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The role of organic nitrogen in the nutrition of *Eucalyptus*

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A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

Nitrogen availability commonly limits plant growth. Addition of N fertiliser alleviates N limitation and increases productivity but can have significant environmental and economic costs, providing incentives to optimise fertiliser application with accurate predictive tests to diagnose N limitation. Traditionally N limitation has been ascertained under the premise that plants are only capable of taking up inorganic nitrogen. We now suspect that organic nitrogen could also play a role in plant nutrition, but additional research is required before organic N can be used as the basis for diagnostic tests of N limitation.

The aim of this thesis is to increase our understanding of the role of organic N in nutrition of two plantation tree species, Eucalyptus nitens and Eucalyptus globulus, to determine a simple and robust method of indicating nitrogen limitation. Protease activity and total exchangeable amino acid concentration are strongly correlated with N-limitation in E. nitens forestry plantation soils. Although there is minimal seasonal variation in protease activity, we measured significant seasonal variation in the absolute and relative abundance of small (<250 Da) dissolved organic and extracted inorganic and organic nitrogen compounds of E. nitens plantations. Strong correlation between nitrogen limitation and the abundance of ammonium, nitrate and amino acids in soil extracts are consistent with a growing body of literature arguing plants can take up organic nitrogen compounds. Our results indicate depolymerisation (protease activity) is the rate limiting step of N availability.

Organic nitrogen is abundant in soil, taken up by plants and strongly related to N-limitation. Protease activity is a strong indicator of N-limitation with great potential as a reliable and ubiquitous indicator of N-limitation in soils.
DECLARATION

This is to certify that:

I. The thesis comprises only my original work towards the PhD except where indicated in the preface,

II. Due acknowledgement has been made in the text to all other material used,

III. The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

Jacquelyn Elizabeth Simpson, 12/12/2015
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GENERAL INTRODUCTION

Thesis background

Nitrogen cycling, forestry plantations and fertiliser use
Nitrogen (N) is essential for plant growth and therefore its availability is one of the major factors which limit plant growth rates (Aggangan et al. 1998). In forestry plantations, similarly to other industrial plantations and crop farms, considerable time, money and effort is invested in augmenting N availability. This is largely achieved through the addition of millions of tonnes of industrial fertilisers, in 2015, 115.1 million tonnes of N fertiliser was applied globally (FAO 2015). The volume of this fertiliser application has substantial environmental and economic consequences.

To accurately determine the N fertiliser requirements of plants we first need to understand what forms of N plants take up. Mounting evidence demonstrating the role of organic N (N$_{\text{ORG}}$) in plant nutrition warrants investigation into the dynamics of N$_{\text{ORG}}$ as an indicator of N limitation. This thesis examines the uptake of N$_{\text{ORG}}$ by Eucalyptus nitens and E. globulus and the role of proteolysis in plant N nutrition. E. nitens plantations in Tasmania have been analysed for soil N content and protease activity on a regular basis. The significance of correlations between these factors, growth rates, and fertiliser treatments are then used to establish a more accurate method of determining fertiliser requirements to maximise plantation productivity while minimising economic and environmental costs.

This general introduction will cover any background information required for the smooth progression through the remainder of the thesis. This includes definitions, explanations and statistics relating to forestry plantations, Eucalyptus species, N cycling, soil microbial biomass, fertilisers and protease activity.
N CYCLE

N is extremely important for plant growth and development as a component in chlorophyll, DNA, RNA, proteins and peptides (Tegeder and Rentsch 2010). Inadequate supply of N reduces rates of plant metabolism and growth. The terrestrial N cycle shows the inputs, outputs, N pools and chemical processes involved in the transformation of atmospheric N through both living and non-living components of an ecosystem (Figure 1).

The earth’s atmosphere is made up of 78% N and 20.09% oxygen as well as small quantities of argon (0.934%), carbon dioxide (0.0383%), other gases and water vapour. This seemingly abundant reservoir of N is inert hence not biologically available to the majority of organisms (Galloway and Cowling 2002).

N fixation

Although 78% of the earth’s atmosphere is N gas the vast majority of plants are not able to directly use this as a N source, instead they rely on the process of N fixation (Equation 1). N fixation requires high energy input to break the triple bond of N₂.

Means by which atmospheric N is fixed include;

- microbial fixation, for example by the free living organisms Diazitrophs and Azobacter, or by Rhizobium and Bradyrhizobium which live within root nodules of N-fixing plants (Rascio and La Rocca 2013);
- atmospheric events, such as lightening which produces more than ten million tonnes (10^{12} g) N each year (Vitousek et al. 1997); and
- industrially using the Haber–Bosch method to fix atmospheric N. Pollution and combustion from human activities also release nitrous oxides into the atmosphere. The N-cycle has been altered by anthropogenic N processing and industrial fertiliser use (Vitousek et al. 1997; Galloway et al. 2008; 2014)

Equation 1

\[ \text{N}_2 + 8\text{H}^+ + 8\text{e}^- \rightarrow 2\text{NH}_3 + \text{H}_2 \]
Figure 1. Summary of the terrestrial N cycle showing the flow of N (arrows) through the atmosphere, soil, plant, animals emphasising the major players and stages (boxes) and processes (light green italicised) involved in plant N uptake, in both N-fixing plants and non-N-fixing plants.
Depolymerisation

Depolymerisation an important step in the breakdown of soil organic matter (from waste and decay of plant, microbe and animal matter) to small N compounds (Equation 2) (Jones and Kielland 2002; Kohli et al. 2012). Depolymerisation is a relatively (compared to mineralisation) slow process with the transformation of proteins to amino acids taking an average of 80 days (Farrell et al. 2011; Jones and Kielland 2002). Numerous enzymes are involved in the proteolysis process, each with a specific purpose and target compound. For example, peptidases catalyse the breakdown of peptides into amino acids.

\[
\text{Equation 2}
\]

\[\text{Soil organic matter (large proteins, } > 1 \text{ kDa) } \rightarrow \text{ Oligo-peptides } \rightarrow \text{ Di and tri-peptides } \rightarrow \text{ amino acids (<1 kDa)}\]

We have focused on one component of depolymerisation, protease activity. Proteases are enzymes which cleave the peptide bonds of proteins and peptides and breakdown proteins to smaller peptides and amino acids (Kohli et al. 2012). There are four major classes of proteases;

- aspartic or fungal proteases;
- metallo proteases from bacterial sources and
- thiol and serine which are general classes of proteases.

Proteases are further classified according to substrate affinity, reaction mechanism and catalytic site affinity within a protein or peptide, such as endo-proteases and exo-proteases (Caldwell 2005).

Mineralisation

Mineralisation occurs both intra- and extracellularly in the soil, soil microbial biomass (SMB) and in plant cells (Schimel and Bennett 2004). We are only discussing extracellular soil mineralisation here as this is the process most directly related to N-availability, although other mineralisation mechanisms make measurement of N availability more complicated. Extracellular soil mineralisation is the process by which enzymes convert low molecular weight (LMW) N\text{ORG} compounds, such as amino acids and small peptides, to N\text{INORG} compounds. Mineralisation of amino acids
to $N_{\text{INORG}}$ compounds is rapid, with an average amino acid half-life of 2.5 hours in some soils (Farrell et al. 2011).

Ammonification is the process resulting in the formation of ammonium and ammonia ions from LMW $N_{\text{ORG}}$ compounds (Equation 3). Nitrification is the oxidation of these ammonium and ammonia ions to nitrites and nitrate (Equations 4-5).

$$\text{Equation 3}$$
$$(\text{Amino Acids} + \text{NH}_2) + \text{H}_2\text{O} + \text{H}^+ \rightarrow (\text{Amino Residues} + \text{OH}) + \text{NH}_4^+$$

$$\text{Equation 4}$$
$$2\text{NH}_3 + 3\text{O}_2 \rightarrow 2\text{NO}_2 + 2\text{H}^+ + 2\text{H}_2\text{O}$$

$$\text{Equation 5}$$
$$\text{NO}_2^- + \text{H}_2\text{O} \rightarrow \text{NO}_3^- + 2\text{H}^+ + 2\text{e}^-$$

**Plant and animal N uptake and N loss from soil ecosystem**

Small molecules of N are found in soil water, in which case it is often referred to as dissolved N (DN); and in soil conglomerates, where it is referred to as extractable N (EN). The flow of soil water carries DN throughout the soil to plant roots, soil organisms and microorganisms and eventually to creeks and rivers. Plant root cell membranes take up a range of N compounds directly from soil water, using transporter proteins. In some plant species, rhizobia and mycorrhizae facilitate N uptake in plant root cells (Berry et al. 2011).

Plants use N in tissue and organ development, such as chlorophyll and other photosynthesis components. Herbivorous animals consume plant tissues (leaves, stems), eventually returning N to the soil through death or waste production. The decomposition process continues with proteolysis of large peptides to smaller peptides and so forth.

Soil N losses occur largely through leaching, denitrification and volatilisation. Leaching occurs when DN flows through soil water to waterways, which in large quantities interferes with the N cycles of aquatic environments (Phillips and Burton 2005). Denitrification and volatilisation chemically alter the N and return it to the atmosphere. Approximately 1-3% of the N is lost to the atmosphere as $N_2$ as a result of volatilisation (Vitousek et al. 1997).
N AVAILABILITY AND UPTAKE

The majority of soil N is insoluble and not available for plant uptake. Of the DN, the greatest fraction is in high molecular weight (HMW) N$_{\text{ORG}}$ compounds, such as protein and peptides (Casals et al. 1995; Huang and Schoenau 1996, 1998; Kalbitz et al. 2000; Jones et al. 2004). These compounds are in the early stages of decomposition and not available for plant uptake. The LMW fraction of N is the second most abundant N fraction (Hill et al. 2011; 2012; Jones et al. 2004; Schimel and Bennett 2004). The LMW N fraction includes DN and EN compounds <1 kDa as N$_{\text{ORG}}$, such as amino acids, osmolytes and some small di- and tri-peptides, and N$_{\text{INGORG}}$ compounds, ammonium and nitrate, many of which are available for plant uptake (Chapin et al. 1993; Hill et al. 2011, 2012; Jones et al. 2004; Näsholm et al. 2009; Schimel and Bennett 2004). Chapter 1 discusses N soil chemistry in more depth.

Traditional model of N nutrition

The traditional model of N nutrition, which has been accepted for over a century, states that plants only take up N$_{\text{INORG}}$, ammonium and nitrate (Schimel and Bennett 2004). In this model (Figure 2) the rate at which N becomes available for plant uptake is limited by the rate of net mineralisation (Vitousek and Melillo 1979) and plants compete poorly for N with the SMB (Schimel and Bennett 2004). Poor plant competitiveness is referred to as the microbial bottleneck of plant N nutrition and the N-limitation of systems is often implied by measurements of net mineralisation and pools of N$_{\text{INORG}}$ (Keeney and Bremner 1966; Smethurst 2000; Schimel and Bennett 2004).

Figure 2. Traditional model of N nutrition. Plants are only able to take up N$_{\text{INORG}}$ and net mineralisation (yellow arrow) is the rate limiting step. Adapted from (Schimel and Bennett 2004).
New model of N nutrition

Research throughout the past two decades established that the ability to take up organic sources of N, mainly amino acids, is widespread among plant species (Jones et al. 2004; Jämtgård et al. 2008; Näsholm et al. 2009; Warren 2009). This has challenged the traditional model of plant N nutrition. In the new model of plant N nutrition, in which $N_{\text{ORG}}$ pool is available for uptake by both plants and SMB, the rate of N-availability is limited by depolymerisation (Figure 3) (Schimel and Bennett 2004).

![Figure 3. New model of plant N nutrition. This shows the incorporation of $N_{\text{ORG}}$ as a N source (green arrows) as well as the role of depolymerisation (yellow arrow) as the rate limiting step in N-availability. Adapted from (Schimel and Bennett 2004).]

The capacity for uptake of $N_{\text{ORG}}$ compounds has been demonstrated in a wide range of plants. For example, uptake of amino acids has been demonstrated in barley (Jämtgård et al. 2008), wheat (Näsholm et al. 2001), Arabidopsis (Paungfoo-Lonhienne et al. 2008) and sugarcane (Vinall et al. 2012) and species of Eucalyptus (Warren 2009; Warren 2013). Field-based experiments have provided insight into the uptake of $N_{\text{ORG}}$ in conditions which are realistic and representative of normal growth conditions of plants (Schimel and Chapin 1996; Näsholm et al. 1998; Näsholm et al. 2009).
FORESTRY PLANTATIONS

Forestry plantations are stands of trees, either native or exotic species, which have been intentionally and purposefully planted in evenly spaced rows as either seeds or seedlings (ABARES 2013). In 2000 it was estimated that there were 187 million hectares of forestry plantations globally (FAO, 2001). This equates to approximately 5% of all global forests and it is expected that each year plantation land area will increase by 2.2% (Aggangan et al. 1998; Boland et al. 2006). The largest plantation land area can be found in China (24% total global forestry plantation land area), India (17%), Russia (9%) and America (9%) (Carle et al. 2002).

Plantations are essential to human life

Wood products play a central role in human activity and are in high demand. This demand is only set to rise with the continuously increasing world population (Figure 4). Presently there are no viable alternatives to using trees to meet this ever growing demand.

Plantation wood is used in building and construction, from shelters and huts in villages, to major structures in the world’s largest cities. Wood is also used for furniture production, fences, in the building of boats, bridges and tools and in processes by which energy is supplied to homes from fossil fuels (Shmulsky and Jones 2011). In many countries wood is the primary source of fuel for cooking and heating. Sustainably sourced wood is considered to be a carbon neutral source of energy and heat when used as a fossil fuel replacement (Kollert 2010).
Figure 4. World population, from 1950 to 2005 with projections to 2050. Showing low (yellow) predictions if population growth decreases from current rate, medium (red) if population rate increase is maintained at today’s rate and high (blue) if there in an increase in population growth rate from the current trends (Institute 2012). The increase in human population is a driving factor behind increasing demand for wood and wood products.

The Food and Agriculture Organisation (FAO) estimates that wood is used at an average rate of 0.6 m$^3$ per person annually. This figure is as high as 2.03 m$^3$ (USA) in more developed countries, with the average Australian consuming 1 m$^3$ wood annually (Shmulsky and Jones 2011). The predicted increase in global population (Figure 4) will require a further two billion cubic meters of wood every year to meet demands (Shmulsky and Jones 2011).

**Hardwood and softwood plantations**

Forestry plantations are classified as being either hardwood (angiosperms) or softwood (gymnosperms) depending on the species of trees planted. Hardwood trees generally produce wood with a firmer structure than softwood trees due to additional support cells and fibers (Desch 1996).

Hardwood products are used for furniture, flooring, construction materials, boat building, pulpwood, tools and wood fuel (Gerrand et al. 1993; Desch 1996; McKenzie et al. 2003; Boland et al. 2006). Softwood plantations supply wood products for structural building and framework, paneling and flooring as well as paper pulp. In Australia hardwood plantation wood is largely used for pulpwood (Lesslie and Mewett 2013).
Australian forestry plantations

In 2013 there were 149 million hectares (ha) of forests in Australia of which approximately 1%, 1.82 million ha, was plantation forests, a 12% increase on 2003 plantation area (ABARES 2013). The rate of conversion of existing forests into plantation forests declined between 2003 and 2008 (ABARES 2013).

Globally about 80% of forests are softwood forests. Australia has increased hardwood plantation establishment (600% since 1995) and the ratio of hard to soft wood plantations is now closer to 50:50. There was a decrease (14%) in timber harvesting from native forests between 2003 and 2008 and forestry plantations provide 66% of the current wood requirements of Australia (ABARES 2013).

Forestry plantations in Tasmania

This thesis has focused on Tasmanian plantations that are either managed by Forestry Tasmania or through joint-venture partnerships of Forestry Tasmania with other companies. In Tasmania, 13% of the land area is dedicated to forestry plantations, which is the largest percentage of plantation area compared to land area of any Australian state or territory (Gavran and Parsons 2010). The majority of Tasmanian forestry plantations are Pinus radiata (softwood plantations) and E. nitens or E. globulus (hardwood plantations). E. nitens are effectively grown at high altitudes and can tolerate colder climates and frost (Smethurst et al. 2004).

Eucalyptus trees

Seven hundred of the eight hundred species of Eucalyptus are native to, and grow exclusively in, Australia. Eucalyptus species are mostly tall, straight, flowering trees that belong to the Myrtaceae family. The flowers grow in clusters and their size depends on the mechanisms of pollination (e.g. wind, insects, and animals), small flowers primarily attract insects and large flowers primarily attract birds and small mammals (ANPSA 2009).

Eucalyptus trees grow in closed, open, woodland (80%, 50-80% and 20-80% crown cover respectively) and mallee forests. Tree heights range from 5-15 m (e.g. E. spathulata), with multiple trunks, to trees which grow over 60 m tall, with long straight trunks (E. regnans) (Slee et al. 2006). Eucalyptus species dominate many habitats in Australia, largely due to adaptations, such as mechanisms allowing rapid recovery following fires to outcompete other plants in the area (Smethurst et al. 2004).
**Eucalyptus** trees are found in both temperate and more extreme environments. For example, *E. pauciflora* has adapted to the cold environment of the mountainous regions of south eastern Australia, such as Mount Hotham in Victoria and Mount Kosciusko in New South Wales (NSW). *E. pauciflora* survives in these alpine climates with features such as crooked trunk and very oily leaves as protection from harsh winter weather. Conversely, species such as *E. loxophleba* have adapted to the dry, acidic and highly saline soils of the plains near Perth in Western Australia (WA) with multiple trunks and altered, shallow root systems (Boland *et al.* 2006; Slee *et al.* 2006).

**Eucalyptus in forestry plantations**

*Eucalyptus* species constitute 95% of hardwood plantation trees in Australia, Acacia and Corymbia species make up the other 5%. Specific adaptations of *Eucalyptus* species determine their suitability to different soils, topography and local climates and are accounted for during forestry plantation establishment (Smethurst *et al.* 2004). *E. globulus* plantations account for half of the hardwood plantations and *E. nitens* (Figure 6) account for one quarter (ABARES 2008; 2013). Other common forestry plantation species include *E. pilularis*, *E. grandis*, *E. regnans*, *E. cloeziana* or *E. dunnii*.

Figure 1. *E. nitens* forestry plantation in north east Tasmania, Australia. This photograph was taken in spring 2012, 1 year after plantation establishment.
Fertiliser use

Fertilisers are sources of nutrients that are added to increase the quantity of plant-available nutrients. Fertilisers are applied to increase plant growth rates, productivity and ultimately crop profitability. For example, heavy applications of fertiliser are often used to overcome gross nutrient deficiencies in agricultural and plantation soils. Selecting the most appropriate fertiliser depends on soil structure and composition, budget, application method, potential loss and specific nutrient deficiencies.

In the present work only N fertilisers will be discussed. An estimated average of 50-70% of N added to soils is lost to the atmosphere or via leaching, as a result of incomplete capture and conversion of N to unavailable N sources (Hodge et al. 2000). As discussed earlier, N loss has serious repercussions for soil and water quality (Phillips and Burton 2005).

Industrial fertiliser production is an expensive, high energy process. During the process of industrial fixation large quantities of nitrous oxides (NO\textsubscript{x}) are released into the atmosphere, contributing to global warming (Masclaux-Daubresse et al. 2010).

Nutrient management practices by Forestry Tasmania

Using *E. nitens* plantations and plantation management strategies employed by Forestry Tasmania as examples this section of the introduction will outline current nutrient management practices, the issues with fertiliser use, N-limitation and eventually lead to a description of the aims of this thesis.

Plantation sites are carefully selected to maximise productivity while reducing the impact on the environment. Forestry plantation sites are subjected to intense mechanical processes, including maintenance and logging. The ability to tolerate mechanical processes is predicted by topography and soil profiles. Historical climate data and soil fertility measurements are used in selecting potentially suitable and productive sites. Today, new plantations are established on land previously used for forestry or agricultural crops, minimising the reduction in natural forests. Prior to establishment/planting all sites are cleared of remnants of previous crops, burned and sprayed with herbicides (Grant et al. 1995).
All plantations managed by Forestry Tasmania are fertilised either at the time of planting or within three to four weeks of planting. There are two main types of fertiliser used by Forestry Tasmania, Di-Ammonium Phosphate (DAP) or Basacote (refer to fertiliser usage section below). Following plantation establishment, one of several management strategies are employed on an ongoing basis, depending on the nutrition requirements and productivity of the specific plantation site. Nutritionally poor plantations, that have lower productivity, are fertilised one year after establishment and then five years later on the poorest sites. In some instances, gross nutrient deficiencies are overcome through the first fertiliser application, negating the need for further application (Grant et al. 1995).

**Significance of accurately overcoming N-limitation**

The productivity of forestry and agricultural crops is the primary concern for crop managers. These crops are often planted in soils with suboptimal N-availability (N-limitation), which is insufficient to achieve maximum productivity (Vitousek et al. 1997). N-limitation is one of the greatest limiting factors to plant productivity. The dynamics of the soil N-cycles, at many forestry and agricultural sites, have been altered by the scale on which N fertilisers are applied to overcome N-limitation (Vitousek et al. 1997; Galloway et al. 2004; Galloway et al. 2008; Galloway et al. 2014). The quantity of N applied, in fertilisers, globally far exceeds the quantity of any other nutrient applied (FAOStat 2014) (Figure 7).

![Figure 2. Fertiliser application rates from 2002 to 2009. This shows that N is applied in greater quantity than total P or K which are the 2nd and 3rd most applied nutrients (FAOStat 2014).](image-url)
The traditional model of N nutrition (Figure 2) has only been challenged relatively recently, with increasing research into the role of N\textsubscript{ORG} in plant nutrition and in the N-cycle (Christou \textit{et al.} 2005). N-limitation is commonly diagnosed using measurements based on N\textsubscript{INORG} pools and other methods that do not account for the possible uptake of N\textsubscript{ORG} (Keeney and Bremner 1966; Smethurst 2000; Gonçalves \textit{et al.} 2004; Moroni \textit{et al.} 2004). N-limitation is used to determine the N fertiliser requirements of sites, inaccuracies therefore result in excessive negative environmental and economic impacts.

Past trends have shown that fertiliser application rates, driven by a need to increase production, tend to increase in line with population growth (Figure 8). To meet increased demand for wood and wood products, through forestry plantations, sites are being selected that are increasingly less suitable for tree productivity (Angell \textit{et al.} 2011). The importance of managing N limitation and N fertiliser application will only increase as global population, therefore demand for forestry and agricultural products, increases.

![Figure 3](image)

Figure 3. Global fertiliser use and population from 1950 to 2011, with population predictions to 2020. This figure highlights the relationship between fertiliser use and human population, as demand for plant (including forestry) products increases.
Call for a new indicator of N-limitation

To reduce the negative environmental and economic consequences associated with N fertiliser application we need to more accurately determine fertiliser requirements. N fertiliser requirements are determined based on N-limitation, and to increase the accuracy of measuring N-limitation we need a new method that accounts for all plant available N compounds. A new indicator is required that is universally accurate, irrespective of soil type, topography, water and nutrient management, altitude, crop species and environmental and climatic conditions. An ideal indicator of N-limitation would be well supported by sound scientific theory and proven correlations with N-limitation, while also being simple and rapid to conduct.
AIMS

The overall aim of this project is to understand the role of $N_{\text{ORG}}$ in the nutrition of *E. nitens* and *E. globulus* trees to establish a more accurate means of estimating N fertiliser requirements. Based on the role of depolymerisation in the breakdown of soil N compounds, a new method of indicating N-limitation is being put forward in this thesis. The first two parts of the thesis (Chapters 1 and 2) provide the science that underpins the proposed indicator that is tested in the final experimental chapter (Chapter 3).

**Aim 1**

Asses the $N_{\text{ORG}}$ compounds in soils, how these vary seasonally and with site productivity. To achieve this aim the LMW DON compounds in soils of *E. nitens* forestry plantations in Tasmania were profiled using soil water samples analysed using capillary electrophoresis mass spectrometry (Chapter 1). Soil water samples were collected in summer, autumn, winter and spring to assess the influence of seasonal variation on LMW DON compound abundance. Samples were also collected from two forestry plantation sites with varying productivity (low productivity and high productivity) to determine the variation due to site productivity.

**Aim 2**

Examine the range of amino acids and small peptides taken up by *E. nitens* and *E. globulus* under different experimental conditions. *E. nitens* and *E. globulus* seeds, seedlings and trees were supplied with a range of amino acids and small peptides to investigate $N_{\text{ORG}}$ uptake capabilities (Chapter 2). Multiple methods (laboratory based, using solution and agar as media, and field based) were used to obtain a more comprehensive understanding of the uptake capabilities of plants.

**Aim 3**

Determine an accurate indicator of N-limitation. The relationships between plant growth or plant response to fertiliser addition and $N_{\text{ORG}}$, depolymerisation (protease activity) or $N_{\text{INORG}}$ pool concentrations were assessed. The overall aim was to identify the most reliable indicator of N-limitation.
REFERENCES LIST


Warren, C. R. (2013) Quaternary ammonium compounds can be abundant in some soils and are taken up as intact molecules by plants. New Phytologist 198(2): 476-485.
CHAPTER 1

Exploring the relationship between soil nitrogen composition and variations due to seasons or site productivity.
ABSTRACT

A recent surge of evidence supporting organic nitrogen (N) as a potentially significant component of plant N nutrition warrants further investigation into which compounds are present in the soil and potentially available for plant uptake. The present study analysed the extractable and dissolved N compounds of two Eucalyptus nitens plantations of contrasting N availability, one high and one low productivity site. We analysed the low molecular weight dissolved organic N (LMW DON) composition of soil water samples using CE-MS. Using colorimetric microplate methods we measured the concentration of exchangeable N in soil extracts (total amino acids, ammonium and nitrate). From these analyses, we investigated the variation in absolute and relative abundance of exchangeable and dissolved N compounds between seasons and between the two sites. (n=2-5 replicates. site⁻¹. season⁻¹. 4 seasons).

The most abundant compounds were protein amino acids (particularly Gln and Glu) followed by quaternary ammonium compounds (namely choline, carnitine and betaine). The absolute abundance of LMW DON compounds increased from summer to winter (e.g. concentration of Gln increased from 5 to 35 μmoles. L⁻¹ soil solution from summer to winter). Although ammonium and nitrate dominated the exchangeable N, the relative abundance of total amino acids in soil extracts peaked during winter. Dissolved and exchangeable organic N was relatively more abundant on the strongly N-limited site than the weakly N-limited site. The DON compounds were highly variable; however, no pattern emerged based on compound class, molecular weight or amino acid properties. The large amounts of exchangeable and dissolved organic N in soils of E. nitens plantations may be representative of a pool of plant available N.
INTRODUCTION

We have known for a century that organic nitrogen (N\textsubscript{ORG}) compounds are abundant in soils (Hutchinson and Miller 1912; Lathrop 1917), but it wasn’t until the 1980s that research into the role of N\textsubscript{ORG} in plant nutrition intensified (Abuarghub and Read 1988; Farrell \textit{et al.} 2013; Hill \textit{et al.} 2011, 2012; Inselsbacher and Näsholm 2012; Jämtgård \textit{et al.} 2010; Kielland 1995). Nowadays it is accepted that N\textsubscript{ORG} compounds are potentially significant sources of N available for plant uptake (Näsholm \textit{et al.} 2009; Schimel and Bennett 2004), but we still know very little about the compounds that comprise the pool of N\textsubscript{ORG}.

Studies characterizing size-classes of N\textsubscript{ORG} have identified that there are large differences in abundance and metabolism of size classes. High molecular weight (HMW) dissolved N\textsubscript{ORG} (DON) compounds, peptides and proteins >1 kDa, are the most abundant size-class of N\textsubscript{ORG} in soils (Jones \textit{et al.} 2004, Casals \textit{et al.} 1995; Huang and Schoenau 1996; 1998; Kalbitz \textit{et al.} 2000). The HMW N\textsubscript{ORG} turns over slowly and is not available for plant uptake (Farrell \textit{et al.} 2011). In contrast, the low molecular weight (LMW, <1 kDa) DON pool is smaller but turned over rapidly and is potentially available for plant uptake (Farrell \textit{et al.} 2011; Jones \textit{et al.} 2004).

Knowing size classes is useful, but understanding the specific compounds in soils is required to understand soil N availability because plants take up some, but not all, LMW DON compounds. Studies on N fluxes and N uptake ought to focus on those LMW DON compounds that are most abundant to determine the significance of these to plant nutrition. At present two amino acids, glycine and alanine, have been the focus of many studies determining the quantitative significance of amino acids to N nutrition (Jämtgård \textit{et al.} 2008; Jones \textit{et al.} 2005; Persson and Näsholm 2001; Vinall \textit{et al.} 2012; Warren 2009), but it seems unlikely these two compounds can faithfully represent the chemical diversity of LMW DON. Clearly, characterisation of the pool of LMW DON is required to help target studies of N uptake.

Studies have indeed revealed that the pool of LMW DON is chemically complex. The LMW DON consists of protein amino acids and peptides <1 kDa (Farrell \textit{et al.} 2013; Hill \textit{et al.} 2011, 2012; Inselsbacher and Näsholm 2012; Jämtgård \textit{et al.} 2010; Kielland 1994; 1995; Warren 2009; 2013a). Many studies have focused solely on protein amino acids and/or peptides, but LMW DON contains a broad suite of other
compounds including non-protein amino acids, quaternary ammonium compounds and nucleic acids (Aseeva et al. 1978; De and Mukherjee 1951; Schreiner 1912; Sowden 1958; Warren 2013c).

For perennial plants, understanding how soil N composition varies seasonally will provide an enhanced understanding of plant N availability. We already know that the relative and absolute abundance of LMW DON compounds can vary among seasons (Falkengren-Grerup et al. 2000; Farrell et al. 2014b; Kielland 1995; Warren and Taranto 2010; Werdin-Pfisterer et al. 2009). Seasonal variations in composition of LMW DON probably relate to turnover of the soil microbial biomass (SMB), production of metabolic byproducts and plant-microbial competition (Buyer and Sasser 2012).

The relative abundance of N compounds varies between sites of high and low productivity in arctic tundra, boreal, grassland and forest soils (Farrell et al. 2014a; Ros et al. 2009; Rothstein 2009). Strongly N limited, low productivity sites, have high concentrations of amino acids relative to ammonium and nitrate (Weintraub and Schimel 2005). Conversely highly productive have relatively high nitrate concentrations and low amino acid concentrations (Rothstein 2009). Variation in the abundance of soil compounds probably relates to variation in microbial activity and turnover. By investigating the abundance of LMW DON compounds and the variation in composition between sites, we may be able to increase our understanding of the drivers of N-limitation in soils.

Compounds are generally primarily localised to either the exchangeable N (EN) or the dissolved N (DN) fractions of soil. Therefore measuring both EN and DN provides complimentary information regarding the potential bioavailability of N compounds in soil and soil water (Laudicina et al. 2013; Ros 2012). For example, in boreal forest soils the DN fraction is dominated by amino acids and the EN by ammonium (Inselsbacher and Näsholm 2012). The constituents of both EN and DN require further analysis.

This study aimed to examine the seasonal variation in LMW DON pools at two forestry plantation sites of E. nitens with contrasting productivity (high vs. low productivity). Seasonal variation was analysed using soil (EN) and soil water (DN) samples collected during summer, autumn, winter and spring. We hypothesised that
the concentration of both total DON and EN would fluctuate throughout the year, peaking prior to the growing season when in low demand by dormant organisms (during winter). We anticipated that amino acids would dominate the EN and $N_{\text{ORG}}$ compounds dominate the DN at the low productivity site, whereas at the high productivity site we expected ammonium to be the dominant EN.
METHODS

Site selection

Nine sites were planted with *E. nitens* at 1100 stems per hectare in spring 2011, and from these we selected two forestry sites representing productivity extremes, low productivity (LP) and high productivity (HP). HP and LP were chosen based on site history and early measurements of N concentrations. The LP and HP sites are described in table 1. Site LP and HP are in relatively close proximity and experience similar climatic conditions, such as rainfall and temperature (Figure 1) (BOM 2014).

Table 1. Field site details. Information for the two field sites of *E. nitens* including geology, latitude, longitude and previous rotation. total N (TN), total P (TP) and organic C (OrgC) (%) were measured in bulked soil samples collected from 5 replicate control plots in October 2012 and analysed by SESL Laboratory (Thornleigh, NSW).

<table>
<thead>
<tr>
<th>Site</th>
<th>Geology</th>
<th>Latitude (S)</th>
<th>Longitude (E)</th>
<th>TN (%)</th>
<th>TP (%)</th>
<th>Org C (%)</th>
<th>Previous Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP</td>
<td>Devonian granite</td>
<td>41°13'13&quot;</td>
<td>147°51'41&quot;</td>
<td>0.48</td>
<td>0.05</td>
<td>10.5</td>
<td><em>Eucalyptus</em></td>
</tr>
<tr>
<td>HP</td>
<td>Ordovician Talus</td>
<td>41°12'36&quot;</td>
<td>147°17'40&quot;</td>
<td>0.25</td>
<td>0.02</td>
<td>4.1</td>
<td><em>Pinus</em></td>
</tr>
</tbody>
</table>

At HP and LP five replicate ten-tree plots (5 replicates x 10 tree plots. site⁻¹) were established for Di–Ammonium Phosphate (DAP) fertiliser treatments (applied 100g. seedling⁻¹) and unfertilised controls.
Site N-limitation indicators

Site N-limitation was indicated based on growth response to fertiliser, as has previously been described in chapter 3. Briefly, tree height was measured on all trees in each of the replicate plots (n=10 trees. plot⁻¹ x 5 plots. treatment⁻¹ x control⁻¹). Trees that had evidently suffered due to external forces were omitted from the study (for example, those that were damaged because of wildlife browsing). The mean height of the trees for each plot and site was determined. The mean tree height at each site of fertilised trees, as a percentage difference to the height of the control trees, was used to indicate N-limitation.

Soil sample collection and extraction

Samples were collected every 4-8 weeks using either an auger or trowel from the top 10-15 cm of soil, within a 20 cm radius of trees on control plots. For each of the five replicate ten tree control plots (n=5 replicates. site⁻¹) 5 to 7 samples of soil were bulked and mixed in a bucket. Each composite sample was then sieved (<5 mm) and 300 g was collected in zip lock bags. Bags of soil were kept cold in an esky until they were stored in the dark in a cool room (4°C) at the University of Sydney for up to two weeks prior to extraction. This process has been described in Chapter 3. Soil samples were extracted using 1M KCl at a ratio of 1 g soil: 8 mL KCl, these were shaken at 100 rpm for 90 mins at 21°C before being centrifuged (3200 g, 10 min) and filtered (Whatman #1 Filter paper).
Soil solution collection

Soil water samplers (1905 Slim Tube Sampler, Soil Moisture Equipment Corp. Santa Barbara, CA) were used to collect soil water samples from one of the control replicates at each of the sites. Soil water samplers were installed into 10-15 cm deep holes in the soil within 20-25 cm of 5 trees on control (unfertilised) E. nitens plots. This was done in September of 2011, with the first of the samples collected a month later. Vacuum pressure (80-90 kPa) was applied using a vacuum hand pump (2005G2, Soil Moisture Equipment Corp. Santa Barbara, CA) and the clamping ring placed over the external extraction tube on the top of the lysimeter. These were left evacuated to maintain adhesive tension around the ceramic cup between the monthly field trips. Tubing from the lysimeter to the vacuum pump and 50 mL screw capped vials was used to collect the water each month, from with a subset of samples was analysed for each season. The vacuum was reapplied after each sampling event. Vials were placed on ice in an esky when in transit, or in freezer (-21°C) overnight and when stationary. Once returned to the laboratory samples were stored in -80°C freezer until analysis.

Ammonium, nitrate and amino acid concentrations

KCl extracts and soil water samples were analysed for ammonium, nitrate and total amino acid concentrations within 2-3 weeks of collection. These were measured using a microplate reader (Biotek Synergy 2 multi-mode, Winooski, USA) according to established methods for ammonium (Baethgen and Alley 1989), nitrate (Miranda et al. 2001) and amino acids (Moore and Stein 1954).

Soil solution analysis using CE-MS

Soil water samples were prepared and analysed as described previously (Warren 2013b) Soil water samples were concentrated by evaporation under reduced pressure (Vacufuge, Eppendorf, Hamburg, Germany). These were dissolved in 25% (v/v) acetonitrile that contained an internal standard (0.4 μg mL⁻¹ methionine sulfone) and 100 mM ammonium formate (pH 9) in 200 μL polypropylene PCR tubes (PCR-02-NC, Axygen Scientific, Union City, USA). Samples were placed in the capillary electrophoresis instrument autosampler (2-4 °C) and analysed within 6 hours.
Untargeted profiling of N\textsubscript{ORG} compound in soil water samples was conducted using capillary electrophoresis–mass spectrometry (CE-MS) the method of which has been previously described (Warren 2013a; 2013b; 2014b). CE-MS was performed with a capillary electrophoresis system (P/ACE MDQ, Beckman–Coulter, Fullerton, USA) equipped with a bare fused silica capillary (50 μm i.d. × 100 cm long, Polymicro Technologies, Phoenix, USA) interfaced via a co-axial sheath-flow sprayer (G1607A, Agilent, Waldbronn, Germany) to an ion trap mass spectrometer (AmaZon SL, Bruker Daltonics, Bremen, Germany). Sheath liquid of 50% (v/v) methanol with 0.1% (v/v) formic acid was delivered at 4 μL. min\(^{-1}\). The mass spectrometer was set to scan a range of 50–255 m/z in enhanced resolution mode (8100 μ/s), with ion source and ion accumulation parameters as described previously (Warren 2013a; 2013b; 2014b). Samples were injected by pressure (3 psi for 30 s) and separated with an electrolyte of 2 M formic acid with 20% (v/v) methanol under 30 kV positive polarity (Warren 2013a).

**Data analysis**

Compound identification and quantification was conducted as described previously (Warren 2013a; 2014b) based on based on comparison of migration times, [M+H]\(^+\), MS\(^2\) and MS\(^3\) with 75 authentic standards run under the same conditions on the same instrument (Warren 2013a).

Quantification of compounds was conducted in Microsoft Excel based on the internal standard methionine sulfone. The contribution of each LMW DON compound class (i.e. amino acids, quaternary ammonium compounds, amines and other) to the N\textsubscript{ORG} pool was determined by accounting for the number of N atoms per molecule, thus acknowledging the potential for compounds yet to be identified to contribute to the LMW DON pool.

Multivariate analysis was performed on the raw data, following Pareto scaling and log transformation, by way of Principle Component Analysis (PCA) in SIMCA-P+ (Umetrics, Sweden) to determine the relationships between the components. 93 compounds were analysed, however only those which were detectable in at least 50% of the samples were analysed.
RESULTS

Site productivity

20 months after site establishment the average height of control (unfertilised) trees on site HP was 4.5 m and on site LP was 1.5 m (Figure 2a). Fertilised trees at site LP grew 46% taller than the unfertilised trees at the same site, which is significantly greater than the 4.5% increase measured on the DAP fertilised trees at site HP (Figure 2b).

![Graph showing tree growth and response to fertiliser treatment.](image)

Figure 2. Tree growth and response to fertiliser treatment. a) Mean *E. nitens* height for sites LP (1.5 m) and HP (4.5 m). b) Mean percentage increase in tree height of DAP fertilised trees greater than mean control tree height. Error bars represent the standard deviation between plots (t=20 months after site establishment, n=5 replicate plots. site⁻¹).
Seasonally averaged compound abundance of LMW DN in soil solution

Amino acids contributed the most N to the LMW DON pool on both LP and HP (42-44%, site LP and HP respectively, Figure 3). Quaternary ammonium compounds contributed 28-30% N (LP, HP respectively). On site HP amines and other compounds were equal contributors (13%), while amines contributed twice as many N molecules per compound as other compounds on site LP (20% and 10% respectively). Other compounds include uroacetate, ectoine, nicotinic acid and other nucleosides, pyrimadines, azoles, amino acid derivatives and unknown compounds.

![Figure 3. Abundance of classes of N\textsubscript{ORG} compounds present in soil water samples. This is a mean of the quantity of detectable compounds each across the 4 seasons and adjusted for N atoms in molecules and the number of molecules in each class of LMW DON. (n=3-5 samples. season\textsuperscript{-1}. site\textsuperscript{-1})](image)

The amino acid class consisted of 20 protein amino acids, 12 non-protein amino acids and 3 peptides. Of this, protein amino acids accounted for 86-95% of the amino acid class on site LP and 77-97% on site HP.

Thirteen of the fifteen most abundant N\textsubscript{ORG} molecules were amino acids, of which ten were proteinaceous (Figure 4a). The most consistently abundant compound was Glu followed by Gln, Asn, Leu, choline, carnitine, Ala and so on as shown in Figure 4b.
Figure 4. Fifteen most abundant DON compounds at site HP and LP as mean value of 4 seasons. a (figure) shows the absolute abundance of each compound (µmol. L⁻¹) and b (table) summarises the percentage contribution (%) to the 15 most abundant compounds (averaged across all seasons) of each NORG compound. Amino acid names are as per common 3 letter symbols and GABA for gamma aminobutyric acid.

Seasonal variation in exchangeable and dissolved N concentrations

Ammonium, nitrate and amino acid concentrations of 1M KCl soil extracts were greater in all seasons at site HP than site LP. Amino acid concentrations peaked during winter (72 µmol. g DW soil⁻¹ at site HP, 31 µmol. g DW soil⁻¹ at LP), ammonium during Autumn (104 µmol. g DW soil⁻¹ HP, 52 µmol. g DW soil⁻¹ LP) and nitrate on site HP during autumn (52 µmol. g DW soil⁻¹) and LP during winter (15 µmol. g DW soil⁻¹) (Figure 5a).

The DN fraction constituted a mix of ammonium, nitrate and amino acids (i.e. no consistently dominant compound). There was also a greater total N concentration (for the measured compounds) at site HP than at site LP, which was also the case with exchangeable N concentration. Due to the limited quantity of soil water collected...
and used for analysing compound abundance (following section) only samples collected during winter and summer were analysed colorimetrically. Although there are fewer sampling times to compare (i.e. two versus four seasons) the seasonal variation in relative abundance of amino acids, ammonium and nitrate was smaller for dissolved N (Figure 5b) than exchangeable N (Figure 5a).

Figure 5. Seasonal variation in the concentration of a) exchangeable and b) dissolved ammonium, nitrate and amino acids in soil extracts from low and high productivity *E. nitens* forest plantation sites.
Seasonal variation in LMW DON pool composition

The absolute concentration (Figure 6a) and number of compounds detectable in the soil water samples (Figure 6b) gradually increased from summer (number of compounds=16 at site HP and 26.4 at LP) to winter (61.2 HP, 58 LP) and reduced again in spring (Figure 6).

Amino acids were the most abundant class of LMW DON in soil water of both sites throughout all seasons (Figure 7a). Non-polar amino acids were the most abundant amino acids during summer, autumn and spring on both site HP and site LP, during winter there was an increase in the abundance of acidic amino acid (Figure 7b).
Figure 7. Comparative analysis of abundance of N compounds in soil solution. a) the relative abundance (% of total measured N\textsubscript{ORG} compounds) of amines, amino acids, quaternary ammonium compounds and other compounds in soil solution samples from high and low productivity sites. b) the relative abundance (% of total amino acid concentrations) of acidic, basic, non-polar and polar protein amino acids in soil water samples.

There were similarities in the seasonal changes at both LP and HP. There was an increase in the relative abundance of amino acids during winter and of amines during summer (Figure 7a). There was an increase in the relative abundance of acidic amino acids at both LP and HP during winter (Figure 7b).

The DON profile of the soil water changed each season as shown by the variation in the top ten most abundant compounds during each season at each of the sites (Figure 8) and by the changes in concentration and quantity of compounds, which peaked in winter samples (Figure 6).
Figure 8. Relative abundance of the top ten most abundant small N\textsubscript{ORG} compounds measured in soil water samples from site HP (right) and LP (left) across the seasons. Common 3-4 letter abbreviations have been used for amino acids.
Figure 10. Score plot for first two principle components, PC1 (climatic variance) and PC2 (site productivity) of the detectable levels of N\textsubscript{ORG} compounds in soil water samples from two forestry plantation sites. The labels for each point show the site – replicate # - season (replicate) where site = high productivity (HP, blue triangle) or low productivity (LP, yellow triangle) season = summer (Su), autumn (A), winter (W) or spring (Sp) and replicate=1–5 (n=2–5 replicates, season\textsuperscript{-1}. site\textsuperscript{-1}). Green (summer), red (spring and autumn) and blue (winter circles have been overlayed to represent the grouping based on PC1 or climatic variation.
PCA produced a model in which 2 significant principle components explained 76% of the variation in X and 62% of the X variables (detectable compounds) were predictable by this model ($R^2_X = 0.76$, $Q^2 = 0.62$). 43 X variables were modelled with 0 Y variables. The first principle component (70.2 % of variation) separated the data into 3 groups, as highlighted by circles, of summer (dotted circle), autumn and spring (solid circle) and winter (dashed circle). This variation was strongly influenced by the concentrations of 10 to 15 amino acids, particularly Gln, some of which were several orders of magnitude more abundant in winter samples than summer samples (not shown). The second principle component led to a partial separation of site LP from site HP and explained 6.1% of the variation in the PCA model. PC2 was positively loaded with the abundance of some amino acids (e.g. His, Glu) and the abundance of quaternary ammonium compounds (e.g. carnitine, betaine and choline) and tertiary amine (triethanolamine) that were more abundant in the soil solution from site LP than that of site HP.
DISCUSSION

Variation in soil exchangeable and dissolved N compounds

The relative abundance of ammonium, nitrate and total amino acids vary as a function of N availability, as observed at two *E. nitens* plantations. For example amino acids dominate the EN of strongly N-limited habitats, such as arctic tundra (Chapin *et al.* 1993; Schimel and Bennett 2004; Weintraub and Schimel 2005). Conversely, in habitats which are weakly N-limited and highly productive ammonium dominates the EN.

The relative abundance of ammonium and nitrate in the EN of *E. nitens* plantations was generally consistent with a moderately N-limited site (Ros *et al.* 2009). Neither ammonium nor nitrate dominated the EN, and amino acids were occasionally as abundant as these $N_{\text{INORG}}$ compounds. The relative abundance of amino acids was greater on site LP than site HP (Figure 7), which was consistent with the measured differences in site productivity and results from other studies (Farrell *et al.* 2014a; Ros *et al.* 2009; Rothstein 2009).

The variation in N compound abundance at site LP and HP suggest that the changes in N compounds in *E. nitens* plantation soils are consistent with the patterns of N compound abundance reported in other soils. From this, we can conclude that the results of this study are transferrable to other soils that also exhibit links between N compound dominance and N-availability.

Measurement of both exchangeable and dissolved N concentrations allowed us to extrapolate more details than possible from either measurement alone. For example, the concentration of exchangeable amino acids increased during winter then decreased rapidly during spring, whereas the concentration of DN increased during summer. The implication of this is that we are able to understand not only what will become available for plant uptake, likely following microbial metabolism (exchangeable N), but the N compounds which are presently available for plant uptake (dissolved N) (Jones *et al.* 2004).
**N\textsubscript{ORG} compound abundance**

Amino acids constitute a large fraction of the LMW DON pool in soils, up to 90% of LMW DON in grasslands (Jones et al. 2004) and 52-88% in alpine soils (Warren 2013b; 2013c). Amino acids contributed 42 to 44% of N molecules in the LMW DON (< 1kDa) detected in soil water samples from *E. nitens* plantations. The abundance of amino acids in the LMW DON indicates they are potentially significant pools of N. Despite representing a large fraction of LMW DON pools, amino acids only contribute ~5% of the total dissolved N\textsubscript{ORG} found in soils (Christou et al. 2006; Paul and Williams 2005; Warren 2014a). The high abundance of amino acids in LMW DON pools, but low contribution of amino acids to total free N (5%) suggests that peptides and proteins are highly abundant in soil water, however the present method was not optimised for detection of peptides (Warren 2013a). Investigating the peptides in soil solution will contribute to advancing our understanding of the organisms and processes involved in N availability.

*We have known the scope of LMW DON compounds in soil solution to extend beyond amino acids since the 1800s (Schreiner 1912; Lathrop 1917; Sowden 1958). In* *E. nitens* soil solution between 28 and 30% of the N detected in LMW DON compounds was in the form of quaternary ammonium compounds, particularly choline, carnitine and betaine. Examining the origins of N the range of compounds detected in soils enhances our understanding of N cycling in soils and comprehension of the potential range of compounds yet to be identified.

The abundance of different compounds indicates the presence, or absence, of microbial turnover and metabolic processes. For example, quaternary ammonium compounds, such as choline, contribute approximately 25% of N in the LMW DON pool in heath, subalpine, grassland and forest (Warren 2013c) and in *E. nitens* forestry plantations. Choline is a component in microbial cell membranes and high concentrations indicate the presence of microbial derived phospholipids and glycerophosphates. Seasonal variation in choline abundance could be indicative of variation in the rate of turnover of the soil microbial biomass (Buyer and Sasser 2012). Further work is required to investigate the significance of these compounds to the N nutrition of plants and microbes.
In demonstrating the diversity of LMW DON, this work represents a significant step towards understanding the interactions and competition between plants, microbes and N compounds in soil. Only recently has research examined the uptake of N compounds to test the significance of LMW DON compounds to plant nutrition (Paul and Williams 2005; Paungfoo-Lonhienne et al. 2012; Warren 2013a; 2013c; 2014a). When investigating plant N uptake capabilities, soil-microbial-plant N fluxes and the soil chemical N cycle we must include a wider scope of relatively abundant N$_{\text{ORG}}$ compounds to account for the diversity of the LMW DON, which we have done in Chapter 2.

**Does the soil N composition vary seasonally?**

The relative and absolute abundance of EN and DN compounds varies seasonally at *E. nitens* plantations where the total LMW DON concentration on both low and high productivity sites, peaked during winter (Figure 6) and total EN concentration peaked during autumn (Figure 5a). Peaks in the absolute abundance of amino acids prior to (winter) and just following (autumn) the growing season suggest that amino acids are a significant component of the N nutrition of arctic tundra natives (Weintraub and Schimel 2005). The abundance of N compounds at wet and dry arctic meadows (Bardgett et al. 2007) and boreal forests (Inselsbacher et al. 2014) peak before and after the growing season. The abundance of EN and LMW DON pools throughout the year indicates long term cycling of N compounds and seasonal N availability.

The relative abundance of individual LMW DON compounds varies from season to season, in at least some soils. For example, in the LMW DON of *E. nitens* plantations, Glu was the most abundant detectable compound during winter, however was not one of the ten most abundant compounds during spring (Figure 8) and PCA indicated multivariate groupings of summer, autumn/spring and winter (Figure 9). The seasonal variation in LMW DON compounds was not based on compound classes, amino acid acidity or polarity (Figure 7). Inconsistencies in the relative abundance of N$_{\text{ORG}}$ compounds has been reported previously in a study of temperate grassland soils (Warren and Taranto 2010). The seasonal variation in composition of the LMW DON pool may indicate variation in microbial origins of the compounds throughout the year.
Comparing the results of the present work with the available literature suggests a variation in the organism or process of origin for LMW DON compounds. The relative abundance of LMW DON compounds varies throughout the year in some systems such as grasslands (Warren and Taranto 2010) but remains generally constant in others (Weintraub and Schimel 2005). Consistencies in the relative abundance of amino acids is suggestive of a common organism or biogeochemical origin, such as amino acid release from bacterial cells or the process of proteolysis (Lipson and Näsholm 2001; Schulten and Schnitzer 1997; Weintraub and Schimel 2005). Seasonal variation in compound abundance indicates independently functioning components of the soil microbial biomass drive the turnover of the detectable LMW DON and the $N_{ORG}$ compounds available for plant uptake change constantly.

**Variation in LMW DON between sites of contrasting N-availability**

The relative abundance of specific compounds in arctic tundra, alpine and heathland ecosystems varies (Abuarghub and Read 1988; Kielland 1995; Kielland et al. 2007; Weintraub and Schimel 2005), however the relationship between site productivity and LMW DON variation is unknown. The present study analysed the differences in the relative and absolute abundance of compounds in terms of site productivity at two *E. nitens* plantations and the relative abundance of ~75 LMW DON compounds (Figure 6). However, the multivariate pattern explained only 6.2% (PC2, site productivity, PCA analysis) with seasonal variation (PC1) accounting 70.2% (Figure 9). Variation in soil N profiles have previously been linked to plant and microbial uptake rates and preferences (Weintraub and Schimel 2005), root exudation (Jones et al. 2005), enzyme activity (Abuarghub and Read 1988), the turnover of roots and mycorrhizae (Ruess et al. 2006) and changes in the soil microbial biomass (Lipson et al. 1999).
Amino acids dominate LMW DON pools in soils at *E. nitens* plantations, but other compounds are also abundant, including amines and quaternary ammonium compounds. This work has highlighted the diversity of compounds in LMW DON pools and potentially available for plant uptake. The diversity of compounds in the LMW DON pool suggests that the pool of plant available N is potentially more complex than previously thought. Future studies would benefit from examining the peptide composition of soils to assess the mechanisms of N availability.

Based on the findings of this study we suggest increasing the scope of N$_{\text{ORG}}$ compounds investigated in N$_{\text{ORG}}$ uptake experiments to include a wider range of amino acids, peptides and other N$_{\text{ORG}}$ compounds. There is also a need to assess the LMW DON composition of a range of soils with different uses, including annual and perennial crops, pastures and unmanaged soils, to determine the relevance of the present work, and N$_{\text{ORG}}$, to other systems. By understanding N-availability, we will be better able to manage the N-nutrition of plants and crops, at forestry plantations but also more broadly in agricultural industries.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bet</td>
<td>betaine</td>
</tr>
<tr>
<td>β-Ala</td>
<td>beta-alanine</td>
</tr>
<tr>
<td>CE-MS</td>
<td>Capillary Electrophoresis Mass Spectrometry</td>
</tr>
<tr>
<td>DON</td>
<td>dissolvable organic nitrogen</td>
</tr>
<tr>
<td>DIN</td>
<td>dissolvable inorganic nitrogen</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>LMW</td>
<td>low molecular weight</td>
</tr>
</tbody>
</table>
REFERENCE LIST


Warren, C. R. (2013c) Quaternary ammonium compounds can be abundant in some soils and are taken up as intact molecules by plants. New Phytologist 198(2): 476-485.


CHAPTER 2

*Eucalyptus nitens* and *E. globulus* take up amino acids and small peptides in laboratory and field based experiments.
ABSTRACT

Inorganic nitrogen (N), ammonium and nitrate, have traditionally been considered the sole source of plant available N. Recently, however, numerous studies have also demonstrated organic N (N\textsubscript{ORG}) uptake occurring in a wide range of plants. The scope of N\textsubscript{ORG} compounds that are taken up by plants is not presently known, with most work focusing on the uptake of simple amino acids such as glycine and alanine. Furthermore the significance of N\textsubscript{ORG} uptake in the field is uncertain because many uptake experiments have involved unrealistic laboratory conditions and N solutions orders of magnitude more concentrated than occurs in soil.

We have employed three methods to investigate N\textsubscript{ORG} uptake in agar (germination and growth), from solution (uptake potential) and \textit{in situ} (uptake potential in competition with soil processes and biota). To increase our understanding of the role of N\textsubscript{ORG} to the nutrition of \textit{E. nitens} and \textit{E. globulus} we have supplied realistic concentrations of N to seeds, seedlings and trees.

The germination and growth of \textit{E. globulus} seeds supplied with one of fourteen amino acids or small peptides in agar was generally comparable to the germination and growth of seeds supplied with ammonium or nitrate at high N concentration (4 mM). Eight of ten amino acids or small peptides (selected based on their relative abundance in soil water of \textit{E. nitens} plantations) provided to \textit{E. nitens} and \textit{E. globulus} seedlings at realistic concentrations (10 µM) were taken up from solution at rates greater than ammonium uptake from solution. The addition of isotopically labelled $^{15}$N glycine, ammonium and nitrate to soils of \textit{E. nitens} plantations \textit{in situ} demonstrated the potential for amino acids to be taken up at rates comparable to inorganic N source and in competition with the soil microbial biomass.

The use of complementary methods has shown that \textit{E. nitens} and \textit{E. globulus} are able to germinate and grow using N\textsubscript{ORG} compounds as well as inorganic N (N\textsubscript{INORG}) compounds. N\textsubscript{ORG} uptake was demonstrated at different stages of growth (germination, seedling maturation and as established trees) and when N was supplied under different conditions (nutrient agar, solution and \textit{in situ}). Suggesting that N\textsubscript{ORG} compounds play a significant role in the N nutrition of \textit{E. nitens} and \textit{E. globulus} and supports the idea that nutritional management strategies on forestry plantations of \textit{E. nitens} should account for uptake of organic N.
INTRODUCTION

Nitrogen (N) plays an important role in the growth and development of plants hence N-availability is one of the greatest limiting factors to plant productivity in forestry and agriculture (Inselsbacher and Näsholm 2012). In the traditional paradigm of plant nutrition, plants were considered only capable of taking up $N_{\text{INORG}}$, ammonium and nitrate (Schimel and Bennett 2004). Recently, studies have shown that plants are also able to take up $N_{\text{ORG}}$ sources (Näsholm et al. 2009), challenging the traditional paradigm of plant N nutrition (Näsholm et al. 1998; Näsholm et al. 2009; Schimel and Bennett 2004). The significance of $N_{\text{ORG}}$ to plant N nutrition is thus far uncertain.

Understanding the soil N composition provides insight into potentially available N sources, thereby providing key information required to understand the biological significance of $N_{\text{ORG}}$ to plant N nutrition. The majority of the N in soils is in the form of $N_{\text{ORG}}$, most of which is high molecular weight (HMW, >100 kDa) and unavailable for plant uptake (Jones et al. 2004). Low molecular weight (LMW, <1 kDa) dissolved (DN) and extractable N (EN) constitute the second most abundant N fraction.

LMW DON and EN pools are potentially available for plant uptake; however we presently have limited knowledge of the scope of LMW DON that plants are able to take up (Chapin et al. 1993, Hill et al. 2011; 2012, Jones et al. 2004, Näsholm et al. 2009, Schimel and Bennett 2004). LMW N pools include $N_{\text{ORG}}$ and $N_{\text{INORG}}$ molecules in soil solution (DON) or bound in soil colloids (EN). We know that amino acids dominate LMW DON pools (0.5 to 50 µM in soil water and 0 to 1000 µM in KCl extracts (Schobert and Komor 1987)). Efforts to determine the range of LMW DON compounds taken up by plants have been hampered by the diversity and number of LMW DON compounds present in soil and thus potentially available for uptake.

The abundance of specific LMW DON compounds varies between soils. For example, in grassland and agricultural soils Ala, Gln, Ser, Leu and Gly were most abundant (Farrell et al. 2014; Perez et al. 2015) whereas in sub-alpine grasslands the most abundant amino acids were Arg, Val, Gln and Try (Warren and Taranto 2010). The LMW DON of forestry plantation soils in Tasmania were dominated by Glu, Gln, Asn and Leu, however the relative and absolute abundance of specific compounds changed seasonally (Chapter 1).
Approximately three hundred peptides have thus far been detected in soils, the majority of which fall in the range of 131 (Gly-Gly) to approximately 600 Da (Farrell et al. 2011a; Warren 2013). There are four hundred possible di-peptides and eight thousand possible tri-peptides, indicating the soil peptide composition likely consists of several hundred more peptides than have been detected thus far (Warren 2013; 2014). Understanding the scope of LMW DON compounds in soils is required to place results of amino acid studies in a broader context.

Without fully understanding the LMW DON composition of soils, $N_{\text{ORG}}$ uptake has been largely studied using a small subset of amino acids, particularly Gly and Ala (Jämtgård et al. 2008; Lipson et al. 1999, Schimel and Chapin 1996; Soper et al. 2011). The subset of $N_{\text{ORG}}$ compounds used in uptake experiments is expanding, incorporating more amino acids (Falkengren-Grerup et al. 2000; Jones and Darrah 1993; Owen and Jones 2001) and small peptides, mainly di-peptides (Hill et al. 2011, Lonhienne et al. 2014; Soper et al. 2011; Tegede and Rentsch 2010; Vinall et al. 2012). The range of amino acids known to be taken up by plants continues to increase.

In defiance of the traditional paradigm of plant N nutrition, plants have been shown capable of uptake of a remarkable range of $N_{\text{ORG}}$ compounds. For example, *Hakea actites* and *Lobelia anceps* seedlings grew greater root biomass when supplied with small peptides in nutrient agar (e.g. Gly-Phe, Ala-Gln, Gly-Gly-Gly-Gly at 10 mM N) compared to $N_{\text{INORG}}$ (10 mM) (Paungfoo-Lonhienne et al. 2008; Soper et al. 2011). Unfortunately the small peptides were provided at unrealistic (10 X) the LMW DON concentration generally present in soil (Schobert and Komor 1987), therefore the biological significance of uptake is unclear. Assessing the quantitative biological significance of $N_{\text{ORG}}$ to plant N nutrition requires investigating plant uptake of compounds ranging in molecular weight, pKa and composition and at realistic soil concentrations.

Uptake experiments are often conducted in the laboratory, through the provision of $N_{\text{ORG}}$ compounds in either agar or hydroponically (in solution). There are advantages to conducting laboratory based N uptake experiments. For example, agar is a useful medium to supply N compounds to seeds and seedlings and assess uptake, germination and growth over a prolonged period of time (weeks rather than hours). Field based experiments demonstrate plant N uptake in competition with the soil.
microbial biomass and processes of cation exchange, adsorption, diffusion and compound loss to the soil stationary phase (Kahmen et al. 2009). There are advantages of using each methodology, but also disadvantages. The use of multiple methods to examine N compound uptake could help to overcome some of the drawbacks of each method. For example, there are significant difficulties encountered in soil based uptake experiments, such as rapid N turnover and the uncertainty in identifying the initial organism to metabolise the N compound being studied (Farrell et al. 2011b; Hill et al. 2011, 2012; Jones and Kielland 2002). However, soil based uptake experiments are important to obtain a sense of the in situ capabilities of plants and could imply uptake of other N\textsubscript{ORG} compounds that behaved similarly in laboratory based experiments.

The aim of the present study was to investigate \textit{E. nitens} and \textit{E. globulus} uptake of N\textsubscript{ORG} compounds To investigate the N uptake, germination and growth of \textit{E. globulus} and \textit{E. nitens} seeds and seedlings supplied with one amino acid (of five to seven treatments), small peptide (two to five amino acids in length, six to eight treatments) or N\textsubscript{INORG} compound in solution or nutrient agar. The amino acids were supplied based on the composition and N\textsubscript{ORG} concentrations (10 \textmu M N) of soil solution at \textit{E. nitens} plantations. N concentrations (4 mM) in excess of native soil N concentrations were supplied to seeds in nutrient agar to ensure continuous N supply for two weeks and make the results relative to other studies. The effect of peptide chain length on growth and N uptake was examined by providing seeds and seedlings with Ala peptides up to Ala penta-peptide.

Isotopically labelled ammonium, nitrate and glycine (Gly) to \textit{E. nitens} trees in forestry plantations to investigate the potential for N\textsubscript{ORG} compounds to be taken up in field conditions. It was hypothesised that the seeds and seedlings provided with lower MW compounds (Gly and Ala) would be taken up, germinate and grow comparatively to the seeds supplied with N\textsubscript{INORG}. The rate of uptake and growth was expected to decrease with compound size and complexity. A slower rate of Gly uptake than N\textsubscript{INORG} uptake by \textit{E. nitens} trees in the field was predicted given the relatively high native concentration of N\textsubscript{INORG} at these sites (Chapter 1).
METHODS

Germination and growth on nutrient agar

Seed preparation

Eucalyptus globulus ssp. globulus (Seed lot: 18894) were purchased from the Australian Tree Seed Centre, CSIRO Plant Industry, Black Mountain ACT. The seeds were surface sterilised with 50% EtOH for 10 mins followed by 3 x 3 min washes in 10-15% NaOCl in dH₂O (1:1). Sterilised seeds were rinsed with 10% EtOH then in dH₂O for 3 x 30 seconds.

Agar preparation and seed sowing

Nutrient agar was prepared in bulk with Hoagland and Arnon Macronutrient solution (Hoagland and Arnon 1938), 10% v/v Murashige and Skoog Basal Salt Micronutrient Solution, 2% w/v sucrose and 1% w/v agar combined in room temperature dH₂O (all chemicals sourced from Sigma Pty. Ltd., Sydney, Australia). This was separated to smaller batches and 4 mM N of one of each of the N compounds was added to each, details in table 1 (purchased from Sigma Pty. Ltd. or Fluka, Germany). Solutions were autoclaved then 25 mL was poured into petri dishes and allowed to cool and set.

Ten seeds were distributed evenly on the surface of the agar (n = 10 seeds. replicate⁻¹ x 2 replicates. treatment⁻¹) Plates were sealed and placed on a flat surface in growth cabinet with 12 hr/12 hr light dark cycle. The plates were observed daily and any with contamination were removed.
Table 1. Summary of N treatments – the molecular weight (Da) and unique property for each of the 18 N treatments is shown. A range of amino acids and peptide were used to cover the various amino acid MWs and classes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MW</th>
<th>Property</th>
</tr>
</thead>
<tbody>
<tr>
<td>No N Control</td>
<td>0</td>
<td>Control</td>
</tr>
<tr>
<td>Nitrate**</td>
<td>62.00</td>
<td>N\text{INORG} control</td>
</tr>
<tr>
<td>Ammonium***</td>
<td>18.04</td>
<td>N\text{INORG} control</td>
</tr>
<tr>
<td>Glycine (Gly)</td>
<td>75.07</td>
<td>Small amino acid</td>
</tr>
<tr>
<td>Alanine (Ala)</td>
<td>89.09</td>
<td>Small amino acid</td>
</tr>
<tr>
<td>Serine (Ser)</td>
<td>105.09</td>
<td>Nucleophilic amino acid</td>
</tr>
<tr>
<td>Glutamine (Gln)</td>
<td>146.14</td>
<td>Amide amino acid</td>
</tr>
<tr>
<td>Tryptophan (Try)</td>
<td>204.23</td>
<td>Aromatic amino acid</td>
</tr>
<tr>
<td>Histidine (His)</td>
<td>155.15</td>
<td>Basic amino acid</td>
</tr>
<tr>
<td>Glutamate (Glu)</td>
<td>147.13</td>
<td>Acidic amino acid</td>
</tr>
<tr>
<td>Gly-Gly</td>
<td>132.12</td>
<td>Di-peptide</td>
</tr>
<tr>
<td>Ala-Ala</td>
<td>160.17</td>
<td>Di-peptide</td>
</tr>
<tr>
<td>Gly-Leu</td>
<td>188.22</td>
<td>Hydrophobic di-peptide</td>
</tr>
<tr>
<td>Gly-Glu</td>
<td>204.18</td>
<td>Acidic di-peptide</td>
</tr>
<tr>
<td>Gly-His</td>
<td>212.21</td>
<td>Basic di-peptide</td>
</tr>
<tr>
<td>Gly-Gln</td>
<td>221.21</td>
<td>Amide di-peptide</td>
</tr>
<tr>
<td>Ala-Ala-Ala</td>
<td>231.25</td>
<td>Tri-peptide</td>
</tr>
<tr>
<td>Ala-Ala-Ala-Ala-Ala*</td>
<td>373.4</td>
<td>Penta-peptide</td>
</tr>
</tbody>
</table>

* only 2 time points were measured due to a shortage of internal standard.
** added as potassium nitrate
*** added as ammonium sulphate

Germination and data collection/ analysis

Seed germination, defined as the presence of at least the radicle from the seed, was counted at 7 and 14 days after sowing. Root and shoot lengths were measured from the original seed mark after 14 days. For long roots and shoots, this was done point to point along the root or shoot.

Analysis of variance (ANOVA) was conducted on the root and shoot measurement data to determine the significance of the variation between treatment and root or shoot growth. Significance was based on a p<0.05 confidence interval using R version 3.1.2 (R Core Team 2014). The average root and shoot length was reported for each treatment and the treatments with no significant variation have been labelled thus.
Uptake potential – N Uptake from solution

Seedling preparation

*Eucalyptus nitens* (seed lot: 20156) and *Eucalyptus globulus* ssp. *globulus* (seed lot: 18724) seeds were purchased from CSIRO Australian Tree Seed Centre. *E. nitens* seeds were cold stratified for three weeks (moist at 4 °C). *E. globulus* and *E. nitens* seeds were germinated in vermiculite. The seeds were grown in vermiculite for approximately two months and were supplied with Hoagland and Arnon nutrient solution (Hoagland and Arnon 1938) every two to three days.

Seedlings were removed from the vermiculite, washed and gently wedged into foam discs. The foam discs were floated on Hoagland and Arnon nutrient solution (Hoagland and Arnon 1938), which was topped up regularly, for five to six weeks in a greenhouse (University of Sydney, Australia). To minimise the exposure of the solution to light and microbial growth, the seedlings and nutrient solution were kept in lidless black tubs, which were cleaned regularly.
Table 2: Treatments used in uptake experiments (part 2) showing the N compound added to nutrient solution, its molecular weight (Da), property and reason for the inclusion of that compound in this part of the study. Nitrate was not included due to the availability of viable seedlings.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MW</th>
<th>Property</th>
<th>Reason For Inclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>No N Control</td>
<td>0</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Ammonium*</td>
<td>18.04</td>
<td>N\textsubscript{INORG} Control</td>
<td>Control</td>
</tr>
<tr>
<td>Glycine (Gly)</td>
<td>75.07</td>
<td>Small amino acid</td>
<td>Commonly used in uptake experiments</td>
</tr>
<tr>
<td>Alanine (Ala)</td>
<td>89.09</td>
<td>Small amino acid</td>
<td>Control</td>
</tr>
<tr>
<td>Glutamine (Gln)</td>
<td>146.14</td>
<td>Amide amino acid</td>
<td>Abundant in forestry plantation soils (CH1)</td>
</tr>
<tr>
<td>Glutamate (Glu)</td>
<td>147.13</td>
<td>Acidic amino acid</td>
<td></td>
</tr>
<tr>
<td>Histidine (His)</td>
<td>155.15</td>
<td>Basic amino acid</td>
<td></td>
</tr>
<tr>
<td>Gly-His</td>
<td>212.21</td>
<td>Di-peptide</td>
<td>Peptide treatment with most growth in agar experiment</td>
</tr>
<tr>
<td>Gly-Glu</td>
<td>204.18</td>
<td>Di-peptide</td>
<td>Amino acid components are abundant in plantation soils (CH1)</td>
</tr>
<tr>
<td>Gly-Gln</td>
<td>221.21</td>
<td>Di-peptide</td>
<td></td>
</tr>
<tr>
<td>Gly-Leu</td>
<td>188.22</td>
<td>Di-peptide</td>
<td></td>
</tr>
<tr>
<td>Ala-Ala</td>
<td>160.17</td>
<td>Di-peptide</td>
<td>Comparison of uptake of peptides</td>
</tr>
<tr>
<td>Ala-Ala-Ala</td>
<td>231.25</td>
<td>Tri-peptide</td>
<td></td>
</tr>
<tr>
<td>Interruption (Gly)</td>
<td>75.07</td>
<td>Seedling removed from solution after 1 hour</td>
<td>Assess N depletion resulting from microbial activity.</td>
</tr>
</tbody>
</table>

*added as ammonium sulphate

**N uptake and N depletion from solution**

Seedlings were weighed individually, and then three to five seedlings with minimum root mass of 50 mg were grouped and transferred into a hole in the lid of a 50 mL tubes (Sarstedt, Gemany). To recover from the process of being transferred each replicate was screwed onto a 50 mL tube containing a weak (10 x dilution) nutrient solution and placed in a rack on the windowsill in the laboratory. After two days each replicate was transferred to a 50 mL tube of fresh 0.01 mM CaCl\textsubscript{2} solution for approximately 1 hour in an effort to gently rinse nutrient solution from the roots while preserving membrane integrity.
A 25 mL tube was secured inside a 50 mL tube (as shown in Figure 1). The 25 mL tubes were filled with one of 15 N sources (10 µM in 0.01 mM CaCl₂). Time 0 samples were taken and 1 replicate (group of seedlings with root mass > 50 mg) was immediately secured to the 50 mL tube, with care taken to enclose the entire root mass within the 25 mL tube, therefore the N solution.

A 0.5 mL sample of solution was collected by inserting a pipette tip through the lid hole containing the seedlings. Samples were collected at t=0, 60 and 240 minutes from root immersion in N solution, in 2 mL Eppendorf tubes and placed in the freezer (-21°C). Once all samples were collected they were thawed and centrifuged for 5 mins at 3200 x g in batches for analysis. 2 µL of internal standard (100 µM ¹³C₆¹⁵N₄ arginine) was added to 100 µL of sample, samples were mixed and then direct infused at 2 µL min⁻¹ into an ion trap mass spectrometer (AmaZon SL, Bruker Daltonics, Bremen, Germany). Quantification was based on multiple reaction monitoring with transitions carefully selected to avoid interferences. All concentrations were calculated based on normalisation with the internal standard ¹³C₆¹⁵N₄ arginine.
Ammonium samples were concentrated, 0.5 mL to 0.2 mL using Eppendorf Concentrator 5301 (eppendorf, Germany) and concentration was measured using colorimetric methods for ammonium (Baethgen and Alley 1989) with absorbance measured using a microplate reader (Biotek Synergy 2 multi-mode). The no N control solution was also analysed using direct infusion mass spectrometry to test for exuded compounds.

The data presented is the mean of four replicates. Analysis of variance (ANOVA) was conducted to determine the significance of the variance in the concentration of N in solution from t0 to t4. Tukey’s post hoc test was used to analyse the significance of the difference between treatments (difference N compounds provided in solution) using R version 3.1.2 (R Core Team 2014).

Soil based N uptake – In situ isotopic labelling experiment

In situ N uptake site set up and substrate application

The sites used for this experiment have previously been described (Chapter 1). In brief, five replicate ten-tree plots (n = 50 trees. site⁻¹) were established, without fertiliser addition, on two Eucalyptus nitens plantations in Tasmania in spring 2011 (1100 stems. hectare⁻¹). In June 2014 one of three labelled N substrates were supplied at 3 mM N in 5 L distilled water to 3 replicate E. nitens trees (n = 3 replicates. treatment⁻¹ x 3 treatments + control. site⁻¹ x 2 sites). Labelled substrates were 60 at.% ¹⁵NO₃⁻, 60 at.% ¹⁵NH₄⁺ and 60 at.% ¹⁵N-¹³C glycine (Isotec Inc., Miamisburg, OH). Control replicates were supplied with 5 L distilled water with no added N source.

Root and soil sample collection and preparation

Root samples were collected prior to treatment addition then at t = 0, 1, 20 and 36 hours. Roots were collected by digging an approximately 15 x 15 cm box of soil to the depth of the shovel (~20 cm) and picking the roots from this. An attempt was made to sample roots evenly from around the tree so as to avoid contamination due to root responses to the damage and changes in uptake resulting from root exudation. Roots were rinsed to remove soil particles and weighed.
Isotopic and data analysis

To prepare samples for isotopic analysis, root samples were freeze dried, ground to a fine powder using a matrix mill (30 s at 25 Hz with a 5 mm stainless steel bead) (TissueLyser; Qiagen, Doncaster, VIC, Australia) (TissueLyser, QIAGEN, Limburg). 3 (±0.1) mg was weighed into a tin capsule (Euro Vector Instruments and Software, Milano), which was manipulated into a ball using tweezers and analysed for $^{13}$C and $^{15}$N by isotope-ratio mass spectrometry (IRMS) at the UC Davis Stable Isotope Facility. The $^{13}$C analysis was only conducted for quality assurance, but has not been reported as only the uptake capability of plants is being assessed, not the fate of N compounds.

The root mass in soil, at.% of added compound and native concentration of compound in soil extract was accounted for when N uptake was calculated from the quantity of $^{15}$N and $^{14}$N (g) in roots and N concentration in the soil samples as in Warren (2009b).

The data presented is the mean of three replicates. Analysis of variance (ANOVA) was conducted to determine the significance of the variance within sites was tested and the significance of variance between each of the treatments using R version 3.1.2 (R Core Team 2014).
RESULTS

Amino acids as N source in nutrient agar

_E. globulus_ was able to germinate and grow with a broad range of amino acids, such that rates of germination and root and/or shoot length did not differ among seeds supplied N\textsubscript{INORG} or six of the amino acids, Gly, Ala, Glu, Gln, Ser or His (7.71 to 9.17 mm shoot length, Figure 2a and 2.1 to 3 mm root length, Figure 2b). With two of the amino acids, Gln and Ala, shoots and roots were longer than with the N\textsubscript{INORG} controls. Only 25% of seeds supplied with Try germinated, that was not significantly different from the No N control seeds (Figure 2c).
Figure 2. Growth of shoots (a) and roots (b) that occurred following E. globulus seed germination (c) in nutrient agar. Lower case letters show treatments that are not significantly different from one another (t = 14 days, error bars = ±1 standard deviation, n = 10 seeds. plate⁻¹ x 2 plates. treatment⁻¹).

Amino acid uptake from solution

Ammonium was supplied to E. globulus and E. nitens seedlings in solution as a representative of an N_{INORG} supply. Over the course of 240 minutes the concentration of ammonium in solution decreased from 10 µM N to 7.4 and 5.8 µM N when provided to E. globulus and E. nitens respectively that is an uptake rate of 0.02 and 0.01 µmoles. minute⁻¹. g root⁻¹ respectively (Figure 3a).
Replicates in which Gly uptake was interrupted, by removing the seeding from solution, assessed whether the depletion of N from solution was solely due to the seedling, or microbial activity. Gly was supplied to seedlings for 60 minutes, then the seedling was removed and the (plant-less) hydroponic solution was incubated for a further 180 minutes. There was a significant decrease in Gly concentration in the first 60 mins by *E. nitens* (uptake = 0.04 µmoles. minute⁻¹ g root⁻¹, p = 0.008) and *E. globulus* (0.02, p = 0.01) however there was no significant change thereafter (p > 0.05) (Figure 3b).

![Figure 3](image)

Figure 3. NINORG (a) and Interrupted Gly (b) controls depletion of N from solution by *E. globulus* and *E. nitens*. Error bars represent 1 standard deviation (n=4. N source⁻¹). Interrupted Gly control had seedlings removed at t₀₆₀ and any continuing depletion is attributable to microbial activity.

Both *E. nitens* and *E. globulus* seedlings took up Gly (Figure 4a), Ala (Figure 4b), His (Figure 4c) and Glu (Figure 4d). There was no depletion of Gln from solution (Figure 4e).

Figure 4 shows the linear regression of N compound depletion over four hours, with subsampling at t = 0, 60 and 240 minutes and is the mean concentration of four replicates for each N source. Table 3 summarises the uptake rates and p values of the results in Figure 4.
Table 3. Amino acids uptake rates by *E. nitens* and *E. globulus* seedlings when supplied in solution (Figure 4). Exclusive of Gln, as no/minimal uptake occurred.

<table>
<thead>
<tr>
<th>N source</th>
<th><em>E. nitens</em></th>
<th>P value**</th>
<th><em>E. globulus</em></th>
<th>P value**</th>
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</thead>
<tbody>
<tr>
<td>Gly</td>
<td>0.029</td>
<td>0.03</td>
<td>0.014</td>
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<td>His</td>
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<td>0.005</td>
<td>0.019</td>
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<td>Glu</td>
<td>0.022</td>
<td>0.002</td>
<td>0.019</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*µmoles. minute⁻¹ g root⁻¹**confidence interval p = 0.05

Figure 4. Amino acid depletion from solution by *E. nitens* (green) and *E. globulus* (blue). Gly (a), Ala (b) depletion is replicated in figure 7, His (c), Glu (d) and Gln (e) are depletion from solution was measured using CE-MS. Error bars represent 1 standard deviation (n=4. N source⁻¹) and values above ten are representative of no depletion from solution or potentially small quantities of elution, however this was accounted for using a no N control. Uptake rates and p values summarised in Table 3.
N uptake (labelled $^{15}$N) by E. nitens roots at forestry plantation sites

Isotopically labelled Gly, ammonium and nitrate was applied to the soils around E. nitens trees in situ. The uptake of Gly, ammonium and nitrate in the first hour after substrate addition was significant ($p < 0.05$, Figure 5). On site HP the uptake of Gly in the first hour was significantly quicker than the uptake of ammonium or nitrate ($p < 0.05$); on site LP the rates of uptake of the substrates were not significantly different.

Figure 5. The concentration in of total N taken up in roots of 2 yr old E. nitens trees on high (a) and low (b) productivity forestry plantations in Tasmania. The N concentration in roots is shown over 36 hours for ammonium (green), nitrate (orange) and glycine (blue) supplied trees. The difference in N uptake rate between Gly and ammonium treatments was most significant in the first hour following N compound application (top p value on each graph) than it was 36 hours after application (bottom p value on each graph)
Peptide chain length and N uptake

*N provided in nutrient agar over 2 weeks*

Seedlings supplied with Ala, Ala tri and penta peptides in agar germinated and grew roots and shoots at least as long as the roots and shoots grown by seeds provided with N\textsubscript{INORG} sources. There was no significant difference in the growth of roots and shoots of Ala-Ala and no N supplied seeds (Figure 6). The branching of the roots of the Ala penta-peptide supplied seeds was extensive in comparison to that of the Ala amino acid and the Ala tri-peptide treatments (not shown).

![Figure 6](image)

**Figure 6.** Growth of *E. globulus* shoots (a), roots (b) and seed germination (c) when provided with Ala as a single amino acid or in a di-, tri- or penta- peptide. Columns with the same letter annotation were not significantly different. (*t* = 14 days, error bars = ±1 standard deviation, *n* = 10 seeds. plate\textsuperscript{-1} x 2 plates. treatment\textsuperscript{-1}).
**Uptake potential from solution**

*E. globulus* and *E. nitens* seedlings took up Ala (uptake $(z) = 0.013$ and 0.023 µmoles. minute$^{-1}$ g root$^{-1}$, $r^2 = 0.85$ and $r^2 = 0.98$, respectively), the Ala di-peptide $(z = 0.018, 0.013$ $r^2 = 0.74, 0.91)$ and Ala tri peptide $(z = 0.038, 0.016$ $r^2$ N/A as only $t_0$ and $t_{240}$), measured as N depletion from solution (Figure 7).

Figure 7 shows the linear regression of N compound concentration depletion over four hours, with subsampling at $t = 0, 60$ and $240$ mins and is the mean concentration of four replicates for each N source. The Ala tripeptide concentration in solution was only measured at two time points due to insufficient internal standard.

![Graph showing N concentration depletion over time for Ala (a), Ala-Ala (b), and Ala-Ala-Ala (c) peptides](image)

Figure 7. Ala and Ala peptides were taken up by *E. nitens* and *E. globulus* seedlings when provided to seedlings in solution. Error bars represent 1 standard deviation (n=4 treatments. N source$^{-1}$) and values above ten are representative of no depletion from solution or potentially small quantities of elution, however this was accounted for using a no N control.
Small peptides as a source of N

*Uptake from nutrient agar over two weeks*

There was no significant difference in the germination rate of seeds supplied with peptides or N\textsubscript{INORG} sources or in shoot length between seeds supplied with N\textsubscript{INORG}, Gly-Leu, Gly-Glu, Gly-Gln, Ala-Ala-Ala or Ala-Ala-Ala-Ala-Ala-Ala (Figure 8a). Root length of *E. globulus* seeds supplied with Gly-Leu, Gly-Gly, Gly-Glu, Gly-His, Gly-Gln or N\textsubscript{INORG} did not differ significantly (Figure 8b). The treatments which varied significantly from the N\textsubscript{INORG} controls did so due to growing either significantly longer shoots (Gly-His) or roots (Ala-Ala-Ala-Ala-Ala).

![Figure 8. Growth of shoots (a) and roots (b) and seed germination (c) rate of *E. globulus* seeds sown in nutrient agar. Lower case letters show treatments that are not significantly different from one another or the treatments in figure 2 (p < 0.05). (t = 14 days, error bars = ±1 standard deviation, n = 10 seeds. plate\textsuperscript{-1} x 2 plates. treatment\textsuperscript{1})](image-url)
**Uptake potential from solution**

N depletion from solution containing 10 µM N bound in peptides revealed that at least 4 peptides can be taken up from solution by *E. globulus* and *E. nitens*; Gly-Leu (Figure 9a), Gly-Gln (Figure 9c), Ala-Ala (Figure 9e) and Ala-Ala-Ala (Figure 7c). There was no significant depletion, therefore uptake, of Gly-Glu or Gly-His from solution (Figure 9b and d).

![Graphs showing depletion of di-peptides](image)

**Figure 9.** Depletion of di-peptide from solution when supplied to *E. nitens* and *E. globulus* seedlings at 10 µM N. Gly-Gln and Gly-Leu were taken up, while Gly-His was not, a small quantity of Gly-Glu was depleted from solution. Error bars represent 1 standard deviation (n = 4 replicates. N source$^{-1}$) and values above ten are representative of no depletion from solution or potentially small quantities of elution, however this was accounted for using a no N control.
DISCUSSION

Amino acids are taken up by *E. nitens*, *E. globulus* and other plants

*E. nitens* and *E. globulus* seeds, seedlings and trees take up a range of amino acids and small peptides in both laboratory and field experiments at similar rates as ammonium and/or nitrate. Similarly to the present study, *E. regnans* seedlings are also able to take up amino acids from solution at rates similar to ammonium uptake rates (ammonium ~6.5 μmol g\(^{-1}\) h\(^{-1}\), glycine ~3.1 μmol g\(^{-1}\) h\(^{-1}\) uptake from solution) (Warren 2009a).

The uptake of amino acids from solution has been demonstrated in a wide range of plant species. For example, plant species native to arctic tundra take up amino acids available at concentrations of 10 to 500 μM N (1.2 to 6.8 μmol g\(^{-1}\) h\(^{-1}\)) (Kielland 1994), and barley when supplied with 2 to 25 μM N amino acids in solution (uptake = 0.6 to 4.6 μmoles amino acid g root DM\(^{-1}\) hr\(^{-1}\)) (Jämtgård et al. 2008). Similar to N\(_{\text{ORG}}\) uptake by *E. nitens* and *E. globulus*, 5 alpine species (Miller and Bowman 2003) and 4 grassland species (Streeter et al. 2000) take up Gly. While there is ample evidence to suggest plants are able to take up N\(_{\text{ORG}}\) compounds few studies have provided N\(_{\text{ORG}}\) compounds based on soil composition and concentration.

Plants are capable of taking up amino acids of varying size and complexity, for example, grasses have been shown to take up Gly, Ser and Arg (Weigelt et al. 2005). However, amino acids with an aromatic side chain have previously been shown to inhibit plant growth as was seen in *E. globulus* seeds provided with Try. For example, toxicity was observed in *Nicotiana silvestris* when supplied with Phe, Try and Trp at concentrations of one to five millimoles (Bonner et al. 1992). The uptake of one amino acid, or other N\(_{\text{ORG}}\) compound, does not imply the uptake of all, stressing the importance of understanding N uptake in regards to composition of the N\(_{\text{ORG}}\) pool.

This study has demonstrated N\(_{\text{ORG}}\) uptake at realistic concentrations and related this to *in situ* amino acid uptake. *E. nitens* and *E. globulus* seedlings, like barley seedlings take up N\(_{\text{ORG}}\) compounds when supplied at realistic concentrations (Jämtgård et al. 2008). In this study we were also able to show that Gly uptake by *E.
*E. nitens* trees at forestry plantation sites was comparable to ammonium or nitrate uptake (Figure 5). Using laboratory and field based methodologies to analyse the uptake of the same compounds (ammonium, nitrate and glycine) by the same species (*E. nitens*) had comparable outcomes.

Excessive N supply is provided to ensure results are measurable and the potential for plants to take up an N sources is optimised. In the present work *E. globulus* seedlings were supplied with a range of amino acids and small peptides in agar to ensure the supply of N over a prolonged (2 week) period and to produce results to compare with previous studies. N\textsubscript{ORG} uptake, when concentrated and supplied in agar, by *E. globulus* seedlings is comparable to ammonium and nitrate uptake under the same conditions, as was the case in *Hakea actites* and *Lobelia aniceps* seedlings (Paungfoo-Lonhienne et al. 2008, Soper et al. 2011). Showing N uptake by *E. globulus* under conditions similar to previous work increases the pool of knowledge on N nutrition in plants.

**Peptide uptake**

*E. nitens* and *E. globulus* are capable of N uptake from at least 7 peptides with chain lengths of two to five amino acids. This is not a unique property of Eucalyptus species, and peptide uptake has been demonstrated previously. For example, Ala tri-peptide uptake in plants native to salt marshes (Quinta et al. 2015) and wheat seedlings (Hill et al. 2011). The significance of peptide uptake to plant N nutrition is difficult to quantify due to the quantity of potential peptides.

Plants are able to take up peptides, but the biological significance of this uptake is difficult to ascertain. The primary difficulties are that four hundred possible combinations of di-peptides exist, and we have a limited understanding as to the peptide composition of soils (Warren 2013; 2014). Further, peptides are rapidly turned over in soil and may be a preferred source of N for the soil microbial biomass, compared to amino acids and N\textsubscript{INORG} compounds (Hill et al. 2012). Conducting laboratory based experiments without understanding the peptide composition of soil or the SMB peptide interactions drastically inhibits future efforts to study the biological significance of peptides to plant N nutrition.
The effect of peptide chain length on N uptake and plant growth

When provided with peptides of the same constituent amino acids but of differing chain length, seedling root morphology and biomass can be altered (Lonhienne et al. 2014; Soper et al. 2011; Vinall et al. 2012). For example, root thickness and branching increases in A. thaliana seedlings supplied with Gly peptides from two to four Gly in length (Soper et al. 2011). Similarly, E. globulus provided with Ala peptides of increasing length (two to five Ala) exhibited increased root to shoot ratio (biomass) and increased root branching. The ability to take up longer peptide chains (3 to 8 amino acids) suggests increases the scope of potential peptides for plant uptake (possible di-peptides amino acid combinations = $20^2$, tri-peptides = $20^3$ etc.).

Peptide properties, such as acidity, conformation and molecular weight vary with amino acid composition and peptide chain length which alters the potential for transport into plant cells due to transporter protein specificity. Variations in seedling morphology and biomass allocation when supplied with N from peptides of increasing length is the result of transporter specificity (Chiang et al. 2004; Hofstetter et al. 2013; Svennerstam et al. 2007; Tegeder and Rentsch 2010) and the role of different peptides in signalling pathways (Kormarova et. al. 2008, Soper et al. 2011; Walch-Liu et al. 2006a; Walch-Liu et al. 2006b). As plants seem to have the capacity to take up a wider scope of peptides than previously thought, indicating that transporter proteins are more promiscuous than originally thought. Studying the scope of peptide transporters, identifying target compounds and uptake mechanisms will increase our ability to manipulate the N management of plants.

N uptake analysis using laboratory and field based experiments

There are advantages and disadvantages of laboratory and field based experiments, however conducting them simultaneously, with the same compounds and species provides meaningful estimates of potential and actual N uptake (Forsum et al. 2008; Harrison et al. 2007; Jämtgård et al. 2008; Näsholm et al. 1998; Persson and Näsholm 2001; Vinall et al. 2012). The present study did use laboratory based experiments for the majority of the investigation, however, the addition of a field based study gives practical relevance to the results. There is a possibility that the amino acids and small peptides which were taken up at rates similar to that of $N_{\text{INORG}}$ compounds may, like Gly, also be taken up in situ. The advantage in the use of even
a small scale field based experiment is that it shows the potential practical relevance of N$_{\text{ORG}}$ uptake in the presence of soil microbial biomass and processes such as cation exchange, adsorption, diffusion and other interactions with the soil stationary phase.

In the context of *Eucalyptus* species, field based studies allow us to assess N uptake in the presence of mycorrhizae. *Eucalyptus* roots form a symbiotic relationship with mycorrhizal fungi (Adams *et al.* 2006). This relationship, between plant and fungi, is an important component of *Eucalyptus* nutrition. In the present study it was of little relevance if uptake was the result of mycorrhizae or the plant, as the relationship occurs in all *E. nitens* or *E. globulus* trees therefore will not alter the significance of N$_{\text{ORG}}$ uptake to tree nutrition (Adams *et al.* 2006). Finally, the focus of this work was to determine if N$_{\text{ORG}}$, amino acids and peptides, are a N source for tree, not the mechanisms involved.

We have enhanced the knowledge on the potential significance of N$_{\text{ORG}}$ in plant nutrition showing that laboratory based uptake results are, to a degree, transferrable to real world results. With this in mind it is reasonable to suggest that field-grown plants are capable of taking up the same N$_{\text{ORG}}$ compounds as were taken up in the laboratory. From the present work it can be inferred that *E. nitens* and *E. globulus* are able to take up a range of amino acids at concentrations both realistic and in excess of soil amino acid concentrations. The ability to take up amino acids which are abundant in soil solution suggests that the pool of LMW DON has a significant quantitative role in the N nutrition of plants.
CONCLUSIONS

$N_{\text{ORG}}$ uptake was demonstrated in short and longer term experiments (one to four hours up to two weeks) and at concentrations akin to $E. \textit{nitens}$ forestry plantation $N_{\text{ORG}}$ concentrations (10 µM in solution) and relative to previous studies (4 mM in agar). The uptake of $N_{\text{ORG}}$ compounds, detected in the soil solution of field sites, did not significantly differ to the rate of uptake of ammonium or nitrate. In the present study, it was shown that $E. \textit{nitens}$ and $E. \textit{globulus}$ take up both $N_{\text{ORG}}$ and $N_{\text{INORG}}$ sources of N under realistic and excessive concentrations of N and the laboratory and field. Using multiple methods was an effective means to increase our understanding of the role of $N_{\text{ORG}}$ in $E. \textit{globulus}$ and $E. \textit{nitens}$ N nutrition and more broadly of $N_{\text{ORG}}$ in plant nutrition.
REFERENCE LIST


CHAPTER 3

Organic nitrogen and protease activity to indicate N-limitation in Eucalyptus nitens plantations
ABSTRACT

N-fertiliser application overcomes N-limitation and maximises plantation and agricultural crop productivity. The main tools required to apply fertiliser judiciously are soil-based tests for N-limitation. Historically, measuring N-limitation uses pools of inorganic N (\(\text{N}_{\text{INORG}}\)) and N mineralisation rates owing to two assumptions: 1) plants only take up \(\text{N}_{\text{INORG}}\) forms, and 2) the rate-limiting step of plant N availability is mineralisation. Gradually more and more evidence challenges these two assumptions. For example, I have shown that \textit{E. nitens} and \textit{E. globulus} take up many of the organic N compounds present in soils of \textit{Eucalyptus} plantations and others have suggested that the rate-limiting step in N availability could be the process of depolymerisation. These shifts in our understanding of which forms of N plants take up and the rate-limiting step in supplying N to plants are currently not included in soil-based N limitation tests.

Our aim was to assess whether amino acid concentration (as a measure of organic N) and potential protease activity (as a measure of the rate of depolymerisation) indicate N-limitation in soils of \textit{E. nitens} plantations. I established field sites at nine \textit{E. nitens} forestry plantations that experienced similar rainfall and temperature, but differed in soil parent material and nutrient availability. I collected soil samples at each of the nine plantations on four occasions over 20 months. We measured the total amino acid, \(\text{N}_{\text{INORG}}\) concentrations, and potential protease activity in soil sample extracts. N-limitation was measured as the increase in tree height with fertiliser application, which revealed N-availability ranged from sub optimal (massive growth response to fertiliser) to supra optimal (no growth response).

Amino acid concentrations and rates of potential protease activity correlate significantly with plant growth (measured using tree height) and N-limitation, whereas \(\text{N}_{\text{INORG}}\) concentrations do not. The strong relationship between protease activity and N-limitation demonstrates the potential for protease activity as a strong indicator of N-limitation in forestry plantations.
INTRODUCTION

Plants require large quantities of N in order to grow and N-limitation is one of the greatest limiters of plant growth (Aggangan et al. 1998; Vitousek et al. 2002). N fertilisers overcome gross N deficiencies in commercial crops (e.g. forestry plantations and agricultural crops) where maximising productivity is imperative. To apply fertiliser wisely requires knowledge of the availability of plant-available forms of N in the soil.

Methods of indicating N-availability have historically focused on the rate at which N$_{\text{INORG}}$ becomes available (Moroni et al. 2004; Smethurst 2006), and the extractable N$_{\text{INORG}}$ compounds in soils (Smethurst 2000; Smethurst et al. 2004). Indices of N-limitation based on N mineralisation have been used on Eucalyptus plantations in Tasmania (Moroni et al. 2004; Smethurst 2006) and other regions of Australia (Aggangan et al. 1998; Hopmans et al. 2005; O’Connell and Rance 1999; White et al. 2009) as well as plantations of a diverse range of tree species around the world (Fisk et al. 2014; Garcia-Montiel and Binkley 1998; Gómez-Rey et al. 2010; Sucre and Fox 2009).

There is massive variation in how well N$_{\text{INORG}}$ and N mineralisation predict N-availability. For example, measurement of N$_{\text{INORG}}$ and N mineralisation have shown to vary with temperature (Andersen and Jensen 2001; Bardgett et al. 2002), water availability (Bardgett et al. 2007; Bejarano et al. 2014), ground cover (Gómez-Rey et al. 2010) and geographic dispersion (Unger et al. 2010). The propensity for variation among indicators suggests N mineralisation is not the rate-limiting step of N-availability and a new robust indicator is required.

Other approaches for developing new indicators of N-limitation have been to characterise the soil microbial biomass (SMB) (Bardgett et al. 2002, 2003; Högberg et al. 2013), measure N use efficiency (López-Bellido and López-Bellido 2001) or other soil chemical properties such as organic C concentrations and C : N ratios (Bauhus et al. 2002; Sucre and Fox 2009). These have had mixed results, with no strong indicator identified.
Indicators of N limitation historically focussed on N\textsubscript{INORG} and mineralisation, but there could be scope for developing indicators based on N\textsubscript{ORG}. Before considering the use of N\textsubscript{ORG} as an indicator of N availability, it is worth considering the two reasons that studies historically focused on N\textsubscript{INORG} and mineralisation. First, N\textsubscript{INORG} compounds, ammonium and nitrate, were considered the only plant available forms of N. Second, the rate-limiting step in supplying those plant-available forms of N was regarded as N mineralisation (Schimel and Bennett 2004). For these two reasons, efforts to develop indicators of N availability focussed firmly on N\textsubscript{INORG}, and N\textsubscript{ORG} has received little attention.

Accruing evidence supports the theory that N\textsubscript{ORG} sources, particularly small peptides and amino acids (from both protein and non-protein sources), play a role in N-nutrition of a variety of plant species (Jämtgård et al. 2010; Jones et al. 2005; Näsholm et al. 2009; Warren 2009, 2013b). A large portion (90%) of total soil N is in the form of N\textsubscript{ORG} (Geisseler and Horwath 2008; Inselsbacher et al. 2014; Schulten and Schnitzer 1997) and low molecular weight (LMW) dissolved N\textsubscript{ORG} (<350 Da) pools constitute N sources which are as abundant as pools of N\textsubscript{INORG} (Jones et al. 2004; Kielland 1995).

Plants have demonstrated an ability to take up some of these N\textsubscript{ORG} compounds (Falkengren-Grerup et al. 2000; Jämtgård et al. 2008; Kielland et al. 2006; Näsholm et al. 2009; Warren 2013a). Logic would dictate that if the plant N diet has the capacity to include N\textsubscript{ORG} then the rate at which the soil microbial biomass produces N\textsubscript{ORG} compounds could be the rate-limiting step in N-availability. The production of N\textsubscript{ORG} compounds occurs via the process of depolymerisation in which extracellular proteases catalyse the hydrolysis of proteins into peptides and amino acids (Jämtgård et al. 2010; Vranova et al. 2013). Taken together these findings suggest that the N\textsubscript{ORG} compounds present in soil and rate of depolymerisation could indicate N limitation.

The present work evaluated whether depolymerisation was the rate-limiting step in supplying N to E. nitens plantations, and thus if depolymerisation could be used as an indicator of N-limitation. I established field sites at nine E. nitens forestry plantations that experienced similar rainfall and temperature, but differed in soil parent material and nutrient availability. Potential protease activity indicated the rate of depolymerisation of soil extracts. Amino acids are abundant in the forestry
plantation soils (Chapter 1) and taken up by *E. nitens* (Chapter 2), hence were measured as an index of N-limitation rather than measuring the entire N\(_{\text{ORG}}\) pool. Soils were also analysed using traditional methods of N-limitation indication for comparison with protease activity and amino acid concentrations. To provide an independent index of N-limitation we used the difference in tree height of fertilised and unfertilised plots of 20-month-old *E. nitens*. 
METHODS

Field site details

Field trials were established on nine *E. nitens* (Deane & Maiden) plantations in September 2011. These sites were chosen that range in productivity (LP-low productivity, MP-moderate productivity and HP-high productivity) or N availability but have similar annual average temperature minima (6 °C to 9 °C) and maxima (15 °C to 18 °C) and annual rainfall (1200 to 2400 mm) as recorded in 2013 (BOM 2014). The nine field sites were previously used for *Eucalyptus, Pinus* or *Acacia* tree plantations.

![Map of Tasmania showing field site locations](image)

Figure 1. Map of Tasmania showing field site locations. The sites in NE Tasmania are part of the Bass district and the sites in central Tasmania are within the Derwent region. These sites are managed by Forestry Tasmania (Image downloaded from d.maps.com, coupe co-ordinates from Forestry Tasmania). Refer to table 1 for field site details.
The field sites ranged in N-availability, from sub optimal (large response to fertiliser application) to supra optimal (minimal or no response to fertiliser application) N-availability. Measured concentrations of total N, P and organic C were above and below the critical levels recommended for Tasmanian forestry plantations. The critical concentrations being the concentrations of organic C (5 %), total N (0.3 %) and total P (0.025 %) in soils of *E. nitens* plantations required for 75 to 99 % of maximum yield (Grant *et al.* 1995). The sites details are summarised in Table 1.
Table 1. Field Site Information. Details for each of the field trial plantation sites, including the district in which they are located (refer to Figure 1), site geology and previous use as provided by Forestry Tasmania. All sites were established in September 2011. Total N, total phosphorus (P) and organic carbon (C) are included which were obtained from a subsample of bulked samples of each of the 5 replicate unfertilised plots at each site (n=1 sample site\(^{-1}\)=9). Site productivity ranking (LP-low productivity, MP-moderate productivity and HP-high productivity) based on visual inspection and the previous rotation productivity (Paul Adams, pers comm). The results discussed with Figure 2 were used to rank sites within each productivity group (e.g. LP1, LP2, LP3)

<table>
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<tr>
<th>Site</th>
<th>District</th>
<th>Geology</th>
<th>Total N (%)</th>
<th>Organic C (%)</th>
<th>Total P (%)</th>
<th>Previous Rotation Species</th>
<th>Site Productivity</th>
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<td>Bass</td>
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<td>Ordovician quartzite</td>
<td>0.28</td>
<td>4.7</td>
<td>0.02</td>
<td><em>Eucalyptus</em></td>
<td>Mid</td>
</tr>
<tr>
<td>HP1</td>
<td>Bass</td>
<td>Ordovician talus</td>
<td>0.25</td>
<td>4.1</td>
<td>0.02</td>
<td><em>Pinus</em></td>
<td>High</td>
</tr>
<tr>
<td>HP2</td>
<td>Derwent</td>
<td>Jurassic basalt</td>
<td>0.53</td>
<td>7.4</td>
<td>0.09</td>
<td><em>Pinus</em></td>
<td>High</td>
</tr>
</tbody>
</table>
Field site set-up

5 replicate plots of 10 seedlings planted at approximately 1100 stems. hectare\(^{-1}\) in September 2011 at every forestry plantation site. At each site there were di-ammonium phosphate (DAP) fertilised plots and 5 replicate unfertilised (control) plots (10 trees. plot\(^{-1}\)). DAP was applied at a rate of 100g. seedling\(^{-1}\) within approximately 1 month of plantation establishment.

Soil sampling and collection

Soil samples were collected from the upper 0-15 cm soil of each of the 5 replicate control plots at each site (n=5 plots. site\(^{-1}\) x 9 sites=45) on 3, 7, 13 and 20 months after plantation establishment. For each of the control plots approximately 500 g of soil was collected within a 30 cm radius of 5-7 trees. The 5-7 samples per plot were combined and sieved (<10 mm) and a subsample of approximately 500 g was placed in a zip-lock bag then transported back to the lab in a cooler bag. Samples were refrigerated at 4 °C until extraction.

Extractable N

Soil samples were extracted in triplicate within 2-3 weeks of sampling. Potassium chloride (1 M KCl) was added to soil samples at ratio of 5:1, KCl (mL): soil (g FW) in 50 mL tubes with screw top caps. These were shaken (100 rpm) for 1.5 hours at room temperature (21°C) then centrifuged for 10 minutes at 3200 x g. Solids were filtered using funnels of Whatman #1 filter paper. Soil extracts were then analysed using colorimetric methods for ammonium (Baethgen and Alley 1989), nitrate (Miranda et al. 2001) and amino acids (Jones and Kielland 2002; Moore and Stein 1954) with absorbance measured using a microplate reader (Biotek Synergy 2 multi-mode).

Potential protease activity

Potential protease activity was determined using a modified protease assay method (Ladd and Butler 1971). Soil sub-samples were extracted using potassium phosphate buffer (50 mM KPO4, pH 7.6) at a ratio of 8: 1 buffer (mL): soil (g FW) shaken (100 rpm) for 90 minutes at room temperature. Samples were centrifuged 3200xg and the supernatant filtered through Whatman #1 filter paper funnels. A 2 mL sub-sample of filtered extract was immediately placed on ice in an eppendorf tube as the blank (no substrate) sample. To the remaining extract 2% casein was added (1: 8
mL casein: extract) and thoroughly mixed. Samples were shaken (100 rpm) for 6 hours at room temperature with 2 mL sub-samples taken at immediately after casein addition then 1, 3 and 6 hours after and placed on ice.

Once the assay was complete the amino acid concentrations in the samples were measured using the ninhydrin reagent (Jones and Kielland 2002; Moore and Stein 1954) with glycine at concentrations from 0 to 200 µM as the standard. Protease activity was determined using the linear regression of the change in amino acid concentration over time.

**Total N, total P and organic C**

In November 2012, sub-samples (400 g) of the bulked soil samples of each of the 5 replicate unfertilised plots for each site were sealed in zip lock bags. These were kept refrigerated until delivery to SESL Laboratory (Thornleigh, NSW) or Allison Laboratory (Hobart, Tasmania). Here they were analysed for total N by dry combustion (LECO), organic C (Dumas C) and total P in acid digest by ICP (SESL 2014).

**N-limitation - tree measurements**

Tree growth response to fertiliser application was used as an indicator of N-limitation. Measuring poles were placed at the base of the tree and the height of the tallest point was recorded for every tree on all of the sites, this was done 20 months after planting. The occasional tree which had been, for one reason or another, removed or considerably disrupted e.g. through animal foraging of a young tree, was excluded from the measurements.

The growth response was calculated as the average increase in height of DAP fertilised trees as a percentage of the average height of control trees for each replicate plot:

\[
\text{Growth Response} (\%) = \frac{\text{Avg DAP tree height} - \text{Avg Control tree height}}{\text{Avg Control tree height}} \times 100
\]
The mean height of each plot was determined, and then the growth response between replicates calculated. The average site height was determined using the mean of five plot replicates. All heights were measured in metres. A greater growth response to fertiliser application was indicative of greater N limitation within the plot/s.

**Statistical analysis**

The results were analysed using a one way ANOVA in SPSS software to test for significance and collated using Microsoft Excel to produce Figures with $R^2$ values to determine the relationships between factors. There were 5 replicate plots per site.
RESULTS

Plant growth and response to fertiliser

The sites at which trees grew tallest without fertiliser addition showed the smallest response to fertiliser addition and vice versa. The percentage by which DAP increased the growth of trees was greatest (46.4% increase) on site LP1, at which control tree height was shortest (1.52 m). The trees which grew the tallest on average (site HP1, 4.48 m) also responded less to the addition of DAP (4.8%).

Figure 2. Final tree height (m) and growth response to fertiliser application. The site average height of *E. nitens* trees at 20 months (a) and the percentage increase in average tree height with DAP fertilisation (b) are displayed in ascending order of the height of control trees at 20 months. The error bars represent the standard deviation between replicate plots on each site (n=5 replicate plots. site⁻¹).
Extractable N and tree growth

The concentration of nitrate and ammonium in the soil extracts show weak ($R^2=0.17$, $p=0.004$) and non-significant correlations ($R^2=0.02$, $p=0.36$) respectively with height of unfertilised trees (Figure 3a and 3b). There was a significant positive correlation ($R^2=0.56$, $p<0.001$) between amino acids in soil extracts and the height of unfertilised trees (Figure 3c).

Figure 3. Indicators of N-limitation results showing the relationship between a) nitrate, b) ammonium and c) amino acid concentrations and the tree height of unfertilised trees at 20 months. These results are average concentrations from soil samples collected 3, 7, 13 and 20 months after plantation establishment in September 2011 (n=5 replicate plots x 9 sites=45).
Protease activity and tree growth

Potential protease activity is an index of depolymerisation rates. There was a positive linear correlation ($R^2=0.68$) between the height of unfertilised trees and the protease activity (Figure 4).

![Protease activity and tree growth](Image)

Figure 4. Protease activity as an indicator of *E. nitens* growth. The relationship between potential protease activity, in soil samples collected from 5 replicate unfertilised plots at 3, 7, 13 and 20 months after planting, and height of unfertilised trees at 20 months after planting. ($R^2=0.68$, $p<0.01$, $n=45$).

Protease activity, soil chemical data and growth response to fertiliser

Among the nine plantations there was a strong negative relationship ($R^2=0.74$, $p=0.006$) between the protease activity and growth response to fertiliser application (Figure 5a). The relationship between growth response and concentrations of total N, P and organic C were weak (Figure 5b and d).

The relationships between ratios of Total N, P or organic C were not significant or strong for either tree height or tree growth response to fertiliser (Figure 6).
Figure 5. The relationship between protease activity, soil chemical data and growth response to fertiliser application. The error bars for a) protease activity represent the standard deviation between averages of samples collected at 3, 7, 13 and 20 months after site establishment. The dotted lines represent critical values for b) Total N, c) Organic C and d) Total P which were measured on composite soil samples for each site collected at the 20th month from each of the 5 replicate unfertilised plots (Grant et al. 1995).
Figure 6. Relationships between ratio of soil chemical data and tree height or growth response to fertiliser. The relationships between ratio of total N to total P (c and d) or organic C to total N (a and b) with tree response to fertiliser application (a and c) or tree height at 20 months (b and d) were determined for the complied soil samples described for soil chemical data in Figure 5 for each of the nine sites.
DISCUSSION

What is the rate-limiting step in the N cycle?

Our study adds to a growing body of literature suggesting that depolymerisation could be the rate-limiting step in the N cycle. In the case of *E. nitens* plantations, among nine field sites the potential protease activity strongly related to the extent of N limitation, as indicated by growth response to fertiliser (Figures 4 and 5a).

Previous studies reported similar relationships between protease activity and N-limitation. For example, protease activity was correlated with total N in soil (Raab *et al.* 1999) and with the microbial properties of soils (Alef *et al.* 1988). Studies in Taiga forests reported an increase in protease activity along successional gradients (Kielland *et al.* 2007), while depolymerisation was also found to be the rate-limiting step in N-availability in black spruce forests (Jones and Kielland 2002). The widespread occurrence of positive correlations between protease activity and N-availability are consistent with the view that depolymerisation is the rate-limiting step in supplying plant-available forms of N (Schimel and Bennett 2004).

Amino acid and oligomers are the immediate products of depolymerisation, and thus correlations of growth rate with pools of amino acids provide further evidence depolymerisation is the rate-limiting step. For example among nine *E. nitens* plantations, extracted amino acid concentrations significantly correlated with tree growth (Figure 3). This result was expected as the ability to take up NORG is widespread among plant species (Jämtgård *et al.* 2008; Jones *et al.* 2004; Näsholm *et al.* 2009; Warren 2009) including *Eucalyptus* (Warren 2006). Taken together, the ability to take up amino acids (Chapter 2) and relationship between amino acid concentration and tree growth (Figure 3), suggests N nutrition of *Eucalyptus* may not be dependent upon mineralisation of NORG to INORG.
Protease activity and amino acid pools as indicators of N limitation

We have established that protease activity and amino acid concentration are strong indicators of N-limitation and tree growth. The correlation between $\text{N}_{\text{ORG}}$, total N, P or organic C concentrations, and tree growth or N-limitation were weak (Figures 3a, 3b, 5b, 5d and 6), especially compared to the strength of the relationship between N-limitation and amino acid concentration (Figure 3c) or protease activity (Figure 5a). Protease activity serves as an indicator of the rate at which $\text{N}_{\text{ORG}}$ compounds potentially become plant available, and plants are able to take up $\text{N}_{\text{ORG}}$ compounds (Chapter 2). Protease activity and pools of amino acids therefore have practical application as new indicators of N-limitation in forestry plantations.

Comparison with other soil indices for N-limitation

Pools of N

Analysing total N concentrations in soil extracts is commonly used to determine N-availability (Smethurst et al. 2004), but it is not successful in all cases. For example, N deficiency was also predictable through the measurements of nitrate, ammonium and total N concentrations on experimental sites of wheat in Colorado (reported by (Smethurst 2000) from (Tisdale et al. 1993)). While total N concentration indicated N-availability in Eucalyptus forests in south east Australia (Hopmans et al. 2005). However, in the present study with plantations of E. nitens, ammonium (Figure 3b), nitrate (Figure 3a) and total N (Figure 5d) correlations were all rather poor indicators of N-limitation, particularly in comparison to protease activity (Figure 5a) and amino acids concentration (Figure 3c).

There are several possible explanations for the inconsistency of relationships between N compounds and N-limitation. Part of the reason for discrepancy among studies could be because different plant species can have preferences for different forms of N (Shedley et al. 1995). How well any particular compound (or class of compounds) predicts N-limitation ought to depend, on which compounds (or classes of compounds) plants take up. Thus, one might predict a strong correlation for compounds readily taken up, and weaker correlations for compounds not taken up.

Another explanation for variation between studies is the difficulty of determining if high N concentrations indicate plant available or rejected N (Schimel and Bennett 2004). Irrespective of why relationships are inconsistent, the often-poor correlations...
seen when using total N, ammonium or nitrate concentrations as indicators of N-limitation demonstrate a limited potential for these as reliable and accurate predictors of fertiliser requirements.

**Organic C**

Some studies have used organic C as an indicator of N limitation, but with inconsistent results. Soil C concentration, C:N ratio and measurements of C mineralisation have in some studies been linked to N mineralisation rates and indirectly with the response of crops to N fertiliser application (Adams and Atwill 1986; Austin and Sala 2002; Garcia-Montiel and Binkley 1998). However, in plantations of *E. nitens*, organic C (Figure 5c) and C:N (Figure 6) were weakly related to N-limitation. Similarly poor correlations were reported for *Eucalyptus* species in eastern Australia (Hopmans *et al.* 2005) and other forest soils (Sucre and Fox 2009). In conclusion, there is no consistent relationship between organic C and C:N and thus only in certain, limited circumstances are organic C and C:N likely to be suitable indicators of N-limitation.

**Total P**

Many terrestrial ecosystems, including *Eucalyptus* plantations (Smethurst 2000), which are N-limited are also P-limited (Harpole *et al.* 2011; Vitousek *et al.* 2010). Thus application of P fertilizer can affect availability of N, and the response of N-availability to the addition of NP fertilizer tends to be greater than to the addition of P or N alone (Ågren *et al.* 2012; Fisk *et al.* 2014; Minick *et al.* 2011). The balance and interaction between N and P levels is mediated by processes at small scales, for example phosphatase activity is increased by increasing N concentration (Fisk *et al.* 2014; Marklein and Houlton 2012) and the converse is also observed (Olander and Vitousek 2000). Despite the overwhelming evidence that there is a relationship between N and P, there is no evidence that P concentration or N:P ratio would be suitable indicators of N-limitation. Just as in *E. nitens*, there was no relationship between total P and N-limitation (Figure 5b) or N:P and tree height at 20 months (Figure 6).
Advantages of using protease activity to predict N-limitation

Protease activity is a logical indicator of N availability because it measures the rate at which N converts into plant-available N forms. Soils contain large amounts of organic N but it is primarily in the form of high molecular weight polymers such as proteins (Lipson et al. 2001; Schulten and Schnitzer 1997) that cannot be directly taken up by plants. Plants can tap into the enormous reservoir of organic N only after polymeric compounds are depolymerised to the oligomers (Farrell et al. 2013) and monomers (Lipson and Näsholm 2001) that plants can take up. Mounting evidence suggest that this depolymerisation step is rate-limiting (Jones and Kielland 2002; Kielland 2007) and thus measurements of protease activity indicate the rate at which soil N compounds become plant available.

Compared with some of the more traditional indicators of N-limitation, there are technical advantages of using protease activity as an indicator of N-limitation. An important aspect of the strong relationships of protease with N-limitation in the present study is that we estimated potential protease activity in the laboratory. Potential protease activity is easy and robust because samples can be analysed in the laboratory, thereby eliminating many environmental factors during the analytical process.

Potential protease activity is in fact easier to measure and potentially more robust than, for example, N mineralisation. The resin bag method of estimating N mineralisation involves burying resin bags in the field for weeks to months during which time the bags are exposed to the elements, such as rainfall, chilling and high heat, which could affect the N mineralisation rates measured (Bejarano et al. 2014). Other methods of measuring mineralisation such as in situ soil cores (Adams and Atwill 1986) involve installation of cores and multiple samplings and thus are more labour intensive than potential protease activity.

Measuring the potential protease activity gives a reliable measurement that could transcend seasonal fluctuations. This is borne out by the observation that there was little seasonal variation in protease activity (Figure 5a, error bars). This low seasonal variation was also reported in a study of amino acid transformations in alpine soils (Lipson et al. 2001) and has been further discussed in Chapter 2.
Limitations and biological context

Other indicators of N limitation, e.g. net N mineralisation and total N in Eucalyptus plantations have been tried and tested extensively, and many of the same tests will need repeating for protease activity. Rigorous testing of net N mineralisation and total N has seen variations of accuracy and reliability between sites based on temperature (Andersen and Jensen 2001; Bardgett et al. 2002), temperature and moisture (Bardgett et al. 2007), rainfall (Bejarano et al. 2014), vegetation cover (Gómez-Rey et al. 2010) and altitude (Unger et al. 2010). In addition to these tests, application of protease activity as an N-limitation indicator needs testing in areas affected by drought and salinity and sites not subject to climatic extremes or nutrient limitations. Such results would validate protease activity and N$_{\text{ORG}}$ as robust indicators of N-limitation and therefore validate their use as indicators of N-limitation.
CONCLUSIONS

Analysis of soils from *E. nitens* plantations indicates there is a strong relationship between protease activity (indicative of depolymerisation), amino acid concentration and the growth response of trees to fertiliser (N-limitation). Taken together these observations suggest that depolymerisation, rather than mineralisation, is the rate-limiting step in production of plant-available forms of N. An important practical outcome of this work is that the strong relationship between protease activity and the growth response to fertiliser shows depolymerisation is a viable indicator of N-limitation in soils of *E. nitens* plantations in Tasmania. In addition, the pool of amino acids in soil extracts was a strong indicator of tree growth potential, especially when compared with the weak or non-significant relationships between N limitation and other measurements of soil chemistry (*viz.* ammonium, nitrate, total N, total P and organic C). Depolymerisation is a simple predictor of differences among sites in N-availability, and thus can help tailor fertiliser application to N-availability. Ultimately, the use of more accurate indicators of N-limitation such as protease activity will help minimise the inaccuracies and waste associated with current methods of matching fertiliser application to N-availability.
REFERENCES


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GENERAL DISCUSSION

Unearthing further questions on the role of organic nitrogen to plant nutrition
Until recently inorganic N (N_{INORG}) was considered the only N source available to plants, plant N availability in soils has been measured based on pools of N_{INORG} (Schimel and Bennett 2004). Organic N (N_{ORG}) plays a role in the N nutrition of a wide array of plant species (Näsholm et al. 2009). Despite a surge in evidence and research into the role of N_{ORG} in plant N nutrition, some key uncertainties persist:

- composition of the low molecular weight (LMW) N pools
- the dynamics and fluxes of N_{ORG} pools;
- the competitiveness of plants with the soil microbial biomass for N_{ORG};
- abundance of N_{ORG} to N_{INORG} pools in soil water and soil extracts and
- determining N requirements and methods of analysing plant N status

To address these uncertainties, this thesis has addressed three key questions:

1. What are the most abundant low molecular weight (LMW) N_{ORG} compounds in soil? Does the relative and absolute abundance of compounds vary seasonally, and between high and low productivity sites?
2. Are plants able to germinate, grow and take up the N_{ORG} compounds which are present in soils? Is N_{ORG} uptake possible in the field as well as the lab?
3. Can measurement of N_{ORG} pools, or protease activity, be used to accurately indicate N-limitation? Does protease activity limit N availability?

The ultimate aim of this thesis was to better understand N_{ORG} uptake in Eucalyptus nitens and E. globulus and use this to determine a method of indicating N-limitation. I successfully addressed the above three questions experimentally; however, this gave rise to more questions (Figure 1). This general discussion will provide some insight into the role of N_{ORG} in plant nutrition, based on extensive literature review and field and laboratory based experiments using Eucalyptus nitens and E. globulus, as outlined in Figure 1. The questions presented in Figure 1 are by no means an exhaustive list and I am unable to provide adequate insight into all of these points. Only the questions in green will be addressed in this discussion.
Figure 1. Summary of the questions investigated in this thesis (blue) the answers (yellow) and the questions which these answers lead to, some of which will (green) be discussed in this general discussion and other which will not (red).
What does the LMW DON pool represent?

The LMW DON represents one or more of three possible scenarios:

1. a pool of N available for plant uptake,
2. a pool of N available for mineralisation or
3. a pool of N which has been rejected by plants and microbes.

Within the scope of this thesis it is possible to conclude that scenario 1 is likely. The rationale behind this conclusion is shown by seasonal variation in the soil LMW DON concentration (Chapter 1). LMW DON compounds accumulate during winter, when plant growth is slowest and nutrient uptake is reduced; then LMW DON compounds rapidly reduce in spring, when plant growth and nutrient uptake increase. *E. nitens* forestry plantations exhibited this pattern of LMW DON accumulation and decline throughout winter and spring (Chapter 1), as has also been reported for boreal forest soils (Inselsbacher and Näsholm 2012a; Inselsbacher *et al.* 2014). *E. nitens* can take up N$_{\text{ORG}}$ compounds, further supporting the LMW DON pool represents a pool of plant available N.

Neither the seasonal variation in N$_{\text{ORG}}$ compound abundance, nor the uptake of N$_{\text{ORG}}$ compounds by plants is evidence that the LMW DON pool is exclusively for plant uptake. The soil microbial biomass (SMB) also reduces in size and activity during colder periods, increasing again with warmer weather (Abuarughb and Read 1988; Jones *et al.* 2005; Lipson *et al.* 1999; Ruess *et al.* 2006; Weintraub and Schimel 2005). While the cycling of the SMB suggests reasons for the variation in LMW DON pool constituents, specifically investigating the relationship between LMW DON compounds and the SMB was beyond the scope of this thesis. Investigating the role of the LMW DON pool through the soil-plant-microbial system using N fluxes is discussed in the following section.
Would N fluxes be more accurate measurements of N availability?

Changes in soil N chemistry, and the movement of N compounds between plants and microbes, are investigated using N flux experiments. Both forms of flux experiments provide valuable insights into plant N nutrition. For example, flux experiments have shown that plants are competitive with the soil microbial biomass for N, \( \text{N}_{\text{ORG}} \) is rapidly mineralised to ammonium and N availability and \( \text{N}_{\text{ORG}} \) uptake is influenced by soil temperature (Inselsbacher and Näsholm 2012b; Jones and Kielland 2002; Schimel et al. 2004).

N flux experiments involve tracing the movement of, often, isotopically labelled N molecules (\( ^{15}\text{N} \)) through the soil and/or between SMB and plant roots (Jones and Kielland 2002). Examining the role of \( \text{N}_{\text{ORG}} \) compounds in plant N nutrition using N flux experiments (either chemical changes or the movement between organisms) seems highly logical. However, such experiments are very specific, focusing on one compound at a time, and expensive to conduct. Thus, without adequately understanding the soil N composition we are potentially studying the movement of irrelevant compounds, wasting both time and money.

Regardless of appropriate compound selection, N flux experimental procedures encounter many issues, both biological and procedural in nature. No robust means of ruling out microbial metabolism as a precursor to plant uptake exists, as LMW DON compounds are rapidly metabolised in soils (Jones et al. 2004). Dual labelled \( ^{13/14}\text{C}^{15}\text{N} \) compounds can identify microbial involvement, however a strong understanding of soil N composition is necessary for dual labelled compounds to be worthwhile (Warren 2014). Hence, in the present work we opted to increase our understanding of the soil N composition so that future studies can conduct more informed \( \text{N}_{\text{ORG}} \) flux experiments.

What are the most abundant N forms in soil?

Most soil N compounds are insoluble and not available for plant uptake; however of the dissolved N (DN) compounds those with a high molecular weight (HMW) constitute the largest fraction (Jones et al. 2004). Via the processes of depolymerisation HMW DN compounds are broken down to LMW DN. Two pools of LMW DN compounds exist in soils, \( \text{N}_{\text{INORG}} \), ammonium and nitrate, and \( \text{N}_{\text{ORG}} \), including amino acids and small
peptides (Vranova et al. 2013). Both LMW DN pools are potentially plant available, and the abundance of each N pool has been linked to site productivity (Rothstein 2009, Schimel and Bennett 2004).

Early studies suggested that a highly abundant LMW DON pool was only a feature of extreme environments, such as arctic tundra and at high altitudes. In the extreme environments cold soils slow mineralisation such that the supply of N\textsubscript{INORG} is inadequate for plant growth (Schimel and Chapin 1996; Hofmockel et al. 2010). However, recent studies indicate that LMW DON is highly abundant in a range of environments, potentially constituting 50-90% of the detectable, bioavailable N in some soils (Friedel and Scheller 2002; Jones et al. 2004; Warren 2013b, Chapter 1). The bioavailability of the LMW DON fraction depends not only on the quantity of N compounds in soil water, but also on the composition of the LMW DON.

In line with previous studies, up to 80% of the LMW DON compounds detected in soil water samples from \textit{E. nitens} forestry plantations were amino acids (Chapter 1). We also found that \textit{E. nitens} are capable of taking up a range of amino acids, detected in soil water samples, in the laboratory, and at least glycine under field conditions (Chapter 1). In arctic tundra, where amino acids constitute a relatively high concentration of the LMW DN fraction, amino acids play a significant role in the N nutrition of native plants (Schimel and Chapin 1996). At \textit{E. nitens} forestry plantation sites, pools of amino acids were as large as pools of N\textsubscript{INORG}, and glycine was taken up as quickly as ammonium and nitrate by \textit{E. nitens} trees. We have therefore shown good reason to conclude amino acids are a source of N for \textit{E. nitens}. 

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Does LMW DON differ among sites?

Site productivity is linked to the relative abundance of ammonium, nitrate and amino acids in soils. LMW DN pools at strongly N-limited sites, such as arctic tundra, tend to be dominated by amino acids (Chapin et al. 1993; Schimel and Chapin 1996). Nitrate tends to dominate highly productive sites, and ammonium dominated sites are moderately productive (Figure 2) (Rothstein 2009). Nitrate is the dominant N compound in many soils, as confirmed by a study of 84 soils across America, indicating moderate N-limitation is a feature of most soils (Hofmockel et al. 2010). An adequate understanding of the N-limitation of soils equips us with the tools to accurately estimate N fertiliser requirements.

Figure 2. Hypothetical representation of the relationship between N source dominance and N-limitation (Rothstein 2009; Schimel and Bennett 2004)
This thesis investigated the N-limitation on forestry plantation sites in partnership with Forestry Tasmania. We measured EN and DN to investigate the relationship between N compound abundance and site productivity within our nine *E. nitens* plantation sites. We found that N\textsubscript{ORG} pools were relatively abundant, compared to N\textsubscript{INORG} pools, at *E. nitens* plantations that ranged in productivity (Figure 3), suggesting moderate N-limitation (Figure 2). The relatively abundant N\textsubscript{ORG} pool at high and low productivity sites slightly contradicts generalisations about N compound abundance and site productivity (Rothstein 2009; Schimel and Bennett 2004), suggesting that N\textsubscript{ORG} pools are abundant on sites that are not N-limited.

**Figure 3.** Concentration of exchangeable N in soil extracts from 9 *E. nitens* plantations in Tasmania. This is in order of tree growth (least to most). Nitrate (blue) ammonium (yellow), and amino acid (green) concentrations have been averaged (columns) with vertical bars representative of the standard deviation between seasons for all measurements (n=2-4 measurements. season\textsuperscript{-1} 4 seasons). Sites are named according to their productivity based on N-limitation (growth response to fertiliser application) - low productivity (LP), medium productivity (MP) and high productivity (HP). In Chapters 1 and 2 LP1 and HP2 have been referred to as LP and HP respectively, as the other sites were not discussed in these chapters.
Plants can take up $\text{N}_\text{ORG}$ including amino acids and small peptides

A wide array of plants can take up $\text{N}_\text{ORG}$ sources particularly amino acids such as Gly, Ser and Glu. N uptake occurs in controlled experiments using wheat (Jämtgård et al. 2008), arctic sedges (Chapin et al. 1993; Kielland 1995), species of pine (Näsholm et al. 1998), grasses (Näsholm et al. 2000) and Eucalyptus species (Warren and Adams 2007). *E. nitens* and *E. globulus* are also capable of $\text{N}_\text{ORG}$ uptake.

*E. nitens* and *E. globulus* take up a range of amino acids and small peptides in laboratory based experiments and least Gly *in situ*. The range of $\text{N}_\text{ORG}$ compounds provided to seeds and seedlings (Chapter 2) was based on detectable compounds in forestry soils (Chapter 1) and provided realistic concentrations and concentrations relative to the previously mentioned uptake studies. Furthermore, we gave real world relevance to our laboratory results by supplying *E. nitens* trees with Gly *in situ*. The uptake of Gly in the field suggests other compounds taken up in the laboratory are also potentially taken up *in situ* and that amino acids and small peptides are N sources for *E. nitens* and *E. globulus*.

What is the quantitative significance of $\text{N}_\text{ORG}$ to plant nutrition?

There are a range of N compounds in soils, both $\text{N}_\text{INORG}$ and $\text{N}_\text{ORG}$. LMW N compounds are the second most abundant soil N compound fraction, after HMW compounds, and include small peptides, amino acids and a wide range of other LMW DN compounds such as osmolytes, amines, tertiary amino acids and quaternary ammonium compounds (Chapter 1; Farrell et al. 2011; Hill et al. 2011, 2012; Inselbacher and Näsholm 2012a; Jämtgård et al. 2010; Jones et al. 2004, Kielland 1994, 1995; Warren 2013a, 2013b). LMW $\text{N}_\text{ORG}$ and $\text{N}_\text{INORG}$ pools were equally abundant at *E. nitens* forestry plantation soils. Plants can take up $\text{N}_\text{ORG}$ compounds (Chapter 2) at rates comparable to $\text{N}_\text{INORG}$. For example glycine was taken up by *E. nitens* trees *in situ* at least as quickly (0.12-0.41 nmoles N. g DW root$^{-1}$ hour$^{-1}$) as ammonium or nitrate uptake (0.08-0.14 and 0.06-0.18 nmoles N. g DW root$^{-1}$ hour$^{-1}$ respectively). $\text{N}_\text{ORG}$ and $\text{N}_\text{INORG}$ uptake rates are comparable in other *Eucalyptus* species (Paulding et al. 2010), barley (Jämtgård et al. 2008) and plants native to boreal forests (Persson and Näsholm 2001). Uptake of $\text{N}_\text{ORG}$ at similar rates to $\text{N}_\text{INORG}$ suggests that $\text{N}_\text{ORG}$ compounds have a significant role in the N
nutrition of plants. We demonstrated that a wide range of amino acids and small peptides were taken up by *E. nitens* and *E. globulus* (Chapter 2), previous studies have also shown amino acid uptake in a wide array of plants (Nåsholm *et al.* 2009).

In systems where N$_{\text{ORG}}$ pools are more abundant than N$_{\text{INORG}}$ pools, such as boreal and arctic tundra, N$_{\text{ORG}}$ plays a significant role in native plant N nutrition, up to 87% of N from N$_{\text{ORG}}$ (Inselsbacher *et al.* 2014; Miller and Bowman 2003; Nordin *et al.* 2001; Persson and Nåsholm 2001; Zhu and Zhuang 2013). N$_{\text{ORG}}$ was approximately as abundant as N$_{\text{INORG}}$ in *E. nitens* forestry soils (Figure 3) and *E. nitens* and *E. globulus* took N$_{\text{ORG}}$ compounds up as rapidly as N$_{\text{INORG}}$ compounds (Chapter 2). The results of this and previous work suggests that in systems with N$_{\text{ORG}}$ pools which are at least as abundant as N$_{\text{INORG}}$ pools, the N$_{\text{ORG}}$ plays a potentially significant role in the N nutrition of native plants (Inselsbacher *et al.* 2014; Miller and Bowman 2003; Nordin *et al.* 2001; Persson and Nåsholm 2001; Zhu and Zhuang 2013).

**Why haven’t we quantified the role of N$_{\text{ORG}}$ to plant N nutrition?**

Uncertainties persist in interpretation of the qualitative significance of N$_{\text{ORG}}$ to plant N nutrition. Much of this uncertainty is the result of assumptions and limitations, which underpin estimates of N$_{\text{ORG}}$ uptake. For example we assume:

- we are conducting an accurate analysis of N uptake when $^{15}$N is applied to soils and the concentration of $^{15}$N is measured in plant tissues,
- isotopically labelled N based experiments are indicative of plant uptake of the intact N compounds and
- the first organism to take up the compound is the plant, and not microbes

A reason to not make assumptions regarding N$_{\text{ORG}}$ uptake is that N compounds are rapidly metabolised prior to translocation to shoots, suggesting analysis of $^{15}$N plant tissues does not reflect N uptake (Warren 2012). N$_{\text{ORG}}$ compounds are also rapidly metabolised in soils with some amino acids having <30 min half-lives, indicating plants are the first organisms to take up N$_{\text{ORG}}$ compounds (Farrell *et al.* 2011b; Jones and Kielland 2002) While research is underway to mitigate the issues related to the assumptions underpinning N uptake experiments, to the best of my knowledge, no solution has been found thus far.
Box 1. The N cycle of forestry plantations of *E. nitens* in Tasmania. This figure is based on experimental work and literature review which have together revealed that NORG compounds are abundant in soils (Chapter 1) and can be taken up by *E. nitens* and *E. globulus* at field relevant concentrations, both in the laboratory and in the field (Chapter 2). In Chapter 3 of the thesis, we demonstrated the strong relationship between plantation productivity and protease activity as an indicator of N-limitation. Protease activity is the process by which HMW N pools are broken down to LMW compounds. This figure summarises the molecular weight based N pools (HMW, LMW) and the pools or compounds within these (HMW-insoluble and soluble, LMW-NORG and NINORG compounds). The relative abundance of LMW N compounds in EN and DN fractions is included for high and low productivity sites. The major enzyme processes are shown as are the major N sources/sinks.
What are characteristics of ideal N-limitation indicators?

\( \text{INORG} \) based measurements have been used to predict N-limitation in forestry and agriculture throughout the last century (Schimel and Bennett 2004). N mineralisation and concentrations of ammonium and nitrate are considered strong indicators of N-limitation, for example, in Australian plantation soils (Bennet \textit{et al.} 1997; Smethurst 2000, Smethurst \textit{et al.} 2003, 2004) and in other regions (Andersson and Berggren 2005; Montemurro \textit{et al.} 2006; Olsson \textit{et al.} 2012; Tisdale \textit{et al.} 1993; Tomé \textit{et al.} 1994). However, with an average of only 30-50% of the N fertiliser applied to crops actually taken up by plants (Tilman \textit{et al.} 2002, Hofmockel \textit{et al.} 2010) it is evident that there is a need to reassess the means by which N-limitation is determined.

There is growing evidence that indicators based on pools of \( \text{INORG} \) and net N mineralisation may not be ideal indicators of N-limitation. An initial issue is that \( \text{INORG} \) based measures are inconsistent across habitats. For example, \( \text{INORG} \) concentration of soil extracts weakly indicated N-limitation for barley crops \((r^2=0.46)\) (Montemurro \textit{et al.} 2006). Furthermore, the relationship is weak between soil N mineralisation and plant N uptake \((r^2=0.45)\) or crop biomass \((r^2=0.37)\) in rice crops (Russell \textit{et al.} 2006). An ideal indicator of N-limitation should not be this variable across sites.

In forestry plantations of \textit{E. nitens} the extractable N concentrations were shown to vary significantly between seasonal samples (Figure 3). This variation in EN concentration possibly explains the variation in the strength of \( \text{INORG} \) based measurements as indicators of N-limitation between habitats. Seasonal variation is known to affect the abundance of N compounds in soils (Abuarghub and Read 1988; Kielland 1995; Kielland \textit{et al.} 2007; Weintraub and Schimel 2005), the uptake of N into roots (Warren 2009) and the proliferation, activity and demise of the soil microbial biomass (Abuarghub and Read 1988; Jones \textit{et al.} 2005; Lipson \textit{et al.} 1999; Ruess \textit{et al.} 2006; Weintraub and Schimel 2005). An ideal indicator should transcend seasonal fluctuations, which \( \text{INORG} \) based measures do not.

In an ideal world N-limitation indicators would use simple, robust methods with easily interpreted results and consistent outcomes, despite the time of sampling, or the technical aptitude of the person conducting the sampling. In addition to the apparent
lack of consistency in NINORG based measurements, there is strong evidence mounting to show that NORG plays a potentially significant role in plant nutrition, as reviewed in (Näsholm et al. 2009; Schimel and Bennett 2004). Considerable work is still required to fully understand the intricacies of the role of NORG to plant nutrition (Figure 1). Nevertheless, it is clear that measurements based solely on the NINORG pools are incomplete indicators of available N. In the following sections I will present a case for protease activity as a more ideal indicator of N-limitation.

What are the characteristics of an ideal indicator? Does protease activity fit this description?

Protease activity is a simple and robust measurement which could be implemented as an indicator of N-limitation. Protease activity when measured as potential protease activity under laboratory conditions is unaffected by short term fluctuations of temperature and rainfall that can alter enzyme activity. A significant advantage of measuring potential protease activity is that soil samples can be collected in the field, stored, and then protease activity can be measured in the laboratory at a time which is more convenient (Appendix 1). As a comparison, the measurement of net N mineralisation requires the collection of multiple soil samples usually over longer periods of time (months) (Adams and Attiwill 1982; 1986).

The most important feature of an ideal indicator of N-limitation is the strength of the relationship between the indicator and N-limitation. An example of the strong relationship between protease activity and N-limitation has been found in soils of E. nitens plantations in Tasmania ($r^2=0.74$, p<0.05) (Figure 4 and Chapter 3). This is stronger than previously reported correlations based on NINORG pool, net mineralisation or total N measurement (Andersen and Jensen 2001; Bardgett et al. 2002, 2007; Bejarano et al. 2014; Gómez-Rey et al. 2010; Unger et al. 2010).

Protease activity could be a useful indicator of N-limitation in habitats other than forestry plantations as it transcends seasonal fluctuations and is based on sound scientific principles. The possibility that potential protease activity is a more suitable metric for N-limitation than traditional NINORG based measurements and measurement of N pools, was demonstrated through the analysis of seasonal variation in concentrations of EN
(vertical error bars in Figure 3 show the magnitude of the variation in N concentration for ammonium, nitrate and amino acids in soil extracts collected seasonally). There was little seasonal (winter, spring, summer, autumn) variation in the relationship between potential protease activity and N-limitation (Figures 4 and 5). In the following section, I have attempted to shed some insight into why protease activity is a strong indicator of N-limitation.

Figure 4. The relationship between protease activity and growth response to fertiliser application. The error bars for protease activity represent the standard deviation between averages of samples collected at 3, 7, 13 and 20 months after site establishment (n=5 rep samples. site⁻¹ month⁻¹). This figure has been repeated from Chapter 3 to enable smoother progression through this chapter and easier reading.
Figure 5. Potential protease activity of the nine *E. nitens* forestry plantation sites. This is the average for each site, shown separately for each season (*n*=5 replicate plots site\(^{-1}\) x 1-2 samples season\(^{-1}\)). The relationship is consistent over the seasons (*r*\(^2\)=0.58-0.65).

**Why is there a relationship between protease activity and N-limitation?**

Depolymerisation, measured using protease activity, is the decomposition of large \(N_{\text{ORG}}\) compounds to smaller N compounds such as peptides and amino acids. Therefore, protease activity represents the rate at which the LMW \(N_{\text{ORG}}\) pool is replenished, whether for direct plant uptake or for mineralisation to nitrate and ammonium.

N-limitation is impacted by the rate at which N compounds become plant available in the soil. Identifying the rate limiting step of N availability is therefore critical to accurately indicating N-limitation. Regardless of which compounds plants take up, the process of HMW \(N_{\text{ORG}}\) breakdown, hence the rate at which N compounds become potentially plant available (summarised in Box 1) involves:

1. depolymerisation (measured using protease activity) of HMW \(N_{\text{ORG}}\) to LMW \(N_{\text{ORG}}\)
2. mineralisation of \(N_{\text{ORG}}\) to \(N_{\text{INORG}}\) compounds (highly simplified in terms of the measureable steps)

Mineralisation occurs rapidly, as has been shown in grassland and forest soils, with an average amino acid half-life of 2.5 hours and ranging from 1 to 12 hours (Jones *et al.* 2005; Jones and Hodge 1999; Jones and Kielland 2002; Kielland *et al.* 2007). By
contrast the rate of transformation of protein to peptides and amino acids, depolymerisation, is quite slow (ca. 80 days) (Farrell et al. 2011a; Jones and Kielland 2002). The rate of depolymerisation compared to mineralisation suggests that once available, amino acids are potentially rapidly mineralised and therefore depolymerisation is the rate limiting step of N-availability, not mineralisation. This slow rate of protein transformation, or depolymerisation, illustrates the rationale behind the current investigation into the use of potential protease activity as an indicator of N-limitation.

Protease activity indicates the rate at which LMW DON compounds are produced, hence the rate at which LMW NO$_{\text{ORG}}$ becomes available either for direct uptake by plants or for mineralisation (Schimel and Bennett 2004; Vranova et al. 2015). In theory, protease activity is an ideal indicator of N-limitation, however, there are limitations to the present study, in which _E. nitens_ plantations have been used as a case study for N-limited systems. These limitations are briefly discussed in the following section.

**What must be done before implementing protease activity as an indicator of N-limitation?**

Protease activity holds great promise as an indicator of N limitation, but additional work is required prior to widespread implementation. Primarily, the system/s in which protease activity was examined in this study was known to be N-limited. The relationship between protease activity and N-limitation should be demonstrated in non N-limited systems.

Systems may be heavily impacted by the availability of nutrients other than N. Nutrient limitation affect difference aspects of the nutrition of organisms, both plants and SMB. As a purely speculative example, if P-limitation reduces protease activity, like P- and N-limitation reduce phosphatase activity (Fisk et al. 2014; Marklein and Houlton 2012; Olander and Vitousek 2000), then the accuracy of indicating N-limitation may be affected by P-limitation. Limitation of nutrients (apart from N) could impact the protease synthesis and activity in soils that was not encountered in this study.

In the present study, protease activity has been investigated in one system, forestry plantations in Tasmania, however the relationship between protease activity and N-
limitation should be tested in other systems. Agricultural soils, for example, often have long histories of heavy fertilisation, dramatically changing the soil chemistry, pH and microbial biomass. Protease activity as an indicator of N-limitation in agricultural soils will require some examination, to confirm depolymerisation as the rate limiting step of N availability in a system which has been so extensively modified and managed.

**Critical values of protease activity for fertiliser requirement estimation**

For widespread implementation of protease activity as a predictive metric of N-limitation in forestry and agricultural soils we also need to define critical values of protease activity. For example, in *E. nitens* forestry plantation soils (Figures 4 and 5):

- Requires fertiliser: Protease activity <0.5 µmole glycine. g soil\(^{-1}\) min\(^{-1}\) indicates strong N-limitation with 43-46% increase in tree growth following fertiliser application
- No fertiliser required: Protease activity >1 µmole glycine. g soil\(^{-1}\) min\(^{-1}\) indicates high productivity and no increase in tree height following fertiliser application

Using these critical values, soils in which protease activity ranges between 0.5 and 1.0 µmole glycine. g soil\(^{-1}\) min\(^{-1}\) would require fertiliser, but not at the same rate as sites with ≤0.5 µmole glycine. g soil\(^{-1}\) min\(^{-1}\) (Figure 6).
CONCLUSIONS

Soils with low mineralisation rates have a relatively low abundance of ammonium and nitrate. Mineralisation, the breakdown of LMW DON compounds to ammonium and nitrate, is the traditional means by which N-limitation is determined. Using N mineralisation rates to indicate N-limitation is based on the now debunked myth that plants are only capable of taking up ammonium and nitrate. Plants are also able to take up LMW DON sources of N (Näsholm et al. 2009; Chapter 2). Our work has looked at the rate of proteolysis, or protease activity as a more accurate indicator of N-limitation. Indicating N-limitation using protease activity is based on the evidence that shows LMW DON compounds are also plant N sources.

![Figure 6. Brief Summary of conclusions including critical levels of protease activity to indicate N-limitation, as represented by growth response to fertiliser application and estimate N fertiliser requirements.](image)

Figure 6. Brief Summary of conclusions including critical levels of protease activity to indicate N-limitation, as represented by growth response to fertiliser application and estimate N fertiliser requirements.
The results presented in this thesis represent an exciting milestone in the development of simple yet robust means of determining N-limitation in soils (summarised in Figure 6). We have shown that the LMW DON compounds detectable in *E. nitens* plantation soils (Chapter 1) are able to be taken up by *E. nitens* seeds, seedlings and trees in controlled and field conditions. Our results suggest that *E. nitens* are competitive with the SMB for N\(_{ORG}\) and N\(_{ORG}\) represents a potential N source for *E. nitens*.

The most innovative and progressive component of this thesis is the demonstration of a strong relationship between N-limitation and protease activity in the soils of *E. nitens* plantations in Tasmania. There is great potential for potential protease activity to be a ubiquitous indicator of N-limitation in soils through the establishment of critical values based on protease activity. That is, fertiliser application will overcome N limitation in soils with protease activity ≤0.5 μmole glycine. g soil\(^{-1}\) min\(^{-1}\). Conversely, soils with protease activity ≥1 μmoles glycine. g soil\(^{-1}\) min\(^{-1}\) are weakly N-limited and the addition of fertilisers will not benefit growth yet will accrue environmental and economic costs. The use of protease as an indicator of N-limitation is recommended as a simple and robust indicator which has strong theoretical underpinning.

**Immediate benefits of this thesis to Forestry Tasmania**

As potential protease activity has been tested across *E. nitens* forestry plantations in Tasmania, there are potential immediate benefits of this work to Forestry Tasmania. Presently fertiliser management strategies are based on N mineralisation rates or the measurements of pools of ammonium and/or nitrate. This thesis shows it is more accurate for Forestry Tasmania to use protease activity, or N\(_{ORG}\) pool abundance, to indicate N-limitation. Further, our results showed that not all sites were in need of fertilisation (HP sites), suggesting massive economic implications for accurately determining N-fertiliser requirements.
REFERENCE LIST


Warren, C. R. (2013a) *Quaternary ammonium compounds can be abundant in some soils and are taken up as intact molecules by plants.* New Phytologist 198(2): 476-485.


APPENDIX 1

The effect of storage conditions on protease activity in moist soil samples from three locations
ABSTRACT

Soil samples require storage when practical reasons prevent their immediate analysis. It is therefore important to know the impact that this storage will have on the samples and to determine soil storage limitations. In this case the limitations of storage for maintenance of the protease activity of the soil is being tested and will be useful for the later work that looks at the protease activity of soils. The literature on this is lacking and the most suitable method of storage varies between soil types and methods being used to analyse the soil. In this experiment protease activity has been determined in 3 samples of soil stored at room temperature (21 °C), in a -80 °C freezer and at 4 °C in the refrigerator for 10 weeks. The three different soil samples maintained a stable protease activity when stored for 10 weeks at 4 °C compared to initial activity levels. The frozen samples showed increased protease activity while there was a decrease in those stored at room temperature.
INTRODUCTION

While many studies have examined the activity of soil enzymes and soil enzymatic processes, very few gauge the reliability of the soil storage methods to which samples are often subjected. Large quantities of soil, distances between collection and analysis sites and field resource availability often make immediate analysis of samples impractical therefore the samples require storage. The few studies that have scrutinised soil storage practices have revealed that there is a difference in the most appropriate method of storage depending on the types of analysis that the sample will be used for (Stenberg et al. 1998). As there is no simple answer to how to best store soil samples some testing of the limitations of sample storage for individual purposes should be determined prior to conducting experiments which will involve storing soil for long durations. For future research in soil nitrogen availability in *Eucalyptus nitens* plantations in Tasmania and a possible relationship between this and protease activity the most appropriate and practical methods of storage are being determined with the present study.

Soil enzyme activity is regulated by the viable cells and colloids in the soil. These have been shown to be present as a result of plant and animal residues, but they primarily originate from the soil microbial biomass (Dinesh, Dubey et al. 1998). Hence factors which affect the soil microbial biomass will also affect the soil enzyme activity, which are important components in nutrient and bio-chemical cycles within the soil-plant ecosystem (Schmidt, Kalbitz et al. 2011). These important enzymatic functions of the soil microbial biomass, which include mineralisation, depolymerisation and nitrification, are vulnerable to environmental changes, such as pH, temperature, light exposure and water content (Čemohlávková, Jarkovský et al. 2009; Wallenius, Rita et al. 2010).

Some studies have looked at the effects of storage on the soil microbial biomass, but very few on soil enzymes. Results of these microbial biomass studies range from soil samples being most stable when stored air dried for 5 days (Luo, White et al. 1996) to frozen for 5 months (Kandeler and Gerber 1988). Others have found that storage of soil samples, either frozen or refrigerated, has no significant effect on the soil microbial biomass after 13 months (Stenberg, Johansson et al. 1998). Different soil
types react differently to storage depending on factors such as pH and moisture content. For example, boreal forest soils which are highly acidic have been reported to be more susceptible to change with freeze and cold storage treatments than mineral agricultural soils (Wallenius, Rita et al. 2010). On the other hand the microbes and enzymes of soils from dry arid areas maintain more activity than soils from wetter environments when they are dried before storage (Černohlávková, Jarkovský et al. 2009).

This present experiment has been done as a prerequisite to other work on the soils of forestry plantations in Tasmania. In this future study large quantities of soil samples will be collected each month and transported to the laboratory in Sydney. The aim is therefore to determine the most appropriate way of packing these samples for travel and then storing them once they are at the lab, before they can be analysed. This experiment also aims to test the reliability and precision of the amended protease assay method (Ladd and Butler 1971) that will be used in future experiments.
Soil samples and sampling method

Soil samples were collected from two sites within the Watagan State Forest (clay loam soils) and a sandy soil from Ku-Ring-Gai National Park near Cowan Creek. Using a spade the top 0-15 cm of a small area of soil was placed in plastic zip lock bags. Leaf litter, rocks, and other large materials/organisms were removed and the samples were sieved (5 mm). The sieved soil was then placed in fresh zip lock bags and transported a short distance, on ice, to the laboratory.

Soil storage

For each of the three locations, 24 aliquots of approximately 20 g were measured into zip lock bags. The 24 bags of soil were randomly allocated into three treatments: refrigerated (4 °C), frozen (-80 °C) and room temperature (21 °C) (n = 8 per treatment and location). Protease activity was measured immediately before storage treatments commenced, and then after one five and ten weeks of storage.

Protease activity assay

5 g soil for each sample was placed in 50 mL tubes (n = 3 tubes.sample⁻¹). These were extracted with 1M KPO₄ buffer for 90 mins then filtered through Whatman #1 filter paper into fresh tubes. 10mL 1% (w/v) casein was added and the tubes shaken thoroughly. The first sub-samples were taken immediately, transferred to a 2mL eppendorf tubes and placed on ice to stop enzyme activity. Additional sub-samples were taken at t = 60, 120, 180, 240 and 360 minutes and placed on ice. This method was modified from (Ladd and Butler 1971).

Ninhydrin reaction

The amino acid concentration was determined using ninhydrin colour reagent [0.3% (w/v) hydindatantin, 2% (w/v) ninhydrin, 1M Na-Acetate in DMSO]. 100 µL of each sub-sample was added to the wells of 96 well plates (n = 5.sub-sample⁻¹). 100 µL Ninhydrin Colour Reagent was mixed with the samples/standards. The plates were incubated for 30 mins at 80 °C. After cooling on ice, 75 µL cold 50% EtOH was added to each well to stop the reaction. Protease activity was determined by measuring the amino acid concentration resulting from casein breakdown overtime,
in terms of glycine concentration (Abs 570 nm using Biotek Synergy 2 multi-mode microplate reader).
RESULTS

Concentrations of the subsamples were determined using the standard curve (Figure 1) from the absorbencies of the glycine standards.

Figure 1. Standard Curve – for 100 µL Ninhydrin Colour Reagent added to 100 µL glycine standards of 0, 10, 20, 40, 80, 120, 160 and 200 µM in KPO4 buffer. These were incubated for 30 mins at 80 °C, cooled on ice, mixed with 50% EtOH and abs measured at 570 nm. The equation of the trendline, \( y = 0.0042x + 0.0517 \), was used to determine the concentrations of amino acids in soil samples. This is one of the standard curves that were used to determine the [glycine] of the samples, each plate had a series of glycine standards.

Table 1. Summary of Protease Activity Results with Standard Deviations – Protease activity is measured as µM glycine.minute⁻¹.g soil(FW)⁻¹ and the standard deviations are for the replicates of each time and location sample (n = 15). The protease activity is all the same for the initial (week 0) location samples as they had not yet been separated. Key: * No significant difference compared to week 0; **Significant difference from week 0; a no significant difference from other storage conditions; b significant difference from other storage conditions

<table>
<thead>
<tr>
<th>Week</th>
<th>Storage Conditions</th>
<th>Room Temperature</th>
<th>Freezer</th>
<th>Fridge</th>
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<tbody>
<tr>
<td>0</td>
<td></td>
<td>0.20NS(±0.085)</td>
<td>0.20NS(±0.085)</td>
<td>0.20 NS (±0.085)</td>
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<tr>
<td>1</td>
<td></td>
<td>0.17 NS (±0.145)</td>
<td>0.25 NS (±0.11)</td>
<td>0.20 NS (±0.090)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.07 NS (±0.025)</td>
<td>0.39”b(±0.081)</td>
<td>0.19 NS (±0.065)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.09 NS (±0.046)</td>
<td>0.75”b(±0.078)</td>
<td>0.18 NS (±0.079)</td>
</tr>
</tbody>
</table>
The protease activities for each sample over the ten-week period are summarised in table 1. For each sample the protease assay was performed on three replicate samples. These were then plated into the 96-well plate with 5 replicates. The standard deviation between all of the replicates \((n = 15)\) was then determined for the replicates of each sample and for the majority of the samples this was found to be relatively small. The majority of the standard deviations are between 1.36 and 6.07 \(\mu\text{M glycine} (1\text{-}10\% \text{ of amino acid concentrations})\). Table 1 shows this as a percentage of amino acid concentration.

The amino acid concentrations were plotted against time, to determine protease activity \((\mu\text{M glycine minute}^{-1} \text{g soil}^{-1})\). These values ranged from 0.04-0.79 \(\mu\text{M glycine minute}^{-1} \text{g soil}^{-1}\). The protease activity values for each sample were normalised then averaged across the three locations. These were plotted against storage duration, shown in figure 2. This data was analysed using repeated measures ANOVA in SPSS software to test for significance. It was determined that there was a significant difference \((p < 0.05)\) between the changes in protease activity after 4 weeks of storage for the samples stored in the freezer to those stored at room temperature and in the refrigerator. There was no significant difference to protease activity for the samples stored at room temperature and in the refrigerator.

The slope values for the trend lines of the normalised data show that there was an increase in protease activity \((0.2584 \, \mu\text{M glycine minute}^{-1} \text{g soil}^{-1})\) in the frozen soil samples and a decrease \((-0.0689 \, \mu\text{M glycine minute}^{-1} \text{g soil}^{-1})\) in the protease activity of the samples stored at room temperature. There was a very small decrease in the protease activity of the samples that were stored in the fridge \((-0.0099 \, \mu\text{M glycine minute}^{-1} \text{g soil}^{-1})\).
Figure 2. The effect on soil protease activity of storing soil samples at different temperature for ten weeks – the protease activity of three different soil samples has been determined using a protease assay followed by ninhydrin colour reaction and absorbance measurement with a microplate reader \((n = 15.\text{soil sample}^{-1}.\text{treatment}^{-1}.\text{week}^{-1})\). The protease activities of each sample were collated for 3 separate protease assays over 10 weeks. The data for each week was normalised and averaged for each storage condition and between the three samples. The trend lines shown here represent the changes in protease activity over 10 weeks compared to initial protease activity.
DISCUSSION

Protease activity and soil storage limitations

The normalised protease activity changes (Figure 2) show that storing soil samples in the refrigerator for 10 weeks has no significant effect on protease activity. This will mean that future experiments will be able to be conducted more efficiently as samples will not require immediate analyses. Furthermore, the analysis of samples will be able to be done on a smaller number of samples at a time, increasing the process accuracy.

The protease activity data that was collected showed that protease activity ranged from 0.04 – 0.79 µM glycine. minute⁻¹. g soil⁻¹. The figures are analogous to those of other studies on protease activity in soils. In a study to determine the effect of nitrogen and sulphur limitation on protease activity in sand cultures, the protease activity was shown to be between 0.08-0.6 µM tyrosine. g soil⁻¹. minute⁻¹ over a period of 6 days. This study also showed a positive correlation between protease activity and N status in the sand cultures (Sims and Wander 2001). The original method by Ladd and Butler (1971) found soil protease activity to range from 0.15-1.78 µM tyrosine g soil⁻¹ hour⁻¹, although the units differ, these findings are still within a similar range to those of this study.

The increase in protease activity that is seen in this study has been shown in studies on the microbial biomass of soil. The significance here is that the activity of enzymes in the soil is regulated and related to that of the microbial biomass. In frozen samples it has previously been observed by another study which showed that after 6 months the microbial biomass of soil samples which were kept in the freezer (-20°C) increased by 25% (Stenberg, Johansson et al. 1998). Similarly, decreases in microbial activity have been noted in samples stored at room temperature (Petersen and Klug 1994).

Other means of analysis; for instance, measuring microbial biomass, the activity of other enzymes such as urease and soil respiration; have also been used to determine soil stability over different periods of storage time. One study, which monitored changes in a range of soil enzymes and microbial biomass C, found that
the samples were negatively affected when dried and that they maintained almost
the same level of enzyme activity when stored refrigerated or frozen for 8 weeks,
with refrigeration being slightly better than freezing (Wallenius et al. 2010).
Refrigeration was again found to be most appropriate for a period of up to 8 weeks in
a wide variety of soil samples when the soil respiration, ammonification and
nitrification rates and microbial biomass C were monitored for 32 weeks
(Černohlávková et al. 2009)

**Protease assay method**

The protease assay method used here was a modification of the methods of Ladd
and Butler (1971) and of Rejsek et al. (2008). The method was tested for precision
and reliability. In order to do this the samples were run through the protease assay in
triplicate and then for each of these the absorbencies of five replicates were
measured in 96 well plates (n=3 x 5 replicates). These replicates were then
compared and the standard deviations between them determined. As shown in table
1, the majority of these standard deviations are quite low, being mostly <7% of the
amino acid concentrations. This shows that the method is precise. The previously
mentioned similarities with results of other studies suggest that the method is also
reliable.
CONCLUSIONS

There was no significant difference found in the results of the samples stored at room temperature or in the refrigerator for the 10-week period that this trial was run for. It can therefore be concluded that it is safe to store soil samples in either of these conditions for this duration of time in order to accurately measure the protease activity of soil samples in future experiments. Although the difference between the results of these conditions was found to be insignificant the data would suggest that refrigeration is perhaps more suitable as the change in protease activity in these samples was less than that of the room temperature stored samples (figure 2). The results and low standard deviations have also shown that the protease assay method that was used is reliable and precise and therefore it will be suitable for use in future experiments.


APPENDIX 2

Summary table of studies which have examined effects of storage on reliability of soil storage methods for different soil analysis methods
## SAMPLE STORAGE SUMMARY

Table 1. Summary of studies which have examined effects of storage on reliability of soil storage methods for different soil analysis methods

<table>
<thead>
<tr>
<th>Soil Components Analysed</th>
<th>Storage Conditions</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Microbial Biomass C</td>
<td>• 32 weeks</td>
<td>• Decrease for -20°C (57%) and freeze dried (88%) from original microbial biomass C measurements.</td>
<td>(Čermohlávková et al. 2009)</td>
</tr>
<tr>
<td>• Respiration (basal and substrate induced)</td>
<td>• -20°C, 4°C and freeze dried</td>
<td>• Soil storage for 8 weeks at 4°C was found to be most appropriate for ammonification and nitrification</td>
<td></td>
</tr>
<tr>
<td>• Ammonification and Nitrification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Denitrification activity (assay)</td>
<td>• 2 and 20°C and assayed 2, 14, 28 and 50 days after collection</td>
<td>• Decrease in denitrification activity of moist samples stored at 20°C seen after 5 days, and after 14 days for samples stored at 2°C.</td>
<td>(Luo et al. 1996)</td>
</tr>
<tr>
<td></td>
<td>• Soils were also air dried and assayed 1 and 7 weeks after collection</td>
<td>• This was shown to be as a result of reduced C in the samples. Denitrification activity did not change significantly over 50 days when the assay was performed with C</td>
<td></td>
</tr>
</tbody>
</table>
- Urease Activity
  - -20°C, 5°C and 20°C both air dried and field moist
  - There was no change (significant) urease activity between samples stored for 1 week and after 5 months of storage, at either 5°C or -20°C. (Kandeler and Gerber 1988)

<table>
<thead>
<tr>
<th>Activity of:</th>
<th>-20°C (moist), 4°C (moist) and air dried (room temp)</th>
<th>Compared to the samples stored at 4°C, enzyme activity in the samples which were stored air dried or at -20°C showed decreased enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease</td>
<td></td>
<td>The amount of difference was dependant on the soil sample and on the time of year that the sample was collected. (Abellan et al. 2011)</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-glucosidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil Components Analysed</td>
<td>Storage Conditions</td>
<td>Results</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>• Microbial Biomass C</td>
<td>• Soils from arid areas were collected and stored for 6 – 9 months, air dried</td>
<td>• 6 months had no effect on these biochemical or microbiological properties</td>
</tr>
<tr>
<td>• Basal Respiration</td>
<td></td>
<td>• After 9 months, the samples showed some difference, mainly in the sample from an area with higher annual rainfall than the other samples.</td>
</tr>
<tr>
<td>• Metabolic quotient</td>
<td></td>
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<tr>
<td>• Acid phosphatase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Urease and B-galactosidase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Soluble carbon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Moisture (%) and pH</td>
<td>• 4°C field moist for 14 months</td>
<td>• Increases were observed in mineral - N after 14 months compared to initial concentrations, otherwise there were no significant changes seen in the samples for any of the other measurements</td>
</tr>
<tr>
<td>• Organic, Extractable and Microbial C</td>
<td>• Samples were analysed 0 and 14 months after collection</td>
<td></td>
</tr>
<tr>
<td>• Total and mineral N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• PFLA profiles of the microbial community</td>
<td>• 25°C, 10°C and 4.5°C for 3 weeks.</td>
<td>• The PFLA profile changed significantly in the samples stored at 25°C and little change observed in the profiles of the samples stored at 4.5°C and 10°C</td>
</tr>
</tbody>
</table>
REFERENCE LIST


