiliser supply on growth parameters. Plants may need up to three growth seasons to deplete these supplies. Another factor is that the soils used in the study to understand the forest plantation
conditions, and may have contained a sufficient N supply within to support plant growth.

**Water Use Efficiency / $\delta^{13}C$**

The forested area of Australia consists of 162.7 million hectares of native forest, and 1.75 million hectares of plantation forest as previously mentioned in Chapter 1. Because of its geography, Australia is prone to droughts that can extend several years. According to the NSW Department of Primary Industries, as of May 2007, 83.3% of NSW is now classified as suffering drought conditions, with only 6.9% being in a satisfactory state. The results are high temperatures and low rainfall, coupled with salinity problems. Young plants in particular, are therefore prone to conditions such as heat stress, water deficit, salinity and hypo-osmotic stress. Drought also has a major impact on the national economy due to crop failure, stock loss, fire and land degradation. Due to the need for conservation of water, more efficient management practices are needed when comes to water use in forest plantations.

The water use efficiency (WUE) of a plant is an indicator as to a plants ability to withstand water shortages. Finding the WUE of a plant is done by taking the ratio of plant biomass produced to water uptake, where high ratios indicate a better WUE. In this thesis the effect the fertiliser N-form used has on the plants WUE has been assessed. Other indicators of improved WUE include, high net leaf photosynthesis, low stomatal conductance and it’s $\delta^{13}C$ composition (Blumfield & Xu, 2006). The $\delta^{13}C$ composition of a plant can be used to determine the WUE of a plant, as during the photosynthesis process, the carbon dioxide having $^{13}C$ is discriminated against in favour of the lighter $^{12}C$ isotope. Therefore if a plant has a high net photosynthesis rate, it will have a higher concentration of $\delta^{13}C$.

In the experiment undertaken by Blumfield & Xu (2006) on hoop pine fertilised with different N-forms and application rates (as mentioned in the previous section), plants were separated into roots, stems and new and old leaves, and were analysed for $^{15}N$ enrichment, total N, $\delta^{13}C$ and total C content. It was found from $\delta^{13}C$ measurements that moderate application rates (150mgNkg$^{-1}$) of nitrate-N fertilisers had slightly higher foliage $\delta^{13}C$. Therefore use of nitrate-containing fertilisers may lead to plants
with improved WUE, however, high rates of application (300mgNkg⁻¹) for all fertilisers were found to have lower δ¹³C, and therefore an adverse effect on WUE. This is possibly due to the high salt concentration, which may damage seedling roots.

Fertiliser N application to higher plants such as trees, has previously shown increases in photosynthesis of well-watered *Pinus taeda L.* (Loblolly pine) seedlings responding to an increased N supply (Green & Mitchell, 1992), while increases in δ¹³C have also been seen in of *Pinus sylvestris* L. (Scots pine) needles (Högberg et al., 1993). Changes in the δ¹³C of stem wood of mature conifers upon N fertiliser addition has also been previously seen (Warren et al., 2001). Along with the potential increase in WUE, increases in δ¹³C also indicate increases in a seedling’s photosynthetic capacity. If fertiliser N additions increase the photosynthetic capability of a plant, unused photosynthetic products, such as carbohydrates in a tree’s stem, could be stored for the future growth needs of the plant. (Wardlaw, 1990), thus also alleviating the plant’s reliance on soil nutrients.

### ¹⁵N and Total N Concentrations

In the split-root system experiments on *Phaseolus vulgaris L.* by Guo and co-workers (2007), which was described in the previous section 3.1.5., leaf N content significantly differed between ammonium fertilised plants (AA=2.91g/m²) and nitrate fertilised plants (NN=1.68g/m²). Leaf area was found to be highly correlated with water uptake. In the experiments also previously mentioned on Hoop Pine, Blumfield & Xu (2006) found that new leaves had a higher total N concentration when compared with older leaves. No trends in total N with respect to fertiliser N-form or rate were observed.

The fertiliser that had been applied to the hoop pine seedlings by Blumfield & Xu, had been isotopically enriched with ¹⁵N in order to follow its movement in the plants. It was found that the ¹⁵N enrichment in plants due to nitrate containing-fertilisers (potassium nitrate and ammonium nitrate) was consistently higher than for nitrate-free fertilisers (in this case, ammonium sulfate and urea). This variation in ¹⁵N enrichment was found at both 150 and 300mgNkg⁻¹ fertiliser application rates. The fact that the total N concentration between fertilisers was the same, but the ¹⁵N
enrichment was different, may be due to the fact that a lot of N was already present in the plants before the experiment had commenced. Therefore significant differences in total N may not be seen. However, because the fertiliser supply was $^{15}$N enriched, the $^{15}$N enrichment of the plants reflects the fertiliser uptake and N-form preferences of the hoop pine. The low $^{15}$N enrichment of seedling fertilised with urea-N could be due to volatilisation of urea from moist soils (Prasertsak et al., 2001a; Prasertsak et al., 2001b).

In this chapter, the effect fertiliser N-form has on plant uptake, assimilation, metabolism and storage was studied using the forest plantations tree species *Eucalyptus pilularis* and *Pinus elliotti*. These plants were grown hydroponically with $^{15}$N-enriched ammonium nitrate, ammonium sulfate, calcium nitrate or urea as their only N source. $^{15}$N and $^{13}$C NMR was then used to analyse different sections of the plants, revealing the fate of the $^{15}$N isotope added, and the N and C composition of the plants. Total C and N analysis was also undertaken as a complementary technique, as well as the use of ion chromatography to analyse the ions present in the left over hydroponic solutions. These forms of analysis, along with the visual observations may then help point out potential indicators of plant health, and give a better understanding as to how these N-fertilizer forms are used by different plant species.

### 3.2. Experimental Methods

#### 3.2.1 Experimental Conditions

**Plant Preparations**

Eucalyptus plants were purchased from the Ku-ring-Gai State Nursery, NSW, Australia, and were approximately 30cm in height. Roots were removed of all soil and washed with de-ionised water. Plants were then placed in 6.5 x 6.5 x 16cm rectangular plastic pots and filled with perlite (Figure 3.1a). Roughly 2cm of clay balls were placed in the bottom of the pots to stop the perlite falling out. Perlite and clay balls were chosen, as they are inert materials that do not provide nutrients.
Figure 3.1: a) Eucalyptus seedlings in hydroponics setup before aluminium covers were put on, b) an example of new leaf samples taken from eucalypts grown in UR solutions, c) the dividing up of sections (from left to right; leaves, stems, roots) of a single eucalyptus seedling before being dried in an oven to constant mass d) Slash pine grown in AS (left) and CN (right) solutions, e) the resulting root systems from slash pine grown in AS (left) and CN (right) solutions, f) an example of a pine stem section removed of its bark. Notches on the ruler are 1mm units.
Because of their porosity, they are able to hold oxygen that is necessary for plant growth, and it also enables the solution to be taken up to the plant’s roots. Slash pine seeds were germinated on wet tissue paper or in rockwool cubes watered with deionised water. They were then transferred to 5 x 5 x 12cm pots filled with perlite (Figure 3.1d). For both species of plant, the tops of the pots were covered in aluminium foil to stop evaporation and light, which may cause algae growth and alter results acquired for water uptake.

**Hydroponics Solutions**

Both species were separated into six groups of five plants. Each group was placed in a separate container, which would hold the hydroponics solutions, with the bottom of the plant pots sitting in holes in the top of a container (Figure 3.1a and d). Containers were blacked out with black electrical tape to stop light from entering the solutions. Each container was then given one of six different nutrient solutions. In these solutions, all macro- and micro-nutrients were kept the same where possible; the only major difference between the solutions was the form of N species present.

Four of the solutions contained either 98%+ double $^{15}$N enriched calcium nitrate (CN), ammonium nitrate (AN), ammonium sulfate (AS) or urea (UR). These nitrogen compounds were chosen for their different chemical forms of N, and would allow observations as to how these different forms are assimilated by the plants using $^{15}$N NMR spectroscopy. Two singly enriched solutions of ammonium nitrate were also used, one with only the ammonium-N enriched (*AN), and the other with only the nitrate-N enriched (A*N). This would allow separate observations of the two different nitrogen forms using $^{15}$N NMR spectroscopy if needed, while still being in the presence of one another. These solutions could also be used as extra replicates to ensure similar results were obtained for solutions with different levels of $^{15}$N enrichment. No isotope effects were observed in the growth parameters measured between the single and double $^{15}$N enrichment levels. All solutions, both doubly and singly enriched, had a level of 112 mgL$^{-1}$ of N present in the full strength solutions. Macronutrient formulas for one litre of full strength solution were as follows:
AS Solution: 0.54g \((^{15}\text{NH}_4)_2\text{SO}_4\), 0.17g K_2\text{HPO}_4, 0.24g MgSO_4, 0.33g CaCl_2
CN Solution: 0.66g Ca\((^{15}\text{NO}_3)_2\), 0.17g K_2\text{HPO}_4, 0.24g MgSO_4
UR Solution: 0.25g H_2^{15}\text{NCO}^{15}\text{NH}_2, 0.17g K_2\text{HPO}_4, 0.24g MgSO_4, 0.52g CaSO_4\cdot2\text{H}_2\text{O}
AN Solution: 0.33g \((^{15}\text{NH}_4)^{15}\text{NO}_3\), 0.17g K_2\text{HPO}_4, 0.24g MgSO_4, 0.52g CaSO_4\cdot2\text{H}_2\text{O}
*A\text{N Solution}: 0.32g \((^{15}\text{NH}_4)\text{NO}_3\), 0.17g K_2\text{HPO}_4, 0.24g MgSO_4, 0.52g CaSO_4\cdot2\text{H}_2\text{O}
A*\text{N Solution}: 0.32g NH_4^{15}\text{NO}_3, 0.17g K_2\text{HPO}_4, 0.24g MgSO_4, 0.52g CaSO_4\cdot2\text{H}_2\text{O}

In order to keep most of the macronutrients at the same concentration between the different solutions, there were some unavoidable differences (Table 3.9). The CN solution had slightly more calcium and less sulfate present, whereas the AS solution had less calcium, and more sulfate and chloride present. Each fertiliser solution was monitored using ion chromatography to see if these differences would effect the experiment in any way. Observations of the plant’s physiology were recorded to understand any effect these ions may have on the well being of the plant, such as any deformities or symptoms of deficiencies. Excess sulfate and chloride, along with calcium deficiencies, were not observed in any of the plants.

<table>
<thead>
<tr>
<th>Fertiliser Solution</th>
<th>Cl&lt;sup&gt;-&lt;/sup&gt;</th>
<th>(^{15}\text{NO}_3\text{^-})</th>
<th>PO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;3^-&lt;/sup&gt;</th>
<th>SO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2^-&lt;/sup&gt;</th>
<th>(^{15}\text{NH}_4\text{^+})</th>
<th>K&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Urea-&lt;sup&gt;\text{i5N}&lt;/sup&gt;</th>
<th>TOTAL&lt;sup&gt;\text{15N}&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>*AN</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>A*\text{N}</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>UR</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>AS</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>CN</td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

A micronutrient solution was made up separately, with one litre consisting of: 2.86g H<sub>3</sub>BO<sub>3</sub>, 0.08g CuSO<sub>4</sub>•5H<sub>2</sub>O, 1.85g MnCl<sub>2</sub>•4H<sub>2</sub>O, 0.02g H<sub>2</sub>MoO<sub>4</sub>•H<sub>2</sub>O and 0.22g ZnSO<sub>4</sub>•7H<sub>2</sub>O. All macro- and micro-nutrient solutions were made using deionised water. Macronutrient solutions were first made. One millilitre of the micronutrient solution was then added to each litre of macronutrient solution, along with 0.03g FeEDTA (13.2%) and 0.06g KCl L<sup>-1</sup>. This resulted in a full strength macro/micro-nutrient hydroponics solution, which could then be diluted with deionised water to
the desired strength. From here onwards in this thesis, these solutions will be referred to as fertiliser solutions.

**Environmental Conditions**
Sets of plants were first set up in a temperature-controlled glasshouse located at the University of Western Sydney’s Hawkesbury campus. After 2 weeks, however, an electricity cut-off caused failure of the air conditioners, allowing the temperature to increase to above 60°C for at least a two hour period. This caused the death of all the eucalyptus seedlings, with the exception of those that were growing in the CN fertiliser solution. This interesting observation will be analysed further in Chapter six of this thesis under the heading Future Research.

A new set of eucalyptus seedlings were grown under natural light and temperature conditions, in a small purpose built greenhouse (1.5 x 0.6 x 0.8m), with a clear vinyl canopy and mesh sides. This provided an environment closer to field conditions, and could not overheat. The positions of the plants were randomly changed every couple of days in order to ensure the same average conditions. Original slash pine seedlings, which had been unaffected by the glasshouse temperature rise, were relocated to the same purpose built greenhouse as the eucalyptus seedlings.

Fertiliser solutions were maintained at a marked level on each of the fertiliser containers throughout the experiment, and were topped up when needed. At first the amount of solution added to each pot was the same for each fertiliser treatment, however, once the differently treated plants started taking up the solution at different rates, this constant addition rate could not be maintained. Measurements of the volume of solution left over allowed the water uptake to be determined. The strength of the solution was also varied over the experiment depending on the age and size of the plant, usually ranging from 1/3 to 1/2 strength. Fertiliser solution concentrations were recorded along with the amounts added in order to calculate the total N added as well as the totals for all macronutrients.

Solutions were monitored for pH, electrical conductivity (EC) and temperature every 2-3 days using a pH/EC/Temp meter from Bluelab. The pH of the solution was at
first adjusted with either potassium hydroxide (to increase the pH) or phosphoric acid (to decrease pH) to obtain a pH between 5.5 and 6.5 for all solutions for the first 20 weeks. It was found that as the plants grew, the pH changed dramatically within the space of one day. Therefore the pH was left to rise or fall to its natural state for the last 3 weeks of experiments. 30mL samples were taken throughout the experiment, therefore the effect of leaving the pH at natural levels could be monitored using Ion Chromatography (IC).

Pine plants were grown for 187 days in the $^{15}$N enriched fertiliser solutions, and eucalypts ranged from 156 to 164 days, depending on how much solution was left over. They were then grown in unenriched versions of the same solutions for a further two weeks and while harvesting was taking place. This allowed enough time for any mobile $^{15}$N in the plant to be assimilated into organic forms.

**Harvesting and Sampling**

Samples of new leaves and new needles from eucalypts and pines respectively were taken throughout the growth period. This was done by taking leaves/needles from the growth tip of the plants (Figure 3.1b). Plant heights were measured from the datum line, which was taken as the top of the pot, to the growth tip of the plant. Eucalyptus leaf areas were estimated for new leaves and older leaves, which were taken from half way down the stem. This was done by tracing leaf perimeters onto paper, which were then cut out and weighed. This was calibrated against the weight of 1cm$^2$ of paper in order to find a reasonable estimate of leaf areas in cm$^2$. For the pine plants, needle lengths were taken for needles found half way down the stem.

Plants were harvested and divided into leaves/needles, stems (this includes branches), and root sections (Figure 3.1c). Some of the stem sections were further sub-sampled to provide small bark and bare stem (stems with no bark) samples (Figure 3.1f). The harvesting of roots was not as quantitative as that of the leaves and stems due to fine roots sticking to the perlite, particularly for the eucalypt samples, where small amounts of fine perlite were getting caught up in the sample. The roots were washed with deionised water to lessen this effect, and also to remove any residue solution in
contact with the roots. This technique was carried out for all root samples, thereby enabling a comparison between samples, which used different fertiliser groups.

The respective sections of each plant were weighed to obtain the mass of fresh material produced by the plant. Samples were then dried in aluminium tins in an oven at 50°C until a constant dry mass was reached. The difference between the fresh mass and the dried mass was taken to represent the sample’s water content. After drying, samples were ground with a mortar and pestle or a food grinder to obtain a fine powder.

### 3.2.2 \(^{15}\)N NMR Spectroscopy

**CPMAS and VCT’s**

\(^{15}\)N NMR CPMAS experiments were run as described in Chapter 2, section 2.4.3, with the exception of contact time (1.5msec), delay time (0.5sec), acquisition time (0.01sec) and the number of scans (15000 to 90000), used to ascertain a spectrum. These parameters were first optimised in order to produce the best spectrum possible. Contact times were first optimised using VCT experiments on eucalyptus leaves grown under the AN regime, and fit with a biexponential model, as described in Chapter 2, Equation 2.4. Preliminary delay time experiments were also carried out in order to pick a delay time that did not cause saturation of spectra. Variable delay time experiments were undertaken and a delay time of 0.5 seconds was found to be sufficient for all peaks in the spectra obtained.

Chemical shifts were measured with respect to glycine as an external standard at -347ppm (Knicker & Hatcher, 2001). Peaks were assigned according to their chemical shift and the spectral region they appeared in. Possible peak regions and assignments are defined in Table 3.10. An example of spectra obtained from CPMAS and Bloch decay pulse sequences are shown in Figure 3.2. The peak and region assignments of the spectra obtained were tentatively assigned to heterocyclic-N (-205ppm), amide-N (-260ppm), guanidino NH groups (-295ppm), amine NH\(_2\)/NR\(_2\) groups (-306ppm), and amino-N groups (-330 to -350ppm). These peak assignments and chemical shifts are also shown in Table 3.11, as well as a comparison of the percentage each peak took up of the total \(^{15}\)N spectrum as acquired by CPMAS and Bloch decay.
Results for VCT’s with biexponential fits show that the hetero-, amide- and guanidine-N peaks have only one $T_1^\rho$H value (Table 3.12), indicating that there is efficient diffusion of proton spin in the rotating frame. As discussed in Chapter 2, this may be due to one crystal structure being present or the presence of high molecular motions that act as a sink for spin diffusion. The latter is more likely, as

<table>
<thead>
<tr>
<th>Region</th>
<th>Sub-Regions</th>
<th>Possible Peak Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitro-N</td>
<td>25 to -25</td>
<td>Nitrate, nitrite, nitro groups, nitro-derivatives</td>
</tr>
<tr>
<td>Hetero-N</td>
<td>-25 to -90</td>
<td>Imine, phenazine, pyridine, Schiff-bases</td>
</tr>
<tr>
<td></td>
<td>-90 to -145</td>
<td>Purine, nitrile groups</td>
</tr>
<tr>
<td></td>
<td>-145 to -220</td>
<td>Chlorophyll-N, purine/pyrimidine, imidazole, in particular substituted pyrroles, N in pyrrole and related ring structures, however these resonances can overlap with amide region, histidine</td>
</tr>
<tr>
<td>Amide N</td>
<td>-220 to -285</td>
<td>Amine/peptide, N-acetylderivatives of amino sugars, tryptophane, proline, lactams, unsubstituted pyrroles, indoles and carbazoles</td>
</tr>
<tr>
<td>Guano N</td>
<td>-285 to -300</td>
<td>NH in guanidine</td>
</tr>
<tr>
<td>Amine N</td>
<td>-300 to -325</td>
<td>NH$<em>2^-$ and NR$<em>2^-$ groups ($N</em>\delta$-arginine and $N</em>\alpha$-citrulline, $N_\varepsilon$-arginine, $N_\omega$-citrulline), urea, nucleic acids, aniline derivatives, side chain N of arginine residues, guanidine residues in DNA, aromatic amines</td>
</tr>
<tr>
<td>Amino N</td>
<td>-325 to -350</td>
<td>Free amino groups in amino acids and sugars, amino-N of terminal amino acids or sugars, and also $\varepsilon$-NH$_2$ in lysine, glycine</td>
</tr>
<tr>
<td>Ammonium N</td>
<td>-350 to -375</td>
<td>NH$_4^+$, some amines</td>
</tr>
</tbody>
</table>
Figure 3.2: The resulting $^{15}$N spectra for eucalyptus leaves run using a CPMAS and Bloch decay NMR pulse sequence, and some examples of the groups which may be present.

Figure 3.3: VCT results for the amide peak (-261ppm) found in the $^{15}$N NMR spectrum of eucalyptus foliage samples.
Figure 3.4: VCT results for the hetero-N, guano-N and amino-N peaks found in the $^{15}$N NMR spectrum of eucalyptus foliage samples.

Table 3.11: Percentage each region took of the total $^{15}$N NMR CPMAS and Bloch decay spectra for a eucalyptus leaf sample, and the percent difference between the two pulse sequences (with respect to CPMAS)

<table>
<thead>
<tr>
<th>Chemical Shift (ppm)</th>
<th>Peak/Region</th>
<th>CPMAS</th>
<th>Bloch Decay</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>-205</td>
<td>Hetero N</td>
<td>1.6</td>
<td>2.0</td>
<td>28%</td>
</tr>
<tr>
<td>-260</td>
<td>Amide N</td>
<td>85.9</td>
<td>68.5</td>
<td>-20%</td>
</tr>
<tr>
<td>-295</td>
<td>Guano-N</td>
<td>2.7</td>
<td>5.9</td>
<td>121%</td>
</tr>
<tr>
<td>-306</td>
<td>Amine N</td>
<td>5.5</td>
<td>8.8</td>
<td>59%</td>
</tr>
<tr>
<td>-338 and -347</td>
<td>Amino N</td>
<td>4.3</td>
<td>14.8</td>
<td>244%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.12: VCT results for Eucalyptus foliage samples from seedlings grown in the AN fertiliser solutions.

<table>
<thead>
<tr>
<th>Peak Region Assignment</th>
<th>Component 1</th>
<th>Component 2</th>
<th>% $I_{0[1]} : I_{0[2]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_{NH}$ (msec)</td>
<td>$T_{1pH}$ (msec)</td>
<td>$\alpha_{[1]}$</td>
</tr>
<tr>
<td>Hetero-N</td>
<td>0.19</td>
<td>5.87</td>
<td>0.97</td>
</tr>
<tr>
<td>Amide-N</td>
<td>0.95</td>
<td>6.37</td>
<td>0.83</td>
</tr>
<tr>
<td>Guano-N</td>
<td>3.34</td>
<td>3.34</td>
<td>0.00</td>
</tr>
<tr>
<td>Amine-N</td>
<td>0.37</td>
<td>3.05</td>
<td>0.88</td>
</tr>
<tr>
<td>Amino-N</td>
<td>0.51</td>
<td>0.53</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Table 3.13: Percentage observability of each $^{15}$N NMR region at 1.5msec, as compared to the signal obtained for each regions optimal contact time.

<table>
<thead>
<tr>
<th>Region</th>
<th>% Observability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero N</td>
<td>89%</td>
</tr>
<tr>
<td>Amide N</td>
<td>100%</td>
</tr>
<tr>
<td>Guano N</td>
<td>87%</td>
</tr>
<tr>
<td>Amine N</td>
<td>80%</td>
</tr>
<tr>
<td>Amino N</td>
<td>95%</td>
</tr>
</tbody>
</table>

The plant material is inherently made up of many different interacting organic structures, ruling out one single crystal being present. Interacting organic structures is also the reason why arginine- and amino-N show two different $T_{1\rho}H$ values, along with the fact that these peaks may represent more than one compound. For example, the amino peak may be made up of more than one type of amino acid. This may also explain the two $T_{NH}$ values each region exhibits, with the exception of hetero-N.

The optimal contact times were found to be 1.5msec for amide-N, 0.75msec for hetero- and guanidine-N, 1msec for amine-N and 3msec for amino-N. A contact time of 1.5msec was chosen for use in all $^{15}$N CPMAS NMR spectra obtained, as it represented the most quantitative spectra that could be obtained with the smallest losses of signal (Table 3.13).

Also of interest is the $\alpha_{[1]}$ values obtained for each of the peaks, where $\alpha = (1-T_{NH}/T_{1\rho}H)$ as discussed in Chapter 2, section 2.2.5. Quantitative NMR spectra can only be obtained if $T_{1\rho}H >> T_{NH}$, or $\alpha$ approaches a value of 1. If $T_{NH}$ approaches similar values to $T_{1\rho}H$, magnetisation transfer can never be completed before some relaxation of the proton spin-lattice has occurred. This will then quantitatively affect the spectrum so only a small fraction of the total signal can be measured. The $\alpha$ value can therefore assist in discovering whether the entire signal for a species is being observed. The $\alpha_{[1]}$ values for both amino-N and guano-N were 0.00 and 0.04 respectively, indicating that transfer of magnetisation was not compete before some relaxation began to occur. Hence, these signals may be under-represented in CPMAS spectra, with possibly only half the peak intensity expected being seen, as indicated by the ratio of $%I_{0[1]}$ to $%I_{0[2]}$. This may help to explain the under-representation of
amino acids in eucalyptus plant material found by Smernik & Baldock (2005), as discussed in section 2.2.7.

**Bloch Decays**

To confirm which peaks were over or underestimated by CPMAS, a $^{15}$N Bloch decay pulse sequence was obtained, and the spectra were compared. The Bloch decay spectrum was obtained using a spin rate of 7k, delay time of 5 seconds, 44000 scans, a 90° pulse width of 4.2usec and an acquisition time of 0.01seconds. The data obtained using the Bloch decay was processed in the same way as the CPMAS data acquired. The resulting spectra and data obtained are shown in Figure 3.2 and Figure 3.13. It was found that both amino-N and guano-N were severely under-represented in the CPMAS spectrum compared to the Bloch decay spectrum, while some under-representation of the hetero and amine-N groups also occurred. This may be in part due to the relative decrease of the amide-N peak measured using the Bloch decay.

**DDCPMAS**

A two point dipolar dephasing experiment was set-up and run as discussed in Chapter 2, section 2.4.1. Parameters used were exactly the same as those used for the CPMAS experiments, with the exception of a dephasing time of 0 and 45µsec. Data was fitted using Equation 2.2, which is based on exponential decay. The resulting $T_{2DD}$ values are listed in Table 3.14. The values presented here are similar to those found in Chapter 2, Table 2.12 for model compounds at ambient moisture levels. While plant sections were dried in a 50°C oven until a constant mass was reached before analysis took place, it is possible that very small amounts of water may still be present in the plant cell walls and cell contents. This could be combated with higher drying temperatures; however this could cause changes or possibly destroy the plant materials chemical structure. For this reason, samples were therefore used without further drying.

Combined with chemical shifts for these regions (Table 3.11), these results help confirm $^{15}$N NMR regions shown in Table 3.10 and tentatively assign specific compounds to peaks observed in all $^{15}$N spectra. For example, the $T_{2DD}$ value of 51.5 µsec obtained for hetero-N is similar to those found for the heterocyclic N containing
compounds histidine and uric acid (Table 2.12), while the guano and amine-N values are similar to the values found for the NH and NH$_2$ groups in arginine. As can be seen in Figure 3.2, the amino N region is occupied by at least 2 peaks, therefore it is expected that the resulting $T_{2DD}$ is an average of at least two amino groups. This may also apply to the amide-N $T_{2DD}$ value of 34.2µsec, as this region is also overlapped by heterocyclic N compounds.

<table>
<thead>
<tr>
<th>Region</th>
<th>$T_{2DD}$ (µsec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero N</td>
<td>51.5</td>
</tr>
<tr>
<td>Amide N</td>
<td>34.2</td>
</tr>
<tr>
<td>Guano N</td>
<td>43.3</td>
</tr>
<tr>
<td>Amine N</td>
<td>20.9</td>
</tr>
<tr>
<td>Amino N</td>
<td>122.0</td>
</tr>
</tbody>
</table>

### 3.2.3 $^{13}$C NMR Spectroscopy

$^{13}$C NMR CPMAS spectra were obtained for this research in order to help describe N processes occurring within the plant material, and to identify whether N fertilisation was having an affect on the carbon (C) cycle. Samples were run on the same Bruker 200MHz spectrometer operating at 50MHz, and were spun at 5 kHz. A pulse width of 4.6µs was used with a contact time of 0.75 msec, acquisition time of 0.01sec, a delay time of 2 sec and a 25 kHz sweep width. The number of scans ranged from 2,500 to 5,000 scans. $^{13}$C CPMAS NMR spectra were integrated into 7 sub-regions depicted in Table 3.15. These region assignments were used as a guide and most often consisted of carboxyl C (185 to 165ppm), phenolic C (165 to 140ppm), aromatic C (140 to 110ppm), di-O-alkyl C (110 to 80ppm), carbohydrate C (80 to 60ppm), methoxyl C (60-45ppm) and alkyl C (45 to 0ppm).

Chemical shifts were measured with respect to adamantane as an external standard at 38.3ppm. All spectra were Fourier transformed with a 50Hz Lorentzian line broadening. Spectra were integrated using MestRec computer software. Absolute intensities were found by integrating peak areas then correcting for the amount of sample present in the rotor during experiments.
Table 3.15: Compilation of possible peak assignments for solid-state $^{15}N$ NMR spectra (Knicker et al., 1996; Kogel-Knabner, 1997; Mathers et al., 2000; Schaefer et al., 1987; Smernik & Oades, 2001; Wikberg & Liisa Maunu, 2004)

<table>
<thead>
<tr>
<th>Region</th>
<th>Sub-regions</th>
<th>Possible Peak Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxyl C (210 -160 ppm)</td>
<td>Carboxyl C</td>
<td>Carboxyl and amide C groups (175ppm), carbonyl C</td>
</tr>
<tr>
<td>Phenolic C</td>
<td>Aromatic COR or CNR groups (140-160ppm), phenolic C in lignin (150ppm)</td>
<td></td>
</tr>
<tr>
<td>Aryl C</td>
<td>Aromatic C-H carbons, guaiacyl C-2, C-6 in lignin, olefinic C (110-140ppm), protonated aromatic C (119ppm) and C-substituted aromatic C in lignin (130ppm)</td>
<td></td>
</tr>
<tr>
<td>Di-O-Alkyl C (110 - 45 ppm)</td>
<td>Carbohydrate C</td>
<td>Carbohydrate derived structures (C-2 to C-5) in hexoses, C-a of some amino acids, higher alcohols (60-90ppm), polysaccharides (72, 65, 80-90ppm),</td>
</tr>
<tr>
<td>Methoxyl C</td>
<td>Methoxyl groups and C-6 of carbohydrates and sugars C-a of most amino acids (45-60ppm), methoxyl C in lignin (56ppm),</td>
<td></td>
</tr>
<tr>
<td>Alkyl C (45 - 0ppm)</td>
<td>Aliphatic C</td>
<td>Terminal methyl groups (0-25ppm), methylene groups in aliphatic rings and chains (25-45ppm), methylenic C in long chain aliphatics, such as fatty acids, lipids, cutin acids etc (30ppm), proteins or peptides (30-55ppm), acetyl groups in hemicelluloses (22ppm), paraffinic C</td>
</tr>
</tbody>
</table>

### 3.2.4 Ion Chromatography

Hydroponic solutions samples were injected via a 45um syringe filter into the 20uL loop a 792 Basic Ion Chromatography (IC) unit from Metrohm.

**Anion Analysis**

The IC was equipped with a Metrosep A Supp 5 150/4.0 anion column, of which an eluent of 3.2mM Na$_2$CO$_3$ + 1.0mM NaHCO$_3$ was pumped through the column with a flow rate of 0.7mL/min. Detection was suppressed with an integrated Metrohm Suppressor Module (MSM), which was regenerated with 100mM H$_2$SO$_4$ and rinsed with milli-Q water. Solutions were analysed for the anions fluoride (F$^-$), nitrite (NO$_2^-$), nitrate (NO$_3^-$), chloride (Cl$^-$), sulfate (SO$_4^{2-}$) and phosphate (PO$_4^{3-}$). The instrument was calibrated by running solutions with different concentrations of the anions to be analysed, to which calibration curves were fitted.
**Cation Analysis**
Cation analysis took place using a Metrosep C2 100/4.0 cation column. An eluent made up of 4.0mM tartaric acid + 0.75mM dipicolinic acid was pumped through the column with a flow rate of 1mL/min. For cation analysis the suppressor was disconnected. Solutions were analysed for the cations sodium (Na$^+$), ammonium (NH$_4^+$), potassium (K$^+$), calcium (Ca$^{2+}$) and magnesium (Mg$^{2+}$). Once again, the instrument was calibrated by running solutions with different concentrations of the cations to be analysed, to which calibration curves were fitted.

**3.2.5 CN Analysis**
Analysis for %C and %N was undertaken on a LECO TruSpec CN analyser. Calibration of the instrument took place before samples were run using varying amounts of the standards EDTA (9.58 ± 0.04% N, 41.05 ± 0.13% C) and glycine (18.66% N, 32.00% C). All samples were loaded into aluminium foil trays for analysis.

**3.2.6 Amino Acid Analysis**
Preliminary thin layer chromatography (TLC) experiments were undertaken to detect the possibility of any amino acids being present in hydroponic solutions. Merck TLC plates used were aluminium sheets (20 x 10cm), pre-coated with polyamide 11 F$_{254}$, with a 0.15mm layer thickness. These sheet were first activated overnight in an oven set to 70°C. Glass capillaries were used to spot solution samples as well as standard amino acid solutions for reference onto the TLC plates. The plates were developed in a square glass container with 1cm of solvent (60:20:20 n-butanol/acetic acid/H$_2$O) covering the bottom, and then put in an oven set to 70°C until completely dry. Visualisation of amino acid spots took place by spraying a solution of 0.2% ninhydrin in acetone onto the TLC plates, which were then dried. Samples of hydroponic solutions were also sent to Australian Proteome Analysis Facility at Macquarie University, Australia, for analysis using an amino acid analyser.

**3.2.7 Statistical Analysis**
Statistical analysis of all plant physiology and NMR results were conducted using ANOVA analysis as part of excelstat software.
3.3. Results and Discussion

The results for the plant N-uptake of different fertilizer N forms by hydroponically grown *Eucalyptus Pilularis* and *Pinus Elliotti* (slash pine) will be presented and discussed with an emphasis on how the different nutrients affect the plant’s general physiology, assimilation, metabolism and storage.

### 3.3.1 Plant Physiology

**Slash Pine**

The resulting fresh and dried weights and % water content for slash pine are shown in Table 3.17. Exposure to different forms of fertiliser N was found to have no effect on the fresh mass of the slash pine section samples. However, the plants grown in CN fertilizer solutions had the lowest combined above ground fresh biomass (needles and stems) without much of a variation in the fresh mass of roots compared with those for the rest of the plants. Some differences were observed in the mass of dried needles and roots, particularly between AS, CN and AN fertiliser solutions, with AS exhibiting the highest average increase in mass for both dried needles and roots. The total dried mass of the stems, however, showed no differences when exposed to the different N–fertiliser solutions, indicating the same amount of total biomass was produced by each fertiliser with a 95% confidence level. The water content of all slash pine sections revealed that the AS fertilised plants also had lower water content when compared with CN fertilised plants.

Height and needle length measurements (Table 3.16) indicate that slash pine heights did not change significantly when exposed to the different fertilisers, with the exception of CN (height = 30.7cm), which was significantly smaller than the heights produced by UR (height = 39.6cm). Differences in needle lengths were observed, with CN once again giving the lowest values (length = 16.6cm) and AS giving slightly higher results than all other fertilisers (length = 20.7cm).

From these results it can be concluded that plants grown under the CN regime were worse off than those grown with other fertiliser N forms, exhibiting the smallest combined above ground biomass, height and needle length. Observations of seedling health changes were also recorded in both written form and through photography (for
example, Figure 3.1). It was found that the CN exposed slash pines changed its colour to more of a green-yellow when compared to a medium-toned green in the other seedlings. This may indicate a smaller uptake of N, which can lead to yellowing or plants having a light green appearance. Sulphur deficiencies can also cause yellowing, however, this usually occurs only in younger leaves, which was not observed here ("Diagnosing nutrient deficiency symptoms in crop plants," 2008; Hosier & Bradley, 1999). A significant difference in root colour was also observed (Figure 3.1e) with pines exposed to CN, producing very dark, thin and unhealthy-looking roots.

Kronzucker and co-workers (1997), have previously found that the uptake of NH$_{4}^{+}$ was 20 times higher than that of NO$_{3}^{-}$ in the conifer white spruce. It was therefore found that white spruce seedlings capacity to use NO$_{3}^{-}$ was reduced. In our experiments, the CN is the solution that had NO$_{3}^{-}$ as the only source of N whereas AN contained both NH$_{4}^{+}$ and NO$_{3}^{-}$. Therefore, it may also explain the poor performance of the CN seedlings in our experiment, if they are unable to utilise NO$_{3}^{-}$ as efficiently as NH$_{4}^{+}$. Kronzucker also pointed out that white spruce needed to be previously exposed to NO$_{3}^{-}$, or “induced”, for 3 days before maximum N uptake to occur, once again limiting the uptake of N when NO$_{3}^{-}$ was the only N source present. Stitt & Scheible (1999) also pointed out that NO$_{3}^{-}$ needed to be converted to NH$_{4}^{+}$ first by nitrate reductase (NR) and nitrite reductase (NiR) within the plant. If there

---

Table 3.16: Average growth measurements for both Eucalyptus and Slash Pine. Letters of the same type indicate that there is no significant difference where p < 0.05

<table>
<thead>
<tr>
<th>Fertilizer</th>
<th>Height * (cm)</th>
<th>Leaf Area (cm$^2$) *</th>
<th>Needle * length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eucalyptus</td>
<td>Slash Pine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Old New</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AN</td>
<td>69.1 a</td>
<td>25.7 a 17.2 a</td>
<td>33.4 ab 19.1 bc</td>
</tr>
<tr>
<td>*AN</td>
<td>66.0 a</td>
<td>28.4 a 14.0 ab</td>
<td>33.8 ab 20.3 ab</td>
</tr>
<tr>
<td>A*N</td>
<td>54.1 ab</td>
<td>20.0 b 12.9 ab</td>
<td>35.4 ab 19.0 c</td>
</tr>
<tr>
<td>CN</td>
<td>61.7 ab</td>
<td>24.7 ab 17.5 a</td>
<td>30.7 b 16.6 d</td>
</tr>
<tr>
<td>UR</td>
<td>46.7 b</td>
<td>19.5 b 13.0 ab</td>
<td>39.6 a 19.5 bc</td>
</tr>
<tr>
<td>AS</td>
<td>60.9 ab</td>
<td>25.9 a 11.3 b</td>
<td>36.3 ab 20.7 a</td>
</tr>
</tbody>
</table>

* $n = 5$ for all fertilizer treatments except pine *AN which has $n = 3$

* $n = 12$ and 15

* $n = 52$ and 62, except *AN which has $n = 35$
Table 3.17: Average slash pine biomass measurements per plant. Letters of the same type indicate that there is no significant difference where 
\( p < 0.05 \) (\( n = 5 \) for all fertilizer treatments except *AN, which has \( n = 3 \)).

<table>
<thead>
<tr>
<th>Fertilizer</th>
<th>Avg. Fresh Masses (g)</th>
<th>Avg. Dried Masses (g)</th>
<th>Avg. % Water Content (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaves</td>
<td>Stems</td>
<td>Roots</td>
</tr>
<tr>
<td>AN</td>
<td>14.22 a</td>
<td>7.38 a</td>
<td>7.04 a</td>
</tr>
<tr>
<td>*AN</td>
<td>13.38 a</td>
<td>5.66 a</td>
<td>8.39 a</td>
</tr>
<tr>
<td>A*N</td>
<td>14.96 a</td>
<td>8.62 a</td>
<td>9.95 a</td>
</tr>
<tr>
<td>CN</td>
<td>9.93 a</td>
<td>5.54 a</td>
<td>8.40 a</td>
</tr>
<tr>
<td>UR</td>
<td>13.87 a</td>
<td>8.18 a</td>
<td>8.46 a</td>
</tr>
<tr>
<td>AS</td>
<td>14.41 a</td>
<td>6.98 a</td>
<td>10.55 a</td>
</tr>
</tbody>
</table>

Table 3.18: Average eucalyptus biomass measurements per plant. Letters of the same type indicate that there is no significant difference where 
\( p < 0.05 \) (\( n = 5 \) for all fertilizer treatments except *AN, which has \( n = 3 \)).

<table>
<thead>
<tr>
<th>Fertilizer</th>
<th>Avg. Fresh Masses (g)</th>
<th>Avg. Dried Masses (g)</th>
<th>Avg. % Water Content (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaves</td>
<td>Stems</td>
<td>Roots</td>
</tr>
<tr>
<td>AN</td>
<td>15.45 a</td>
<td>9.44 ab</td>
<td>7.97 a</td>
</tr>
<tr>
<td>*AN</td>
<td>17.40 a</td>
<td>10.24 a</td>
<td>9.75 a</td>
</tr>
<tr>
<td>A*N</td>
<td>16.06 a</td>
<td>9.13 ab</td>
<td>9.49 a</td>
</tr>
<tr>
<td>CN</td>
<td>17.25 a</td>
<td>10.98 a</td>
<td>-</td>
</tr>
<tr>
<td>UR</td>
<td>10.67 a</td>
<td>5.36 b</td>
<td>6.65 a</td>
</tr>
<tr>
<td>AS</td>
<td>12.71 a</td>
<td>8.92 ab</td>
<td>17.83 b</td>
</tr>
</tbody>
</table>

Key: AN = Ammonium Nitrate (double \( ^{15} \)N enrichment)  
A*N = Ammonium Nitrate (where only the nitrate is \( ^{15} \)N enriched)  
*AN = Ammonium Nitrate (where only the ammonium is \( ^{15} \)N enriched)  
CN = Calcium Nitrate (double \( ^{15} \)N enrichment)  
UR = Urea (double \( ^{15} \)N enrichment)  
AS = Ammonium Sulfate (double \( ^{15} \)N enrichment).
was low NR activity, then uptake of NO$_3^-$ supply was restricted, resulting in excess nitrate not converted and therefore unable to be used. These may be reasons for the low N content of CN slash pine seedlings.

Apart from longer needle length and a lower water content, AS fertilised pines displayed relatively similar biomass and plant heights as for those treated with UR, AN, *AN and A*N. This may indicate that AS fertilisers produce seedlings with a slightly denser biomass. It has also previously been observed that NH$_4^+$ toxicity can lead to restricted growth (Monselise & Kost, 1993).

**Eucalyptus**

In the eucalyptus seedling hydroponics experiment, there were no differences between total average mass for the different fertiliser N forms with the exception of seedlings in the UR fertilizer solutions (Table 3.18). The seedlings exposed to UR had the lowest total dry mass, almost half the average value compared to those of other seedlings. It was of interest to note that the AS exposed roots, whose fresh roots mass was almost double that found for the other fertilisers. Upon drying, however, the only differences found were in the UR treated eucalypts, which gave consistently lower masses. UR exposed plants appeared to be in poor growth condition, with three plants dying in the preliminary experiments, and one plant dying before the end of the current experiment. This is most likely due to the fact that the eucalypts are unable to take up urea directly; hence the urea needs to be broken down into inorganic N compounds first. The presence of such inorganic minerals was confirmed by the IC studies carried out during the experiment and this aspect of uptake of nutrients will be discussed later in the chapter.

The leaf and stem water content of UR treated plants, was significantly higher than those treated with most other fertilisers. The AS treated eucalypts contained a significantly higher fresh root mass compared to all seedlings and a major portion of the weight was due to water present in the roots. The total water content in the plant for UR and AS treatment was found to be significantly higher than those treated with the AN, *AN and A*N fertilisers. While with CN treatment the total water content could not be found (root fresh mass was not recorded), its leaf and stem water contents are similar to the AN fertilised eucalypts.
UR fertilised eucalyptus plants were found to give smaller seedling heights and reduced older leaf areas (Table 3.16). It is of interest however, to observe that such growth limitations in the corresponding UR fertilized slash pines were not observed. The possible cause for such growth patterns may be linked to the inability of the plant species to utilise urea as a nutrient source. It is also important to note that a smaller average new leaf area was found for AS treated eucalypts. It was further observed that the leaves on the AS treated plant seemed thicker when touched. The average old and new leaf areas for CN treated seedlings agree with all other fertiliser treatments except new leaves produced using AN. The observations taken throughout these experiments however, showed that with CN treatment, random large leaves (both old and new) were produced that were over double the area of the largest leaf found on the other fertilised plants. These leaves were also very soft to touch compared to other fertilisers. Further studies on insect-plant interactions and studies carried out on toughness and its N content is discussed towards the end of this section.

Guo and co-workers (2007) have also found that plants fertilised with nitrates only, produce larger leaves, while ammonium fertilised plants have reduced leaf areas. It has also been suggested that excess nitrates that have not been reduced to ammonium accumulate in a plants shoots, signals possibly involving plant hormones such as cytokinin are sent to the roots, resulting in inhibited root growth (Stitt & Scheible, 1999; Walch-Liu et al., 2005). A larger shoot to root ratio is hence found for plants grown on nitrates only. Shoot to root ratios were found for the research results, but due to large variations in plant growth and therefore dried weights, all ratios were found to be in agreement within uncertainties. This may also be due to the age of the seedlings, as they were all under a year old and may not have had enough time for plants in the different fertilisers to differentiate themselves. Also the eucalypts had been purchased after 6 months of growth in the same soils. As pointed out by Blumfield & Xu (2006), they may still be relying on stored N accumulated before the experiment and therefore less dependency on the supplied N in the fertiliser solutions. Hence large differences may not be seen.
3.3.2 Total N and Total C content by weight

Total N and total C content were found by taking the resulting average dried mass for each plant component, and multiplying it by the respective %N and %C concentrations (w/w) measured on the CN analyser.

Results for %N and %C concentrations for each component (needles, stems and roots) are shown for slash pine (Table 3.19) and eucalyptus (leaves, stems and roots) (Table 3.20). The %N and %C for each component of the plant varied based on the N-source in the fertilizer solution. The overall %C concentration was highest for slash pines grown in AN fertiliser solutions, with all sections of the AN pine contributing approximately equally to the carbon concentration. CN and AS showed lower %C concentrations in their stems (38.7% and 34.2% respectively) compared to the other sections. Slash pine in AS solutions gave the lowest needle and stem %C concentrations, as well as the lowest %N concentrations for all plant components. %N was highest in UR needles and stems, with CN producing the highest results for roots. When considering the overall N distribution in slash pines in all fertilizer solutions, %N was lowest in the stems compared to those present in needles and roots. The %N distribution in needles and roots however, varied based on the N-source in the fertilizer.

Table 3.19: The average % N and % C concentration (w/w) found for slash pine sections in each of the fertiliser solutions

<table>
<thead>
<tr>
<th>Fertiliser</th>
<th>Needles</th>
<th>Stems</th>
<th>Roots</th>
<th>Needles</th>
<th>Stems</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>1.90</td>
<td>0.68</td>
<td>1.56</td>
<td>44.6</td>
<td>44.7</td>
<td>44.5</td>
</tr>
<tr>
<td>CN</td>
<td>1.41</td>
<td>0.49</td>
<td>2.01</td>
<td>42.6</td>
<td>38.7</td>
<td>41.3</td>
</tr>
<tr>
<td>UR</td>
<td>2.02</td>
<td>0.79</td>
<td>1.99</td>
<td>41.7</td>
<td>41.3</td>
<td>43.2</td>
</tr>
<tr>
<td>AS</td>
<td>1.33</td>
<td>0.49</td>
<td>1.52</td>
<td>40.3</td>
<td>34.2</td>
<td>42.5</td>
</tr>
</tbody>
</table>

Table 3.20: The average % N and % C concentration (w/w) found for eucalyptus sections in each of the fertiliser solutions

<table>
<thead>
<tr>
<th>Fertiliser</th>
<th>Leaves</th>
<th>Stems</th>
<th>Roots</th>
<th>Leaves</th>
<th>Stems</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>2.13</td>
<td>0.48</td>
<td>1.33</td>
<td>42.5</td>
<td>42.0</td>
<td>42.2</td>
</tr>
<tr>
<td>CN</td>
<td>1.73</td>
<td>0.47</td>
<td>1.54</td>
<td>40.0</td>
<td>40.5</td>
<td>38.5</td>
</tr>
<tr>
<td>UR</td>
<td>1.82</td>
<td>0.56</td>
<td>1.68</td>
<td>38.0</td>
<td>41.8</td>
<td>41.2</td>
</tr>
<tr>
<td>AS</td>
<td>2.10</td>
<td>0.69</td>
<td>1.93</td>
<td>36.3</td>
<td>39.8</td>
<td>42.6</td>
</tr>
</tbody>
</table>
The average total N content per plant for slash pine sections is shown in Figure 3.5. Total N was found to be highest in UR slash pine, with the majority of N being found in needles (0.080g). This, combined with the higher N concentrations suggests that UR slash pines were making more efficient use of their N supply. It also implies that slash pine may be able to directly take up urea. This will be discussed further in section 0. CN was found to have the lowest total N; however it exhibited the highest total N content in roots (0.036g). This indicates that CN may be partitioning more N to its roots than the rest of the plant sections. Since CN also exhibited the lowest heights, needle dried mass and needle length, more N may be sent to the roots as a response to a lower N uptake compared to AN and AS fertilised plants, which can be seen in Figure 3.11. Total C content for slash pine sections (Figure 3.6), shows a similar trend to that found for total N, with the exception of AS pine, which showed the highest total C content.

The %N and %C concentrations of eucalyptus sections are given in Table 3.20. Concentrations were found to be similar to those found for the slash pine seedlings. The %N concentration of eucalyptus stems was lower than that for leaves and roots for all fertilisers. The highest leaf N concentrations were found in the AN eucalypts, however these seedling also exhibited the lowest N concentrations for stem and roots. This trend is also seen to a certain extent in the total N content of the AN eucalypts, implying that these seedlings may be partitioning a larger amount of N to their leaves. This same was not seen in total C content however, with AN eucalyptus having the highest root and stem content (3.59 and 7.14g respectively).

The highest %N concentrations in root and stem components and total N content were found in the AS fertilised seedlings. Once again this may indicate partitioning, in this case more towards the lower half of the tree. This partitioning change was not seen in total %C content. Both total N and total C contents were lowest in UR plants, in most cases being less than half that found in the other fertilisers. This along with the low dried mass, height and leaf area, indicates that eucalypt seedling may not be able to use efficiently, or utilise directly, N in the form of urea.
Figure 3.5: Total N results for slash pine sections under different N-fertiliser regimes. Numbers given are the total N in grams for that particular section.

Figure 3.6: Total C results for slash pine sections under different N-fertiliser regimes. Numbers given are the total C in grams for that particular section.

Figure 3.7: Total N results for eucalyptus sections under different N-fertiliser regimes. Numbers given are the total N in grams for that particular section.

Figure 3.8: Total C results for eucalyptus sections under different N-fertiliser regimes. Numbers given are the total C in grams for that particular section.
The %N in eucalyptus leaves has a direct link to the insect-plant interactions associated with the nutrition of the insect herbivore larvae. Earlier studies by Fox and Macauley (1977) reported that the growth rate of *Paropsis atomaria* Oliver larvae was directly related to N concentrations in *Eucalyptus* foliage and not affected at all by a wide range in concentrations of phenols. They concluded that N could be a limiting factor for the early stages of the insect since N concentrations in naturally-occurring eucalypt foliage were low.

Effects of food quality, particularly nitrogen concentrations, of *Eucalyptus blakelyi* foliage on the growth of *Paropsis atomaria* larvae has been studied by Ohmart and co-workers (1985). In their studies the eucalyptus seedlings were differentially fertilized to obtain a range of N concentrations from 0.8–3.0% dry wt in the foliage. The growth of the insect larvae did not differ between N levels of 1.7–3.0% but when the foliage N below this range led to a significant decrease in pupal weight and increase in development time for larvae. Our experimental studies show that the %N content in leaves range from 1.73-2.13% based on the N-form in the fertilizer solution. Therefore, %N is an important factor that could be used to regulate the survival of the larval stages.

Another feature that is associated with the %N is the physical characteristics of the leaves. The investigations by Ohmart and co-workers have reported that all larvae which hatched on foliage with < 1% N died during the first instar. Later it was found that the low %N resulted in tough leaves and therefore mortality probably due to inability of first instar larvae to feed rather than by N starvation. These findings also suggested that N is not limiting for *P. atomaria* in most years since new foliage is normally abundant, soft and high in N. It would be limiting in years when new foliage is scarce and most foliage available is old, tough and low in N. (Ohmart et al., 1987)

### 3.3.3 Plant Hydroponic Fertiliser Solutions

**Water Uptake**

Water uptake results for slash pine and eucalyptus seedlings (Figure 3.9) were corrected for the number of plants and the number of days in solutions. This
correction was done as some plants were in solutions for a slightly longer time period due to varied harvest times. It was found that the slash pines did not show much of a difference in water uptake between the different N-fertilizers. While eucalyptus, on the other hand, showed large differences between results for the AN solutions, and the other fertilizers. All of the seedlings in the three AN solutions used almost double the amount of water compared to those in the other fertilizers. This could be due to either increased transpiration rates as water uptake of a plant is controlled by transpiration, or the plants being able to hold larger amounts of water. The latter explanation can be ruled out, as the water content shown in Table 3.18 found that the AN fertilised eucalypts in fact had a lower water content than AS and UR. Eucalypts grown with UR, CN and AS gave similar results for water uptake.

Transpiration usually takes places during photosynthesis. Photosynthesis uses light energy and CO$_2$ which is taken in through open stomata to synthesise biomolecules, such as, carbohydrates and amino acids, needed for plant growth. This, however, comes at the expense of transpiration, as while the stomata are open, water molecules are lost. If a plant underwent increased transpiration, it would be expected that there is also increased photosynthesis, which would lead to increased carbon content and hence biomass accumulation. There can however, be other processes that would lead to carbon loss from the plant. These include C respiration by the plant organs and the exudation of carbon compounds via the root systems. Therefore, these carbon losses

![Figure 3.9: Water uptake of Eucalypts in different fertilizer solutions (data has been corrected for number of plants and days in the solutions).](image-url)
Figure 3.10: The water use efficiency (WUE) of both slash pine and eucalyptus seedlings in different fertilizer solutions (data has been corrected for number of plants and days in the solutions).

would have a negative impact on WUE. Similarly, there can be other mechanisms to lose water from the plant and/or hydroponic medium. For example, night time transpiration via the incomplete closure of the stomata will have a negative impact on the WUE. In our experiments, the loss of water due to evaporation from the hydroponic medium was minimised as all were covered with aluminium foil. It is therefore important to consider the overall effect of all these processes impacting on the net C gain of the biomass and the water loss to evaluate the efficiency of water usage. The experimental data shown in Figure 3.10 indicated that AN treated eucalypts lost the most amount of water, yet their C content and fresh and dried biomass were found to be roughly the same as those treated with CN and AS seedlings.

The answer to this problem may be similar to research results found by Guo and co-workers (2007) for Phaseolus vulgaris L. (French bean), which was discussed in section 3.1.6 of this chapter. Research findings showed that plant roots in vessels containing nitrate took up more N during the day, however, for vessels containing ammonium roots took up the same amount of N during night, as they did during the day. As discussed in section 3.1.2, nutrient uptake relies on water uptake, which in turn relies on transpiration. If the AN treated eucalypts were transpiring more during
the night, this transpiration would be taking place in the absence of light and therefore the absence of photosynthesis. This means that CO$_2$ taken in by the plant during the night is not used for the synthesis of biomolecules, and hence C-containing biomass.

The water use efficiency (WUE) was also assessed by plotting the average total dry mass produced versus the average water uptake per day for each fertiliser solutions. Both the slash pine and eucalyptus results are shown in Figure 3.10. While the slash pine data points fell in roughly the same area, eucalyptus showed significant differences. Data points show that while all eucalyptus produced roughly the same biomass, with the exception of UR, the fertilisers CN and AN produced it with almost half the amount of water, with CN having the best WUE. This could possibly be due to the slightly higher Ca$^{2+}$ level present in the CN solution, which was unavoidable in order to keep all other nutrients the same between fertilisers. Ca$^{2+}$ is important in the functioning of stomata on a plant's leaves, and is also involved in plant signaling when the plant is under stress (Ridolfi et al., 1994; Ruiz et al., 1993). It may be that the stomata of the CN are more often signalled to close, and therefore water is retained. This aspect will be explored further in the Chapter 6 of this thesis.

**Total N uptake**

Figure 3.11 and Figure 3.12 show the total N uptake of slash pine and eucalyptus seedling respectively. The total N uptake shown in these graphs has been separated into uptake from ammonium and uptake from nitrates as total N uptake was found using ion chromatography (IC). The IC could only analysed the inorganic forms of N, and hence urea-N uptake could not be found. It was found however, that small amounts of ammonium and nitrates were present in the eucalyptus UR solutions, even though they had not been added. This may be explained by the presence of a combination of chemical and microbial activity, which converts the urea to inorganic forms as discussed in section 3.1.1.

It was found that seedlings in the presence of ammonium only (AS) took up slightly more N than seedlings exposed to nitrates only (CN) for both slash pine and eucalyptus seedlings. It is interesting that when ammonium and nitrates are both
present (AN), the uptake of each is roughly 50:50. This could be due to the presence of nitrates inhibiting the ammonium uptake (Aarnes et al. 1995). It has been found that when both of these form of N are present together in solution, total N uptake is increased (Guo et al., 2007). This is confirmed from our study, where the eucalypts in the AN solutions seem to benefit from both N types being present, as more N is taken up by the seedlings (Figure 3.12).

![Figure 3.11: Average total N uptake by slash pine plants.](image1)

![Figure 3.12: Average total N uptake by eucalyptus plants.](image2)
The N uptake of eucalypt seedlings in AN solutions however, is not double that of other fertilisers as the water uptake was found to be (Figure 3.9). If night transpiration is occurring in these plants more than the other fertilised plants a major difference in N uptake would be expected, as more N and other nutrients should be taken up with the increase of water. The fact that this increase in N uptake has not occurred with increased water uptake suggests that there may be a selective barrier that regulates the uptake of N so that excessive amounts cannot be taken up. Aarnes and co-workers (1995) have previously shown that tissue \( \text{NH}_4^+ \) levels in Norway spruce (\textit{Picea abies}), grown in hydroponic solutions peaked when N concentrations in the solution were 5mM, but no increases were observed when the concentration was raised to 50mM. This indicates that there must be some sort of metabolic process controlling the levels of \( \text{NH}_4^+ \) in plant tissues, which may also explain the results found in this study. Alternatively, N may not have been taken in by roots due to the roots needing to maintain electroneutrality. This will be discussed in more detail in the subsequent section on influx and effluxes.

**Potential Losses**

It is likely that some N losses may have occurred due to volatilisation during these experiments. This was minimised by covering the tops of each seedling pot, however seals were not airtight, so some losses may still have occurred. Although the amounts of N added to the hydroponic solutions were recorded along with the N uptake and N content of the plants, the N content of the starting eucalyptus seedling and slash pine seeds was not recorded. Therefore the exact amounts of N loss from the system could not be found. As slash pine seeds should have a small N content compared to a six-month-old seedling, N losses were estimated and shown in Table 3.21. Losses were shown to be small compared to losses of at least 20-40\% usually found in soil systems (Blumfield & Xu, 2006; Prasertsak et al., 2001a). By using hydroponics instead of soil for this research, no clay was present and all solutions were kept, therefore ruling out losses due to leaching and binding of cations to clay particles.

The ion \( \text{NH}_4^+ \) undergoes volatilisation at high pH’s (section 3.1.1). As the CN solution only contains \( \text{NO}_3^- \), losses should be minimal. However, if denitrifying bacteria that use \( \text{NO}_3^- \) as their oxygen supply are present, they may be converting
some N into dinitrogen, which is then released back into the atmosphere. This may explain the N loss from CN solutions. The pH of AS solutions for both slash pine and eucalyptus seedling solutions (Table 3.21), was found to be low (pH < 3.5), therefore volatilisation should also be low. Denitrification should not be occurring here.

Table 3.21: Estimated nitrogen losses from slash pine hydroponic solutions and the average unadjusted pH for slash pine and eucalyptus fertiliser solutions.

<table>
<thead>
<tr>
<th>Fertiliser</th>
<th>Slash Pine N Loss</th>
<th>Average pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>12.1%</td>
<td>4.2</td>
</tr>
<tr>
<td>UR</td>
<td>5.8%</td>
<td>5.6</td>
</tr>
<tr>
<td>AS</td>
<td>8.4%</td>
<td>2.7</td>
</tr>
<tr>
<td>CN</td>
<td>13.6%</td>
<td>7.1</td>
</tr>
</tbody>
</table>

UR solutions on the other hand, had higher average pH’s. If the urea present in these solutions was hydrolysed to $\text{NH}_4^+$ acidic solutions, then higher amounts of volatilisation would occur from these solutions. Urea has been found to be moderately volatile when applied to soils due to higher soil pH, with approximately 20-30% being lost to volatilisation (Blumfield & Xu, 2006; Prasertsak et al., 2001a). However, if the slash pine seedling were able to utilise urea-N directly, without the need for it to be mineralised, this would minimise losses and may explain the loss of only 5.8% from UR solutions. Eucalyptus seedlings may have experienced much larger losses from UR solutions as they were possibly relying on the urea to be hydrolysed first, and therefore having a greater possibility of volatilisation.

Lastly AN solutions may also experience both volatilisation and denitrification, as both $\text{NH}_4^+$ and $\text{NO}_3^-$ are present. The average pH for AN solutions should not be high enough for any major losses through volatilisation, therefore most of the 12.1% loss may be from denitrification.

Another form of potential N loss considered here was the exudation of amino acids from the plant roots. This loss could not be monitored by ion chromatography, as only inorganic ions could be observed. Root exudations of amino acids and amides have been previously reported for *Eucalyptus marginata* and *Eucalyptus calophylla*.
(Bowen, 1969) and Pinus radiata (Malajczuk & McComb, 1977) species. Malajczuc & McComb (1977) found that these two species of Eucalyptus exuded up to 20 different amino acids over 42 days of sampling.

Samples of hydroponic solutions were first tested using thin layer chromatography (TLC). Very faint spots could be seen on TLC plates after developing with ninhydrin, indicating that there may be trace amounts of amino acids in hydroponic solutions. Samples were then sent for amino acid analysis and quantitation at the Australian Proteome Analysis Facility at Macquarie University. Although the presence of amino acids was not detected in the hydroponic samples, however, this may be due to the levels of sensitivity of the instrument. In any case, if there are either no amino acids present or trace amounts, amino acid exudation will not account for a significant N-loss in these experiments.

**Macronutrient Uptake**

Ion chromatography was used in our study to monitor the uptake of N as well as the uptake of other macronutrients. The total uptake of all anions/cations at the end of seedling growth in $^{15}$N-enriched solutions was found for slash pine and eucalyptus seedlings. Results for total uptake are shown in Table 3.22 and Table 3.24 respectively. Table 3.25 and Table 3.23 show the relative percentages of macronutrients taken up by slash pine and eucalyptus plants respectively, with respect to the total nutrient uptake. These percentages were then used to find any trends between fertilisers and macronutrient uptakes. Figure 3.13 and Figure 3.14 show trends that were found with $R^2 > 0.95$ (where $R^2$ is the correlation coefficient).

The same trends were found for both slash pine and eucalyptus. These trends and their positive or negative correlation gradients are shown in Table 3.26. It is interesting to note that not only were the same trends found for both slash pine and eucalyptus seedling uptake, but the gradient of the line of the trendline fitted was very similar for each species (Figure 3.23). The position of the different fertilisers in Figure 3.13 and Figure 3.14, are also the same between plant species, with the exception of a slight difference in the position of UR in the N-containing graphs (Figure 3.13d,e and Figure 3.14d,e). In all positively correlated graphs, AS shows the lowest uptake of all nutrients, while UR exhibits the highest uptakes. AN, *AN, A*N
Table 3.22: Total uptake of macronutrients by slash pine expressed in mmol.

<table>
<thead>
<tr>
<th>Fertilizer</th>
<th>Cl⁻</th>
<th>NO₃⁻</th>
<th>PO₄³⁻</th>
<th>SO₄²⁻</th>
<th>NH₄⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>12.8</td>
<td>13.1</td>
<td>3.2</td>
<td>13.7</td>
<td>12.8</td>
<td>18.6</td>
<td>8.9</td>
<td>6.0</td>
<td>88.9</td>
</tr>
<tr>
<td>*AN</td>
<td>14.6</td>
<td>14.4</td>
<td>3.6</td>
<td>17.3</td>
<td>14.0</td>
<td>21.6</td>
<td>10.6</td>
<td>7.1</td>
<td>103.2</td>
</tr>
<tr>
<td>A*N</td>
<td>14.2</td>
<td>14.4</td>
<td>3.6</td>
<td>16.0</td>
<td>13.9</td>
<td>21.2</td>
<td>10.2</td>
<td>6.8</td>
<td>100.3</td>
</tr>
<tr>
<td>UR</td>
<td>13.9</td>
<td>-0.1</td>
<td>3.4</td>
<td>15.5</td>
<td>0.0</td>
<td>20.5</td>
<td>9.7</td>
<td>6.6</td>
<td>69.4</td>
</tr>
<tr>
<td>AS</td>
<td>32.6</td>
<td>-0.01</td>
<td>3.4</td>
<td>18.9</td>
<td>26.9</td>
<td>19.9</td>
<td>9.9</td>
<td>6.6</td>
<td>118.1</td>
</tr>
<tr>
<td>CN</td>
<td>12.1</td>
<td>23.5</td>
<td>2.9</td>
<td>5.6</td>
<td>0.0</td>
<td>17.6</td>
<td>11.6</td>
<td>5.7</td>
<td>78.9</td>
</tr>
</tbody>
</table>

Table 3.23: Percentage of the total uptake of all macronutrients by slash pine for each element

<table>
<thead>
<tr>
<th>Fertilizer</th>
<th>Cl⁻</th>
<th>NO₃⁻</th>
<th>PO₄³⁻</th>
<th>SO₄²⁻</th>
<th>NH₄⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>14.4</td>
<td>14.7</td>
<td>3.6</td>
<td>15.4</td>
<td>14.4</td>
<td>20.9</td>
<td>10.0</td>
<td>6.7</td>
<td>100</td>
</tr>
<tr>
<td>*AN</td>
<td>14.2</td>
<td>13.9</td>
<td>3.5</td>
<td>16.8</td>
<td>13.6</td>
<td>20.9</td>
<td>10.3</td>
<td>6.9</td>
<td>100</td>
</tr>
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<td>A*N</td>
<td>14.2</td>
<td>14.4</td>
<td>3.6</td>
<td>16.0</td>
<td>13.9</td>
<td>21.1</td>
<td>10.1</td>
<td>6.8</td>
<td>100</td>
</tr>
<tr>
<td>UR</td>
<td>20.0</td>
<td>-0.2</td>
<td>4.9</td>
<td>22.3</td>
<td>0.0</td>
<td>29.5</td>
<td>14.0</td>
<td>9.5</td>
<td>100</td>
</tr>
<tr>
<td>AS</td>
<td>27.6</td>
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<td>2.9</td>
<td>16.0</td>
<td>22.8</td>
<td>16.8</td>
<td>8.4</td>
<td>5.6</td>
<td>100</td>
</tr>
<tr>
<td>CN</td>
<td>15.3</td>
<td>29.8</td>
<td>3.6</td>
<td>7.1</td>
<td>0.0</td>
<td>22.3</td>
<td>14.7</td>
<td>7.2</td>
<td>100</td>
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</tbody>
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Table 3.24: Total uptake of macronutrients by eucalyptus expressed in mmol.

<table>
<thead>
<tr>
<th>Fertilizer</th>
<th>Cl⁻</th>
<th>NO₃⁻</th>
<th>PO₄³⁻</th>
<th>SO₄²⁻</th>
<th>NH₄⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
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<td>26.9</td>
<td>25.8</td>
<td>6.6</td>
<td>32.6</td>
<td>25.2</td>
<td>40.0</td>
<td>19.8</td>
<td>13.2</td>
<td>190.1</td>
</tr>
<tr>
<td>*AN</td>
<td>23.6</td>
<td>23.0</td>
<td>5.8</td>
<td>29.2</td>
<td>22.2</td>
<td>35.3</td>
<td>17.5</td>
<td>11.7</td>
<td>168.3</td>
</tr>
<tr>
<td>A*N</td>
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<td>19.5</td>
<td>5.0</td>
<td>24.9</td>
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<td>30.2</td>
<td>14.9</td>
<td>10.0</td>
<td>143.9</td>
</tr>
<tr>
<td>UR</td>
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<td>-3.8</td>
<td>4.2</td>
<td>18.4</td>
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<td>25.5</td>
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<td>CN</td>
<td>17.7</td>
<td>33.8</td>
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<td>25.9</td>
<td>17.0</td>
<td>8.4</td>
<td>115.4</td>
</tr>
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Table 3.25: Percentage of the total uptake of all macronutrients by eucalyptus for each element

<table>
<thead>
<tr>
<th>Fertilizer</th>
<th>Cl⁻</th>
<th>NO₃⁻</th>
<th>PO₄³⁻</th>
<th>SO₄²⁻</th>
<th>NH₄⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>14.2</td>
<td>13.6</td>
<td>3.5</td>
<td>17.2</td>
<td>13.2</td>
<td>21.0</td>
<td>10.4</td>
<td>6.9</td>
<td>100</td>
</tr>
<tr>
<td>*AN</td>
<td>14.0</td>
<td>13.6</td>
<td>3.5</td>
<td>17.3</td>
<td>13.2</td>
<td>21.0</td>
<td>10.4</td>
<td>6.9</td>
<td>100</td>
</tr>
<tr>
<td>A*N</td>
<td>14.1</td>
<td>13.6</td>
<td>3.5</td>
<td>17.3</td>
<td>13.3</td>
<td>21.0</td>
<td>10.4</td>
<td>6.9</td>
<td>100</td>
</tr>
<tr>
<td>UR</td>
<td>22.5</td>
<td>-4.7</td>
<td>5.2</td>
<td>22.9</td>
<td>-2.4</td>
<td>31.8</td>
<td>14.8</td>
<td>9.9</td>
<td>100</td>
</tr>
<tr>
<td>AS</td>
<td>28.2</td>
<td>0.0</td>
<td>3.2</td>
<td>15.0</td>
<td>22.9</td>
<td>17.3</td>
<td>8.0</td>
<td>5.5</td>
<td>100</td>
</tr>
<tr>
<td>CN</td>
<td>15.3</td>
<td>29.3</td>
<td>3.7</td>
<td>7.2</td>
<td>0.0</td>
<td>22.5</td>
<td>14.7</td>
<td>7.3</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 3.13: Relationships between the percentage uptakes of macronutrients in slash pine solutions, based on the results found in Table 3.23. Negative percentages indicate an output of nutrient rather than an uptake.
Figure 3.14: Relationships between the percentage uptakes of macronutrients in Eucalyptus solutions, based on the results found in Table 3.25. Negative percentages indicate an output of nutrient rather than an uptake.
and CN solutions gave similar results, showing moderate uptakes compared to AS and UR.

All of these correlations can be explained by the seedlings need to maintain electroneutrality in its roots (Mengel & Pilbeam, 1992). For example, it was found here that as the roots of each plant took more negative charges in the form of $\text{PO}_4^{3-}$, it also took up more positive charges in the form of $\text{K}^+$ (Figure 3.13a and Figure 3.14a) or $\text{Mg}^{2+}$ (Figure 3.13b and Figure 3.14b). A positive correlation was also found between the uptake of $\text{K}^+$ and $\text{Mg}^{2+}$, a correlation in which there is no balance of charges. The increasing uptake of these positive charges however, is balanced by the uptake of $\text{PO}_4^{3-}$, as shown in Figure 3.13c and Figure 3.14c. Peuke & Jeschke (1993) have also found that $\text{Cl}^-$ acts as a counter-ion, and therefore balance, to $\text{NH}_4^+$ uptake. This however, was not observed here.

Negative correlations were seen between like charges. As the roots took up more the positively charged ions in the form of $\text{NH}_4^+$, it took up less positive charge in the form of $\text{Ca}^{2+}$ (Figure 3.13d and Figure 3.14d). Both negative correlations that were found involved nitrogen species. It has been previously found that plants took up less $\text{Cl}^-$ when $\text{NO}_3^-$ uptake was higher, and vice versa (Peuke & Jeschke, 1993). In the experiments undertaken for this thesis, it was found that $\text{NO}_3^-$ uptake was balanced by the total uptake of $\text{PO}_4^{3-} + \text{SO}_4^{2-} + \text{Cl}^-$. Correlations were not found between $\text{NO}_3^-$ and these individual ions, only the total.

It is interesting to note that not only were the same trends found for both slash pine and eucalyptus seedling uptake, but the gradient of the line of the trendline fitted was very similar for each species (Figure 3.23). The position of the different fertilisers in Figure 3.13 and Figure 3.14, are also the same between plant species, with the exception of a slight difference in the position of UR in the N-containing graphs (Figure 3.13d,e and Figure 3.14d,e). In all positively correlated graphs, AS shows the lowest uptake of all nutrients, while UR exhibits the highest uptakes. AN, *AN, A*N and CN solutions gave similar results, showing moderate uptakes compared to AS and UR.
Table 3.26: Positive and Negative correlations found for trends in slash pine and eucalyptus macronutrient uptake and their corresponding trendline gradients rounded to 2 decimal places.

<table>
<thead>
<tr>
<th>Positive Correlations</th>
<th>Gradient of trendline</th>
<th>Negative Correlations</th>
<th>Gradient of trendline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slash Pine</td>
<td>Eucalyptus</td>
<td></td>
</tr>
<tr>
<td>PO₄³⁻ and Mg²⁺</td>
<td>1.90</td>
<td>1.89</td>
<td>NH₄⁺ and Ca²⁺</td>
</tr>
<tr>
<td>PO₄³⁻ and K⁺</td>
<td>6.14</td>
<td>6.51</td>
<td>NO₃⁻ and (PO₄³⁻+SO₄²⁻+Cl⁻)</td>
</tr>
<tr>
<td>PO₄³⁻ and (Mg²⁺+ K⁺)</td>
<td>8.03</td>
<td>8.39</td>
<td></td>
</tr>
<tr>
<td>K⁺ and Mg²⁺</td>
<td>0.31</td>
<td>0.29</td>
<td></td>
</tr>
</tbody>
</table>

Negatively correlated graphs show a different order in the fertilisers, which is due to the fertiliser N type the solution was made with. As CN solutions were made up with NO₃⁻ as its only source of N, it is not surprising that these plants took up the most NO₃⁻ (Figure 3.13e and Figure 3.14e). This large uptake of the negatively charged NO₃⁻ anion comes at the expense of other negatively charged anions, in this case the combined total of PO₄³⁻, SO₄²⁻ and Cl⁻. It was also expected that AS solutions would take in the most NH₄⁺, while the AN solutions showed moderate uptake of both forms of nitrogen (Figure 3.13d and Figure 3.14d). The same trend is seen here, with the uptake of the positively charged NH₄⁺ cation, coming at the expense of Ca²⁺ in order to maintain electroneutrality of the seedling roots.

UR solutions have resulted in exceptional trends as urea-N is a neutral organic molecule. Therefore if a plant is able to directly utilise this form of N, it should not come at the expense of another charged molecule. It is interesting to note that in the slash pine solutions, the UR seedlings have taken up the highest percentage of K⁺, Mg²⁺ and Ca²⁺, and also the combination of negative charges comprising of PO₄³⁻, SO₄²⁻ and Cl⁻. This uptake of both positive and negative ions may be necessary to maintain root electroneutrality. This could then indicate that urea-N is being taken up directly by the slash pine, as there is no real indication of NH₄⁺ or NO₃⁻ ions being produced in these hydroponic solutions that could balance the charge. The direct uptake of urea is consistent with those observed by Aarnes et al (1995) for conifer Norway Spruce. There may be, however, another explanation where urea is being hydrolysed and those inorganic N ions are taken up by the pine seedlings fast and therefore not detected in the hydroponic solutions. Alternatively, it is possible that both of these processes are taking place in the UR hydroponic solutions set up for the slash pines.
Eucalyptus UR solutions on the other hand, do exhibit small amounts of NH$_4^+$ and NO$_3^-$ being present in solutions. The presence of NH$_4^+$ ions could be as a result of hydrolysis of urea under mild acidic conditions. i.e.:

$$\text{CO(NH}_2\text{)}_2 + \text{H}^+ + 2\text{H}_2\text{O} \rightarrow 2\text{NH}_4^+ + \text{HCO}_3^-$$

If the urea is converted to ammonium ions, then the pH of the solution will increase due to loss H$^+$. These ammonium ions may be converted to NO$_3^-$ by nitrifying organisms present as a contaminant in solutions used to study nitrogen assimilation by plants (Padgett & Leonard, 1993). If plants are then using these NH$_4^+$ ions, the pH will decrease, as it should be accompanied by either an influx of negatively charged ions and possibly an efflux of positive charged ions (Mengel & Pilbeam, 1992). This is consistent with the experimental observations where the pH of the UR fertilizer solution decreased on average from 6.3 to 5.0. This pH decrease was not large compared to for example AS solutions (6.6 to 3.3) and may indicate NO$_3^-$ has indeed been formed and used, which would raise the pH. It is possible however, that there is more than one of these processes taking place and are therefore counteracting each other. This will result in a net effect on a solution’s overall pH.

Since these ions are being produced in the medium with time the IC data obtained are seen as negative numbers. This further implies that eucalyptus seedlings may not be able to directly use urea-N, hence relying on this breakdown of urea, as mentioned. This breakdown of urea may not be fast enough to supply an adequate amount of N for eucalyptus growth, hence their poor health and survival rate.

**Influx and Effluxes**

The preceding section provided an overall uptake of nutrients by plants for each of the N fertilizer solution. In this section, the rate of the macronutrients uptake along with their fluxes will be analysed. The experimental solution samples were collected during the three weeks leading towards the project completion. The solutions samples (30mL) were taken in every couple of days in order to follow the rate of macronutrient uptake. It was found that not only was there an influx of nutrients into plant roots, but also effluxes of nutrients where nutrients have been exuded out of plant roots back into the hydroponic solutions.
Results for slash pine macronutrients Mg$^{2+}$, NH$_4^+$, K$^+$ (Figure A.1a-c), Ca$^{2+}$, Cl$^-$, NO$_3^-$ (Figure A.2a-c), PO$_4^{3-}$ and SO$_4^{2-}$ (Figure A.3a-b) are given. The high fluctuations of macronutrients observed in these figures once again reflect the electroneutrality balance on seedling roots. It appears in the slash pine seedling roots, that in order to take in a charged molecule, a molecule of opposite charge is exuded out. These high amounts of influx and efflux were found in all macronutrient solutions except for those containing N. The AN, *AN and A*N solutions exhibited influxes only for the macronutrients NH$_4^+$ and NO$_3^-$. Only influxes of NH$_4^+$ were observed for AS seedlings, while influxes of NO$_3^-$ were found in CN solutions. AS and CN showed negligible effluxes of NH$_4^+$ and NO$_3^-$ respectively.

Samples taken from UR solutions showed what appear to be large influxes and effluxes of N containing macronutrients. The average total N uptake measured (Figure 3.11) found no evidence of NH$_4^+$ and NO$_3^-$, however, both NH$_4^+$ and NO$_3^-$ were present in UR slash pine solutions when more regular sampling was carried out. As there was no inorganic-N added to these solutions, and IC cannot analyse samples for urea, it is not possible to monitor the formation and uptake of NH$_4^+$ and NO$_3^-$ in the hydroponic solution. Hence, what looks like an efflux of NH$_4^+$ and NO$_3^-$ from plant roots in Figure A.1b and Figure A.2c respectively, may actually be the conversion and accumulation of these N forms by chemical and microbial activity. The presence and fluctuations of N seen here suggests that slash pine may be relying on the breakdown of urea into inorganic forms by microbial activity in the solutions, and that these conversions are fast enough that this supply of N is sufficient for plant growth. Alternatively, the UR seedlings may be using both the urea directly and these inorganic by-products simultaneously.

Results for eucalyptus macronutrients Mg$^{2+}$, NH$_4^+$, K$^+$ (Figure A.4a-c), Ca$^{2+}$, Cl$^-$, NO$_3^-$ (Figure A.5a-c), PO$_4^{3-}$ and SO$_4^{2-}$ (Figure A.6a-b) are given. While large fluctuations of macronutrients were observed in the slash pine solutions, almost no macronutrients were found to exude out of eucalyptus roots back into solutions. This would suggest that rather than exchanging a charge from inside the seedlings root for a nutrient of opposite charge, the eucalyptus roots reduce the uptake of a particular charge in order to increase its uptake of an opposite charge. This may also explain the lack of fluctuations for N-containing macronutrients in the slash pine seedlings.
In the case of N, by decreasing N uptake rather than exuding N in exchange for an oppositely charged molecule, the seedling is not losing any N, which is the most limiting macronutrient needed for healthy plant growth.

Eucalyptus UR solutions were the only ones to exhibit any major increases of inorganic N. Once again, this may be due to chemical and microbial activity and the accumulation of NH\(_4^+\) and NO\(_3^-\) in solutions as previously mentioned, rather than an efflux of these macronutrients.

### 3.3.4 \textsuperscript{15}N and \textsuperscript{13}C NMR Spectroscopy: Quantitative Results

The absolute intensity per gram of sample used was calculated by integrating the entire \textsuperscript{15}N or \textsuperscript{13}C NMR spectrum and dividing the integrals (arbitrary units) by the mass of sample (in grams) loaded into the rotor. This resulted in a value for absolute intensity per gram. These results were then multiplied by the average dried biomass (in grams) for the particular plant component, i.e., leaves/needles, stems and roots (Table 3.17 and Table 3.18.) to give the resulting \textsuperscript{15}N or \textsuperscript{13}C enrichment of these sections. When the results for all sections were added together, the total \textsuperscript{15}N and \textsuperscript{13}C enrichment of the plant could be estimated. Results for \textsuperscript{15}N and \textsuperscript{13}C enrichment for slash pine seedlings are given in Figure 3.15 and Figure 3.16 respectively. Similarly, \textsuperscript{15}N and \textsuperscript{13}C enrichment for eucalyptus seedlings are given in Figure 3.17 and Figure 3.18 respectively.

\textsuperscript{15}N CPMAS NMR spectra are shown in Figure 3.20 through to Figure 3.25 slash pine sections and Figure 3.29 through to Figure 3.34 for eucalyptus sections. Here the spectra have been scaled in order to compare the chemical environment around \textsuperscript{15}N, or its distribution of peaks in the spectrum for each N-fertilised plant. The qualitative results taken from these distributions are discussed in the subsequent sections (Sections 3.3.5 and 3.3.6). Similarly \textsuperscript{13}C CPMAS NMR spectra (which can be found in the appendix of this thesis) are shown for slash pine sections in Figures A.7 to A.12 and for eucalyptus sections in Figures A.13 to A.18.

The slash pine total \textsuperscript{15}N enrichment as found by \textsuperscript{15}N NMR for each fertilizer group follows the same trend as the results found for total N (Figure 3.5, page 127) using the CN analyser. It also reflects a similar trend observed for total N uptake
determined using ion chromatography results (Figure 3.11, page 132) excluding urea-N uptake which could not be detected. Any differences between these three sets of results may be due to N losses from the system, due to ammonium volatilisation or denitrification depending on the fertiliser solution used. These losses are thought to be small (<14%) as discussed on page 134. Results for eucalyptus total $^{15}$N enrichments for each of the fertiliser groups (Figure 3.17) were also similar to results found for total N (Figure 3.7, page 127) within uncertainties.

Although the trend is the same, the $^{15}$N enrichment of roots for both the slash pine and eucalyptus seedlings, the total N appears to be smaller than those found for all fertilised plants. This may be due to N-species in the pine roots being underestimated by CPMAS NMR, more than in the other sections. As discussed in chapter 2, section 2.2.7, previous results found by Smernik & Baldock (2005), have shown that while the observability of eucalyptus leaves and stem samples was approximately 80%, roots had only 69% observability using CPMAS. This may therefore explain the smaller root $^{15}$N enrichment compared to total N for both the slash pine and eucalyptus plants. Alternatively, there is also the possibility that higher amounts of unlabelled-N ($^{14}$N) being found in the root because of the plants being exposed to unenriched fertilisers for 2 weeks prior to harvesting took place.

While the $^{15}$N enrichment trend between fertilisers for slash pine and eucalyptus followed that for total N, the $^{13}$C enrichment (Figure 3.16 and Figure 3.18 respectively) however, did not represent a similar trend with that of total C (Figure 3.6 and Figure 3.8 respectively). It was found that UR and AS fertilised slash pine seedlings had higher amounts of $^{13}$C than the CN and AN fertilised plants. The total C results obtained using the CN analyser measured both $^{12}$C and $^{13}$C isotopes, whereas NMR experiments determine only the $^{13}$C. Therefore differences between the two results could reflect the extent of photosynthesis and water use efficiencies (WUE) as explained in section 3.1.6, page 103 taken. Higher $^{13}$C levels compared to total C levels may be due to increases in photosynthesis in the seedlings, therefore, the UR and AS slash pine seedlings may have a higher photosynthetic capacity. The eucalyptus seedling $^{13}$C enrichment were roughly the same as the total C results, with the possible exception of the CN eucalypts, which may have lower $^{13}$C enrichments compared to total C. This would indicate less photosynthetic activity.
Figure 3.15: Total $^{15}$N enrichment of Slash Pine

Figure 3.16: Total $^{13}$C enrichment of Slash Pine.

Figure 3.17: Total $^{15}$N enrichment of Eucalyptus

Figure 3.18: Total $^{13}$C enrichment of Eucalyptus
3.3.5 $^{15}$N NMR Spectroscopy: Qualitative Results

$^{15}$N CPMAS NMR spectra were obtained for slash pine and eucalyptus seedlings grown in the doubly $^{15}$N enriched solutions of AN, AS, UR and CN. New leaf samples were collected during the experiment (Figure 3.1b, Section 3.2.1), while plants were harvested and divided into bulk leaves, stems and bark (Figure 3.1c). A small section of stem from the base of each plant (approx. 2.5cm), was then separated into bare stem-wood and bark (Figure 3.1f). Spectra obtained for slash pine sections are shown in Figure 3.20 through to Figure 3.25. Eucalyptus sections are represented in Figure 3.29 through to Figure 3.34. All spectra have been scaled in order to compare the relative peak distributions between fertilisers. Comparisons of the distributions of peak areas (%) relative to the total peaks in the spectrum can be found in Table 3.27 and Figure 3.27/Table 3.28 for slash pine and Table 3.29 and Figure 3.36/Table 3.30 for eucalyptus. Absolute intensities, and hence relative $^{15}$N enrichments, have been given in the previous section (section 3.3.4.).

As explained in Chapter 1, higher plants such as slash pine and eucalyptus follow the GS/GOGAT pathway for nitrogen assimilation (Figure 3.19). During this process, all N taken up by the plant roots needs to be converted to ammonium, which plants can then metabolise. Plants that take up nitrates directly will therefore spend more energy converting it to ammonium. This conversion, which can be undertaken within the plant via nitrate reductase (NR) and nitrite reductase (NiR) activity, commonly occurs in plant roots, but can also take place in the stems or on arrival at the plants leaves (Stitt & Scheible, 1999). Once converted to ammonium, N is incorporated into amide groups (eg. glutamine) and then converted into amino groups (eg. glutamate) via reactions with ketoacids, enzymes, ATP and ADP. These free aminos can then be incorporated back into proteins and amino acids depending on whether the plant needs them for growth or storage.

As plants were grown in unenriched $^{14}$N versions of their respective fertiliser solutions for two weeks prior to harvesting, the $^{15}$N peaks found in these $^{15}$N NMR spectra represent N used for plant growth and incorporated into plant material, medium-long term N storage and N still involved in cycling. This allows an insight into where N is more likely to accumulate as it grows, and hence what organic N species are put back into soils in harvest residues.
An amide peak resonating at -260ppm dominates all $^{15}$N spectra for both slash pine and eucalyptus sections. This is to be expected, as plant material is inherently made up of a large amount of protein structures. The amide containing amino acids of glutamine and asparagine as well as the amino acids glutamate and aspartate, have also been found to dominate plant solutes in both eucalyptus and conifer species (Radomiljac et al., 1998). These amino acids are important in assimilatory, metabolic and storage processes. Glutamine and asparagine contribute to peaks found in the amide-N region, and hence the peak at -260ppm. Peaks located at -295 and -306ppm can be assigned to the respective guanidino NH and amine NH$_2$ groups of arginine (Smernik & Baldock, 2005). Arginine is also important in plant processes and also storage of N within the plant (Canovas et al., 2007). Two major peaks were observed in the amino-N region of the $^{15}$N spectra are most likely due to free amino -NH$_3^+$
groups (-338ppm) and NH$_3^+$ amino groups found in lysine, glycine and other amino acids incorporated into proteinaceous material (-347ppm).

The $^{15}$N NMR peaks were not observed for nitrates (25 to –25ppm) or ammonium (-350 to –375ppm), therefore these regions are not shown. Plants were fertilised with unenriched N solutions for two weeks prior to harvesting allowing maximum assimilation of the $^{15}$N-enrichment, into organic forms in the plant material. This way, it was easier to characterise the chemical environments of $^{15}$N structures, perhaps the final chemical compounds incorporated in the plant seedling. It is therefore possible that there is unenriched ammonium and nitrate present in the plant material that is not seen by NMR. Smernik & Baldock (2005), have previously studied $^{15}$N enriched eucalyptus seedlings using $^{15}$N Bloch decay NMR. It was found that nitrates accounted for a maximum of 8% of the total spectrum for root sections, with leaves and stem section accounting for 2% and 0% of their respective spectra. In the spectra acquired for each section there was no evidence of an ammonium peak. Therefore the amount of unobservable unenriched N in inorganic forms during these experiments is thought to be small compared to $^{15}$N-enriched organic N forms.

**Slash Pine Spectra**

The resulting $^{15}$N NMR spectra for slash pine new needles (Figure 3.20), bulk needles (Figure 3.21), and roots (Figure 3.23), were similar in composition. This can also bee seen in Table 3.27. No significant differences were found between the different fertilisers; however, small differences between plant components have been identified. A slightly wider peak at -338ppm corresponding to free amino groups is exhibited in the new needles spectra compared to the bulk needles. More free amino groups may be present in the new needles, as they are still growing. As the bulk needles have stopped their growth, the lack of peak at -338ppm in the bulk needle spectra may be due to any free amino acids being transported to elsewhere in the plant, where they can be either stored or used for further growth. The slash pine root spectra also have a reduced amino-N region.

It was observed that the $^{15}$N NMR spectra of slash pine whole stem sections differed between fertilisers (Figure 3.22). UR seedling displayed a higher percentage of
Figure 3.20: $^{15}$N NMR spectra of new pine needle samples, taken while the plant was still growing in a) UR, b) AS, c) AN, and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure 3.21: $^{15}$N NMR spectra of bulk pine needle samples taken after harvesting. Spectra are from plants grown in a) UR, b) AS, c) AN, and d) CN solutions. All spectra have been scaled in order to compare N composition.
SLASH PINE: Whole Stem and Roots

Figure 3.22: $^{15}$N NMR spectra of pine whole stem samples taken after harvesting. Spectra are from plants grown in a) UR, b) AS, c) AN, and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure 3.23: $^{15}$N NMR spectra of pine root samples taken after harvesting. Spectra are from plants grown in a) UR, b) AS, c) AN, and d) CN solutions. All spectra have been scaled in order to compare N composition.
SLASH PINE: Bare Stem and Bark

Figure 3.24: $^{15}$N NMR spectra of pine bare stem samples (where the bark has been removed), taken after harvesting from pines grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure 3.25: $^{15}$N NMR spectra of pine bark samples, taken after harvesting from pines grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.
Table 3.27/Figure 3.26: Percentage each region took of the total $^{15}\text{N}$ NMR spectrum for Slash Pine sections. Results are averages from all fertilisers, as no significant differences were found between the fertilisers for these sections. Parentheses indicate standard deviations based on n=6.

<table>
<thead>
<tr>
<th>Section</th>
<th>Hetero-N</th>
<th>Amide-N</th>
<th>Guano-N</th>
<th>Amine-N</th>
<th>Amino-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Needles</td>
<td>2.23 (0.35)</td>
<td>83.82 (2.10)</td>
<td>2.94 (0.27)</td>
<td>5.63 (1.23)</td>
<td>5.39 (1.03)</td>
</tr>
<tr>
<td>Bulk Needles</td>
<td>2.64 (0.32)</td>
<td>84.4 (1.10)</td>
<td>3.44 (0.33)</td>
<td>5.51 (0.35)</td>
<td>4.01 (0.76)</td>
</tr>
<tr>
<td>Bare Stems</td>
<td>-</td>
<td>70.50 (3.39)</td>
<td>6.70 (0.97)</td>
<td>13.00 (1.95)</td>
<td>9.80 (1.37)</td>
</tr>
<tr>
<td>Roots</td>
<td>2.81 (0.89)</td>
<td>84.07 (0.85)</td>
<td>3.35 (0.76)</td>
<td>6.04 (0.74)</td>
<td>3.73 (0.31)</td>
</tr>
</tbody>
</table>
Figure 3.27/Table 3.28: Percentage each region took of the total $^{15}$N NMR spectrum for Slash Pine bark sections, in each fertiliser solution. Singly enriched solutions have been included here for comparison.

<table>
<thead>
<tr>
<th>Fertiliser</th>
<th>CN</th>
<th>UR</th>
<th>AS</th>
<th>AN</th>
<th>*AN</th>
<th>A*N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero-N</td>
<td>2.07</td>
<td>3.12</td>
<td>3.40</td>
<td>2.43</td>
<td>2.55</td>
<td>3.31</td>
</tr>
<tr>
<td>Amide-N</td>
<td>56.99</td>
<td>70.78</td>
<td>83.85</td>
<td>78.31</td>
<td>71.75</td>
<td>74.10</td>
</tr>
<tr>
<td>Guano-N</td>
<td>10.06</td>
<td>5.95</td>
<td>2.59</td>
<td>4.18</td>
<td>6.79</td>
<td>4.62</td>
</tr>
<tr>
<td>Amine-N</td>
<td>21.00</td>
<td>13.60</td>
<td>5.68</td>
<td>7.58</td>
<td>11.62</td>
<td>9.90</td>
</tr>
<tr>
<td>Amino-N</td>
<td>9.87</td>
<td>6.54</td>
<td>4.48</td>
<td>7.49</td>
<td>7.28</td>
<td>8.07</td>
</tr>
</tbody>
</table>

% $^{15}$N as N-Source
0  unknown  100  50  100  0

Figure 3.28: Percentage each region took of the total $^{15}$N NMR spectrum for Slash Pine bark sections in each fertiliser solution, verse the percentage of the $^{15}$N solution supplied that originated from $\text{NH}_4^+$ (Urea has not been included here).
guano-, amine-, and amino-N compared to the other fertiliser solutions, with AS seedlings showing the least. Differences could also be seen in the amino-N section, with the -339ppm peak dominating in the UR, AN and CN spectra, while the largest peak was found at -347ppm in AS whole stems. It was unknown, however, why these changes were seen in the whole stem. In order to find out more on $^{15}$N incorporation in the stem, small sections from the bottom of the seedling’s stem were therefore taken, and were separated into bare-stem and bark sub-samples (Figure 3.1f). This division also corresponds with the separation of the seedling’s xylem (the bare-wood material) and phloem (the bark).

$^{15}$N NMR spectra of the bare stem samples showed no real differences between the fertilisers (Figure 3.33). The bark samples on the other hand (Figure 3.34), do show significant differences similar to those found in the whole stem spectra. Therefore it can be assumed that the differences seen in the whole stem spectra are due to differences in the seedling’s bark, not the bare stem. This result makes sense as the woody bare stem of a plant has a much lower N content, and therefore shouldn’t affect the whole stem spectrum as much as the bark, which should have a higher N content. As the bark contains the phloem, it is likely these changes between fertilisers reflect the cycling of organic nutrients in the plant at the time of sampling, as well as the storage status of the plant. As N moves from roots, up through the xylem to the seedling’s needles, it is then cycled back down through the phloem where it can be used for growth in different areas of the plant, or recycled and stored until needed. This is where we are seeing differences in N composition.

To establish whether these changes in slash pine bark composition were due to the different sources of N in fertiliser solutions, plots were made showing the percentage each region took up in the $^{15}$N NMR bark spectrum versus the percentage of the solution supplied that originated from $^{15}$N-enriched NH$_4^+$ (Figure 3.28). UR bark has not been included in the plot, as it is unknown how much urea was taken up directly and how much was converted to either NH$_4^+$ or NO$_3^-$ The singly $^{15}$N-enriched solution *AN and A*N have been included, as $^{15}$N NMR spectrum of *AN will have originated only from the $^{15}$N labelled ammonium-N, while A*N had only the nitrate-N labelled. As a result of this single labelling it was possible to follow these two different N-species while still in the presence of each other. The percentage of the
solution supplied that originated from $^{15}$N-enriched NH$_4^+$ in the AS, CN, AN, *AN and A*N solutions is 100%, 0%, 50%, 100% and 0% respectively.

No real trends were found between fertiliser N-source and bark composition (Figure 3.28). The amino-N region exhibited the most potential with a trendline regression of $R^2 = 0.63$. It is possible however, that the differences in bark N composition could act as an indicator of plant health. In the bark sections, CN has the smallest amide-N peak, which represents amide/peptide present in proteins. As the CN pines showed signs of poor-health (sections 3.3.1 and 3.3.2), with slower growth patterns compared to the other fertilised seedlings, smaller amounts of protein could be due to a lack of excess N being stored as storage proteins. This was also exhibited by results for the uptake of N from the slash pine fertilizer solutions, where CN had the least N uptake (Figure 3.11). In contrary, the pine seedling bark from the UR fertilizer solution also exhibited lower protein levels. If one analyse these data in conjunction with its good health of the plant, then it could indicate that the seedling is accessing its stored N reserves for growth. Alternatively, as the seedlings could not be harvested at exactly the same time, differences could be due to timing of harvesting. The phloem N composition could be constantly fluctuating due to demands of the plants at different times of the day or days of the week.

If bark and therefore whole stems produce significantly different chemical compositions of N-structures depending on the fertiliser N-type used, then this may also cause changes in their rates of decomposition. This could then impact the return of nutrients to the soil in a plantation forest, if these sections are left to decompose with harvest residues. These results however are qualitative. While the composition is different, the quantity of N in these sections also needs to be taken into account, as it could further exaggerate differences in N-forms put back into the soil or removed with harvesting.

**Eucalyptus**

The resulting $^{15}$N NMR spectra for the eucalyptus new leaves (Figure 3.29), bulk leaves (Figure 3.30), and roots spectra (Figure 3.32), were found to have basically the same composition, between both fertilisers and sections. Changes in the major peak
in the amino-N region could be seen in the new leaf samples, with the major peak falling at -339ppm for the UR eucalypts, -347ppm for AN and CN eucalypts, and an even distribution of the two peaks for the AS eucalypts.

It is interesting that the two nitrate-N containing solutions (AN and CN) lack a peak at -339ppm, which corresponds to free amino acids. A lack of peak in this region may suggest that free amino acids have been used for growth quite rapidly, as the AN and CN eucalypts also exhibited larger new leaf areas (17.2 and 17.5cm$^2$ respectively) compared to the UR and AS seedlings (13.0 and 11.3cm$^2$ respectively) (Table 3.16). Alternatively, AN and CN seedlings may have used any free amino groups to convert NO$_3^-$ ions into NH$_4^+$, as this conversion is necessary as the plant can only metabolise NH$_4^+$. UR and AS seedlings have not used this source of N, possibly due to them taking up more NH$_4^+$ in the first place, where in the case of UR, urea has been converted to NH$_4^+$ in solutions via hydrolysis under slightly acidic pH. The NMR spectra for bulk leaves show almost no free amino groups, suggesting it has been used for growth, or transported elsewhere for storage.

Olsen and Bell (1990), have previously found that the N content of undeveloped leaves and the three youngest mature leaves belonging to 6 eucalyptus species, were the most sensitive leaves to tree biomass increases. It was therefore suggested that these leaves were the best indicators of N status of a plant, and could therefore be used for determination of N. The N content in stems was found to be the least sensitive to biomass increases. These results are also reflected in the thesis research, with the new leaves showing differences in the abundance of free amino groups. While the bare stem, and indirectly the whole stem also show differences, their %N concentration and N content is much lower compared to that of the new leaves. The whole stems showed approximately 0.55%N and leaves approximately 1.95%N (Table 3.20, page 125). Therefore, our results also confirmed that the new leaves would make the best indicator of growth.

The eucalyptus whole stem spectra (Figure 3.31) showed differences between the fertilisers used. The whole stems from AS gave the largest distribution of N into guano- and amine-N groups, followed by UR. The amino region showed similar
Figure 3.29: $^{15}$N NMR spectra of new Eucalyptus leaf samples, taken while the plant was still growing in a) UR, b) AS, c) AN, and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure 3.30: $^{15}$N NMR spectra of bulk Eucalyptus leaf samples taken after harvesting. Spectra are from plants grown in a) UR, b) AS, c) AN, and d) CN solutions. All spectra have been scaled in order to compare N composition.
Figure 3.31: $^{15}$N NMR spectra of Eucalyptus whole stem samples taken after harvesting. Spectra are from plants grown in a) UR, b) AS, c) AN, and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure 3.32: $^{15}$N NMR spectra of Eucalyptus root samples taken after harvesting. Spectra are from plants grown in a) UR, b) AS, c) AN, and d) CN solutions. All spectra have been scaled in order to compare N composition.
Figure 3.33: $^{15}$N NMR spectra of Eucalyptus bare stem samples (where the bark has been removed), taken after harvesting from Eucalypts grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure 3.34: $^{15}$N NMR spectra of Eucalyptus bark samples, taken after harvesting from Eucalypts grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.
Table 3.29/Figure 3.35: Percentage each region took of the total $^{15}$N NMR spectrum for Eucalyptus sections. Results are averages from all fertilisers, as no significant differences were found between the fertilisers for these sections. Parentheses indicate standard deviations based on n=6.

<table>
<thead>
<tr>
<th>Section</th>
<th>Hetero-N (SD)</th>
<th>Amide-N (SD)</th>
<th>Amine1-N (SD)</th>
<th>Amine2-N (SD)</th>
<th>Amino-N (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Leaves</td>
<td>1.64 (0.17)</td>
<td>86.35 (1.75)</td>
<td>2.30 (0.25)</td>
<td>4.99 (0.69)</td>
<td>4.71 (1.14)</td>
</tr>
<tr>
<td>Bulk Leaves</td>
<td>2.48 (0.55)</td>
<td>84.33 (0.73)</td>
<td>3.13 (0.33)</td>
<td>5.29 (0.34)</td>
<td>4.77 (0.55)</td>
</tr>
<tr>
<td>Bark</td>
<td>3.21 (0.54)</td>
<td>81.44 (3.19)</td>
<td>3.71 (0.92)</td>
<td>7.05 (1.30)</td>
<td>4.66 (1.17)</td>
</tr>
<tr>
<td>Roots</td>
<td>2.25 (0.84)</td>
<td>86.02 (2.86)</td>
<td>2.38 (0.78)</td>
<td>4.94 (0.88)</td>
<td>4.41 (1.46)</td>
</tr>
</tbody>
</table>
Figure 3.36/Table 3.30: Percentage each region took of the total $^{15}$N NMR spectrum for Eucalyptus bare stem sections, in each fertiliser solution. Singly enriched solutions have been included here for comparison.

![Graph of Eucalyptus Bare Stems](image)

<table>
<thead>
<tr>
<th>Fertiliser</th>
<th>CN</th>
<th>UR</th>
<th>AS</th>
<th>AN</th>
<th>*AN</th>
<th>A*N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero-N</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amide-N</td>
<td>61.50</td>
<td>73.26</td>
<td>48.76</td>
<td>56.64</td>
<td>48.44</td>
<td>63.82</td>
</tr>
<tr>
<td>Guano-N</td>
<td>10.33</td>
<td>7.28</td>
<td>11.99</td>
<td>11.70</td>
<td>13.93</td>
<td>9.50</td>
</tr>
<tr>
<td>Amine-N</td>
<td>15.73</td>
<td>12.92</td>
<td>27.41</td>
<td>21.62</td>
<td>28.86</td>
<td>16.68</td>
</tr>
<tr>
<td>Amino-N</td>
<td>12.43</td>
<td>6.55</td>
<td>11.84</td>
<td>10.04</td>
<td>8.77</td>
<td>9.99</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% NH$_4^+$ as N-Source</th>
<th>0</th>
<th>unknown</th>
<th>100</th>
<th>50</th>
<th>100</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio Amide/Amine</td>
<td>3.9</td>
<td>5.7</td>
<td>1.8</td>
<td>2.6</td>
<td>1.7</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Figure 3.37: The percentage of the fertiliser solution supplied that originated from $^{15}$NH$_4^+$, verse a) the percentage each region took of the total $^{15}$N NMR spectrum for Eucalyptus bare stem sections b) the ratio of amide-N/amine-N. (Urea has not been included in each graph, as it is unknown how much urea was converted to NH$_4^+$).
results to those found for new leaves, with smaller peaks located at -339ppm. Once again, whole stem samples were separated into sub-samples of bare stem and bark. Contradictory to slash pine results, the eucalyptus bark showed little difference between the fertiliser solutions. Differences in the bare stem were observed however, and can therefore be assumed to cause the changes seen in the whole stem spectra. In the bare stem spectra, the AS and AN solutions showed larger guano-N and amine-N peaks, while UR showed little in these regions, and almost no peaks in the amino-N region. The major peak for all spectra in the amino-N region was at -339ppm, once again showing free amino acids, although UR showed peaks with little intensity in the amino-N region.

In eucalyptus xylem, most of the woody material is in the form of cellulose, hemicellulose and lignin, all of which do not contain N. Therefore it is assumed that most of the $^{15}$N NMR signal for the bare stem is coming from the xylem sap. The absence of the amino acids peak (-347ppm) and the smaller amide-N region, both of which represent proteinaceous material, may therefore be expected. These N-forms are more likely to be present in the plants structure rather than in the soluble forms found in xylem sap. In a previous study which included *Eucalyptus camaldulensis*, N found in the eucalyptus xylem sap mostly came from amino acids (Radomiljac et al., 1998). The amino acid composition was co-dominated by glutamate, glutamine, aspartate and arginine.

To establish whether these changes in eucalyptus bare stem composition were due to the different sources of N in fertiliser solutions, plots were again made showing the percentage each region took up in the $^{15}$N NMR bark spectrum versus the percentage of the solution supplied that originated from $^{15}$N-enriched NH$_4^+$ (Figure 3.37a). As with the slash pine bark plots, UR bare stem results have not been included for similar reasons. The singly $^{15}$N-enriched solution *AN and A*N have been included, as $^{15}$N NMR spectrum of *AN will have originated only from the $^{15}$N labelled ammonium-N, while A*N had only the nitrate-N labelled. The percentage of the solution supplied that originated from $^{15}$N-enriched NH$_4^+$ is shown in Figure 3.36/Table 3.30.
Strong positive correlations were found for guano-N \((R^2=0.80)\) and amine-N \((R^2=0.99)\), while a strong negative correlation was found for amide-N \((R^2=0.98)\). Therefore it appears that these N-forms are changing in the eucalyptus bare stem depending on the fertiliser N-species used. For example, the results given in Figure 3.36/Table 3.30 show that the nitrate-N in the A*N is acting similarly to the nitrate-N in the CN solution, while the ammonium-N in *AN is acting similar to the ammonium-N in the AS solution. The ratio of amide-N/amine-N was then taken, as to find out more on what N-source the eucalyptus plants are using for growth. This ratio was also found to have a strong negative correlation with the amount of \(\text{NH}_4^+\) used in the fertilisers.

These correlations may be explained by processes which take place in the GS/GOGAT cycle (Figure 3.19). In a study following \(\text{NH}_4^+\) assimilation and amino acids formation in the floating aquatic agiosperms *Wolffia arrhiza* (L.) and *Lemna gibba* L., Monselise and Kost (1993) have previously shown that plants that are able to regenerate glutamate supplies quickly, are able to cope with excessive \(\text{NH}_4^+\), as glutamate acts as a \(\text{NH}_4^+\) “trap”. By way of the GS/GOGAT pathway, this can then be converted into glutamine which in turn can produce two new glutamate molecules, which can either go back into the GS/GOGAT cycle to assimilate more \(\text{NH}_4^+\). Alternatively, some of this glutamate will be synthesised into other N-containing compounds such as amino acids and proteins, which can either be used in the growth of new plant material, or stored/cycled in the plant until needed. Therefore, in the presence of ample glutamate supply, the more \(\text{NH}_4^+\) that is trapped and metabolised, the more amino acids and proteins should be formed and either used in new growth or stored/cycled.

In the eucalyptus bare stems it was found that the \(\text{NH}_4^+\) solutions AS and *AN had high amounts of particularly amine-N, possibly due to larger amounts of arginine and other -\(\text{NH}_2\)/-\(\text{NR}_2\) groups. This may be as a result of higher \(\text{NH}_4^+\) uptake leading to higher synthesis of glutamate, and therefore additional synthesis of amino acids and proteinaceous material. Seeing as the growth of eucalyptus seedlings was not significantly different between the fertilisers, it is more likely any excess organic-N produced would be either stored in the phloem or cycled around the plant. As mentioned previously, the \(^{15}\text{N}\) NMR signal for bare stems is most likely coming from
the xylem sap, therefore any amino acids found in this section would be cycling around the plant, acting as a mobile sink, rather than being stored in proteinaceous material such as found in the phloem. Therefore cycling would best explain the higher levels of amino acids in the AS and *AN bare stems. Canovas and coworkers (2007) have previously pointed out that N fertilisation increased the arginine content of needles and wood in conifers and may therefore reflect the N status of the plant. Therefore if storage proteins are rich in arginine, this would correspond to more intense amine-N and guano-N regions, as seen in this study.

The N-uptake for CN was found to be low (Figure 3.12), however, plant growth was not found to be significantly different to the other fertilisers. Therefore it could be reasoned that the N-supply was adequate as growth was not affected, however, this would affect the amount of excess N which could potentially be stored or cycled. This may explain the higher ratio of amide-N to amine-N, as more N may have been used in plant growth and not cycled around the plant. Further, any NO$_3^-$ taken up by the plant roots needs to be reduced to NO$_2^-$ and then to NH$_4^+$ before it can be used by the plant. These processes involve both energy and time. Therefore, it also slows the amount of NH$_4^+$ entering the GS/GOGAT cycle, therefore explaining the decrease in products containing amine-N. This would also be the case for A*N fertilised seedlings.

3.3.6 $^{13}$C NMR Spectroscopy: Qualitative Results

$^{13}$C CPMAS NMR spectra were accumulated for each of the plant sections for each of the fertilisers to see if N fertiliser type had an effect on C composition. It has already been shown that fertiliser N-species had an effect on total C and $^{13}$C enrichment (section 3.3.4). $^{13}$C NMR spectra collected for slash pine and eucalyptus sections are shown in the Appendix of this thesis (Figures A.7 – A.12 and Figures A.13 – A18 respectively).

No significant differences were found between the $^{13}$C NMR peak distributions between different fertilisers for each section, with one exception. This exception involved a decrease in the aliphatic-C region of CN fertilised eucalyptus new leaves (Appendix: Figure A.14). This result coincides with the observation that the CN
Table 3.31/Figure 3.38: Percentage each region took of the total $^{13}$C NMR spectrum for different sections of Slash Pine. Results are averages from all fertilisers, as no significant differences were found between the fertilisers for these sections. Parentheses indicate standard deviations based on n=6.

<table>
<thead>
<tr>
<th>Section</th>
<th>Carboxyl</th>
<th>Phenolic</th>
<th>Aryl</th>
<th>Di-O-Alkyl</th>
<th>Carbohydrate</th>
<th>Methoxyl</th>
<th>Alkyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Needles</td>
<td>8.26 (0.81)</td>
<td>5.98 (1.18)</td>
<td>4.97 (0.61)</td>
<td>8.58 (1.20)</td>
<td>50.66 (1.99)</td>
<td>2.58 (0.62)</td>
<td>18.97 (2.03)</td>
</tr>
<tr>
<td>Bulk Needles</td>
<td>8.38 (2.39)</td>
<td>6.00 (2.39)</td>
<td>5.65 (1.11)</td>
<td>9.28 (1.64)</td>
<td>49.31 (3.99)</td>
<td>2.75 (0.47)</td>
<td>18.63 (1.91)</td>
</tr>
<tr>
<td>Whole Stems</td>
<td>3.72 (0.34)</td>
<td>4.02 (0.47)</td>
<td>8.22 (0.59)</td>
<td>11.07 (0.21)</td>
<td>60.85 (1.00)</td>
<td>4.57 (0.54)</td>
<td>7.55 (0.64)</td>
</tr>
<tr>
<td>Bare Stems</td>
<td>2.22 (0.46)</td>
<td>4.25 (1.15)</td>
<td>9.71 (1.28)</td>
<td>10.86 (0.51)</td>
<td>63.22 (2.20)</td>
<td>5.51 (0.54)</td>
<td>4.23 (0.45)</td>
</tr>
<tr>
<td>Bark</td>
<td>4.23 (0.48)</td>
<td>7.20 (1.58)</td>
<td>4.58 (0.72)</td>
<td>10.85 (0.57)</td>
<td>59.33 (1.40)</td>
<td>3.25 (0.52)</td>
<td>10.56 (1.36)</td>
</tr>
<tr>
<td>Roots</td>
<td>5.57 (0.51)</td>
<td>9.80 (1.67)</td>
<td>4.44 (0.76)</td>
<td>8.17 (1.84)</td>
<td>48.20 (3.04)</td>
<td>4.30 (0.65)</td>
<td>19.52 (1.00)</td>
</tr>
</tbody>
</table>
Table 3.32/Figure 3.39: Percentage each region took of the total $^{13}$C NMR spectrum for different sections of Eucalyptus. Results are averages from all fertilisers, as no significant differences were found between the fertilisers for these sections. Parentheses indicate standard deviations based on n=6.

<table>
<thead>
<tr>
<th>Section</th>
<th>Carboxyl</th>
<th>Phenolic</th>
<th>Aryl</th>
<th>Di-O-Alkyl</th>
<th>Carbohydrate</th>
<th>Methoxyl</th>
<th>Alkyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Leaves</td>
<td>7.82 (1.57)</td>
<td>8.44 (1.92)</td>
<td>3.67 (0.64)</td>
<td>14.48 (2.15)</td>
<td>38.44 (2.30)</td>
<td>3.57 (0.56)</td>
<td>23.57 (4.13)</td>
</tr>
<tr>
<td>Bulk Leaves</td>
<td>9.49 (1.46)</td>
<td>9.14 (1.14)</td>
<td>3.86 (0.42)</td>
<td>11.53 (1.10)</td>
<td>41.00 (2.21)</td>
<td>3.03 (0.42)</td>
<td>21.95 (2.86)</td>
</tr>
<tr>
<td>Whole Stem</td>
<td>4.90 (0.81)</td>
<td>3.70 (0.33)</td>
<td>3.68 (0.28)</td>
<td>14.01 (0.33)</td>
<td>59.97 (2.17)</td>
<td>5.12 (0.81)</td>
<td>8.62 (1.79)</td>
</tr>
<tr>
<td>Bare Stems</td>
<td>3.13 (0.66)</td>
<td>3.41 (0.44)</td>
<td>3.15 (0.59)</td>
<td>15.61 (0.52)</td>
<td>65.29 (2.05)</td>
<td>6.44 (0.70)</td>
<td>2.96 (0.29)</td>
</tr>
<tr>
<td>Bark</td>
<td>5.10 (0.85)</td>
<td>10.11 (1.70)</td>
<td>3.07 (0.83)</td>
<td>14.16 (1.25)</td>
<td>48.78 (1.06)</td>
<td>3.64 (0.81)</td>
<td>15.14 (1.09)</td>
</tr>
<tr>
<td>Roots</td>
<td>8.48 (0.90)</td>
<td>6.42 (2.34)</td>
<td>4.50 (1.38)</td>
<td>8.73 (1.55)</td>
<td>50.78 (3.19)</td>
<td>5.81 (0.86)</td>
<td>15.28 (1.70)</td>
</tr>
</tbody>
</table>
leaves tended to feel soft, possibly due to lower leaf wax contents. It was also observed in the $^{13}$C spectra of new leaves and bulk leaves (Figure A.13 and A.14 respectively), that there were changes in the major peak residing in the $^{13}$C di-O-alkyl region between the fertilisers. The major peak shifted between approximately 105 and 110ppm, however, the percentage this region occupied of the total spectrum was not significantly different.

As there were no major changes in $^{13}$C distributions between the fertilisers, the average $^{13}$C percentage distribution was taken across all fertilisers for each plant section, as shown in Table 3.31 and Table 3.32 for slash pine and eucalyptus respectively.

### 3.4. Conclusions

#### 3.4.1 Consequences of Fertiliser N-type

Results for N uptake were similar for both slash pine and eucalyptus seedlings grown in $^{15}$N-enriched fertilisers containing either ammonium sulfate (AS), calcium nitrate (CN), ammonium nitrate (AN) or urea (UR). Uptake of N in AS was greater than CN for both species, as was their total N content and $^{15}$N enrichment. This would suggest that both species prefer uptake of NH$_4^+$ over NO$_3^-$. When both of these inorganic forms were present as in AN solutions, N uptake originating from the individual sources was roughly 50:50. The mechanism for the uptake of NH$_4^+$ and NO$_3^-$ and how it happens for the oppositely charged ions under these conditions needs further investigation. In slash pine fertilised with AN, the total N and $^{15}$N enrichment was slightly higher than CN and AS, while for eucalyptus AN total N and $^{15}$N enrichment fell in between that found for CN and AS pine seedlings.

The highest total N and $^{15}$N enrichment for slash pine resulted from the UR solutions. This is an interesting observation and it is possible that the urea-N in these solutions breaks down into inorganic N-forms fast enough for the slash pine to use, or that the slash pines can utilise the urea-N directly. Alternatively both of these processes for N uptake may be taking place. It is most likely, on the other hand, that eucalyptus
seedlings were unable to directly use urea-N, and therefore had to depend on the conversion of urea to mineral forms for the plant to take up the N containing nutrients. This would explain why, in general, the eucalypts grown in UR hydroponic solution exhibited poor health and low survivability.

While uptake and total content of fertiliser-N shows what N-form these species prefer, this is not the only factor that needs to be taken into account when choosing which fertiliser is best for a particular plant species. If a plant can produce the same amount of above-ground biomass while using less N and still remain healthy, then, it would provide a better economic viability as not as much fertiliser-N is needed. Also the water use efficiency (WUE) is important, especially in Australia, when drought conditions are frequently experienced by forest plantations.

In these experiments, there were no significant differences observed between the N-fertilizer source and total dried biomass for both species, with the exception of eucalyptus UR seedlings which had a slightly lower total dried biomass. It must be remembered however, that young plants (less than a year old) were used in these experiments and therefore, may not have had enough time in the different N-fertilizer solutions. Alternatively they may have utilized any N already stored within the plant and haven’t had time to experience any change associated with the different N-sources. Therefore, to see a change due to the different N-source, we speculate that long term (eg. in the order of years) experiments are needed in order to observe variation in biomass accumulation in older plantation trees.

In the case of the eucalyptus seedlings, it is of interest to observe the variation in the uptake of water, which was found to be almost doubled for the AN seedlings compared to the rest of the plant groups. This is most likely due to incomplete closure of stomata during night times and therefore transpiration taking place. However, the plant is not photosynthesising to accumulate more C and hence biomass. Alternatively, it could be due to heavy C respiration that uses up the accumulated C as a source of energy. Therefore the water use efficiency (WUE) is poor for Eucalyptus seedlings grown in AN hydroponic solutions compared to the other N-fertiliser sources. Therefore, our initial studies for the growth of eucalyptus seedlings under hydroponic conditions show that CN fertilized plants take up less N,
and less water. However this is done while producing the same biomass as those in AS and AN fertilisers, which had larger N uptake and water contents.

N content is also important in a forest plantation when considering what is going to be put back into the soil after harvesting takes place. In the subsequent chapter the affect of windrowing, where leftover tree components are gathered in mounds on the plantation site and allowing it to decompose and return nutrients to the soil, will be discussed. It is possible that N content can affect the rates of decomposition of plant residues and therefore the total N content in plant components needs to be taken into consideration during decomposition and its impact on N cycling.

Apart from the major nutrient uptake such as N, it is also of interest to find out what other nutrients; both macro- and micro-nutrients are potentially taken up by the plant. It was found that the net amount of macronutrient ions taken up by eucalyptus and slash pine seedling in hydroponic solutions was dependent on the N species present in the hydroponic solutions. Strong positive correlations were found between PO$_4^{3-}$ vs. Mg$^{2+}$, and PO$_4^{3-}$ vs. (Mg$^{2+}$ + K$^+$), while negative correlations were found between NH$_4^+$ vs. Ca$^{2+}$, and NO$_3^-$ vs. (PO$_4^{3-}$ +SO$_4^{2-}$+Cl$^-$). These correlations are believed to be due to plant root’s requirement to maintain electroneutrality.

Frequent sampling of hydroponic solutions found that this electroneutrality could be maintained either through the efflux of nutrients from the plant back into the solution, or by lowering the uptake of specific like charges in order to let a similar charged nutrient enter the root. While these changes are relatively easy to monitor in hydroponic solutions, soil substrate provides a more complex system and therefore it is difficult to get an understanding of the ions available for the plant’s uptake and actual uptake by the plant, taking into consideration the potential to adsorb to soil particles, volatilization and leach into ground water.

The $^{15}$N NMR spectra obtained for all of the eucalyptus and slash pine components were dominated by the amide-N peak (-261ppm). The distribution of peaks was the same between the different fertiliser N-forms with the exception of the plant whole stems. In the case of slash pine, differences in $^{15}$N NMR distribution in the stems were attributed to changes in bark N-composition. As the bark contains the phloem,
changes could be due to the plant’s health status and therefore reflecting the cycling and storage of N. Alternatively, changes could be due to fluctuations in cycling of organic nutrients in the plant at the time of sampling. Eucalyptus stems on the other hand, showed that the changes in N compositions were due to the bare stem, and hence the plant xylem. Changes are thought to be due to differences in assimilation of the different N species provided in the solution, and storage/cycling of excess organic N produced. For these plant components, the ratio of percentage amide-N to amine-N was found to be a good indicator of the N-source used by the plant.

### 3.4.2 Hydroponics versus Soil Systems

The differences between a simple hydroponic system and a complex soil system must be taken into account when considering the outcome obtained in this chapter. Hydroponics represents an ideal environment that is free from mechanisms which may inhibit uptake of any particular macronutrients. Therefore, hydroponics make a good starting point for studying $^{15}$N enriched fertilizer uptake using NMR techniques to understand how it assimilate in the plant and contributes to the N cycling in forest plantations especially under optimal or extreme conditions. In soil, the presence of exchange sites such as charged clay and mineral particles will selectively immobilize certain charged inorganic and organic molecules and therefore will not be available to the forest ecosystem.

This would mean that not all fertiliser applied to the plantation soils may be available to reach the plants. Apart from this abiotic immobilisation, microbial immobilisation will also occur and thereby, reduce the nutrients available for plant uptake. Fertilisers applied to soil will also experience losses due to processes such as leaching, ammonification and nitrification. These processes were either reduced or absent under hydroponic conditions. The difficulties in applying NMR characterisation techniques in order to study the chemical transformations in nutrient dynamics during decomposition will be discussed more closely in the subsequent chapter.
3.5. References


CHAPTER FOUR

THE DECOMPOSITION OF PLANT COMPONENTS IN SOIL

4.1 Plantation Management of Harvest Residues

In the last chapter, the need for fertilisation in forest plantations was discussed. As tree stems are removed during harvest, this represents an export of potential nutrients from the forest ecosystem’s N cycle that could have been returned via decomposition (Beever et al., 2007; Lewis & Ferguson, 1993). The extent of these nutrient losses has been clearly shown in Figure 3.1, accounting for losses in the order of hundreds of kilograms per hectare with each harvest in radiata pine plantations (Lewis & Ferguson, 1993). The large extent of these losses therefore needs to be supplemented in part by man-made N fertilisers. Excess inorganic fertilisers however, can be detrimental to environmental health, as they are prone to leaching into groundwater. This inorganic input due to leaching, however, can be reduced through effective management practices.

During the harvesting process a machine is used on site which extends an arm that wraps around the tree trunk and cuts the stem to a specific size (Figure 4.1). The machine then loads the stem onto a truck (Figure 4.1) which then transports the wood to its next destination (eg. a lumber yard). The rest of the tree parts, such as leaves, small branches and roots, are all left behind on site to decompose. Previously these leftover plant residues were burnt on site (McMurtrie & Dewar, 1997) but it is now common practice to mound these residues in long lines which follow the contour lines of the plantations (Blumfield et al., 2004; Bubb et al., 1999; Mathers et al., 2000). These long mounds are known as “windrows”, and allow the natural return of nutrients to the plantation soil system via decomposition and mineralization. This practice therefore reduces the loss of N from the N cycle and the reliance of plants on man-made fertilisers. The next rotation of trees is planted in between windrows.
Figure 4.1: TOP: The machine used to harvest stems. The arm wraps around the trunk and the saws located at the top and bottom of the arm attachment cut the tree stem to size. MIDDLE: Stems cut to size are then loaded onto trucks for transportation. BOTTOM: The remaining tree parts (harvest residues) are piled up into long mounds known as “windrows”, which follow plantation contour lines.
The decomposition process in Australian soil is usually slow due to limitations in moisture and temperature (Williams & Woinarski, 1997). Slow decomposition rates brought about by these conditions therefore lead to large amounts of nutrients being “trapped” in the decomposing dead plant material for an extended period of time and are therefore unavailable for plant use. Blumfield and co-workers (2004), have previously monitored temperature and moisture conditions under windrows compared to non-windrowed areas of a Queensland hoop pine plantation. It was found that temperatures were more stable under windrows, whereas between windrows they were highly variable and dependant on the time of day. Moisture retention levels were also increased under windrows. The stable temperature and improved moisture conditions under windrows therefore provide a better environment for microbial populations to thrive. Hence decomposition rates are improved under windrows.

4.2 **Decomposition of Plant Material on Soils**

4.2.1 **Effects of the Chemical Composition of Plant Residues**

As mentioned previously, decomposition under windrows includes the left over small branches, leaves and roots. This is in contrast to non-windrowed areas of a plantation, which will have a decomposing layer of mostly leaf litter from the plantation trees. The differences between the windrow and non-windrowed areas therefore include differences in the initial plant residues and hence the chemical composition of the plant residue available for decomposition. Due to the physical structure of the different plant residues, there may also be changes in the surface area of plant material that is in contact with the soil. This is not always the case, as the harvesting process produces a lot of sawn and chopped up tree components. Hence it is quite clear that these plant components left on site may not always be intact and therefore altering the surface area to soil ratio compared to a natural forest. This pulverizing of plant material may also affect decomposition rates, and increase the amount of soluble plant residue nutrients that are released into the soil system.

In soil solutions, dissolved organic C (DOC) usually derives from biological degradation of plant residues or leaching from dead plant tissues. Due to its soluble
nature, it can therefore be easily removed from the forest ecosystem by way of leaching. Michalzik and Matzner (1999), have previously studied DOC, and found that it mostly contained hydrophilic acids, hydrophobic acids (which contain less N) and neutral substances. The chemical composition of dissolved organic N (DON) was found to contain NO$_3^-$, NH$_4^+$ as well as amide-N, amino-N and N in amino sugars. DON and DOC were found to be weakly correlated with decomposition; therefore mechanisms for these two groups may be different.

Valenzuela-Solano & Crohn (2006) have previously undertaken research into the decomposition of various types of organic mulches and the effect their chemical composition has on decomposition. It was found that chemical composition did indeed affect decomposition rates; with short term decomposition being positively correlated with increases in the content of polar extractable C and N. Decomposition was negatively correlated however, with the acid insoluble fraction content, which consisted of mostly lignin. This fraction was found to be the best predictor of remaining mulch mass in the long term. It was concluded that decomposition rates of N depends on the initial chemical composition of plant material, and also on the behaviour of the microbial populations relying on these plant materials as a source of energy. Bargali (1996) has also pointed out that the C-N ratio of eucalyptus wood was found to increase with eucalyptus plantation age. Therefore new rotations of seedlings in a plantation will cause a change in the availability of N in the ecosystem.

Differences in the chemistry of slash pine and eucalyptus plant components can be seen in the $^{15}$N NMR (Chapter 3, Figures 3.30-3.25 and 3.29 to 3.35) and $^{13}$C NMR (Appendix, Figures A.7 to A-18) spectral data analysed during the thesis research. The eucalyptus plants grown in hydroponic solutions containing ammonium nitrate (AN) as the fertilizer is of particular interest, as these plant components were used in the decomposition experiments presented in the current chapter. It is also of interest to note that not only the N and C composition of plant components can differ but also the total N and C content of the plant itself depending on the availability of N and other nutrients, plantation management and environmental conditions.

An experiment undertaken by Prescott (1995), studied the decomposition rates of needles from N fertilised Lodgepole pine trees, as well as those from unfertilised
control trees in soil. Although the fertilised trees have 5 times the N content, this study found that the decomposition rates did not significantly differ between the fertilised and unfertilised plots. This suggests that N content is not the major factor deciding the rates of decomposition; rather it depends on the overall chemical composition of the samples.

4.2.2 Effects of Soil Mineral Content

Soil chemistry inherently involves the inclusion of mineral particles such as clay minerals, metals and therefore paramagnetics. In chapter 3, hydroponic systems were used in order to allow the plants studied have free access to nutrients. In a soil system, however, access can be restrained by adsorption to charged particles, such as NH$_4^+$, to clay and metal exchange sites. Therefore, the mineral chemistry needs to be taken into account when studying soil and its nutrient cycles. The use of different soil extraction procedures can help identify the quantity of soil nutrients involved in adsorption, and also the amount that is present as soluble and therefore more labile nutrients.

In the current research investigation, the focus will be on removal of organic and inorganic N during the extractions. The hot water extractions remove the readily decomposable fraction of soil organic matter, which includes N originating from microbial biomass, root exudates and other forms of organic N. Therefore, it reflects the more labile organic N that exists in soil solutions and macropores (Burton et al., 2007). It will also remove small amounts of free inorganic compounds, which are not bound to charged sites. Lipson & Nasholm (2001) found that only a small amount of free amino acids are dissolved in soil solution during various extraction methods including water (0.04 – 24 µg N g$^{-1}$). This may be due to amino acids having strong interactions with soil particles. Proteins are usually extracted at higher levels, and indicate a stable source of amino acids. Any amino acids in soil solution may originate from leaking, decaying plant structures (Lipson & Nasholm, 2001).

The KCl extraction procedure involves the removal of free inorganic compounds present in the sample as well as inorganic compounds from charged exchange sites including clays and paramagnetics. Analysis of KCl extracted solutions can allow the determination of mineral N levels in soil. McTaggart & Smith (1993) have pointed
out that if a prediction of the amount of mineral N available for plant uptake can be made, then N-fertilisers can be applied accordingly leading to better site management and use of limited amounts of mineral N which can otherwise be potentially detrimental to the environment. In their research, it was found that good correlations existed between KCl extracted mineral N content and the uptake of N found in the plant tissue of crops grown in the soils analysed.

Both hot water and 2M KCl extraction procedures have been used in the current research undertaken, in order to monitor and understand the fluctuations in N and C in organic and inorganic forms. This will therefore allow observations as to how much is possibly adsorbed to charged soil particles, as well as changes in the labile fraction easily dissolved in soil solutions with respect to short-term decomposition.

4.2.3 Use of NMR as a Soil Quality Indicator

Many studies have been undertaken using $^{13}$C NMR to observe the chemical composition of plant and soil material, as well as decomposition of the two (Almendros et al., 1992; Baldock et al., 1992; Gerasimowicz & Byler, 1985; Gonçalves et al., 2003; Keeler & Maciel, 2003; Knicker et al., 1996; Kogel-Knabner, 1997; Mathers et al., 2000; Schmidt et al., 1997; Smerik, 2006). From $^{13}$C NMR spectra, it has been found that ratios of certain peak regions may be used as indicators of soil health or quality and to understand the factors contributing towards humification and mineralization. Firstly the percentage of the spectrum which falls into the aromatic-C regions, as compared to the total spectrum intensity, can be used to identify humification of organic matter taking place (Dai et al., 2001; Wilson, 1987). This is shown in Equation 4.1

$$\text{% aromaticity} = \frac{\text{aromatic peak area (110 - 160 ppm)}}{\text{Total spectrum area (0 - 160 ppm)}} \times 100$$  \hspace{1cm} \text{Equation 4.1}

$$\text{A/O-A ratio} = \frac{\text{alkyl peak area (0 - 50 ppm)}}{\text{O-alkyl peak area (50 - 110 ppm)}}$$  \hspace{1cm} \text{Equation 4.2}

$$\text{CC/MM} = \frac{\text{carbohydrate peak area (65 - 95 ppm)}}{\text{methoxyl peak area (45 - 65 ppm)}}$$  \hspace{1cm} \text{Equation 4.3}
However, Baldock & Preston (1995) have proposed that the ratio of peak intensities in the alkyl and O-alkyl regions may better describe decomposition (Equation 4.2). They indicated that while the alkyl-C region increased, the O-alkyl-C region decreased during decomposition of plant litter. However, it is not necessary for the aromaticity to increase with decomposition. Blumfield and co-workers (2004) have previously found that the correlations with either mass loss, total N, or $^{15}$N enrichment and either carbohydrate-C or methoxyl-C opposed each other. For this reason it was suggested that the ratio of carbohydrate-C to methoxyl-C (CC/MM), may be a useful indicator of decomposition.

4.2.4 The Presence of Paramagnetics in Soil Samples

When studying soils however, care must be taken with respect to the paramagnetic content in the sample, and its possible affects on the NMR spectral peak intensities obtained. The affect paramagnetics have on the signal intensity of NMR spectra, and the relaxation parameters acquired, has been previously discussed in Chapter 2, section 2.3.5. (page 48). The paramagnetic ions of iron, copper and manganese have been found to contribute strongly to signal loss, with iron producing the greatest adverse effect (Mathers et al 2002; Smernick & Oades, 2002). However, the paramagnetic content of soils is not limited to these three metals. The presence of paramagnetics can affect spectral intensity in various ways. If the paramagnetics are present as free ions, then the total spectrum intensity may be reduced evenly. However if present in complexes with organic material, the paramagnetics can selectively diminish the corresponding $^{13}$C peaks in the NMR spectrum obtained (Smernik & Oades, 2002). Therefore, the reduction of the NMR peak intensities provides an idea of the proximity of these carbons to the paramagnetic species in soil.

Treatment with hydrofluoric acid (HF) to remove mineral matter such as paramagnetics is commonly used. When the mineral matter is removed, it also increases the concentration of organic matter and hence the observability of the $^{13}$C peaks in the resulting NMR spectrum. The HF treatments used by various researchers involve treating samples containing paramagnetic concentrations usually between 2 – 10%. However, it has been concluded that this process if not 100% efficient and that a small amount of paramagnetics may still remain in the sample. Mathers and co-workers (2002) have previously used 2%HF treatment on soil samples, and measured
the HCl extractable iron content present before and after HF treatment. It was found that 86.9-99.3% of iron was removed by HF treatment, leaving only 0.7-13.1%. It is possible however, that these small amounts present will still affect NMR spectra obtained. More importantly, as previously stated, where the left over paramagnetics are located will determine the effect they may have on spectral distribution.

With decomposition experiments, comes the loss of mass through CO$_2$ respiration of microbial populations, as well as the breakdown of compounds present. Through these processes it is possible that certain organic or inorganic compounds present in soils will therefore move closer to paramagnetic centres or form organic-metal complexes. This again will only affect the NMR regions of the functional groups involved. It is therefore important to consider these processes, as when comparing decomposition of samples. For example, when using ratios such as the A/O-A ratio, the increase in one functional group may not be due to degradation of samples, but instead the particular compounds becoming closer to paramagnetic centres.

In a recent investigation by Heike and co-workers (2008), the very short-term to short-term dynamics (1-6 days duration) of N in soil were studied using the $^{15}$N CPMAS NMR and soil sterilization. The results are very encouraging, reporting for the first time that biotic processes causing $^{15}$N immobilization are dominant in the very short-term, typically within a few days. It also emphasises the need for further studies in order to understand the biotic and abiotic immobilization processes and to improve all methods for the identification and quantification of the N components in soil by $^{15}$N CPMAS spectroscopy.

In the current thesis research, a short-term, 8-week experiment involving the decomposition of eucalyptus leaves mixed with Queensland soil has been compared with that of a mixed sample of eucalyptus plant components in soil. The experiments have been designed to reflect the chemical species found under and between plantation windrows. By using an 8-week experiment, the focus was mainly on the short-term dynamics of nutrients that includes the decomposition of the more labile nutrients. It is thought that the time lag between harvesting including the formation of windrows, and the planting of the next rotation of trees, may not allow these newly decomposed products to be used up by the plants. This would mean that it is
possible these chemical species in the harvest residues may be prone to processes such as leaching, volatilisation and microbial and abiotic immobilization and therefore lost from the forest plantation ecosystem.

These short-term N dynamics are studied using ion chromatography (IC), C/N analyses, elemental data and $^{13}$C and $^{15}$N NMR spectral data. The use of IC, elemental and C/N analyses would provide a more quantitative understanding of the chemical changes while NMR will provide a relative qualitative understanding of these changes in nutrients with time. Attempts will also be made to understand and compare the involvement of paramagnetic species interacting with the $^{13}$C and $^{15}$N chemical functional groups in the decomposing soil samples.

4.3 Experimental Methods

4.3.1 Experimental Conditions

Soil Properties and Preparation
The soil used in this experiment was taken from a hoop pine forest plantation in the Imbil State Forest in southeast Queensland, Australia. This soil was used as it provides a more realistic substrate to study decomposition of eucalyptus material. The soil samples were taken from a depth of 0-10cm from random positions around the site, and then sifted through a 2mm sieve to remove rocks and large plant material. Samples were kept in sealed airtight plastic bags, and stored in a potable cooler (Esky) for transport back to Sydney and until further use. Soil physical and chemical properties have previously been recorded by Blumfield & Xu (2006).

The soil’s ability to hold water, otherwise known as the field capacity (FC), was found by bounding 10 weighed samples of soil into cloths with a rubber band. These were then saturated in deionised water overnight (approx. 16 hrs) and were then hung up by string and allowed to drip until downward forces ceased to release bound water, and thus dripping stopped (approx. 2 days). Samples were then weighed, and assumed to be at 100% FC. The soil was then allowed to air-dry in aluminium tins until a constant mass was reached and then oven dried to remove all moisture (0% FC) in an oven set to 50°C. At each stage the mass was recorded, which was used to
find the percentage capillary and hydroscopic water present using Equations 4.4 and 4.5.

\[
\text{% Hydroscopic Water} = \frac{(\text{air dried mass} - \text{oven dried mass})}{\text{air dried mass}} \times 100 \quad \text{Equation 4.4}
\]

\[
\text{% Capillary Water} = \text{% Water at Field Capacity} - \text{% Hydroscopic Water} \quad \text{Equation 4.5}
\]

The percentage capillary and hydroscopic water present were found to be 31.2% and 2.2% respectively. The hydroscopic water content is important to note for NMR experiments, as in Chapter 2 of this thesis, any moisture present can have an affect not only on time constant but also NMR peak observability. Soil to be used in the decomposition experiment was then made to 60% FC, by adding deionised water.

**Plant Material**

Leaves, whole stems and root samples taken from the $^{15}$N-enriched *Eucalyptus Pilularis* seedlings described in Chapter 3, were used here for the decomposition experiment. The eucalyptus plants used were grown in the doubly $^{15}$N enriched AN (ammonium nitrate) hydroponics solutions. The macro- and micro-nutrient content is given in Chapter 3 section 3.2.1, while $^{15}$N and $^{13}$C NMR CPMAS spectra of these sections are shown in section 3.3.5. All plant material to be used was dried in aluminium tins in an oven at 50°C until a constant dry mass was reached, and then ground to a powder.

**Sample Preparation**

In order to simulate different conditions that may be found in a plantation environment, different sample types were made for the decomposition study. These samples consisted of soil mixed with leaves (SL), which is what would usually be decomposing in the form of ground litter during a stand rotation. A second set of samples were made with soil added to a mix of leaves, whole stems and root (SM). These samples reflect more closely what may be found under a plantation windrow, where harvest residues are pushed into mounds, which run along the sites contour lines, and left to decompose and return their nutrients. In the field, windrow composition depends on what is left behind after harvesting as well as what litter was already present. The ratio of leaves, stems and roots found in windrows is therefore highly variable.
Table 4.1: Mass of plant and soil material used in each sample for the decomposition study, initially containing a total N and total C content as found by C/N analysis (see pg 189 for experimental conditions)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Leaves (g)</th>
<th>Stems (g)</th>
<th>Roots (g)</th>
<th>Soil (60% FC)</th>
<th>Total Mass (g)</th>
<th>N Content (g)</th>
<th>C Content (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL (Soil + Leaves)</td>
<td>1.1</td>
<td>-</td>
<td>-</td>
<td>10.0</td>
<td>11.1</td>
<td>0.049</td>
<td>0.773</td>
</tr>
<tr>
<td>SM (Soil + Mix)</td>
<td>0.5</td>
<td>0.4</td>
<td>0.2</td>
<td>10.0</td>
<td>11.1</td>
<td>0.040</td>
<td>0.696</td>
</tr>
<tr>
<td>SO (Soil Only)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.0</td>
<td>10.0</td>
<td>0.027</td>
<td>0.303</td>
</tr>
<tr>
<td>LO (Leaves Only)</td>
<td>1.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.1</td>
<td>0.022</td>
<td>0.465</td>
</tr>
<tr>
<td>MO (Mix Only)</td>
<td>0.5</td>
<td>0.4</td>
<td>0.2</td>
<td>-</td>
<td>1.1</td>
<td>0.013</td>
<td>0.456</td>
</tr>
</tbody>
</table>

The ratio of sections in this mix were based on the ratio of dry plant matter partitioning of these sections found in the AN eucalyptus seedlings (Chapter 3, section 3.3.1). This ratio may not be exactly what is found in field conditions, but serves as a starting point for experiments. In each of these sample types, the total amount of plant material was kept constant (1.1g), as was the amount of soil (10g at 60% FC). The SL and SM sample therefore will also represent two slightly different N contents, as the mix, which includes stems and roots, inherently has less N than leaves. Table 4.1 shows both the masses of plant and soil material used in each sample, as well as their total N and total C content.

Samples consisting of soil only (SO), leaves only (LO), mix only (MO) were made as standards, and were used in order to compare decomposition rates with the SL and SM mixed samples. Sample compositions are shown in Table 4.1. All samples containing soil were made at 60% FC, which was the equivalent of 7.683g oven dried soil. For the SL and SM samples, 10.0g of this soil was put into 50mL centrifuge tubes along with finely ground eucalyptus plant material. The unmixed samples were made to equal the amounts used in the mixed samples. Enough samples were made so that an individual sample (in separate centrifuge tubes) could be taken at 0, 2, 4, 6 and 8 weeks. A replica of each sample was also made in order to reproduce results.

**Decomposition**

A method similar to Eneji and co-workers (2002) and was used for the decomposition of samples. Samples were capped to avoid excessive moisture loss and put into an incubator kept at 27°C. Lights were timed to come on for 12 hours each day after which they switched off for 12 hours, therefore replicating day and night. Samples were taken out of the incubator once a week and allowed to air for 10
minutes before being re-capped and put back in. Moisture losses were minimised this way. Samples were then collected for analysis after 0, 2, 4, 6 and 8 weeks, and separated into three parts. Each part was used for either hot water extraction to extract any soluble forms of N present, 2M KCl extraction to extract any mineral forms of N present or was left untouched. A flow chart depicting the separation and analysis of these different sections is shown in Figure 4.2.

**4.3.2 Sample Extractions**

The methods for sample extractions used were based on those methods used by Chen and co-workers (2005). KCl extractions were carried out using 5g of field moist soil (the equivalent of 3.93g air-dried soil) which was weighed into centrifuge tubes along with 50mL of 2M KCl (Chen et al., 2005). These were then capped and placed on their side for 1 hour in a water bath set to 30°C, which simulated an end-to-end

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**Figure 4.2:** An experimental flow chart depicting the separation of the decomposed samples, and the corresponding chemical analysis.
shaking motion. The samples were then removed and vacuum filtered through Whatman 42 filter paper. Soil extracts were stored at -4 °C until analysis, while the residue solids were air-dried. Residues were then quickly washed in cold deionised water to remove as much left over KCl as possible, and were then oven dried. Sub-samples of the extracted KCl solutions were freeze-dried in order to obtain the extracted solid. C/N analysis was then undertaken on these samples in order to obtain the total N and total C extracted.

Hot water (HW) extractions were performed using 4g of air-dried soil weighed into glass jars along with 20mL of deionised water (Chen et al., 2005). These were then capped, shaken and incubated in oven set to 70°C for 18 hours. The jars were then removed and shaken in an end-to-end manner for 5 minutes, then filtered through Whatman 42 filter paper. Soil extracts were then stored at -4 °C until analysis, while the left over solids were oven dried. Sub-samples of the extracted HW solutions were freeze-dried in order to obtain the extracted solid. These solids were weighed in order to determine the extracted mass. Due to the small amounts extracted, C/N analysis could not be undertaken. One large bulk SL sample at 0 weeks was therefore made (20g soil + 2.2g leaves), that would enable a larger amount of HW extractions to collected. The extract from this sample was freeze-dried and analysed with the C/N analyser and \(^{15}\)N and \(^{13}\)C NMR spectroscopy. This would then give us an idea of what organic compounds may be extracted.

All extracted solutions as well as the residual solid soil samples were stored at -4 °C in a freezer. Freezing soil samples for storage reasons may kill micro-organisms contained in the sample, and therefore increased mineralisation and immobilisation may occur when thawed (Jansson & Persson, 1982). This, however, should be less than what would occur if the samples were not frozen and stored. Also, all samples are treated in the same way, so all samples are still comparable (Prasertsak et al., 2001). In addition, all soil samples and soil samples after extractions were oven dried before freezing to remove moisture and therefore to minimise any mineralisation that may occur when thawed. As all soil samples and residues were oven dried before storage, this should also minimise any mineralisation that may occur when thawed.
4.3.3 Ion Chromatography

Samples of the extracted HW solutions were injected via a 45µm syringe filter into the 20µL loop of a 792 Basic Ion Chromatography (IC) unit from Metrohm. The calibration, anions and cations analysed and equipment setup are discussed in detail in Chapter 3, section 3.2.4. As expected, all the KCl extracts inherently contained extremely high levels of K\(^+\) and Cl\(^-\), and were therefore not analysed with IC.

4.3.4 Elemental Analysis

Analysis for %C and %N was undertaken on a LECO TruSpec CN analyser at University of Western Sydney, Hawkesbury campus. Calibration of the instrument was carried out using varying amounts of the standards EDTA (9.58 ± 0.04% N, 41.05 ± 0.13% C) and glycine (18.66% N, 32.00% C). All samples were loaded into aluminium foil trays for analysis.

Some samples were also sent to Microlab at the University of Otago, New Zealand for elemental analysis and to verify the results obtained on the CN analyser. Metal analysis of some of the 0 week samples were carried out at the Microlab in New Zealand in order to get an idea of the extent of paramagnetics that may be present particularly in the soil samples. Samples were analysed using a digestion method, where 10mg of sample was digested in 3ml HCl and 1ml HNO\(_3\) at 95°C for 2 hours. Solutions were decanted from the residues and diluted for analysis. Due to the very high Fe concentration found in the solutions, samples needed to be diluted, which put Co below reporting limits.

4.3.5 \(^{15}\text{N} \)NMR Spectroscopy

Solid-State NMR analysis was carried out on untouched samples (samples before extractions) as well as the remaining solid residues obtained after hot water (HW) and KCl extractions. All samples were oven dried and ground before analysis.

Solid-state \(^{15}\text{N} \)NMR CPMAS spectra were obtained using the same experimental parameters outlined in Chapter 3, section 3.2.2. The only exception was the accumulation of 130,000 scans for all \(^{15}\text{N} \)spectra, as the concentration of the \(^{15}\text{N} \)was low compared to samples used in Chapter 3. The addition of soil also meant there was now a large presence of paramagnetics in the samples. All time domain spectra
were Fourier transformed with a 30Hz Lorentzian line broadening to give frequency domain spectra. Chemical shifts were measured with respect to glycine as an external standard at -347ppm (Knicker & Hatcher, 2001) which is equivalent to nitromethane at 0ppm. DDCPMAS spectra obtained were obtained using a pulse sequence with the same parameters used in the CPMAS experiments, along with a $t_{1/2}$ of either 0 or 45 µsec. This 45µsec delay was shown in Chapter 2 to be sufficiently long enough to allow the complete disappearance of the peak intensity for protonated N-species (section 2.4.2). For these experiments 260,000 scans were collected.

All spectra were integrated using MestRec computer software. Absolute intensities were found by integrating peak areas then correcting for the amount of sample present in the rotor during experiments to give the absolute intensity per gram of sample. $^{15}$N CPMAS NMR spectra were integrated into the same five regions as used in Chapter 3 (see section 3.2.2). Peak region assignments were hetero-N (-190 to –220ppm), amide-N (-220 to –285ppm), guano-N (-285 to –300ppm), amine-N (-30 to –325ppm) and amino-N (-325 to -350ppm). These region assignments were used as a guide. Spectra regions were integrated from the low points found between peaks. Further assignments for chemical structures containing N-compounds can be found in Table 3.10.

4.3.6 $^{13}$C NMR Spectroscopy
Solid-state $^{13}$C NMR CPMAS spectra were obtained using the same experimental parameters outlined in Chapter 3, section 3.2.3. Exceptions were the accumulation of 40,000 scans for all $^{13}$C spectra 130,000, and the use of 150Hz Lorentzian line broadening for all Fourier transformations to give frequency domain spectra. The number of scans has been increased compared to those in chapter 3 to accommodate the low levels of $^{13}$C nuclei and increased paramagnetic content. Chemical shifts were measured with respect to adamantane as an external standard at 38.3ppm and corrected relative to tetramethylsilane (0 ppm).

Spectra were integrated and the absolute intensities corrected using the same method as described for the $^{15}$N NMR spectra. $^{13}$C CPMAS NMR spectra were integrated into 7 sub-regions depicted in Table 3.15 in Chapter 3, which consists of regions for carboxyl C (185 to 165ppm), phenolic C(165 to 140ppm), aromatic C (140 to
110ppm), di-O-alkyl C (110 to 80ppm), carbohydrate C (80 to 60ppm), methoxyl C (60-45ppm) and alkyl C (45 to 0ppm). Once again the assignment of these chemical shifts to possible structures was used as a guide. Spectra regions were integrated from the low points found between peaks.

### 4.4 Results and Discussion

#### 4.4.1 Decomposition Rates

After 8 weeks the overall mass lost from SL and SM samples were the same, the SM samples seem to have decomposed at a more constant rate, whereas SL varied (Figure 4.3). Both the SL and SM mixed samples decomposed more quickly than the SO sample which was expected. The LO and MO samples showed no mass loss over the 8 week period. The total mass lost from each sample is shown in Table 4.2, and are most likely due to CO$_2$ respiration of the microbial biomass, indicating that decomposition is taking place.

The SL and SM samples had different initial N contents with the SL sample containing 22.5% more N (Table 4.1, page 186), and with each sample having comparable decomposition rates indicates that it is possible the total N content has had no effect on the decomposition rate during the 8-week time period. This, however, may be because the plant material used in the decomposition experiment comes from two different sources, one being just leaves mixed with soil and the other being a mixture of plant components in soil. When decomposition is considered in a forest plantation situation, the chemical composition as well as the material structure may be different.

It has been reported that both physical structure and chemical composition influence the microbial community, which participate in the decomposition process (Chen et al., 2003). Therefore, for the current study, the attention was drawn to compare decomposition rates based on differences in the assimilation within the plant components grown under similar nutrient uptake. This means that the study would imply how the decomposition is affected by the overall chemical composition of the
plant residue. To enable this comparison, the plant material was ground to a fine powder and mixed with the soil and thus significantly reducing the effect of physical structure on the decomposition rate.

Due to time limitations imposed on the decomposition experiments, the study was restricted to a short term of 8 weeks. This is relatively a very short time duration compared to previous studies on decomposition rates which extended for periods of years (Blumfield et al., 2004; Prescott, 1995). The shorter experiment times may present a reason why no decomposition rate differences were seen in this study. Over a longer time the more labile N or C may be consumed first leaving the microbial communities a longer period of time to decompose the more recalcitrant compounds. This would lead to an averaged decomposition rate that would be significantly different to the separate rates required for the labile and recalcitrant compounds.

Table 4.2: Calculations of the remaining mass, and the mass lost in each sample over 8 weeks.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Week</th>
<th>Remaining Mass (g)</th>
<th>Mass Lost (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL</td>
<td>0</td>
<td>8.78</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.72</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.66</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8.67</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8.57</td>
<td>0.21</td>
</tr>
<tr>
<td>SM</td>
<td>0</td>
<td>8.78</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.72</td>
<td>0.06</td>
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<tr>
<td></td>
<td>4</td>
<td>8.69</td>
<td>0.09</td>
</tr>
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<td>6</td>
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<td>0.21</td>
</tr>
<tr>
<td>SO</td>
<td>0</td>
<td>7.68</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.61</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>8</td>
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</tr>
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</tr>
<tr>
<td></td>
<td>8</td>
<td>1.10</td>
<td>0</td>
</tr>
</tbody>
</table>
4.4.2 CN Analysis of Soil before and after HW Extractions

A C/N analyser was used to determine the amount of total N and total C in SL and SM samples before and after HW extractions. HW extraction removes labile organic matter from the soil and soil macropores. As the samples underwent shaking during the HW extraction process, it is possible to remove small amounts of organic matter present in the micropores of the soil. Most of the organic matter extracted from soils usually has its origin from microbial activity and/or from plant roots and dead plant exudates. Some free, unbound, inorganic compounds may also be removed by HW extraction.

The total N and total C measurements, for the untouched SL and SM samples at different time intervals during decomposition, are shown in Figure 4.4 and Figure 4.5 respectively. These diagrams also display the total N and total C measurements for the HW extraction residues for the SL and SM samples. The plots show similar trends for all cases. Data for soil only after HW extraction does not vary in N or C content as much as the untouched soil over the 8 weeks. This may indicate that there is a base soil structure which remains relatively constant over the 8 week time period and could possibly due to more recalcitrant N and C compounds not being extracted.
Therefore the difference between the varying untouched N and C content and the relatively stable C and N content after HW extractions, represents more labile N and C compounds.

For SL samples, the same amount of N and C is present in both the untouched and HW residue samples after 6 weeks. This may represent immobilisation of N and C at this point in time, leading to a null in the availability of labile N and C compounds that can be extracted. After 6 weeks, the total N level in the untouched SL appears to be increasing while the total N in the soil after the HW extraction remains relatively constant. It is possible that there is enhanced N-fixing microbial activity increasing the total N in the sample. However, after the 6 weeks, the total extracted N has increased to maintain relatively constant N content throughout even when the untouched total N has increased. The same response is seen for total N and C in the SM sample over the 8-week period; however the null point occurs at 4 weeks in contrast to 6 weeks for the SL sample. This is an interesting observation and it could mean that the N and C immobilization takes place faster in SM compared to SL samples. In general, both total N and C has fluctuated during the 8-week period while the total N and C after the HW extraction has not significantly changed throughout the period.

4.4.3 CN and IC Analysis of HW Extracts

Analysis of the extracted solutions using IC was undertaken in order to better understand quantitatively the extent of inorganic anions and cations removed. The total mass of both inorganic N compounds and non-N containing inorganic compounds removed by extraction are shown in Table 4.3. These amounts were then compared with the total mass extracted, which was found by weighing HW extracts after freeze-drying. The difference between the total mass extracted and the total amount of all inorganic compounds has been identified here as “unknown”, and could be represented by any of the following species described below:

- Inorganic compounds not detected by the IC equipment. e.g., IC does not detect inorganic C, such as carbonates, which should be present in all samples.
- Any organic compounds, which also cannot be detected by IC analysis.
Figure 4.4: Total N found in a) SL and b) SM samples when untouched and after hot water extraction at 0, 2, 4, 6 and 8 weeks.

Figure 4.5: Total C found in a) SL and b) SM samples when untouched and after hot water extraction at 0, 2, 4, 6 and 8 weeks.
Table 4.3: Mass removed during hot water extraction and Total C and N present in KCl extractions for each sample over the 8 weeks

<table>
<thead>
<tr>
<th>Sample</th>
<th>Week</th>
<th>Inorganics a (non-N)</th>
<th>Inorganic b N</th>
<th>Unknown c</th>
<th>Total Mass d Extracted</th>
<th>KCl Extracted Masses (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total N e</td>
</tr>
<tr>
<td>SL</td>
<td>0</td>
<td>53.9</td>
<td>0.57</td>
<td>106.2</td>
<td>160.8</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>89.4</td>
<td>135.5</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>49.7</td>
<td>0.10</td>
<td>57.2</td>
<td>106.9</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>52.2</td>
<td>0.10</td>
<td>0.4</td>
<td>52.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>47.4</td>
<td>0.12</td>
<td>4.6</td>
<td>52.1</td>
<td>2.3</td>
</tr>
<tr>
<td>SM</td>
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<td>0.43</td>
<td>30.1</td>
<td>65.0</td>
<td>2.3</td>
</tr>
<tr>
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<td>36.2</td>
<td>0.07</td>
<td>26.6</td>
<td>62.9</td>
<td>2.1</td>
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<tr>
<td></td>
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<td>0.13</td>
<td>38.0</td>
<td>80.1</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
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<td>71.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>39.5</td>
<td>0.13</td>
<td>-9.5</td>
<td>30.1</td>
<td>3.4</td>
</tr>
<tr>
<td>SO</td>
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</tr>
<tr>
<td></td>
<td>4</td>
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<td>0.24</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
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<td>0.47</td>
<td>26.0</td>
<td>30.5</td>
<td>1.4</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
</tr>
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<td>83.5</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>60.1</td>
<td>0.21</td>
<td>-2.4</td>
<td>58.0</td>
<td>-</td>
</tr>
</tbody>
</table>

a Total non-N inorganic mass removed as determined by IC., including F-, Cl-, SO₄²⁻, PO₄³⁻, Na⁺, K⁺, Ca²⁺ and Mg²⁺.
b Total inorganic-N mass removed as determined by IC., including NO₂⁻, NO₃⁻, and NH₄⁺.
c Total mass of unknown compounds, taken as the difference between total inorganic and total mass extracted.
d Total mass extracted was found by taking the HW extract mass after freeze-drying, and correcting for the whole sample mass.
e Mass found by freeze-drying KCl extracts, and running C/N analysis to determine total C and N. Results were corrected for whole sample mass.
Figure 4.6: The total mass extracted with hot water from a) SL and b) SM samples over 8 weeks. Total mass has been divided into two regions representing mass from all inorganic compounds detected by IC and all other compounds such as inorganic not detected by IC and all organic matter removed.

It can be seen in Table 4.3 that the total mass extracted decreases over the 8 week period for SL, but remains at a relatively constant level for SM until week 8 when a large decrease is seen. In Table 4.3 the total mass extracted has been broken up into three groups representing non-N containing inorganic compounds, inorganic N, and an unknown fraction identified here as “unknown”. The total inorganic compounds removed by HW extraction have remained relatively constant during the 8 weeks period (Figure 4.6a and b). Analysis of the total species, which include; non-N containing inorganic F, Cl, SO$_4^{2-}$, PO$_4^{3-}$, Na$^+$, K$^+$, Ca$^{2+}$ and Mg$^{2+}$, and the N-containing inorganic NO$_2^-$, NO$_3^-$, and NH$_4^+$. These species were observed for both SL and SM samples.

The amounts of individual anions and cations present in each of the hot water extracted solutions are recorded in the thesis Appendix (Figure A.19 and A.20). Most of the inorganics removed came from sulfate, chloride, potassium and calcium, all of which did not change by weight much over the 8 week period and would explain why the amounts of inorganics extracted are fairly constant throughout the experiment. The total amount of inorganic N extracted from each of the samples is shown in Figure 4.7. This shows that there are changes in the availability and
production of inorganic N. It can be seen that both SL and SM samples show a rapid decrease in the inorganic N extracted at 2 weeks, followed by a slow increase over the next 6 weeks. The SO sample showed a steady increase over the 8 week period, while LO and MO remained at a constant level. The amounts of inorganic N extracted by HW are very small compared to the total inorganics extracted, so any variations seen here, will not affect total levels significantly.

The contributions of NO$_2^-$, NO$_3^-$, or NH$_4^+$ to the total inorganics are shown in Figure 4.8. Here it can be seen that the majority of the total N at 0 weeks is made up of NO$_3^-$ for both SL and SM samples. The amount of NO$_3^-$ falls to zero by week 2 and and remains at zero till the end of the experimental time period. This would indicate that NO$_3^-$ has been rapidly converted to other forms. It was different for the SO sample, which showed a steady increase in NO$_3^-$ levels during the experimental period, while LO and MO extracted NO$_3^-$ amounts remained at a constant level. HW extracted NH$_4^+$ levels for SL and SM samples both fell at 2 weeks, after which they slowly rise again.

![Figure 4.7: Comparison of total hot water extracted inorganic N from SL, SM, SO, LO and MO samples over 8 weeks. These represent free inorganic N found in the samples.](image)

The unknown fraction seen in Table 4.3, is shown to decrease over time for SL samples and this result is echoed in Figure 4.6. This may demonstrate that the leaves in these samples provide a source of dissolvable, labile organic compounds or inorganic C when the leaves are initially mixed with soil. Over time these forms may be used as an energy source by microbes and converted to less recalcitrant forms or
lost as CO\textsubscript{2}. The SM samples on the other hand had a more stable “other” fraction. This may be due to less labile fractions being present in the stem and roots sections.

![Figure 4.8: Hot water extracted mineral forms of N found in a) SL, b) SM, c) SO, d) LO, and e) MO samples over 8 weeks.](image-url)
In order to gain a better view of the type of organic compounds that may be present in HW extracts, a larger bulk SL sample at 0 weeks was made, as mentioned in section 4.3.2., these extract samples were freeze-dried and analysed using $^{15}$N and $^{13}$C NMR spectroscopy. After multiple attempts, a satisfactory $^{15}$N spectrum could not be obtained and its quite possible that there is low extractable $^{15}$N content in the sample. Any N extracted that originated from the soil component of the SL samples, will be in the form of $^{14}$N, which is unobservable by $^{15}$N NMR. A $^{13}$C spectrum, however, was acquired, and is shown in Figure 4.9.

The $^{13}$C spectrum shows a very sharp peak at 74ppm thought to represent carbohydrate C, however a strong corresponding peak at 105ppm representing anomeric C is not observed. It is possible that the 74ppm peak may represent a combination of carbohydrate C and other alkyl-C-O- functional groups (Buchanan and Rastegar, 2001), for example ethylene oxides (Spevacek and Baldrian, 2008). It has previously been found that ethylene oxide is present in broad beans as an ethylene metabolite (Jerie and Hall, 1978). Eucalypts are known to contain small amounts of ethylene present as a plant signalling molecule (Correa et al, 2005). Therefore it is possible that ethylene oxide was extracted from the bulk SL sample, originating from the eucalypt leaves. A broad region with peaks at 27 and 21ppm represents aliphatic terminal C moieties.

Figure 4.9: $^{13}$C NMR of the mass extracted from a bulk SL sample with hot water.
These C species represent a labile C fraction extracted from the SL samples. Analysis disclosed that the stems and roots contained less of these labile species, and more aromatic-C species such as tannins. This may explain why there was a smaller amount of “other” groups removed from SM samples as seen in Table 4.3. Furthermore, it may also explain why the amounts of HW extract from SM samples were more consistent over the 8 week period, as the less labile forms of C should be more stable and less prone to breakdown.

The \( ^{13} \text{C} \) spectrum displayed peaks in regions that represent a small amount of carboxyl-C groups (181 and 175ppm), which had been removed by HW extraction. This may indicate that a small amount of soluble protein has been removed, and therefore a small amount of N. Evidence of N in the form of amino acids was found qualitatively using thin layer chromatography (TLC) analysis.

On the other hand, KCl extractions removed any free inorganic compounds and inorganic compounds adsorbed on charged particles such as clays and metals. Therefore these inorganic species represent the exchangeable fraction that was associated with organic matter. It is also possible that a small amount of charged organic molecules could have been removed. Extracted KCl solutions were freeze dried then analysed with a C/N analyser to determine the total content of extracted N and C. The resulting amounts extracted with KCl are also shown in Table 4.3 on page 196.

A comparison of the total inorganic C and N removed for SL and SM samples, by KCl extraction, is shown in Figure 4.10a) and b) respectively. Compared to the amount of inorganic N removed by HW extraction (0.57mg for SL at 0 weeks), the KCl extracted inorganic N is much larger (3.5mg for SL at 0 weeks). This shows that a large portion of inorganic N is adsorbed to charged particles.

The amount of inorganic C removed with KCl is also interesting when compared to the unknown fraction in Table 4.3. The KCl extracted inorganic C, which would include free inorganic C as well as that adsorbed to charged particles, ranged from 9.6 up to 14.9mg. The free inorganic C pool is removed by both HW and KCl extraction. Therefore, from the HW extracts, the largest amount of the unknown fraction that is
accounted for by inorganic C, would be the amount found by KCl extraction, assuming 100% of this is free inorganic C, which is unlikely. Hence the unknown fraction from the HW extracts must be mostly made up of organic substances.

4.4.4 Decomposition Analysis Using NMR Spectroscopy

Soil plus leaves (SL) and soil plus mix (SM) samples when left untouched, after HW extractions and after KCl extractions, were analysed at 0 and 8 weeks using $^{15}\text{N}$ and $^{13}\text{C}$ NMR spectroscopy. The distribution of peaks in each spectrum could then be compared in order to observe any changes in samples as they decomposed. The differences in distribution, or sample compositions, are discussed in this section. Initial spectra obtained for soil only (SO) samples showed no peak for either $^{15}\text{N}$ or $^{13}\text{C}$ NMR spectra. Therefore it can be assumed that any spectral peaks obtained for SL or SM samples are originating from the plant material, either directly or after decomposition and incorporation into soil or the microbial biomass.

The relative intensities of NMR spectra obtained for the SL and SM samples were also compared to leaves only (LO) spectra obtained before and after extractions, and spectra for untouched mix only (MO) samples. By comparing these spectra relative to the LO spectra, the effect of paramagnetics, in soil, have on NMR observability.
and possibly their relaxation parameters can be observed. This is discussed in the subsequent section 4.4.5. The differences in relative spectral intensities are also discussed in section 4.4.6 in relation to the extraction procedures undertaken.

\textit{\textsuperscript{15}N NMR Peak Region Distributions}

The \textsuperscript{15}N NMR spectra obtained for SL samples at 0 and 8 weeks when left untouched, after HW extractions and after KCl extractions are shown in Figure 4.11, Figure 4.12 and Figure 4.13 respectively. Corresponding SM spectra are shown in Figure 4.14, Figure 4.15 and Figure 4.16. Spectra obtained have not been scaled; hence peak intensities can be directly compared. The resulting distribution of peaks in the \textsuperscript{15}N spectrum for SL and SM are shown in Table 4.4 and Table 4.5. These distributions have been compared to those of unmixsed samples LO and MO in the respective tables.

The amino-N region of both SL and SM untouched samples were seen to decrease between 0 and 8 weeks, most likely indicating decomposition of this group. This trend was also seen after HW and KCl extraction. Decreases were also seen in the amount of amine-N after 8 weeks. Apart from these changes, the rest of the spectrum remains relatively unchanged between 0 and 8 weeks between each of the sample condition, when considering the poor signal to noise ratio.

The lack of decomposition observed in \textsuperscript{15}N NMR spectra may be due to several reasons. Firstly, these experiments were setup to observe short term decomposition, therefore not enough time has been experienced for a large amount of decomposition to take place. CN analysis of the 0 and 8 week SL and SM untouched samples and the corresponding samples after HW extractions also showed that the N content was roughly the same. This is coupled with the fact that although IC and CN analysis of extracts showed some N forms being removed, these were only small amounts, and therefore not enough to see a change in the \textsuperscript{15}N spectra. These extracts may also have had lower \textsuperscript{15}N enrichments which would give lower NMR signal intensities, which is further reduced by the presence of paramagnetics.

Another reason why significant amounts of decomposition may not have been observed may be due to the discrimination of \textsuperscript{15}N during decomposition processes.
Hogberg and co-workers (1995) has pointed out that N cycling processes such as volatilisation, nitrification and subsequent leaching, and denitrification discriminate against $^{15}$N. Therefore the remaining $^{15}$N may become more concentrated as $^{14}$N is lost from the system. The experiments for this thesis however, were not long enough for a large amount of $^{14}$N to be lost from the system; therefore there would be no increases in $^{15}$N concentration. As IC and CN analysis observe total N ($^{15}$N and $^{14}$N), this may explain why IC and CN analysis are able to show changes in N during decomposition over 8 weeks.

$^{13}$C NMR Peak Region Distributions

As $^{15}$N NMR spectra did not show significant differences between 0 and 8 weeks, $^{13}$C NMR were accumulated in order to compare decomposition rates between the two samples types SL and SM. $^{13}$C NMR spectra for SL untouched samples and those taken after HW and KCl extractions are shown in Figure 4.17, Figure 4.18, and Figure 4.19 respectively and the corresponding $^{13}$C NMR spectra for SM samples are shown in Figure 4.20, Figure 4.21 and Figure 4.22 respectively. The resulting C composition obtained from $^{13}$C NMR spectra for SL and SM are shown in Table 4.6 and Table 4.7 respectively. Common indicators of decomposition were also found. These include % aromaticity (Dai et al., 2001; Wilson, 1987), and the alkyl-C/O-alkyl-C (A/O-A) (Baldock & Preston, 1995) and carbohydrate-C/methoxyl-C/(CC/MC) (Blumfield et al., 2004; Mathers et al., 2007) ratios as shown in equations 4.1, 4.2 and 4.3. These have been previously described in section 4.2.3.

Biodegradation generally involves the increase of alkyl C, decrease of O-alkyl C, while aromatics remaining roughly constant. For this reason Baldock & Preston (1995) suggested using an A/O-A ratio as an index to reflect the extent of decomposition. Kogel-Knabner (1997) has also pointed out that the deeper down through organic soils layers the percentage of aliphatics present increases, while aromatics and carbohydrates decrease. This is due to presence of less plant litter as soil depth increases, which is also what is mostly found in sand sized fractions of soil. It is also of importance when comparing two different samples; in this case SL and SM, indicators and ratios cannot be compared between the samples without taking into account the parent material (Baldock & Preston, 1995). For example the A/A-O ratio for SL-8 is 0.94 while it is 0.51 for SM-8. This would suggest that SM-8
Figure 4.11: \textsubscript{15}N NMR SL-0 untouched (top) vs. SL-8 untouched (bottom)

Figure 4.12: \textsubscript{15}N NMR SL-0 HW (top) vs. SL-8 HW (bottom)

Figure 4.13: \textsubscript{15}N NMR SL-0 KCl (top) vs. SL-8 KCl (bottom)
Figure 4.14: $^{15}$N NMR SM-0 untouched (top) vs SM-8 untouched (bottom)

Figure 4.15: $^{15}$N NMR SM-0 HW (top) vs. SM-8 HW (bottom)

Figure 4.16: $^{15}$N NMR SM-0 KCl (top) vs. SM-8 KCl (bottom)
Figure 4.17: $^{13}$C NMR SLO untouched (top) vs. SL8 untouched (bottom).

Figure 4.18: $^{13}$C NMR SLO HW (top) vs. SL8 HW (bottom).

Figure 4.19: $^{13}$C NMR SLO KCl (top) vs. SL8 KCl (bottom)
Figure 4.20: $^{13}$C NMR SMO untouched (top) vs. SM8 untouched (bottom).

Figure 4.21: $^{13}$C NMR SMO HW (top) vs. SM8 HW (bottom).

Figure 4.22: $^{13}$C NMR SMO KCl (top) vs. SM8 KCl (bottom)
Table 4.4: Comparisons of the percentage of each $^{15}$N NMR region between soil + leaves samples after 0 and 8 weeks (SLO and SL8) and between extraction procedures

<table>
<thead>
<tr>
<th>Region</th>
<th>Untouched</th>
<th>HW residues</th>
<th>KCL residues</th>
<th>Untouched</th>
<th>HW residues</th>
<th>KCL residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero-N</td>
<td>2.3</td>
<td>1.3</td>
<td>18.0</td>
<td>1.0</td>
<td>37.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Amide-N</td>
<td>87.4</td>
<td>87.6</td>
<td>84.7</td>
<td>90.6</td>
<td>87.5</td>
<td>89.2</td>
</tr>
<tr>
<td>Guano-N</td>
<td>2.8</td>
<td>3.2</td>
<td>3.4</td>
<td>2.4</td>
<td>3.4</td>
<td>3.2</td>
</tr>
<tr>
<td>Amine -N</td>
<td>5.0</td>
<td>4.0</td>
<td>6.8</td>
<td>3.5</td>
<td>3.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Amino-N</td>
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<td>4.0</td>
<td>3.3</td>
<td>2.5</td>
<td>2.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 4.5: Comparisons of the percentage of each $^{15}$N NMR region between soil + mix samples after 0 and 8 weeks (SMO and SM8) and between extraction procedures

<table>
<thead>
<tr>
<th>Region</th>
<th>Untouched</th>
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<th>KCL residues</th>
<th>Untouched</th>
<th>HW residues</th>
<th>KCL residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero-N</td>
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<td>0.0</td>
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<td>1.6</td>
<td>0.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Amide-N</td>
<td>87.0</td>
<td>100.0</td>
<td>85.7</td>
<td>88.8</td>
<td>100.0</td>
<td>84.7</td>
</tr>
<tr>
<td>Guano-N</td>
<td>2.5</td>
<td>0.0</td>
<td>2.4</td>
<td>2.5</td>
<td>0.0</td>
<td>4.7</td>
</tr>
<tr>
<td>Amine -N</td>
<td>5.4</td>
<td>0.0</td>
<td>6.0</td>
<td>4.3</td>
<td>0.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Amino-N</td>
<td>3.5</td>
<td>0.0</td>
<td>3.6</td>
<td>3.1</td>
<td>0.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
### Table 4.6: Comparisons of the percentage of each $^{13}$C NMR region between soil + leaves samples after 0 and 8 weeks (SLO and SL8) and between extraction procedures

<table>
<thead>
<tr>
<th>Region</th>
<th>SL LO-0 weeks</th>
<th>SL- 0 Weeks</th>
<th>SL- 8 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untouched</td>
<td>Untouched</td>
<td>HW residues</td>
</tr>
<tr>
<td>Carboxyl</td>
<td>8.8</td>
<td>11.5</td>
<td>12.2</td>
</tr>
<tr>
<td>Aromatic</td>
<td>12.4</td>
<td>9.1</td>
<td>11.3</td>
</tr>
<tr>
<td>Di-O-Alkyl</td>
<td>13.7</td>
<td>5.3</td>
<td>6.2</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>37.7</td>
<td>34.6</td>
<td>26.1</td>
</tr>
<tr>
<td>Methoxyl</td>
<td>3.0</td>
<td>6.8</td>
<td>6.7</td>
</tr>
<tr>
<td>Aliphatic</td>
<td>24.3</td>
<td>32.7</td>
<td>37.5</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

| % aromaticity | 13.6% | 10.3% | 12.9% | 8.5% | 9.3% | 10.8% | 7.6% |
| A/O-A ratio   | 0.45  | 0.70  | 0.96  | 0.68 | 0.94 | 0.97  | 0.88 |
| CC/MC ratio   | 12.6  | 5.1   | 3.9   | 3.8  | 5.7  | 4.3   | 4.7  |

### Table 4.7: Comparisons of the percentage of each $^{13}$C NMR region between soil + mix samples after 0 and 8 weeks (SMO and SM8) and between extraction procedures

<table>
<thead>
<tr>
<th>Region</th>
<th>SM MO-0 weeks</th>
<th>SM- 0 Weeks</th>
<th>SM- 8 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untouched</td>
<td>Untouched</td>
<td>HW residues</td>
</tr>
<tr>
<td>Carboxyl</td>
<td>6.1</td>
<td>9.6</td>
<td>9.2</td>
</tr>
<tr>
<td>Aromatic</td>
<td>11.2</td>
<td>7.3</td>
<td>6.8</td>
</tr>
<tr>
<td>Di-O-Alkyl</td>
<td>15.0</td>
<td>8.3</td>
<td>8.0</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>47.2</td>
<td>46.0</td>
<td>45.0</td>
</tr>
<tr>
<td>Methoxyl</td>
<td>2.6</td>
<td>5.6</td>
<td>6.4</td>
</tr>
<tr>
<td>Aliphatic</td>
<td>17.9</td>
<td>23.1</td>
<td>24.7</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

| % aromaticity | 11.9% | 8.1% | 7.5% | 8.8% | 8.0% | 6.5% | 5.2% |
| A/O-A ratio   | 0.28  | 0.39 | 0.42 | 0.36 | 0.51 | 0.37 | 0.35 |
| CC/MC ratio   | 18.2  | 8.2  | 7.0  | 8.5  | 5.1  | 5.0  | 5.3  |
has undergone more decomposition, however it needs to be noted that the mixed parent material has a lower starting A/O-A ratio, and therefore may not have decomposed as much as thought.

The CC/MC ratio as was also calculated for 0 and 8 week samples. For all SL samples this ratio was found to slightly increase after 8 weeks. This however, may be due to the poor signal to noise ratio of $^{13}$C spectra obtained or the lack of separation between the carbohydrate-C and methoxyl-C peaks. The SM samples on the other hand showed a significant decrease after 8 weeks both before and after extractions, suggesting that they have undergone a higher amount of decomposition.

The aromatic-C region has remained approximately the same after 8 weeks, a closer look at the spectra in Figure 4.17 for SL untouched samples and Figure 4.20 for SM untouched samples, shows some slight variations in this region. There has been a loss of definition of peaks in this region, which includes phenolic-C groups such as syringyl (155ppm) and lignin (146ppm) and aromatic and unsaturated C (136 and 131ppm). In particular the peak at 146ppm corresponding to the methoxy- or hydroxy-substituted aromatic carbon in guaiacyl lignin and/or tannins has greatly reduced. This decrease in peak intensity could be explained by the cleavage of substituents from aromatic rings, the formation of quinones, conjugation reactions involving aromatic rings and those C in aromatic rings moving into closer proximity of paramagnetic centres as mass is lost through decomposition of other groups.

At this point it should also be considered that the SM samples used here were based on the ratio of leaves, stems and roots found in the plants grown for these experiments. In actual field windrows, the proportions of these components will most likely be different. Due to the exportation of stems to be used for wood products, larger amounts of roots and leaves will be left behind. A high amount of roots for example, may result in higher amounts of lignocellulose thereby altering the C/N ratio and slowing decomposition rates. Higher amounts of leaves could show an increase in the terpene content of windrows, and therefore also affecting decomposition rates. These differences need to be taken into account when studying decomposition of mixed plant material and windrows.
4.4.5 Observed Effects of Paramagnetics

The effects of paramagnetics in samples containing soil can be immediately seen upon mixing leaves with soil when comparing half peak height widths (Table 4.8). Upon mixing with soil, the peak width of the amide-N peak in $^{15}$N NMR CPMAS spectra was seen to increase from 14.0 to 16.9 ppm. An increase was also found between MO and SM-0 (13.6 to 16.8 ppm). This increase in width is brought about by the presence of paramagnetics, and can indicate increases in magnetic field inhomogeneity and changes in $T_1\rho$ relaxation times due to interactions between paramagnetics and organic molecules (Gonçalves et al., 2003; Mathers et al., 2002; Schmidt et al., 1997; Smernik & Oades, 2002).

![Table 4.8: Comparison of the half peak height widths of amide-N peak in $^{15}$N NMR CPMAS between untouched samples, HW extraction residues and KCl extraction residues in ppm at either 0 or 8 weeks.](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Untouched</th>
<th>After HW</th>
<th>After KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>LO-0</td>
<td>14.0</td>
<td>17.2</td>
<td>17.1</td>
</tr>
<tr>
<td>SL-0</td>
<td>16.9</td>
<td>18.8</td>
<td>18.5</td>
</tr>
<tr>
<td>SL-8</td>
<td>17.2</td>
<td>18.6</td>
<td>19.1</td>
</tr>
<tr>
<td>MO-0</td>
<td>13.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SM-0</td>
<td>16.8</td>
<td>19.5</td>
<td>19.7</td>
</tr>
<tr>
<td>SM-8</td>
<td>17.8</td>
<td>18.2</td>
<td>19.4</td>
</tr>
</tbody>
</table>

Definite changes due to the presence of paramagnetics were also seen when comparing the relative intensities of their resulting $^{15}$N and $^{13}$C NMR spectra. In Table 4.9a, the intensities of the total $^{15}$N spectra obtained for untouched samples SL-0, SM-0, LO and MO have been compared. The intensity of LO untouched has been adjusted to equal the intensity which would be expected for the mass of leaves present in the SL sample at 0 week. The adjusted intensity of LO was then normalised so that it would equal 100. The intensities of all samples, both untouched and after extractions, have then been given relative to LO. This procedure was also undertaken for all $^{13}$C spectra obtained.
Table 4.9: Comparisons of the intensities of each $^{15}$N NMR region between soil + leaves samples at 0 and 8 weeks (SL-0 and SL-8) and soil + mix samples at 0 and 8 weeks (SM-0 and SM-8) after a) untouched, b) HW extraction and c) KCl extractions. All intensities are relative to LO-0 untouched, which has been made to total 100.

<table>
<thead>
<tr>
<th>Region</th>
<th>LO-0 *</th>
<th>SL-0</th>
<th>SL-8</th>
<th>MO-0 *</th>
<th>SM-0</th>
<th>SM-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero-N</td>
<td>2.3</td>
<td>1.6</td>
<td>0.8</td>
<td>1.1</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Amide-N</td>
<td>87.4</td>
<td>59.6</td>
<td>64.7</td>
<td>62.7</td>
<td>37.4</td>
<td>34.5</td>
</tr>
<tr>
<td>Guano-N</td>
<td>2.8</td>
<td>1.2</td>
<td>1.7</td>
<td>1.8</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Amine -N</td>
<td>5.0</td>
<td>3.5</td>
<td>2.5</td>
<td>3.9</td>
<td>2.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Amino-N</td>
<td>2.5</td>
<td>3.4</td>
<td>1.7</td>
<td>2.5</td>
<td>2.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>69.2</td>
<td>71.4</td>
<td>72.0</td>
<td>44.6</td>
<td>39.7</td>
</tr>
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</table>

b) After Hot Water Extraction

<table>
<thead>
<tr>
<th>Region</th>
<th>LO-0 *</th>
<th>SL-0</th>
<th>SL-8</th>
<th>MO-0 *</th>
<th>SM-0</th>
<th>SM-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero-N</td>
<td>3.0</td>
<td>0.3</td>
<td>1.0</td>
<td>-</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Amide-N</td>
<td>134.5</td>
<td>20.1</td>
<td>23.3</td>
<td>-</td>
<td>13.6</td>
<td>11.1</td>
</tr>
<tr>
<td>Guano-N</td>
<td>3.7</td>
<td>0.7</td>
<td>0.9</td>
<td>-</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Amine -N</td>
<td>8.0</td>
<td>0.9</td>
<td>0.8</td>
<td>-</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Amino-N</td>
<td>3.1</td>
<td>0.9</td>
<td>0.6</td>
<td>-</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>152.4</td>
<td>22.9</td>
<td>26.7</td>
<td>-</td>
<td>13.6</td>
<td>11.1</td>
</tr>
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</table>

c) After KCl Extraction

<table>
<thead>
<tr>
<th>Region</th>
<th>LO-0 *</th>
<th>SL-0</th>
<th>SL-8</th>
<th>MO-0 *</th>
<th>SM-0</th>
<th>SM-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero-N</td>
<td>2.3</td>
<td>2.4</td>
<td>1.1</td>
<td>-</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Amide-N</td>
<td>132.3</td>
<td>108.6</td>
<td>96.3</td>
<td>-</td>
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<td>30.9</td>
</tr>
<tr>
<td>Guano-N</td>
<td>4.8</td>
<td>4.4</td>
<td>3.4</td>
<td>-</td>
<td>1.1</td>
<td>1.7</td>
</tr>
<tr>
<td>Amine -N</td>
<td>11.1</td>
<td>8.7</td>
<td>4.9</td>
<td>-</td>
<td>2.7</td>
<td>2.3</td>
</tr>
<tr>
<td>Amino-N</td>
<td>4.9</td>
<td>4.2</td>
<td>2.2</td>
<td>-</td>
<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Total</td>
<td>155.4</td>
<td>128.4</td>
<td>108.0</td>
<td>-</td>
<td>44.5</td>
<td>36.5</td>
</tr>
</tbody>
</table>

* The intensity of LO and MO samples has been adjusted to equal the intensity which would be expected for the mass of leaves present in the SL or SM sample at 0 week. The adjusted intensity of LO was then normalised so that it would equal 100.
Table 4.10: Comparisons of the intensities of each $^{13}$C NMR region between soil + leaves samples at 0 and 8 weeks (SL-0 and SL-8) and soil + mix samples at 0 and 8 weeks (SM-0 and SM-8) after a) untouched, b) HW extraction and c) KCl extractions. All intensities are relative to LO-0 untouched, which has been made to total 100.

<table>
<thead>
<tr>
<th>Region</th>
<th>LO-0 *</th>
<th>SL-0</th>
<th>SL-8</th>
<th>MO-0 *</th>
<th>SM-0</th>
<th>SM-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxyl</td>
<td>8.8</td>
<td>5.9</td>
<td>5.4</td>
<td>6.6</td>
<td>4.4</td>
<td>3.8</td>
</tr>
<tr>
<td>Aromatic</td>
<td>12.4</td>
<td>4.7</td>
<td>3.8</td>
<td>12.2</td>
<td>3.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Di-O-Alkyl</td>
<td>13.7</td>
<td>2.7</td>
<td>2.8</td>
<td>16.3</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>37.7</td>
<td>17.9</td>
<td>13.7</td>
<td>51.3</td>
<td>21.3</td>
<td>19.5</td>
</tr>
<tr>
<td>Methoxyl</td>
<td>3</td>
<td>3.5</td>
<td>2.4</td>
<td>2.9</td>
<td>2.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Aliphatic</td>
<td>24.3</td>
<td>16.9</td>
<td>17.9</td>
<td>19.4</td>
<td>10.7</td>
<td>13.9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100</td>
<td>51.6</td>
<td>46.0</td>
<td>108.7</td>
<td>46.3</td>
<td>48.3</td>
</tr>
</tbody>
</table>

b) After Hot Water Extraction

<table>
<thead>
<tr>
<th>Region</th>
<th>LO-0 *</th>
<th>SL-0</th>
<th>SL-8</th>
<th>MO-0 *</th>
<th>SM-0</th>
<th>SM-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxyl</td>
<td>14</td>
<td>2.6</td>
<td>2.3</td>
<td>-</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Aromatic</td>
<td>19.8</td>
<td>2.8</td>
<td>2.3</td>
<td>-</td>
<td>2.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Di-O-Alkyl</td>
<td>22.4</td>
<td>2.0</td>
<td>1.9</td>
<td>-</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>68</td>
<td>8.2</td>
<td>7.1</td>
<td>-</td>
<td>14.1</td>
<td>11.8</td>
</tr>
<tr>
<td>Methoxyl</td>
<td>7.1</td>
<td>1.9</td>
<td>1.7</td>
<td>-</td>
<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Aliphatic</td>
<td>45.9</td>
<td>11.7</td>
<td>7.6</td>
<td>-</td>
<td>7.7</td>
<td>6.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>177.2</td>
<td>29.1</td>
<td>22.9</td>
<td>-</td>
<td>31.3</td>
<td>26.3</td>
</tr>
</tbody>
</table>

c) After KCl Extraction

<table>
<thead>
<tr>
<th>Region</th>
<th>LO-0 *</th>
<th>SL-0</th>
<th>SL-8</th>
<th>MO-0 *</th>
<th>SM-0</th>
<th>SM-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxyl</td>
<td>11.7</td>
<td>4.7</td>
<td>5.2</td>
<td>-</td>
<td>4.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Aromatic</td>
<td>14.3</td>
<td>3.7</td>
<td>3.1</td>
<td>-</td>
<td>3.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Di-O-Alkyl</td>
<td>11.4</td>
<td>4.0</td>
<td>2.7</td>
<td>-</td>
<td>4.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>48.9</td>
<td>15.7</td>
<td>14.4</td>
<td>-</td>
<td>22.8</td>
<td>21.5</td>
</tr>
<tr>
<td>Methoxyl</td>
<td>4.2</td>
<td>4.1</td>
<td>3.0</td>
<td>-</td>
<td>2.7</td>
<td>4.1</td>
</tr>
<tr>
<td>Aliphatic</td>
<td>35.4</td>
<td>16.3</td>
<td>17.8</td>
<td>-</td>
<td>10.5</td>
<td>10.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>125.9</td>
<td>48.6</td>
<td>46.2</td>
<td>-</td>
<td>48.5</td>
<td>46.0</td>
</tr>
</tbody>
</table>

* The intensity of LO and MO samples has been adjusted to equal the intensity which would be expected for the mass of leaves present in the SL or SM sample at 0 week. The adjusted intensity of LO was then normalised so that it would equal 100.
It can be seen that the relative $^{15}$N spectrum intensity originating from the leaves in the SL-0 sample (69.2) is diminished compared to the expected intensity observed for LO (100). The same decrease in intensity is observed between MO and SM-0 (72.0 and 44.6 respectively). The paramagnetic content was also seen to affect $^{13}$C nuclei. Table 4.10a shows the resulting decreases in $^{13}$C NMR intensities between the samples LO and SL-0 (100 and 51.6) and MO and SM-0 (108.7 and 46.3).

When comparing the N chemical environment of these samples, as shown in Table 4.4, it is evident that roughly the same distribution of peaks is seen between either LO and SL-0 and MO and SM-0, with the possible exception of the amino-N region. This region is seen to increase in intensity. However, with the poor signal to noise ratio obtained in the $^{15}$N spectra, it is hard to say if this is a real increase or not, although the same trend is seen between the MO and SM samples at 0 weeks (Table 4.5). This possible increase in amino-N may work in favour of obtaining a more quantitative spectrum, as it was found in Chapter 3 that this region was greatly underestimated (section 3.2.2, Table 3.11). Therefore, an increase in intensity due to paramagnetics would make it closer to the intensity it should be. Taking this into consideration, it appears that the presence of paramagnetics has an even affect on the reduction in intensity of all regions in a $^{15}$N spectrum.

While comparisons of N composition showed that intensity loss from the spectra obtained due to paramagnetics was mostly evenly distributed between the $^{15}$N regions, $^{13}$C NMR spectra showed uneven signal intensity losses. Comparisons of LO and SL-0 (Table 4.6) and MO and SM-0 (Table 4.7) show that presence of paramagnetics increased the intensity of the carboxyl, methoxyl and aliphatic-C regions, while decreasing the aromatic and di-O-alkyl-C regions. The carbohydrate-C region remains relatively the same. As the distribution of $^{13}$C signal intensity has changed unevenly, potential indicators of soil quality, such as % aromaticity, the A/O-A ratio and the M/C ratio will also change. These indicators therefore will not accurately reflect the content of C at the starting contained plant material, however for the purpose of monitoring the relative degree of decomposition of materials while in soil, they can still be compared.
The fact that N distribution in $^{15}$N spectra was slightly affected by paramagnetics, while C distributions in $^{13}$C spectra were highly affected may indicate that C nuclei in certain structures are interacting more with paramagnetic centres. In this case the loss of signal in the aromatic and di-O-alkyl-C may suggest that these structures are interacting with metals in complexes, or that metals are residing in cation exchange site where these structures are bound to (Smernik & Oades, 2002). In the case of N nuclei, it may be that these nuclei are interacting with the fraction of more free paramagnetic elements, which will therefore cause a more even signal loss across NMR spectra (Smernik & Oades, 2002).

### Table 4.11: Samples analysed for metal content, and the corresponding levels found for aluminium, manganese, iron, cobalt and copper.

<table>
<thead>
<tr>
<th>Sample Analysed</th>
<th>Al</th>
<th>Mn</th>
<th>Fe</th>
<th>Co</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO-0 Untouched</td>
<td>19.3</td>
<td>2.04</td>
<td>61.2</td>
<td>&lt;50</td>
<td>29</td>
</tr>
<tr>
<td>SL-0 Untouched</td>
<td>17.2</td>
<td>1.87</td>
<td>51.8</td>
<td>&lt;50</td>
<td>29</td>
</tr>
<tr>
<td>SL-0 HW</td>
<td>16.9</td>
<td>1.83</td>
<td>53.6</td>
<td>&lt;50</td>
<td>26</td>
</tr>
<tr>
<td>SL-0 KCl</td>
<td>17.4</td>
<td>1.66</td>
<td>49.7</td>
<td>&lt;50</td>
<td>25</td>
</tr>
</tbody>
</table>

In order to identify the extent to which paramagnetics were present, 4 samples were sent for metal analysis at the University of Otago, New Zealand. The resulting content of various metals is shown in Table 4.11. Smernik & Oades (2002) have previously shown that the presence of the metal cations Cu$^{2+}$, Fe$^{3+}$ and Mn$^{2+}$ in soil organic matter samples (with concentrations of 1.66, 2.77 and 1.02% by weight), reduced spectral intensities by 50, 68 and 77% respectively. It was found in the results for this thesis that Cu$^{2+}$, Fe$^{3+}$ and Mn$^{2+}$ were present at average concentrations of 2.7, 5.4 and 0.2% respectively. These levels are definitely high enough to have a large affect on signal intensities, and are discussed further in relation to samples before and after extractions in the subsequent section.

#### 4.4.6 Effect of Extraction Procedures on $^{15}$N and $^{13}$C NMR Spectra

Significant changes in the intensity of both $^{15}$N and $^{13}$C NMR spectra were observed before and after HW and KCl extractions, for all samples at both 0 and 8 weeks. This can be seen in Figure 4.11 through to Figure 4.22. It was found that all of the peaks
in the $^{15}$N and $^{13}$C NMR spectra decreased in intensity after HW extractions took place for SL and SM samples. For example, the $^{15}$N NMR spectrum total intensity of SL-0 relative to LO-0 is 69.2 when left untouched (Table 4.9a). After HW extractions the intensity falls to 22.9 (Table 4.9b). An increase in total intensities, however, was seen for LO (Table 4.9b and Table 4.10b). In the case of samples analysed after KCl extraction, $^{15}$N NMR spectra of SL and LO samples were the only samples to show a significant increase in signal intensity (Table 4.9c). SM $^{15}$N NMR spectra total intensities remained the same as they were before KCl extractions, as did the $^{13}$C spectra obtained for both SL and SM (Table 4.10c).

**Changes in Signal Intensities: The Effect of Paramagnetic Content**

An attempt was first made to explain these differences in total signal intensities with respect to the paramagnetics present, as shown in Table 4.11. Samples analysed for metal content included soil only (SO), SL untouched, SL after HW extractions, and SL after KCl extractions. As the leaf material included in these samples should have a negligible metal content, any metals present should be coming from the soil component of each sample. Therefore, these metal contents were assumed to be the same for samples which include mixes of plant material (SM samples). Plots were made of metal content versus the observability (or total intensity) for each set of samples (eg. SL-0, SL-0 HW and SL-0 KCl). This was undertaken for the three metals manganese, iron and copper, as well as the total content of these three, as Smernik and Oades (2002) have previously pointed out their effects on NMR signal observability. The resulting graphs for $^{15}$N and $^{13}$C NMR spectra are shown in Figure 4.23 and Figure 4.24 respectively. The resulting correlation coefficients ($R^2$) are shown in Table 4.12.

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>Sample Set</th>
<th>$R^2$ values for Metals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$^{15}$N</td>
</tr>
<tr>
<td>$^{15}$N</td>
<td>SL</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>SM</td>
<td>0.11</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>SL</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>SM</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Figure 4.23: Plots of the content of Mn, Cu, Fe or the total of these three metals, versus the relative percentage observability of the $^{15}$N NMR total signal intensity obtained for SO, SL, SL after HW and SL after KCl (Data taken from Table 4.11 and Table 4.9 for 0 week samples)
Figure 4.24: Plots of the content of Mn, Cu, Fe or the total of these three metals, versus the relative percentage observability of the $^{13}$C NMR total signal intensity obtained for SO, SL, SL after HW and SL after KCl (Data taken from Table 4.11 and Table 4.9 for 0 week samples)
Strong linear correlations were found between iron ($R^2=1.00$) and total metal content ($R^2=1.00$) with SL $^{15}$N NMR signal observability, and hence total signal intensity. This may indicate that N nuclei are interacting, or are in close proximity to iron nuclei in particular. This is an interesting observation considering that these are all 0 week samples, hence no decomposition has taken place; only dry mixing of the soil and leaf material. The correlation for $^{13}$C SL spectra and the iron and total metal content however, was not as strong ($R^2=0.59$ and 0.56 respectively), suggesting that C nuclei are not interacting with these metal groups as much as N. This could explain why the presence of paramagnetics only affected some regions of the $^{13}$C NMR spectra obtained, whereas the whole $^{15}$N NMR spectrum was affected by paramagnetics (section 4.4.5).

The correlation between SM spectra obtained for both $^{15}$N and $^{13}$C NMR observability versus iron and total metal content were all found to be significant, with better correlation for $^{13}$C observability than those found for SL samples ($R^2=0.81$ and 0.78 respectively). Poor correlations were found for all samples and nuclei when plotted against either manganese or copper content, with the exception of manganese and $^{15}$N NMR observability of SL ($R^2=0.65$). Result taken in their entirety would suggest that iron is by far contributing most to the loss of total signal intensity in both the $^{13}$C and $^{15}$N NMR spectrum for all samples.

Alternate Explanations for KCl Extractions

Apart from the $^{15}$N NMR spectra of SL samples, data points plotted for NMR observability versus the paramagnetic content did not fit trendlines precisely. This may be due to other paramagnetics which may be present that were not measured in the current study. Alternatively, the differences in signal intensity between the different extraction procedures may be due to other mechanisms and processes taking place in the sample. These alternate suggestions are discussed here.

As mentioned throughout this chapter, the KCl extractions procedure involves the removal of mostly inorganic compounds and charged particles including clay and paramagnetics, as well as free inorganic compounds present throughout the sample. It may be that KCl is removing a small amount of free paramagnetic ions, for example $\text{Fe}^{3+}$, from the samples. Losses of manganese, iron and copper content after KCl
extractions were observed in samples as shown in Table 4.11. This would therefore increase NMR signal intensities.

The presence of paramagnetics in $^{13}$C spectra, however, were shown to affect only certain $^{13}$C NMR regions, which would suggest that C nuclei in these regions are forming organic-metal complexes, or that these structures are bound to cation exchange sites involving metals. Since signal intensity changes were seen between SL untouched samples and the corresponding samples after KCl extractions at 0 weeks, the latter explanation would be more likely, allowing the formation of organic-metal complexes. In any case, this would suggest that C nuclei are not interacting with the free paramagnetic fraction, of which part may have been removed with KCl, but the fraction that has remained in the sample. This is further backed up by the fact that after KCl extractions, the $^{13}$C NMR distribution of SL and SM samples was not the same as that obtained for LO and MO, and hence is still affected by paramagnetics. This was expected though, as previous researchers have shown that even after the strong treatment of samples with HF, not all paramagnetics are removed.

Another explanation for increases in signal intensity of NMR spectra may be due to the replacement of ions at exchange sites, such as clays and paramagnetics. For example, as NH$_4^+$ cation is removed from a paramagnetic surface, it is most likely replaced with a K$^+$ cation during KCl extraction. In this process, a molecule that has protons attached is replaced with one that does not. This would imply some change in links there may have been between paramagnetic centres to any surrounding molecules via the proton spin lattice. Therefore organic molecules which may have been affected by paramagnetics via proton spin lattice diffusion through the NH$_4^+$ cation will be cut off and therefore, will increase the NMR signal intensity. Also, inorganic compounds that are removed, such as NH$_4^+$ and NO$_3^-$, are not easy to see using NMR spectroscopy. NH$_4^+$ adsorbed to clay particles will be rigid, and therefore likely to relax slowly with longer T$_1$H’s, therefore decreasing its observability if long enough delay times are not used.

In Chapter 2 of this thesis, the effect of mixing the more rigid molecule urea with the mobile molecule glycine was observed. It was found that as the amount of glycine in
the mix increased, the $^{15}$N NMR observability of urea-N increases (section 2.5.2 and 2.5.3). This was found in part to be due to the dilution of urea in the sample mixes, and also due to more mobile $\text{--NH}_3^+$ amino groups being provided by the glycine. This meant that urea interacting with these groups could use them for proton spin diffusion, and hence allow the rigid urea to relax more quickly, improving its signal intensity. This may be the case also with KCl extractions. As KCl removes the more inorganic fraction from the samples, it is removing the more rigid molecules that are adsorbed to charged clay and mineral sites. This fraction is also more likely to be rigid, and hence harder to see using NMR. By removing this fraction, we are increasing the content of more mobile compounds which can act as relaxation sinks during NMR, hence improving signal acquisition parameters and therefore signal intensity. Also the dilution of inorganics left may also increase their observability, as shown in section 2.5.3 of Chapter 2.

In order to further test these theories, a small experiment was undertaken where $^{15}$N CPMAS NMR VCT’s were used to examine $^{15}$N-enriched eucalyptus leaf material and its relaxation parameters and NMR observability when mixed with either urea or ammonium sulfate (Figure 4.25). Samples were made so that the same amount of eucalyptus material was present in each of the mixes. Urea and ammonium sulfate mixed with eucalyptus material were added so that the same N content was present in mixes. The $^{15}$N NMR amide-N peak at -261ppm has been observed here, and a biexponential equation (Equation 2.4, page 55) was used to find the resulting two sets of time constants $T_{1\rho H}$ and $T_{NH}$ (Table 4.13). Intensities have been corrected for the amount of eucalyptus sample present in the mixes.

It was found that at a contact time of 1.5msec, where all VCT curves were at their maximum, the percentage observability for the leaves only and the leaves when mixed with urea was roughly the same (100 and 102% respectively). However the observability of eucalyptus leaves in the presence of ammonium sulfate decreased from 100 to 84%. This drop in observability may be explained by the $\alpha_{[1]}$ value that was found ($\alpha_{[1]} = -0.03$) for one of the sets of relaxation parameters. This would indicate that some of the nuclei present are relaxing before a significant build up of magnetisation can be transferred. Therefore this component of the resulting peak
intensity may not be seen or underestimated. This would support the theory that the presence of \( \text{NH}_4^+ \) may lower signal intensities.

Figure 4.25: \(^{15}\text{N}\) NMR VCT results for the amide-N peak in spectra obtained for either \(^{15}\text{N}\)-enriched eucalyptus only samples, or \(^{15}\text{N}\)-enriched eucalyptus when mixed with either urea or ammonium sulfate. Intensities have been corrected for the amount of eucalyptus sample present in the mixes.

Table 4.13: VCT results for the amide-N peak in spectra obtained for either \(^{15}\text{N}\)-enriched eucalyptus only samples, or \(^{15}\text{N}\)-enriched eucalyptus when mixed with either urea or ammonium sulfate.

<table>
<thead>
<tr>
<th>Mix</th>
<th>Component 1</th>
<th>Component 2</th>
<th>%I(<em>{01}) : %I(</em>{02})</th>
<th>% Obs.(^{\wedge})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( T_{\text{NH}} ) (msec)</td>
<td>( T_{\text{1H}} ) (msec)</td>
<td>( \alpha_{[1]} )</td>
<td>( T_{\text{NH}} ) (msec)</td>
</tr>
<tr>
<td>Euc Only</td>
<td>1.0</td>
<td>6.4</td>
<td>0.83</td>
<td>0.12</td>
</tr>
<tr>
<td>Euc + Urea</td>
<td>1.5</td>
<td>3.1</td>
<td>0.52</td>
<td>0.13</td>
</tr>
<tr>
<td>Euc + \text{SO}_4(\text{NH}_4^+)(_2)</td>
<td>2.5</td>
<td>2.4</td>
<td>-0.03</td>
<td>0.20</td>
</tr>
</tbody>
</table>

\(^{\wedge}\) The percentage observability’s are compared to the eucalyptus only sample, which made to equal 100%.

**Alternate Explanations for HW Extractions**

HW extractions, as explained throughout this chapter, remove any labile organic compounds from macropores, as well as free inorganic compounds. Therefore after HW extractions, larger concentrations of paramagnetics can be expected, which has been shown in this chapter in Table 4.11. This will decrease signal intensities, as was found here for SL and SM sample (Figure 4.12, Figure 4.15, Figure 4.18, Figure
4.21, Table 4.9 and Table 4.10). As mentioned previously, trendlines applied to plots
of metal content verse NMR observability were not always precise, therefore
suggesting that other processes may be altering NMR signal intensities.

With the removal of HW extracts, concentrations of organic/inorganic compounds
which are adsorbed to charged particles may increase. This is the opposite to what
was seen for KCl extractions. With HW extractions the number of organic, more
mobile groups may decrease, while the rigid molecules and hence those adsorbed to
exchange sites, will increase. This results in a diminished signal intensity, especially
if the remaining compounds are more closely associated with the paramagnetics. This
may also explain why HW extraction of LO samples saw an increase in their spectra.
In this sample, there should be basically no paramagnetics and no clays present, and
therefore no exchange sites for ions to adsorb to. Therefore, for example, paramagnetic
content is not increased with the removal of HW extractable
compounds. Also with the lack of exchange sites at hand, a larger amount of
inorganic compounds should also be removed with HW, hence improving the
resulting spectra obtained.

Also contributing to decreased signals in samples with soil components may be the
type of organic molecules removed. As previously discussed, it has been shown that
amino acids can be removed with HW extraction (Lipson & Nasholm, 2001), and it
was also demonstrated here that significant amounts of alkyl-C may be removed as
well (Figure 4.9). For example, both the mobile amino -NH₃⁺ and terminal methyl -
CH₃ groups have been shown to act as sinks for spin lattice relaxation, as discussed
in Chapter 2. If these mobile groups are removed, any other more rigid molecules
which may have been transferring their energy to these more mobile groups will no
longer have a sink for relaxation. This may therefore decrease their NMR
observability, as was shown in Chapter 2 with the different mixes of the rigid
molecule urea, and the mobile glycine. This may therefore also explain the decrease
in both the ¹⁵N and ¹³C NMR spectra acquired for all samples after HW extraction.

4.4.7 Use of ¹⁵N DDCPMAS to Show Sample Changes
In these decomposition experiments, because the samples were mixed together and
observed after 0 and 8 weeks, it is hard to tell how much decomposition has taken
place. This is due to both the original material and any decomposition products produced being seen in the same spectra. It is also possible that the decomposed products produced have either similar N-species to the original plant material, or that the functional groups produced have spectral peaks which overlap in the 15N spectrum. Therefore it is difficult to detect subtle differences in NMR signals. 15N NMR dipolar dephasing experiments (DDCPMAS) were undertaken in order to see if there has been any changes in structure of compounds found in the same chemical shift regions. If any significant changes occur, as a result of molecular environment changes, a corresponding change in the relaxation processes or time constants should also occur. The $T_{2DD}$ values are sensitive to changes in the protonation and mobility of functional groups. However, it is also important to note that the presence of paramagnetics in samples will also affect $T_{2DD}$ values.

For these decomposition experiments, 15N DDCPMAS spectra were acquired for SL samples at 0 and 8 weeks, and following KCl extraction at the respective times. A two point dipolar dephasing experiment was set-up and run as discussed in Chapter 2, section 2.4.1. Parameters used for studies described in chapter 2 are exactly the same as those used for CPMAS experiments undertaken in this chapter, with the exception of using the dephasing times of 0 and 45$\mu$s. Data was fitted using Equation 2.2, which is based on exponential decay. The resulting $T_{2DD}$ values are listed in Table 4.14. The spectra acquired are shown in Figure 4.26 and Figure 4.27. Results for LO $T_{2DD}$ values have been taken from Chapter 3, section 3.2.2 where they

| Table 4.14: $T_{2DD}$ results (in $\mu$s) for 15N DD-CPMAS NMR spectra of LO and SL samples before and after KCl extractions. |
|------------------|----------------|----------------|----------------|----------------|----------------|
| Region           | LO-0           | SL-0           | SL-0 KCl       | SL-8           | SL-8 KCl       |
| Hetero-N         | 51.5           | 35.3           | 27.4           | 76.0           | 75.4           |
| Amide-N          | 34.2           | 27.7           | 21.6           | 27.1           | 25.7           |
| Guano-N          | 43.3           | 69.0           | 20.3           | 90.0           | 25.7           |
| Amine-N          | 20.9           | 31.2           | 17.9           | 43.6           | 19.6           |
| Amino-N          | 122.0          | 51.6           | 50.8           | 86.4           | 78.3           |
Figure 4.26: $^{15}$N DD-CPMAS NMR for SL-0 (Left) and SL-8 (Right) untouched samples with dephasing times of  a) $t = 0 \mu $sec, b) $t = 45 \mu $sec.

Figure 4.27: $^{15}$N DD-CPMAS NMR for SL-0 (Left) and SL-8 (Right) samples after KCl extractions with dephasing times of  a) $t = 0 \mu $sec, b) $t = 45 \mu $sec.
have been further discussed. When analysing SM samples and SL samples after HW extractions the NMR signal intensities were so low that it was not possible to observe satisfactory spectra, therefore $T_{2DD}$ values for these samples were not found. Clearly, significant effects are immediately seen when leaf material is mixed with soil (SL sample), which exhibited changes in $T_{2DD}$ values when comparing LO-0 and SL-0. These changes are most likely due to the paramagnetic content of soil. Peaks found in the hetero, amide and amino-N regions show decreases in $T_{2DD}$ values, while guano and amino-N show increases. The decreases in $T_{2DD}$ values signify that groups in this region have become more protonated, or more rigid. As protonation should result in a chemical shift, therefore it is more likely that rigidity is affecting the $T_{2DD}$ values of these groups, as is the case with most changes in $T_{2DD}$ values seen in Table 4.14.

The decrease in $T_{2DD}$ values for the hetero, amide and amino-N regions seen in SL-0 samples compared to LO-0, may therefore indicate that these groups have formed associations with exchange sites, such as metals and clays. This decrease was most significant for amino-N, which would make sense as the $-\text{NH}_3^+$ amino group would make an ideal candidate for adsorbing to negatively charged sites.

After 8 weeks, the $T_{2DD}$ values for the untouched SL samples have increased for all regions except the amide-N region, which has remained constant. This increase in $T_{2DD}$'s may indicate microbial activity reducing molecular structures or stopping adjacent molecules interacting, as a result making them more mobile. This does not need to directly involve compounds containing N. Close associations between N groups and other compounds which have been broken down, such as carbohydrates that have been broken down into shorter chains by microbes, may affect $^{15}\text{N} T_{2DD}$ values.

KCl extractions of SL samples at 0 weeks, showed significant decreases in $T_{2DD}$ values compared to the untouched precursor. Decreases were found for all regions except amino-N, which remained constant at 50.8 $\mu$sec. These decreases once again may show an increase in rigidity. It is possible that as inorganics were removed during KCl extractions, rather than be replaced at exchange sites by, for example, K+ cations, they have instead been replaced by charged organic functional groups. This would cause them to become more rigid, and hence decrease their $T_{2DD}$ values.
Values obtained after KCl extractions at 0 and 8 weeks are roughly the same, with the exception of hetero-N. The values for hetero-N are not reliable, as the signal to noise ratio for this area was poor.

4.5 Conclusions

A short-term, 8-week decomposition experiments were undertaken for this thesis in which mixtures of either leaves and soil (SL), or leaves/stems/roots and soil (SM) were compared. The use of different plant component mixtures is thought to mimic decomposition in the forest plantation ecosystem that consist of windrows and leaf litter. To understand the affect of plant chemical composition on decomposition, it was important to utilize the same plant species (*Eucalyptus pilularis*) grown under similar $^{15}$N-enriched fertilizer (ammonium nitrate) containing hydroponic solutions. The mass loss from both samples during incubation was found to be the same after 8 weeks even though the two samples differed in the initial N content. Therefore, it was evident that the different chemical compositions of the plant samples in soil need to be taken into consideration when having a closer look at the short-term nutrient dynamics during decomposition. No loss of mass was observed for those samples containing leaves only and mixture only. The use of $^{15}$N and $^{13}$C NMR spectral data along with total C and N analyses for the samples have been very helpful in gaining insight to these initial processes.

The total C and N content revealed similar trends between fluctuations in these two elements for both samples. This implies that there were periods of immobilisation occurring, with SM samples showing immobilisation of C and N was taking place faster than in SL samples. This may therefore indicate that SM samples are decomposing faster. These results agree with the indicators of decomposition such as the A/O-A and CC/MC ratios, which were calculated using the corresponding $^{13}$C NMR spectra data for SM and SL samples.

Hot water and KCl extractions were undertaken on samples incubated for 8-weeks and compared with extractions undertaken on the 0-week samples. All of the hot water extracts were examined for their chemical species by IC, total C, total N and elemental analyses. The KCl extractions were carried out to identify the removal of
N and C, most likely in inorganic forms. It is possible, however, that some organic compounds have also been removed as well. It was revealed that the extraction of C was mostly stable over the 8-week period; however, fluctuations in inorganic N were observed. The hot water extractions were expected to remove the soil organic matter, often the more labile organic N originating from microbial biomass, root exudates and other forms of organic N. The total mass removed by hot water extractions decreased over time for SL samples, but remained relatively constant for SM which may possibly indicate the extent of active decomposition taking place in the samples. Any fluctuations seen in both samples were found to be due to the “unknown” fraction removed, which most likely consists of mostly organic compounds and some inorganic C. The total non-C containing inorganic fraction was found to remain relatively constant.

The $^{13}$C NMR spectral data for the hot water extract of 0-week SL samples revealed that NMR peaks corresponding to carbohydrates and aliphatic C groups have been removed along with very small amount of carboxyl and aromatic C. These compounds therefore represent the more labile fraction of the sample. In plantation soils, these compounds would be rapidly mineralised and used by plants. If not used, due to their soluble nature, it is possible that these labile compounds could potentially be leached into ground water and therefore would not be available for the plants in the forest ecosystem.

The $^{13}$C NMR spectra of the untouched SL and SM samples showed possible decomposition taking place, with decreases in carbohydrate-C and increases in the alkyl-C regions. It is important however, to consider the paramagnetic content of the sample, as it may be that peak intensities are changing due to changes in proximity to paramagnetic centres rather than decomposition of that particular functional group. No significant changes were observed in $^{15}$N spectra data for both untouched SL and SM samples. However, this may be due to discrimination of $^{15}$N in the decomposition process, or the insensitivity of NMR to small changes in the N content.

The $^{15}$N and $^{13}$C spectra of leaves only (LO) and the leaves/stem/roots mixture only (MO) were compared with those spectra obtained for the samples mixed immediately
with soil (SL and SM). The intensities of both $^{15}$N and $^{13}$C NMR peaks decreased dramatically. The $^{15}$N signal intensity appeared to decrease evenly over the entire spectrum and therefore relative peak distributions remained comparable except for the amino-N peak, which increased its intensity. However, this may cancel out the decrease in intensity observed for this region due to mobility as previously discussed in chapter 3. The $^{13}$C peak intensities, on the other hand, were reduced selectively, with carboxyl, methoxyl and aliphatic-C intensities increasing relative to carbohydrates, while, the intensity of aromatic and di-O-alkyl- decreased.

As the $^{15}$N and $^{13}$C NMR spectra for the SL and SM behaved differently compared to those of LO and MO, the reduction in peak intensity was attributed to the presence of paramagnetic species in soil. This effect was even throughout $^{15}$N NMR spectral peaks and could be due to the presence of free paramagnetics, as they would affect the whole sample and therefore the whole spectrum. In $^{13}$C NMR peaks, however, the effect was seen only in selective peaks. This may be explained by considering that only certain functional groups are either forming complexes with paramagnetic species and/or in closer proximity to these centres. This would explain the selective reduction of the peak intensities in the NMR spectra.

The effect of paramagnetics was also exhibited when comparing NMR spectral data for SL and SM samples both before and after HW or KCl extractions. There was a significant reduction of intensity of peaks after the HW extraction. It was proposed that the HW extractions concentrated these paramagnetic species by removing the more mobile organic compounds, therefore increasing the inorganic content of the sample, of which most are likely to be adsorbed to an exchange site. This would therefore coincide with an increase in the concentration of rigid molecules in the sample. In chapter 2, it was shown for mixes of glycine and urea that as the concentration of the mobile compound (glycine) decreases, the observability of urea in the NMR also decreased. This is most likely due to a decrease in mobile groups which could act as sinks for proton spin relaxation, and may also be the case for samples after HW extraction. The opposite is true for after KCl extractions, as KCl removes the inorganic content, leaving more mobile groups. Hence this may explain the increase in some of the spectral peak intensities.
$^{15}$N DDCPMAS NMR has also been used in the study for this thesis to observe changes in plant material when mixed with soil. Changes in mobility of N functional groups were observed immediately upon mixing with soil, and were found to change further after 8 weeks. This could be due to direct associations and disassociations with paramagnetic ions, or interactions with other non-N containing compounds which may have decomposed and become more mobile.

The results obtained in this chapter further confirm the complexities associated with paramagnetic species in obtaining qualitative and quantitative $^{13}$C and $^{15}$N NMR spectral information on a dynamic sample undergoing change with time. It is important therefore to use NMR spectral data in conjunction with other chemical analyses data such as total C and N content and elemental analyses to get a better understanding of the nature of processes taking place during decomposition in soil.

### 4.6 References


CHAPTER FIVE

THE ENVIRONMENTAL IMPACT OF AN INSECT INFESTATION ON THE NITROGEN CYCLE

5.1. Introduction

Australia has had a rapid expansion of its hardwood plantations in recent years, which consist of various species of eucalyptus. The area these plantations cover has increased by almost 50% since 2000, to reach 740,161 hectares by 2005 (Parsons et al., 2006). Despite the relatively low nutritional value and high fibre content of eucalypt foliage, there are many species of animals and insects that feed on this plant. The advantage of consuming eucalypt foliage is the sheer abundance of this resource in Australia. The early stages of the life cycle of insects, for example, larval and caterpillars as well as some adult insects, are therefore of concern to the forestry industry, as they can be detrimental to the health of the plantation when present in large numbers. An environmental condition such as an insect infestation can limit tree growth and establishment, with the most damaging being chewing insects, which defoliate plantation trees. For example, in 2005 the California pine aphid (*Essigella Californica*), damaged 60,500 hectares of plantation trees in NSW (Eldridge, 2007).

Figure 5.1: Juvenile leaves of Eucalyptus globulus damaged by *Mnesampela privata* larvae of different developmental stages: I) first- and second-instar larvae, skeletonized leaf. II) third- and fourth-instar larvae, lamina between major veins eaten. III) fourth and fifth-instar larvae, lamina completely eaten (Steinbauer & Matsuki, 2004).
The eucalyptus leaf beetle, *Paropsis atomaria* (Coleoptera: Chrysomelidae) is a native beetle with approximately 700 different species. Both adult and larval stages are major pests that have the ability to adapt and attack a broad range of eucalypt species, the main species being *E. cloeziana*, *E. pilularis*, *E. dunnii* and *E. grandis*. The distribution of this pest covers a wide area in Australia, from central Queensland down to southern Victoria and across to South Australia (Lawson & McDonald, 2005). These insects can defoliate eucalypts (Figure 5.1), both young and old leaves, causing serious environmental damage, which in turn has a dramatic effect on the ecosystem when insects are present in large numbers. An example is rural dieback, where insects progressively defoliate tree canopies (Figure 5.2), until the tree eventually dies (Ohmart & Edwards, 1991). The loss of leaves can cause a decrease in the transpiration of the plant and stunted stem growth, therefore increasing plant mortality rates (Lovett et al., 2002; Ohmart & Edwards, 1991).

![Figure 5.2: Characteristic defoliation of upper crown by larvae (Collett, 2001; Lawson & King, 2006)](image)

The reduced transpiration rates can also have an impact on the soil. One such example is a higher soil water content, which can alter rates of decomposition and nutrient turnover. Vitousek and co-workers (1979) has reported that nitrification, which results in the formation of nitrate from ammonium, is often stimulated by severe forest disturbance. Considerable nitrate leaching has been observed from Appalachian forested water sheds and is linked to the defoliation by gypsy moth larva (Eshleman et al in 1998; Webb et al 1995). Such nitrogen losses may not only result in stream water acidification and net catchment export of calcium ions, but also lead to potential effects on forest net primary productivity and nutrient retention of
the forest ecosystem. However, there are several effective mechanisms in place to conserve N in the forest ecosystems affected by insect infestation.

One such mechanism involves the active root uptake of nutrients by defoliated plants reducing the levels of nutrients available for leaching (Ayres et al 2004). The second involves immobilization by insect or microbial activity. Data on the fate of nitrogen in years of high and low forest defoliation affected by a gypsy moth infestation in a north-eastern United States is reported in Figure 5.3 (Lovett et al., 2002). It demonstrates how under high defoliation conditions, a significant portion of leaf nitrogen was diverted into insect faeces (known as frass) or part of the insect biomass rather than leaves falling to the ground and adding nitrogen to the soils. The insects also caused an increase in the loss of young leaves (both eaten and uneaten), resulting in a decrease in nitrogen resorption, which usually takes place before a tree sheds its leaves.

A third mechanism, which complements N retention in the ecosystem, arises as a result of the digestive physiology of the leaf-eating larva and caterpillars. It is reported that digestion of foliage under high pH in the insect’s midgut would limit

![Fate of Nitrogen in an Oak Tree Forest](image_url)

**Figure 5.3:** Fate of nitrogen in years of high and low defoliation in an oak forest in the north-eastern United States (Lovett et al., 2002).
the ammonia production and acidic pH of the hindgut is expected to trap most of the ammonia that is produced. Therefore, the frass pellets will contain the ammonium by-products of N which will result in extremely low N losses via volatilization during insect infestation (Lovett et al 1998).

The nitrogen in the frass pellets produced by insects, and also in the insect itself, may differ both qualitatively and quantitatively to that of the host plants foliage. Insect frass is also believed to be analogous to other animal manures in its potential to mineralize and volatilize ammonia (Sommer et al 1991). Studies by Lovett and co-workers concluded that carbon in the insect frass decomposes at high rates, hence fuelling a rapidly increasing microbial community in the soil and therefore high amounts of nitrogen become immobilised in the microbial biomass (Lovett et al., 2002). This would mean that nitrogen is unavailable for mineralisation and consequently to the defoliated trees, creating a change in the nitrogen cycle.

Ohmart and co-workers (1983a; 1983b), have reported that the number of leaf eating insects in an *Eucalyptus pauciflora* forest canopy could be up to 91,000 insects per hectare. Although this level was not high enough to cause serious defoliation, the high levels of insect biomass and the frass produced could lead to loss of nitrogen, which would otherwise have been incorporated in some form or another in the soil nitrogen content. This clearly gives an idea of how an insect infestation in a forest plantation could lead to severe limitations and changes to the nutrient levels available to the plants and other dependents. This however, is based only on the number of insects present in the tree canopy. In a study undertaken by Holt & Spain (1986), the number of insects present in litter and soil layers per m$^2$ in a rainforest and a hoop pine forest were compared. As shown in Table 5.1, the number of insects in these layers was found to be up to $4.66 \times 10^8$ insects per hectare, which is far greater than numbers found in a tree canopy.

It has been reported that larvae from the Lepidopteran species (moths) are devastating when present in large numbers, with an outbreak of *Sathomorrhopa aphotista* (Lepidoptera: Geometridae) completely defoliating 1000 hectares of eucalyptus forest in Tasmania. Lepidoptera can also make use of leaf litter, thereby
Table 5.1: Mean abundances of the major groups of arthropods found in a Queensland hoop pine plantation and rainforest, in both the litter and soil layers. Results are based on modified results from tables presented by Holt & Spain (1986). Totals are also shown in insects/ha.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Average in litter (insects/m²)</th>
<th>Average in soil (insects/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hoop Pine</td>
<td>Rainforest</td>
</tr>
<tr>
<td>Cryptostigmata</td>
<td>813</td>
<td>1113</td>
</tr>
<tr>
<td>Prostigmata</td>
<td>31</td>
<td>21</td>
</tr>
<tr>
<td>Mesostigmata</td>
<td>213</td>
<td>482</td>
</tr>
<tr>
<td>Acari larvae</td>
<td>529</td>
<td>354</td>
</tr>
<tr>
<td>Poduroidea</td>
<td>506</td>
<td>277</td>
</tr>
<tr>
<td>Entomobryoidea</td>
<td>43</td>
<td>49</td>
</tr>
<tr>
<td>Sminthuridae</td>
<td>229</td>
<td>99</td>
</tr>
<tr>
<td>Coleoptera (adult)</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Coleoptera (larvae)</td>
<td>19</td>
<td>33</td>
</tr>
<tr>
<td>Diptera larvae</td>
<td>49</td>
<td>75</td>
</tr>
<tr>
<td>Araneida</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>2461</td>
<td>2530</td>
</tr>
</tbody>
</table>

Total in hoop pine litter + soil 46,595 m² (4.66 x 10⁸ ha⁻¹)
Total in rainforest litter + soil 21,084 m² (2.11 x 10⁸ ha⁻¹)

competing with soil microbes for organic matter to break down (Ohmart & Edwards, 1991).

Research by Frost and Hunter (2007) highlighted the dynamics of frass when introduced to the soil. ¹⁵N enriched frass has been used to monitor the recycling process of nitrogen from frass into plants and future plant eating herbivores. An interesting finding of their research is that the large majority of the frass N leached from the soils during decomposition was in the organic form. However, the exact nature of the chemical entities present in frass at the start of the process not known. It is also of interest to study the nature of the chemical constituents that would be introduced to the soil in the form of insect frass and to compare against leaf litter. Though the larval and caterpillar frass has the potential to be incorporated back into the forest soils, the adult insects however, can relocate away from a specific forest ecosystem. It would therefore be of interest to get a qualitative understanding of such export of nutrients by carrying out a comparative study between the chemistry of herbivorous insect biomass and the foliage consumed.
The aim of this chapter will be to follow nitrogen and carbon transformations from eucalyptus foliage through to the metabolism and deposition of waste material in such insects, known to be pests in Australian eucalyptus plantations. Insects from the Chrysomelidae and Geometridae families belong to this category, and therefore have been chosen to follow the chemical transformations. This will be done by using C/N analyser and also $^{15}$N and $^{13}$C solid-state nuclear magnetic resonance (NMR) spectroscopy as a technique for following the chemical environments and relative abundance of the different chemical functional groups in insect metabolites. The environmental impact that these insect infestations may cause on a eucalyptus forest ecosystem, in particular the nitrogen cycle, could also be ascertained.

The differences in chemical profile of the insect biomass, frass and the eucalyptus leaves have been investigated. Any significant changes in chemical compositions could have an impact on the plantation’s ecosystem, and therefore the N cycle. This may also be the case when comparing young and older foliage. Eucalyptus in particular is known for producing leaves which are markedly different to that of the adult foliage, with differences in shape, texture, toughness, arrangement and surface characteristics such as wax content (Ohmart & Edwards, 1991). All these features have the potential to pose some difficulty to the insects feeding on them.

Eucalyptus leaves contain high levels of plant secondary metabolites (PSMs), such as essential oils, terpenes, phenols and formylated phloroglucinols (FPGs), some of which are of high commercial interest. These PSMs act as anti-feedants for marsupials such as koalas and possums, and can be toxic when ingested and hence need to have mechanisms for detoxification. Eucalyptus leaves are also high in tannins, a water soluble phenolic compound which can precipitate large amounts of foliage proteins (Martin & Martin, 1984). This means that nitrogen found in protein or specific amino acids may be limited or unavailable for animal and insect metabolism (Cork, 1996). This, together with the low nutrient levels eucalyptus leaves already have, identifies these as poor source of nutrients available to the dependent. Eucalyptus feeding insects, however, have the ability to digest this foliage and survive on it, but little is known of the digestion process.
A review by Cooper (2001), has highlighted several reasons for the insects’ ability to access nutrients present in eucalypt leaves. Firstly, the high gut pH of some insects may affect the activity of digestive enzymes. *Paropsis* and lepidopteran larvae have high gut pH’s ranging from 8.5 to 9.8. At this pH the ability for phenols such as tannins to bind with proteins in the foliage decreases, making more nutrients, such as amino acids, available for the insect. (Fox & Macaulay, 1977; Martin & Martin, 1984). Secondly, the peritrophic membrane, a tube which provides a barrier between food in an insect’s midgut and the midgut cell membrane, may help stop lipophilic compounds such as phenols in the eucalypt foliage from interacting with the insect proteins (Barbehenn, 1999). This could account for why various insects can pass through some leaf material compounds undigested and unchanged (Ohmart & S., 1989). Another study found that insect guts contain surfactants that interfere with the precipitation of proteins with tannins, explaining why insects with neutral or acidic gut pH’s may still be able to metabolise proteins in the presence of tannins (Martin & Martin, 1984).

Solid state NMR spectroscopy has previously been used (Schaefer et al., 1987), but has not been a common technique used to study insects, particularly using $^{15}$N NMR. Schaefer used both $^{15}$N and $^{13}$C NMR to follow chemical compositional changes in the exoskeleton of *Manduca sexta* pupae (tobacco hornworm), during tanning and hardening, also known as sclerotisation. It was found that the unsclerotised cuticle from a newly ecysed pupae (pupae which have just shed their skin), had a high protein content with smaller amounts of lipids and chitin. During the growth of pupae the cuticle becomes stiffer, drier and resistant to chemical and physical degradation. The concentrations of chitin, protein and polyphenols such as catechol, tend to increase substantially with increased sclerotisation. The analysis of the intact cuticle specifically labelled with $^{13}$C and $^{15}$N revealed direct linkages between the ring nitrogens of the protein histidyl residues and ring carbons derived from catecholamine dopamine. These studies supported the hypothesis that the stiffening of insect cuticle during sclerotisation results primarily from the deposition of proteins and chitin polymers and their cross linking by quinonoid derivatives of catecholamines, and have also been studied more recently (Andersen & Roepstorff, 2007; Kramer et al., 2001). These compounds also act as a pigment to the insect’s exoskeleton (Kramer & Hopkins, 1987)
The research involved in this chapter will focus on understanding how nitrogen transposes from eucalyptus foliage through to metabolism and deposition of wastes by these insects, and how these changes affect the surrounding environment, especially the nitrogen cycle. According to our knowledge, this aspect has not been studied previously using $^{15}$N and $^{13}$C NMR spectroscopic data for an insect from the Chrysomelidae and Geometridae families. These are, however, important issues that need foremost consideration to understand the eucalyptus plantation ecology that will help find remedies to nullify any adverse effects of unexpected insect infestation in forest plantations.

5.2. Experimental method

In these experiments $^{15}$N enriched Eucalyptus plants were grown according the method described in chapter 3 and were used to feed the larvae of *Paropsis atomaria* and a *Mnesampela privata* (Guenée). A brief description of the life cycles of these insects is presented to help understand the samples collected at different growth stages used in the experiment.

5.2.1 Life Cycle of Insects Species Used

*Paropsis atomaria*
Order: Coleoptera  
Family: Chrysomelidae  
Common name: eucalyptus leaf beetle

*Paropsis atomaria* is the most common species of leaf beetle in Southeast Queensland (Lawson & King, 2006). Its life cycle from the egg to emergence of adult usually takes about 31-57 days. It takes approximately 8 days for eggs to hatch, which is followed by four larval instars. Each instar finishes with the shedding of its skin (ecdyses). During the larval stage, larvae feed on mostly new leaves of various eucalyptus species. It is believed that most damage to the eucalyptus foliage is done by the 3rd and 4th instars (about 90% of total consumed during the two instars) and the adults. The four instars take approximately 22 days and are followed by a pre-pupal and a pupal stage (averaging 4.5 and 9.5 days respectively), which usually takes
place in the soil beneath the host tree (Miles et al., 1982; Tanton & Epila, 1984). After this period an adult beetle emerges. Adult beetles can feed on both new and older foliage. *Paropsis* adults can live anywhere up to 90 days or more (Edwards & Wightman, 1984).

*Paropsis atomaria* have 2-3 generations/year. All stages (adults, eggs and larvae), can be present at any time. In southeast Queensland there are usually two incidence of defoliation per year, usually occurring in late December-early January and late March to May. Management recommendations by the QDPI-Forestry include biological, manual and chemical control depending on the situation, along with regular inspections (Lawson & King, 2006).

**Mnesampela privata** (Guenée)
Order: Lepidoptera  
Family: Geometridae  
Common name: Autumn gum moth.

*Mnesampela privata* (Guenée) are known to inhabit up to 41 different eucalyptus species, and larvae can cause severe defoliation depending on their instar stage as shown in Figure 5.1, page 234 (Steinbauer & Matsuki, 2004). Geometrid insects go through four stages during their life. Eggs are first layed, from which the larvae, also known as caterpillars, emerge. These caterpillars are leaf-eaters, and have a sclerotized head capsule, chewing mouthparts, and a soft body. They can also be distinguished by their three pairs of true legs, and additional prolegs. Caterpillars of *Mnesampela privata* (Guenée) go through five instar stages.

Caterpillars pupate in a cocoon, undergoing metamorphosis until they emerge in their adult stage, as a moth. The moth does not feed on foliage, instead relying on nutrients found in its own blood such as amino acids (Gilmour, 1965).

**5.2.2 Preparation of ¹⁵N-Enriched Plant Samples**

*Eucalyptus pilularis* plants were purchased from the Cumberland State Nursery, NSW, Australia, and were approximately 30cm in height. Soil was removed from roots and washed the roots with de-ionised water, then placed the plants in plastic
pots filled with perlite, with roughly 2cm of clay balls at the bottom to stop the perlite falling out. Pots were then placed with their bottoms sitting in holes in the top of a black sealed container, which was filled with a hydroponic nutrient solution containing calcium nitrate (CN) as its source of N, made up as per procedure outlined in Chapter 3, section 3.2.1.

For the Paropsis atomaria, these plants grown under hydroponic conditions were then placed into (0.55m x 0.55m x 0.55m) insect enclosures (Figure 5.4a) containing a cloth sheet so that insect frass pellets could be collected for analysis. The lepidopteran caterpillars were placed in a round 27cm diameter x 9cm tall container with a cloth lid, and were fed with leaves from the hydroponic plants.

5.2.3 Collection of $^{15}$N-Enriched Insect Samples

Paropsis atomaria

A group of 12 Paropsis atomaria larvae, referred to here as leaf beetle larvae, were collected from a eucalyptus plantation grown at the University of Western Sydney’s Hawkesbury campus. Larvae were collected from the same leaf to ensure they were of similar age. The age of the caterpillars when captured was estimated to be approximately 2 weeks old.

Nine of the twelve larvae were transferred to the insect enclosure and allowed to feed on $^{15}$N enriched eucalyptus plants (Figure 5.4b). The other three larvae were put in a separate insect enclosure and allowed to feed on the leaves of the unenriched eucalyptus plants. Two larvae died during the experiment, one from the enriched plants and the other from the unenriched, leaving eight enriched larvae and two unenriched for the experiment. Once larvae had reached the 4th instar (Figure 5.4c), and just before pupation the larvae were harvested and frozen. Frass was also collected and dried. The leaf beetle larvae were then defrosted and dissected in order to remove their digestive track (Figure 5.4e). Previous reports have shown up 50% of an insect’s dry weight can be from material found in an insect’s gut (Edwards & Wightman, 1984). As the gut contents can contain undigested leaf material, as well as semi/fully digested material, removal of the digestive tract would ensure that any NMR spectra
Figure 5.4: a) Insect enclosure with eucalyptus plants inside, b) leaf beetle larvae, c) 4th instar leaf beetle larvae, d) dissection and removal of the digestive track of a leaf beetle larvae, e) leaf beetle pupae with shed skin, f) and g) top view of the leaf beetle adult and h) bottom view of a leaf beetle adult.
Figure 5.5: a) and b) Parasitic fly ovary which emerged from a leaf beetle larvae, c) and d) the parasitic fly which emerged, e) and f) moth caterpillars, g) two moth cocoons and h) the moth which emerged.
obtained would not include any eucalyptus material still inside the insect gut. The resulting spectra were of the insect’s body material only. Two bulk samples were prepared, one from all the enriched larvae digestive tracks, the other from the remaining sections of the larvae. One $^{15}$N enriched larva was left intact to be used as a reference. Both unenriched larvae were also left fully intact. All samples were freeze dried to remove all water and ground into a powder to enable analysis by solid-state NMR spectroscopy.

The two remaining $^{15}$N enriched leaf beetle larvae left to feed on the eucalypts and were allowed to pupate (Figure 5.4e) and mature into adult beetles (Figure 5.4 f-g) in order for us to identify the species better and also study the differences between the larvae and adult forms. Of the two leaf beetle larvae left to mature, a parasitic fly (species: Tachinidae) had infected one of the larvae and this infected larva hatched into an ovary (Figure 5.5a and b), and later into the fly itself (Figure 5.5c and d). Previous research has found that approximately 19.2% of Paropsis atomaria larvae may be infected by tachinid flies (Tanton & Epila, 1984). As the fly does not feed on eucalypts, it did not survive long. Both the ovary and fly were kept and freeze-dried for possible analysis. This left one enriched leaf beetle larva to fully mature into an adult leaf beetle (Figure 5.4f-h). It was left to feed on the eucalyptus plants before also being sampled. Frass pellets from the adult leaf beetle were collected for analysis.

Mnesampela privata (Guenée)

Four Mnesampela privata (Guenée) caterpillars (referred to here as moth caterpillars) were fed in a similar way as the leaf beetle larvae. Of the four moth caterpillars (Figure 5.5e and f), two were harvested before pupation occurred and two were left to mature into adult moths (Figure 5.5h). After approximately 2 weeks, the moths and also the cocoons from which they hatched (Figure 5.5g) were sampled. Frass from the moth caterpillars was collected and dried for NMR analysis. All insect samples were freeze-dried and ground to a powder following a similar procedure to that for the leaf beetles.
5.2.4 $^{15}$N NMR Spectroscopy

Solid-state $^{15}$N NMR spectra were obtained on a Bruker 200MHz spectrometer operating at 20.28MHz, using cross polarization, magic angle spinning (CPMAS). Samples were loaded into 4mm zirconium oxide rotors and spun at 7 kHz. A Pulse width of 4.6µs was used with a contact time of 1.5 msec, acquisition time of 0.01sec, a delay time of 0.5 sec and a 25 kHz sweep width. The number of scans ranged from 35,000 to 200,000 scans. These parameters were used as they are the same as those used for the eucalyptus leaves (Chapter 3, section 3.2.2). Insect spectra obtained were therefore comparable to those of eucalyptus leaves. The only exception is the number of scans acquired.

Absolute intensities were found by integrating peak areas then correcting for the amount of sample present in the rotor during experiments. $^{15}$N CPMAS NMR spectra were integrated into five regions and peak regions have been assigned as in Table 5.2. Relative $^{15}$N enrichment per gram was found for all samples (insect, frass and plant material), by taking the total integrated area of a spectrum, and dividing it by the mass present in the NMR rotor. Results, however, were not included for the moth even though a moth spectrum was obtained. This was due to the limited amount of sample collected, filling less than an eighth of the sample rotor. It was therefore hard to weigh accurately, so the $^{15}$N enrichment was not obtained.

**Table 5.2: $^{15}$N NMR peak assignments (Knicker et al., 2002; Ma et al., 2004; Smernik & Baldock, 2005, Schaefer et al., 1987)**

<table>
<thead>
<tr>
<th>Region (ppm)</th>
<th>$^{15}$N NMR Peak Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero-N (-190 to -240)</td>
<td>Heterocyclic N in purines, chlorophyll, uric acid, allantoin, allantoic acid, N in histidine rings.</td>
</tr>
<tr>
<td>Amide-N (-240 to -290)</td>
<td>Amide N, peptide N in proteins</td>
</tr>
</tbody>
</table>
| Guano-N (-290 to -303) | $\text{RHN} \equiv \text{NH}$  
                          | NH groups, $\text{H}_2\text{N}$ (guanidino NH)                        |
| Amin-N (-303 to -330)   | $-\text{NH}_2$ and $-\text{NR}_2$ groups                                |
| Amino-N (-330 to -360)  | $-\text{NH}_3^+$ and $-\text{NR}_3$ groups, $\text{NH}_4^+$, free amino groups in amino acids and amino sugars |
5.2.5  $^{13}$C NMR Spectroscopy

$^{13}$C NMR spectra were obtained on the same Bruker 200MHz spectrometer operating at 50MHz, using CPMAS. Samples were spun at 5 kHz. A Pulse width of 4.6µs was used with a contact time of 1.5 msec, acquisition time of 0.01sec, a delay time of 0.5 sec and a 25 kHz sweep width. The number of scans ranged from 2,000 to 25,000 scans. $^{13}$C CPMAS NMR spectra were integrated into 6 regions as shown in Table 5.3.

<table>
<thead>
<tr>
<th>Region (ppm)</th>
<th>$^{13}$C NMR Peak Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonyl C</td>
<td>Carbonyl C in chitin, protein and lipids</td>
</tr>
<tr>
<td>(185 – 165ppm)</td>
<td></td>
</tr>
<tr>
<td>Phenolic C</td>
<td>Phenolic C in catechols, proteins, tannin and lignin,</td>
</tr>
<tr>
<td>(165 – 140ppm)</td>
<td></td>
</tr>
<tr>
<td>Aromatic C</td>
<td>Oxygenated aromatic C in catechols, aromatic C in protein, tannin and lignin, unprotonated aromatic C</td>
</tr>
<tr>
<td>(140 – 110ppm)</td>
<td></td>
</tr>
<tr>
<td>di-O-Alkyl C</td>
<td>Anomeric C1 in cellulose, condensed tannins, flavonoids and chitin components.</td>
</tr>
<tr>
<td>(110-80)</td>
<td></td>
</tr>
<tr>
<td>O-Alkyl C</td>
<td>Carbohydrate C in cellulose, alcohol and amino sugars, broad peptide $\alpha$-carbons, methoxyl C.</td>
</tr>
<tr>
<td>(80-40)</td>
<td></td>
</tr>
<tr>
<td>Alkyl C</td>
<td>Aliphatic C in lipids, methyl C in chitin, protein, lipid, catechol, fatty acids, waxes and amino acids</td>
</tr>
<tr>
<td>(40 – 0)</td>
<td></td>
</tr>
</tbody>
</table>

5.3. Results and Discussion

5.3.1. Proteins

The qualitative analysis of the $^{15}$N NMR spectra reveal that N species found in the insect biomass material from the leaf beetle larvae, leaf beetle adult, moth caterpillar and moth (Figure 5.6a,b,c and d respectively) and that of eucalyptus foliage (Figure 5.7a), were all very similar. This is also shown in Table 5.4, where the N composition (the percentage each N region takes up in the whole spectrum) is compared. The relative intensity of the two peaks found at -296 and -308ppm showing guano-N and amine-N groups respectively are basically the same for all leaf and insect spectra, with only the leaf beetle larvae showing a slightly higher overall intensity.
Figure 5.6: $^{15}$N NMR spectra of a) 4th instar leaf beetle larvae, b) leaf beetle adult, c) moth caterpillars and d) adult moth. All spectra have been scaled in order to compare N composition.

Figure 5.7: $^{15}$N NMR of a) untouched eucalyptus foliage and frass pellets from, b) 4th instar leaf beetle larvae, c) leaf beetle adult and d) moth caterpillars All spectra have been scaled in order to compare N composition.
Figure 5.8: $^{13}$C NMR spectra of a) 4th instar leaf beetle larvae, b) leaf beetle adult, c) moth caterpillars and d) adult moth. All spectra have been scaled in order to compare $^{13}$C composition.

Figure 5.9: $^{13}$C NMR of frass pellets from a) 4th instar leaf beetle larvae, b) leaf beetle adult c) moth caterpillars and d) eucalyptus foliage. All spectra have been scaled in order to compare $^{13}$C composition.
Table 5.4: Comparison of percentage composition of $^{15}$N NMR spectra taken.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hetero N</th>
<th>Amide N</th>
<th>NH</th>
<th>NH$_2$/NR$_2$</th>
<th>Amino N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eucalyptus Leaves</td>
<td>2.0</td>
<td>80.5</td>
<td>3.8</td>
<td>9.0</td>
<td>4.7</td>
</tr>
<tr>
<td>LB Larvae</td>
<td>2.1</td>
<td>84.3</td>
<td>2.5</td>
<td>5.3</td>
<td>5.8</td>
</tr>
<tr>
<td>LB Adult</td>
<td>1.7</td>
<td>90.1</td>
<td>1.7</td>
<td>2.9</td>
<td>3.6</td>
</tr>
<tr>
<td>Caterpillar</td>
<td>2.9</td>
<td>82.6</td>
<td>2.3</td>
<td>5.9</td>
<td>6.4</td>
</tr>
<tr>
<td>Moth</td>
<td>3.5</td>
<td>85.5</td>
<td>3.0</td>
<td>5.2</td>
<td>2.7</td>
</tr>
<tr>
<td>LB Larvae frass</td>
<td>16.9</td>
<td>67.5</td>
<td>2.8</td>
<td>6.6</td>
<td>6.3</td>
</tr>
<tr>
<td>LB Adult frass</td>
<td>24.7</td>
<td>59.6</td>
<td>3.0</td>
<td>5.8</td>
<td>6.9</td>
</tr>
<tr>
<td>Avg. Caterpillar Frass</td>
<td>7.9</td>
<td>74.0</td>
<td>8.9</td>
<td>7.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Caterpillar Frass $^{(1)}$</td>
<td>5.7</td>
<td>72.7</td>
<td>10.4</td>
<td>9.4</td>
<td>1.9</td>
</tr>
<tr>
<td>Caterpillar Frass $^{(2)}$</td>
<td>10.1</td>
<td>75.2</td>
<td>7.4</td>
<td>5.8</td>
<td>1.5</td>
</tr>
</tbody>
</table>

(1) Sampled on the 23/8/06
(2) Sampled on the 28/8/06

Table 5.5: Comparison of percentage composition of $^{13}$C NMR spectra taken.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Carboxyl C</th>
<th>Phenolic C</th>
<th>Aromatic C</th>
<th>C1 of Glucose</th>
<th>Carbohydrate C</th>
<th>Aliphatic C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eucalyptus Leaves</td>
<td>9.8</td>
<td>6.4</td>
<td>9.7</td>
<td>12.8</td>
<td>41.6</td>
<td>19.7</td>
</tr>
<tr>
<td>LB Larvae</td>
<td>19.3</td>
<td>2.2</td>
<td>8.1</td>
<td>5.8</td>
<td>34.1</td>
<td>30.7</td>
</tr>
<tr>
<td>LB Adult</td>
<td>21.4</td>
<td>8.1</td>
<td>12.2</td>
<td>7.2</td>
<td>25.0</td>
<td>26.0</td>
</tr>
<tr>
<td>Caterpillar</td>
<td>19.2</td>
<td>3.2</td>
<td>10.2</td>
<td>4.7</td>
<td>31.2</td>
<td>31.5</td>
</tr>
<tr>
<td>Moth</td>
<td>19.9</td>
<td>4.6</td>
<td>14.4</td>
<td>5.6</td>
<td>18.8</td>
<td>36.7</td>
</tr>
<tr>
<td>LB Larvae frass</td>
<td>10.1</td>
<td>7.0</td>
<td>15.0</td>
<td>18.3</td>
<td>29.4</td>
<td>20.2</td>
</tr>
<tr>
<td>LB Adult frass</td>
<td>6.3</td>
<td>6.1</td>
<td>18.7</td>
<td>20.7</td>
<td>27.4</td>
<td>20.9</td>
</tr>
<tr>
<td>Caterpillar frass</td>
<td>6.5</td>
<td>4.8</td>
<td>12.0</td>
<td>18.0</td>
<td>43.8</td>
<td>14.9</td>
</tr>
</tbody>
</table>
The major peak in all the spectra acquired is positioned at approximately -261 ppm, corresponding to amide-N. This indicates a transfer and incorporation of $^{15}\text{N}$ from the eucalyptus leaves consumed by the insects in structures such as proteins found in its body. Table 5.4 shows that there is slightly more amide-N in the insects than the eucalyptus leaves. Between the insects, the adults tend to have slightly more amide-N than the larvae/caterpillar. This increase in amide-N agrees with results found by Schaefer et al. (1987), who showed protein levels and chitin (Figure 5.10) increase substantially as an insect becomes an adult and its cuticles harden (sclerotisation).

![Figure 5.10: Chemical structure of chitin](image)

The $^{13}\text{C}$ CPMAS NMR spectra of all insect materials on the other hand (Figure 5.8) were both qualitatively and quantitatively different to that of the eucalyptus leaves (Figure 5.9d) and the frass produced by the insects (Figure 5.9a, b and c). The $^{13}\text{C}$ eucalyptus leaves spectrum shows the low-field carbonyl-C peak to be positioned at 175 ppm. This could be due to either carbons bonded to N such as in proteinaceous material or those not directly bonded to N, such as glycerate, serine and glycolate compounds. This peak however, is not present in the insect spectra. Instead a large carbonyl peak at high field 169 ppm can be found in all insect $^{13}\text{C}$ spectra (Figure 5.8), which is characteristic of carbonyl C. This suggests that a new protein may have been formed in the insects’ body, as the chemical environment around N is different to the one found in the eucalyptus material. The $^{15}\text{N}$ NMR spectral data shows that during the growth of larvae to adult leaf beetle, the relative distribution of amide N in the proteins is increased at the expense of other forms of N. Table 5.5 also shows the adult insects have a slightly higher percentage of carbonyl groups than the larvae/caterpillar, also agreeing with the trend observed in $^{15}\text{N}$ amide chemical environments.
The metabolism of leaf proteins by the insects is shown in the $^{15}$N and $^{13}$C NMR data of the insect frass. The $^{15}$N NMR spectra of frass (Figure 5.7b,c and d) show a loss of signal intensity in the amide-N region compared to the eucalyptus foliage (Table 5.4). This suggests that some proteins, rather than becoming bound to tannins and therefore unavailable for metabolism, have been used by the insects and are incorporated into insect biomass. The $^{13}$C frass spectra (Figure 5.9) does show that some amide-C present in foliage has gone through the insect untouched, with the presence of the 175ppm peak in all insect frass collected. However, the presence of the 169ppm peak in frass, characteristic of the carbonyl of insect proteins suggests that some of the insect’s biomass is excreted with the frass.

5.3.2 Fats and Lipids

The $^{13}$C NMR spectra for all insects show a peak at 24ppm, most likely due to methyl carbons adjacent to carbonyls such as those found in chitin (Smernik & Oades, 2001). The intensity of this peak is reduced in the leaf beetle adult spectrum (Figure 5.8b) but has a more pronounced peak at 14ppm. The region less than 20ppm is defined by terminal methyl carbons on long chain fatty acids. The presence of these two peaks along with the lack of peak in all insect spectra around 32ppm, which corresponding to mid-chain methylene carbons, suggests that any waxes present have small chain lengths, as mainly terminal end groups are seen. The smaller intensity in general of the aliphatic-C region in the adult leaf beetle spectra may be due to smaller amounts of fats being present in the adults’ biomass compared to the larvae, as these reserves are used for energy during activities such as flight. The moth caterpillar and moth have roughly the same relative distribution in the aliphatic-C region, but the total intensity of this region is slightly larger in the moth $^{13}$C NMR spectrum.

The aliphatic-C region of the frass for all insects shows slight differences from that of the leaves spectra. The frass shows a sharp peak at 32ppm which corresponds to long chain fatty acids, waxes and resins present in foliage. It seems the insects have passed these compounds with minimum metabolism, whereas the diminished peaks to the right (less than 30ppm) suggest these forms of aliphatic-C have been digested and metabolised.
5.3.3 Anti-feedants

The difference between the $^{13}$C NMR spectra of the eucalyptus leaves (Figure 5.9d) and the insect frass (Figure 5.9a, b and c) shows what has been metabolised and used by the insect (decrease in peak intensity), or the by-products this process has produced (increase in peak intensity). For example the $^{15}$N frass spectra (Figure 5.7b, c and d) showed the production of uric acid due to the insect’s metabolism process. The chemical profile of the leaf material and insect frass, as shown by $^{13}$C NMR spectra, are very similar. This indicates that a large amount of the carbon found in the eucalyptus material has passed through the insects mostly untouched.

The phenolic and aromatic-C regions, which include peaks at 155, 145, 138, 131 and 119ppm, are slightly higher in intensity in the leaf beetle larvae and adult frass than the moth caterpillar (Table 5.5). This region in the eucalyptus foliage $^{13}$C NMR spectra includes phenolic-C groups (155ppm), lignin (145ppm) and aromatic and unsaturated C (138 and 131ppm) which corresponds to the PSMs and can act as antifeedants. Therefore the results of the $^{13}$C NMR spectra for frass would suggest that these compounds are passing through the insect digestive system possibly untouched, and are therefore becoming more concentrated as other compounds are metabolised. Table 5.5 also shows that there has been an increase in aromatic C groups. The passing of these leaf material compounds undigested and unchanged may be due the peritrophic membrane, as mentioned in the introduction to this chapter (section 5.1). It is possible that some of this membrane has been excreted as well. Tellam and co-workers (2000), have previously found that Lucilia cuprina larvae excreted the protein perithrophin-95, which originates in the insect’s peritrophic matrix membrane. If peritrophic proteins are excreted from the insects studied here, this may then explain the peak found in the carboxyl-C region at 169ppm for all $^{13}$C NMR spectra for insect frass.

The di-O-alkyl region of the eucalyptus foliage suggests the presence of PSM’s such as tannins and lignin. The moth caterpillar frass spectra show a similar split peak at 104 and 109ppm to that of the eucalyptus leaves spectra. This suggests that the di-O-alkyl C peaks at 104 and 109ppm, which correspond to C-1 of cellulose and C-2 of guaiacyl units in lignin respectively in leaves, have passed through the moth caterpillar untouched. The leaf beetle larvae however do not show a split peak but a
wide peak covering this range. This may be due to a reduced 104ppm peak, as the peak is asymmetrical and is more pointed at 109ppm, showing a shoulder to the right hand side. The fact that the intensity of the 104ppm peak seems to have been reduced suggest the leaf beetles larvae and adult are able to partly break down the C-1 bond in cellulose better than the moth caterpillars. This is also reflected by peaks in the carbohydrate region of 90 – 60ppm, which show cellulose levels, all being smaller in the leaf beetle frass spectra compared to the moth caterpillar frass spectra (Table 5.5).

The peak at 97ppm in the $^{13}$C NMR spectrum seems more defined in the leaf beetle adult and larvae spectra. This peak corresponds to the presence of chitin, and it is most likely that some of this is excreted as by-products in the insect frass as evidenced by the appearance of this peak.

### 5.3.4 Sclerotisation

Evidence of sclerotisation can be seen in the $^{15}$N and $^{13}$C insect spectra with the increase in protein levels as previously described. Evidence of tanning, hardening can also be seen in the $^{13}$C spectra of the insect biomass. The $^{13}$C region that represents aromatic-C (160–110ppm) showed significant differences particularly between the leaf beetle larvae and adult insect material (Figure 5.8a and b). The adult $^{13}$C NMR spectra show four peaks in this region at 150, 138, 123 and 112ppm. The peaks present in all insect spectra at 150, 123 and 112ppm reflect aromatic carbons belonging to protein residues. The adult leaf beetle once again has slightly higher intensity in this area compared to that of the larvae. (Table 5.5). In the larvae $^{13}$C NMR spectra the peak located at 138ppm has disappeared. This peak reflects catechol levels, as the peak is due to oxygenated aromatic-C (Schaefer et al 1989). When catechols are oxidised, they go from colourless to reddish-brown benzoquinone derivatives, and are therefore known as tanning agents. Pictures of the leaf beetle larvae and adult (Figure 5.4) show that the adult has become speckled brown, and therefore, as shown by the presence of the 138pm peak, has higher catechol levels. The moth caterpillar and moth spectra (Figure 5.8c and d) have an aromatic-C region similar to that of the leaf beetle larvae, with no real peak at 138ppm. This could be due to their colour, with the caterpillar being green and the moth mostly white/pale brown/grey, or they may not have as hard a shell as the adult leaf beetle.
As the insect cuticle hardens supramolecular structures consisting of protein amino groups cross-linked by C-N linkages with catechols are formed (Kramer et al., 2001; Schaefer et al., 1987). If the adult leaf beetle spectrum shows the catechol peak at 138ppm, this may suggest the adult has a harder exoskeleton than the larvae, caterpillar and moth. It has also been suggested that the phenolic amino acid tyrosine, and its precursor phenylalanine, may be a provider of phenyl rings needed for sclerotisation (Hopkins & Kramer, 1992). Tyrosine is usually stored during larval feeding in the hemolymph (an insect’s blood), and levels have been reported to increase just before sclerotisation (Kramer & Hopkins, 1987). Its phenyl rings are then metabolised and used to form catechols and tanning agents as the exoskeleton hardens. This may be why we see a reduction in the amine-N peak at –308ppm in the adult leaf beetle $^{15}$N NMR spectrum as compared to the larvae, as this is where we would find the tyrosine/phenylalanine NH$_2$ group. If the larvae are storing tyrosine we would expect a more intense peak.

Peaks found at 98, 77, 70 and 67ppm reflect the chitin content in the insect samples. As an insect grows and its exoskeleton hardens, the result is also higher amounts of chitin being formed (Schaefer et al., 1987). In the leaf beetle adult, the peak at 98ppm is larger than the other insects and very sharp, suggesting that it is more crystalline/highly ordered chemical structure in the sample. The peak at 77ppm is also larger for the leaf beetle adult. Peak 66ppm appears to have a higher intensity in the leaf beetle larvae and moth caterpillar than in the corresponding adult leaf beetle and moth. The leaf beetle adult’s peak is split into two peaks 70 and 67ppm.

Peaks found at 54 and 49ppm also reflect the broad peptide backbone $\alpha$-carbons, which show protein levels. These peaks were found to be roughly the same in all insects, although the moth does not show a strong peak at 54ppm peak.

5.3.5 Amino Acids

In the $^{15}$N NMR spectra, changes were observed in the two peaks found in the amino end groups region at -339 and -347ppm between the leaves and the spectra of all the insects. In the leaves spectra, the two peaks are of equal intensity. However, in the insect spectra, the -347ppm peak is prominent while the moth spectra (Figure 5.6d) showing no peak at -339ppm, which is due to free amino acids. This could be due to
the fact that the moth does not feed during this stage of its life, relying instead on 
nutrients found in its own blood such as amino acids. Therefore all $^{15}$N incorporated 
into N forms in the moth has evolved from when the moth was a caterpillar. The loss 
of the peak at -340ppm may be due to the moth using up and metabolising this 
particular type of amino-N.

5.3.6 Heterocyclic N

The peak corresponding to heterocyclic N forms in the $^{15}$N NMR spectra for insects, 
found at -205ppm, was slightly more pronounced in the eucalyptus leaf spectrum 
(Figure 5.7a). A small peak was found in the moth caterpillar at -230ppm (Figure 
5.6c and d), which may also be due to heterocyclic N found in compounds such as 
uric acid and its derivatives (Figure 5.12).

The $^{15}$N spectra of frass material collected from the leaf beetle larvae and adult, and 
the moth caterpillars are shown in Figure 5.7b, c and d respectively. The moths do 
not produce frass pellets, but a spray that could not be collected; therefore no $^{15}$N or 
$^{13}$C NMR spectra could be obtained. The leaf beetle larvae and adult and the moth 
caterpillar’s frass were found to be very different from that of the leaves and insect 
spectra. Most obvious is the extra peaks located in the hetero-N region at -208, -227 
and -248ppm in the leaf beetle adult and larvae frass spectra (Figure 5.7b and c), 
which are due to the insects metabolising the leaves and converting N into 
heterocyclic forms, most likely uric acid and its derivatives.

To identify these peaks, frass spectra were compared to $^{15}$N NMR spectra from uric 
acid, allantoin and L-histidine (Figure 10a, b and c respectively). Uric acid and 
allantoin were chosen, as they are known to be by-products of insects’ digestive 
system, and histidine allowed us to look at different forms of N. Histidyl has also 
been shown to be involved in the hardening process of the insects’ exoskeleton 
(Schaefer et al, 1987).

$^{15}$N NMR peak assignments showed that none of these model compounds seem to 
correlate with peaks found in the insect frass spectra. The $^{13}$C NMR peak 
assignments however, suggest that uric acid may be present (Figure 5.9), as these 
peaks can be found in the frass $^{13}$C NMR spectra. It may be possible that the $^{15}$N uric
acid peaks have shifted due to the pH the uric acid is present at in the frass material. As discussed in the introduction, insects have high a midgut pH and an acidic pH in the hindgut (Lovett et al 1998). Differences in pH will affect N nuclei more than C nuclei due to their lone pair of electrons. This therefore may explain why, if uric acid is present, the peaks have shifted by approximately 20ppm in the $^{15}$N NMR spectrum, and not the $^{13}$C NMR spectrum.

Figure 5.11: Chemical structure of a) uric acid, b) allantoin, c) L-histidine

Figure 5.12: $^{15}$N NMR spectra of a) Uric Acid, b) Allantoin and c) L-Histidine (* denotes ssb)

Figure 5.13: $^{13}$C NMR spectra of a) Uric Acid, b) Allantoin and c) L-Histidine
The moth caterpillar frass (Figure 5.7d) has a different heterocyclic N region, with a very small peak at -208 ppm, a larger peak at -227 ppm and a shoulder at roughly -248 ppm. It was found that caterpillar frass collected on different days showed different N distributions, particularly in this region (Table 5.4). Figure 5.14a and b shows frass that has been sampled 6 days apart, and shows significant differences in both the heterocyclic N region and at -301 ppm. The samples on two different occasions, however, did not show any significant differences in their $^{13}$C spectra. The caterpillar frass spectrum was also different to the leaf beetle frass spectra, in that it had an extra peak at -356 ppm that corresponds to the chemical shift for ammonium. This could be linked to a way of retaining N without losing to the atmosphere as discussed in the introduction. i.e., there is some formation of ammonia when exposed to high pH in the mid-gut of moth caterpillars and it is been trapped by the high acidity in the hind-gut forming ammonium by-products in the frass.

![Figure 5.14: Moth caterpillar frass sampled on a) 23/08/06 and b) 29/08/06.](image-url)
The amide/peptide N peak found at -261ppm has been greatly reduced in the frass spectra of all insects compared to that of the insect themselves or that of the eucalyptus leaves. This shows that the leaf amide/peptides are being metabolised by the insects, and incorporated into the insect biomass or passed out as frass. The frass of the leaf beetle adult and the moth caterpillar showed the greatest reduction in the amide peptide peak.

In the moth caterpillar frass spectra, the peak at -297ppm has almost disappeared, and a peak at approximately -301ppm had appeared. The leaf beetle adult and larvae frass spectra show peaks at -297 and -308ppm, which is similar to the insect itself and the leaf spectra. For the leaf beetle adult and larvae insect frass pellets, the amino-N region is dominated by the peak found at -338ppm, with a small peak at -346ppm. As these two peaks were of similar intensity in the leaf spectrum, and the -346ppm peak was dominant in the insect spectrum, it would therefore seem that the insect is able to metabolise and use the amino end groups found at -346ppm, but not -338ppm, which is why these have been excreted in the leaf beetle frass pellets. The moth caterpillar frass pellets show a similar reduction of the -346ppm peak, with a new peak arising at -356ppm. This chemical shift corresponds to that of ammonium.

5.3.7 Nitrogen and Carbon Use Efficiencies

By looking at the relative $^{15}$N and $^{13}$C enrichment per gram for all of the insects and plant material used in this study (Figure 5.15 and Figure 5.16), in particular the frass spectra results, we can compare the nitrogen and carbon use efficiency of the insects. For example, as the leaf beetle larvae and moth caterpillar are still growing, they make use of a greater amount of nitrogen compared to the adult leaf beetle. Therefore their frass has a lower intensity per gram of sample packed in the rotor. The leaf beetle larvae frass has a lower amount of $^{15}$N present than the eucalyptus foliage, showing a lot of nitrogen has been used in insect growth or as energy. The leaf beetle adult and moth caterpillar frass show higher amounts of $^{15}$N than the eucalyptus foliage, suggesting that either the nitrogen is not being used, or any unused nitrogen is being concentrated due to other nutrients from the foliage being metabolised by the insects. Concentration of nitrogen is likely as the adult leaf beetle itself has become
more enriched in $^{15}$N than the larvae, and therefore must still be using some nitrogen from the eucalyptus foliage.

Figure 5.15 also shows that $^{15}$N enrichment is highest in the caterpillar material compared to the leaf beetle larvae and adult, indicating that the caterpillar may be more efficient at using metabolising and storing nitrogen taken from the eucalyptus leaves. All of these results, however, rely upon how much was eaten by the insects and for how long, as the more they eat, the more they have access to $^{15}$N in the eucalyptus foliage. Also, because the larvae go through instars where their skin is shed, the amount of nitrogen lost during this process was not accounted for, and may be why the leaf beetle larvae are less enriched. Therefore the $^{15}$N enrichment of the insect bodies cannot be directly compared.

![Figure 5.15: Relative $^{15}$N enrichment per gram of insect biomass, insect frass or eucalyptus foliage as found by $^{15}$N NMR. All results were corrected for the number of scans used.](image1)

![Figure 5.16: Relative $^{13}$C enrichment per gram of insect biomass, insect frass or eucalyptus foliage as found by $^{13}$C NMR. All results were corrected for the number of scans used.](image2)

Because the $^{13}$C seen in the carbon NMR spectra was not enriched (it is natural abundance), the $^{13}$C spectra of the insect bodies, frass and the eucalyptus material can be directly compared. Both the insect bodies and frass were found to be more
enriched than the eucalyptus leaves. This is expected for the insect bodies, as carbon is metabolised and stored for energy use of used in the insect’s body structure. Table 5.5 confirms that a large percentage of carbon in the insects was in the form of carbonyl C (found in protein structures) and aliphatic C (found in lipids). The high carbon content in the frass may be due to concentration, where other nutrients have been taken in high amounts from the eucalyptus leaves. This may also be due to the insects passing through indigestible forms of carbon as explained earlier in this chapter.

5.3.8 Effect of an Insect Infestation on a Plantation Ecosystem

Ohmart and co-workers (1983a; 1983b), have previously reported that up to 91,000 insects per hectare were present in a *Eucalyptus pauciflora* forest canopy. They have also recorded the number of insects present in various eucalyptus plantations in Australia, as well as the amounts of leaf litter, other litter (including twigs and bark), and insect biomass (including frass and bodies), which fall to the ground during defoliation by insects. Here, these results have been collected and modified to show the total N (kg/ha/yr) found in these different segments, based on the masses and %N presented by Ohmart and co-workers (Table 5.6). All of the N found in these results represent the amount of N which is being returned to the plantation soils. It has been found here that the N returning to soils in the form of insect bodies and frass is small compared to that returned in the form of leaves and other litter.

Experimental results found in this thesis research, however, revealed that a large portion of the N found in insect frass is in the form of heterocyclic N structures (an average of 16.5 %). It was also found in section 3.3.5 that heterocyclic N accounts for only a very small portion of N in all tree sections (1.6 to 3.2%). Hence it was thought, that even though Ohmart and co-workers show that only a small amount of N is returned to the soil via insect bodies and frass, this may still account for a significant portion of N in the form of heterocyclics which end up in the soil. Therefore the average data obtained in Chapter 3 for the %hetero-N found in leaves and whole stems (Table 3.29), along with averages for the %hetero-N found in insect frass and bodies (Table 5.4), was used to estimate the amount of heterocyclic N that may be present in Ohmart and co-workers results, and hence returned to the soil.
These results are shown in Table 5.7 and compared in Figure 5.17. It was discovered that while the amount of heterocyclic N from insect material is small, it is still significant, especially considering that these results are for a low level infestation. If a higher number of insects were present, then this portion may increase compared to the leaf and other litter segments.

It also needs to be taken into consideration that these results are based on insect numbers found in a plantation canopy only. Results for insect numbers could be a lot higher if insects present in ground litter and soil are also counted, therefore also increasing the amounts of potential heterocyclic N in soils. For example Holt & Spain (1986), found that up to 466,000,000 insects per hectare were present in the

<table>
<thead>
<tr>
<th>Forest Species</th>
<th>Leaves (kg/ha/yr)</th>
<th>Other (kg/ha/yr)</th>
<th>Insects (frass/bodies)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. delegatensis</em> (1981)</td>
<td>15.96</td>
<td>15.26</td>
<td>1.02</td>
</tr>
<tr>
<td><em>E. delegatensis</em> (1982)</td>
<td>10.77</td>
<td>19.00</td>
<td>0.90</td>
</tr>
<tr>
<td><em>E. dives</em> (1981)</td>
<td>12.32</td>
<td>7.48</td>
<td>0.58</td>
</tr>
<tr>
<td><em>E. dives</em> (1982)</td>
<td>8.33</td>
<td>10.48</td>
<td>0.55</td>
</tr>
<tr>
<td><em>E. pauciflora</em> (1982)</td>
<td>8.25</td>
<td>11.81</td>
<td>0.53</td>
</tr>
</tbody>
</table>

* Based on Ohmart et. al. (1983a) results where leaves=0.58%N, other=0.54%N, insects=2.113%N (where frass =0.97% and bodies=8.3%)

# values in parentheses show the total N originating from insect frass and bodies sections

<table>
<thead>
<tr>
<th>Forest Species</th>
<th>Leaves (kg/ha/yr)</th>
<th>Other (kg/ha/yr)</th>
<th>Frass/Bodies (kg/ha/yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. delegatensis</em> (1981)</td>
<td>0.26</td>
<td>0.43</td>
<td>0.08</td>
</tr>
<tr>
<td><em>E. delegatensis</em> (1982)</td>
<td>0.18</td>
<td>0.53</td>
<td>0.07</td>
</tr>
<tr>
<td><em>E. dives</em> (1981)</td>
<td>0.20</td>
<td>0.21</td>
<td>0.05</td>
</tr>
<tr>
<td><em>E. dives</em> (1982)</td>
<td>0.14</td>
<td>0.29</td>
<td>0.05</td>
</tr>
<tr>
<td><em>E. pauciflora</em> (1982)</td>
<td>0.14</td>
<td>0.33</td>
<td>0.04</td>
</tr>
</tbody>
</table>

^ Using results found in Chapters 3 and 5 (where leaves = 1.64%, other = whole stems = 2.81%, average frass = 16.50%, average bodies = 2.55%).

Table 5.6: Results for total N (kg/ha/yr) either lost from the canopy of various eucalyptus plantations, or diverted into insect bodies/frass. Results have been taken from Ohmart et al (1983a)

Table 5.7: Modified results from Table 5.6 (Ohmart et al (1983a), where the amount of heterocyclic N (kg/ha/yr) has been estimated using results for %hetero-N found in this thesis for leaves and whole stems (chapter 3) and insect frass and bodies (chapter 5).
combined litter and soil layers of a hoop pine plantation. This is huge compared to the 91,000 insects per hectare that were found by Ohmart and co-workers in a eucalyptus plantation canopy. However the amounts of heterocyclic N produced by these ground dwelling insects, where the largest portion of heterocyclic N is found in the insect’s frass, will depend on insects present. A large portion of the insects found by Holt & Spain in litter and soil layers were from the acari taxon, which consists for example of mites, ticks and spiders, all of which may produce frass of a different kind, or not at all. A large portion of these soil insects may also be in their pupation or metamorphosis stages, and therefore also not producing frass. It would therefore be hard to estimate the amounts of heterocyclic N being returned to the soil by this community of insects. However, if any is produced, results for heterocyclic N from insects in Table 5.7 and Figure 5.9 can only be increased.
5.4. Conclusions

The use of solid-state $^{15}$N and $^{13}$C NMR spectroscopy has been an effective tool for analysing the metabolism of various insects. Results from $^{15}$N NMR CPMAS spectra showed that the $^{15}$N enriched eucalyptus leaf material eaten by the insects is being metabolised and incorporated into the insect body. A slight increase in the leaf beetle adult and moth amide-N peak at -260ppm agrees with previous reports of protein levels increasing as an insect’s cuticles harden. $^{13}$C NMR CPMAS spectra of the insects were dramatically different to that of the eucalyptus material, which was to be expected. Carbon was shown to be bound up in protein, chitin, catechol and lipid structures. The moth and leaf beetle adult showed significant increases in aromatic C reflecting protein and catechol levels, and a decrease in O-alkyl C and aliphatic C which corresponds to mostly chitin and lipid levels respectively.

$^{15}$N spectra of the frass collected from each of the insects showed significant conversions of amide-N forms from the leaves into heterocyclic forms of N such as uric acid and its derivatives in the leaf beetles as well as ammonium in the moth caterpillar. The dominance of the -340ppm peak to that of the -348ppm peak in the amino-N region of the insect frass spectra shows the insects are unable to use this form of N, and it has passed through undigested. The moth does not feed during its adult life, but lives off the amino acids in its blood. This may explain the complete loss of the peak found at -340ppm in the moth spectra.

$^{13}$C spectra of insect frass showed for all the insects, aromatic C forms such as those found in eucalyptus leaf antifeedant compounds, are passing through the insect undigested and possibly becoming more concentrated in the frass excreted. Aliphatic C found in the leaves’ waxes and resins also seem to have been undigested. The carboxyl-C peak found at 169ppm in the insect body and frass $^{13}$C spectra, as opposed to the 175ppm peak found in leaves, suggests that proteins from the insects peritrophic membrane may be excreted, also suggesting that this membrane may provide a barrier between the insect and any antifeedants compounds present in the eucalyptus leaves.
The NMR results obtained here show qualitative differences between the eucalyptus leaves, insect bodies and insect frass. This qualitative change could have a significant effect on a forest plantation’s nutrient cycle. During a forest insect infestation, the insects eat a substantial amount of a tree’s new leaves, and as adults, older leaves also. If the trees are putting most of their N resources into new growth, then the insect infestation represent a major loss of N. There is also the loss of the trees photosynthetic capacity and the occurrence of a stunted growth tip. The N eaten by the insects has been shown here to be converted to much higher levels of heterocyclic N forms in the insects’ frass than what was originally present in the eucalyptus leaves. This is coupled with the fact that part of the N consumed will be immobilised in the insects’ body, eventually returning to the soil when the insect dies. $^{13}$C spectra have shown that the C composition of the insects themselves is also quite different to that of the leaves.

These changes give rise to changes in the N and C cycle in a forest plantation when insects are present in large amounts. Whether this is a positive or negative change for the forest depends on various factors. For example, the large amount of heterocyclic N in the insect frass deposited may decompose and be mineralised more rapidly by microbes in the soil. Therefore a tree that has lost its new foliage may recover better due to access to this mineral N pool. The higher amounts of aromatic C in the insect frass should break down more slowly in the soil, and therefore provide a more long term C pool for the tree in the future. The recovery of the plant, however, would depend on its access to any resultant nutrient pools, and whether they are sufficient for a plant that needs extra amounts to recover from the infestation.

5.5. References


CHAPTER SIX

FUTURE RESEARCH

The research undertaken for this thesis has opened up a broad range of questions which may provide the basis for future research work. The ideas stemming from this thesis are briefly discussed in this chapter.

6.1. $^{15}$N NMR Spectroscopy

$^{15}$N NMR spectroscopy is used for the analysis of samples originating from many different sources including organic matter as demonstrated in this thesis, materials research, polymers and medicine just to name a few. As the $^{15}$N isotope has a low natural abundance and is quite insensitive to NMR, it is not as commonly used as other isotopes such as $^1$H, $^{31}$P and even $^{13}$C. It would therefore be useful if a comprehensive account of $^{15}$N NMR spectroscopic parameters such as time constants for synthetic and naturally occurring compounds was obtained. It was clear from the thesis NMR experiments, the intensity and the chemical shifts of the NMR peaks depend on the physical state of the compounds as well the temperature, the presence of moisture and paramagnetic species etc. Therefore, in gathering and interpreting NMR spectral data, it is equally important to make a note of the other associated information.

The use of biexponential equations has also been proven to be beneficial for teasing out more information on N functional groups and their environment, as well as pointing out where signal losses may be occurring. Further work utilising $^{15}$N and $^{13}$C NMR could also be undertaken as to the effects of paramagnetics on spectral intensities and distributions. This could involve further work with plant residues both before and after mixing with different species of paramagnetics at different quantities. Complete effective removal of the paramagnetic species from the sample is reported.
to be not possible and therefore, it is important to verify what effect very small amounts of paramagnetics, such as those found after HF treatments, have on spectral intensity, distribution and relaxation time parameters.

6.2. Use of Hydroponics for Growth of Seedlings

It has been shown in this thesis that hydroponic solutions can be altered to provide only specific nutrients or forms to plants. These solutions can therefore be altered to provide optimal conditions for plant growth. Losses of fertilisers through processes such as leaching and volatilisation are also dramatically reduced, making the hydroponic system highly efficient. It may therefore be of interest to undertake further research into the viability of growing plantation seedling hydroponically rather than in soil. Hydroponics is now widely used for growing fresh produce such as lettuce and tomatoes, as it provides faster growth rates and healthier plants, where the quality of the produce can be relatively easily controlled in greenhouses.

If tree seedlings were initially grown hydroponically, the process of transplantation to soil systems would be of the most concern. During this process of transition, the plants will be going from an ideal system with free access to nutrients, to a soil system where the availability of nutrients may be limited. This situation is further exacerbated as the plant would require a time period to establish the root system in order to uptake nutrients. This may provide a heavy load on the soil system when plants are initially transplanted. The resilience of the hydroponically grown trees in stress conditions, such as droughts, would also need to be taken into account as is discussed in the next section.

6.3. Calcium Signalling in Drought Conditions

The hydroponics experiments initially set up for this thesis research were originally located in air-conditioned, temperature controlled greenhouses at the University of Western Sydney, Hawkesbury campus. This initial set of plants including the eucalyptus and slash pine were growing in the AN, UR, AS, CN, *AN and A*N fertiliser solutions as described in Chapter 3. There was however, some slight
differences between the fertiliser solutions make up when controlling the concentration of $^{15}$N in the fertilizer solution which could not be avoided. For example, the CN containing hydroponic solutions contained more calcium ions than the rest to maintain constant levels of other major nutrients.

On a hot summer day, the temperature of the greenhouse increased to about 58 °C (caused by a power failure), burning and killing almost all of the Eucalyptus plants, except, the plants with a slightly higher calcium level (Figure 6.1). The plants having a higher level of $\text{Ca}^{2+}$ in the hydroponic solution survived remarkably well and had only burnt new growth while the rest of the plant was green and healthy. Also, the plant that had burnt had no solutions left, whereas the CN plants had a full container of solutions still present.

![Figure 6.1: Eucalyptus plants after being subjected to temperatures over 58°C. The survived plants on the left had a slightly higher concentration of calcium applied than the dead plants on the right](image)

This serendipitous research outcome prompted us to further investigate the role of calcium ions as a strategy of survivability of these plants to stressful conditions. For this reason a UWS sustainability grant has been awarded in order to carry out further research to identify the critical links between calcium ions and plant’s ability in
meeting environmental challenges such as heat stress and drought conditions. The aims and objectives of the project are to investigate the role of Ca$^{2+}$ in high temperature conditions on the growth of native plant seedlings and how the ion uptake effect the cell wall composition and the stomatal opening in plant tissues grown in the absence and presence of supplemental Ca$^{2+}$ ions. While many have studied the effect of environmental conditions on calcium levels and signalling in plant cells, and *vice versa*, few studies have been done on the potential to modify fertilizers with supplemental calcium to enhance plants survivability in drought conditions.

### 6.4. Soil N composition in Close Proximity to Roots

It may be of interest to study the decomposition and mineralisation of N in soils in relation to the distance of the soil sample from plant roots. Most soils used in NMR spectroscopy studies are bulk soils taken from different layers of the soil horizon, however their relation to any plants growing in the region are not usually mentioned. There are many interactions between soil and plant roots, such as microbial activity and mycorrhiza associations. This thesis has also discussed in detail a plant’s ability to maintain root electroneutrality, leading to influxes and effluxes of nutrients and possible amino acid exudations. This is also coupled with changes in microbial populations and soil structure, such as aggregation, which are usually associated with plant roots. Therefore further studies of soils at different distances from the plant roots may help to better explain N cycling processes and any links to mycorrhiza associations helping the influxes and effluxes of nutrients that take place in the soil.

### 6.5. Decomposition of Insect Frass Containing Heterocyclic N

Further research on the role of insects in a plantation or natural forest ecosystem is needed. As discussed in chapter 5 of this thesis, insects can potentially have a major impact even at low infestation levels. Of particular interest would be the decomposition of insect frass on soils. It was found in this thesis that insect frass had substantial amounts of heterocyclic-N present, compared to eucalyptus tree foliage. This represents a change in what forms of N are being returned to the soil. Therefore,
further research into how this heterocyclic N decomposes, how it affects the microbes connected with the decomposition and the rate at which it is mineralised or incorporated into soil organic matter will provide a better understanding of the N cycling. This may also help determine other factors associated with defoliation of plants such as uptake of nutrients, the ability of the plant to recover from an insect attack minimising the environmental impacts associated with these insect infestations.
Figure A.1: Influx and efflux of (a) magnesium, (b) ammonium, and (c) potassium ions in hydroponic solutions containing slash pine seedlings. Uptake values are the average taken for the time period shown above each column and have been corrected for the number of plants present. Positive values represent influx into the roots, while negative values represent efflux out.
Figure A.2: Influx and efflux of (a) calcium, (b) chloride and (c) nitrate ions in hydroponic solutions containing slash pine seedling. Uptake values are the average taken for the time period shown above each column and have been corrected for the number of plants included in each solution. Positive values represent influx into the roots, while negative values represent efflux out.
Phosphate uptake forSlash Pine

17/3 - 20/3

17/3 - 20/3

17/3 - 20/3

17/3 - 20/3

20/3 - 24/3

20/3 - 24/3

20/3 - 24/3

20/3 - 24/3

24/3 - 28/3

24/3 - 28/3

24/3 - 28/3

24/3 - 28/3

24/3 - 28/3

24/3 - 28/3

24/3 - 28/3

24/3 - 28/3

28/3 - 3/4

28/3 - 3/4

28/3 - 3/4

28/3 - 3/4

28/3 - 3/4

28/3 - 3/4

28/3 - 3/4

28/3 - 3/4

3/4 - 7/4

3/4 - 7/4

3/4 - 7/4

3/4 - 7/4

3/4 - 7/4

3/4 - 7/4

3/4 - 7/4

3/4 - 7/4

7/4 - 10/4

7/4 - 10/4

7/4 - 10/4

7/4 - 10/4

7/4 - 10/4

7/4 - 10/4

7/4 - 10/4

7/4 - 10/4

-1.0

-0.5

0.0

0.5

1.0

1.5

2.0

2.5

3.0

3.5

4.0

AN

A*N

AS

UR

Fertilizer

Sulfate uptake for Slash Pine

Positive values represent influx into the roots, while negative values represent efflux out. Shown above each column and have been corrected for the number of plants present. Concentrations slash pine seedling. Uptake values are the average taken for the time period. Figure A.3: Influx and efflux of (a) phosphate and (b) sulfate ions in hydroponic solutions.
Figure A.4: Influx and efflux of a) magnesium, b) ammonium and c) potassium ions in hydroponic solutions containing eucalyptus seedlings. Uptake values are the average taken for the time period shown above each column, and have been corrected for the number of plants present. Positive values represent influx into the roots, while negative values represent efflux out.
Figure A.5: Influx and efflux of a) calcium, b) chloride and c) nitrate ions in hydroponic solutions containing eucalyptus seedlings. Uptake values are the average taken for the time period shown above each column and have been corrected for the number of plants present. Positive values represent influx into the roots, while negative values represent efflux out.
Figure A.6: Influx and efflux of a) phosphate and b) sulfate ions in hydroponic solutions containing eucalyptus seedling. Uptake values are the average taken for the time period shown above each column and have been corrected for the number of plants present. Positive values represent influx into the roots, while negative values represent efflux out.
Figure A.7: $^{13}$C NMR spectra of Slash Pine bulk needles samples, taken after harvesting from pines grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure A.8: $^{13}$C NMR spectra of Slash Pine new needles samples, taken after harvesting from pines grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.
Figure A.9: $^{13}$C NMR spectra of Slash Pine whole stem samples, taken after harvesting from pines grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure A.10: $^{13}$C NMR spectra of Slash Pine root samples, taken after harvesting from pines grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.
Figure A.11: $^{13}$C NMR spectra of Slash Pine bare stem samples, taken after harvesting from pines grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure A.12: $^{13}$C NMR spectra of Slash Pine bark samples, taken after harvesting from pines grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.
Figure A.13: $^{13}$C NMR spectra of Eucalyptus bulk leaves samples, taken after harvesting from eucalypts grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure A.14: $^{13}$C NMR spectra of Eucalyptus new leaves samples, taken while eucalypts were grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.
Figure A.15: $^{13}$C NMR spectra of *Eucalyptus* whole stem samples, taken after harvesting from eucalypts grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure A.16: $^{13}$C NMR spectra of *Eucalyptus* root samples, taken after harvesting from eucalypts grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.
Figure A.17: $^{13}$C NMR spectra of *Eucalyptus* are stem samples, taken after harvesting from eucalypts grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.

Figure A.18: $^{13}$C NMR spectra of *Eucalyptus* bark samples, taken after harvesting from eucalypts grown in a) UR, b) AS, c) AN and d) CN solutions. All spectra have been scaled in order to compare N composition.
Figure A.19: Hot water soluble anions found in SL, SM, SO, LO, and MO samples over 8 weeks. Graphs show results for a) nitrite, b) nitrate, c) phosphate, d) sulfate, e) flouride and f) chloride.
Figure A.20: Hot water soluble cations found in SL, SM, SO, LO, and MO samples over 8 weeks. Graphs show results for a) ammonium, b) potassium, c) calcium, d) magnesium and e) sodium.