

**Commercial Lignite Coal-Derived  
Amendments For Improved Pasture Growth  
And Soil Health.**

Karen R. Little

BSc (Biotechnology) (Hons) Monash University

A thesis submitted to the Faculty of Science, Monash University,  
in fulfilment of the requirements for the degree of  
DOCTOR OF PHILOSOPHY

School of Chemistry  
Monash University  
Australia  
February 2015



# Table of contents

<b>Table of contents</b> .....	<b>i</b>
<b>Abstract</b> .....	<b>v</b>
<b>General Declaration</b> .....	<b>ix</b>
<b>Acknowledgments</b> .....	<b>xi</b>
<b>Abbreviations</b> .....	<b>xiii</b>
<b>1 Introduction</b> .....	<b>1</b>
1.1 <i>Background</i> .....	1
1.2 <i>The structure and chemistry of humic substances</i> .....	5
1.3 <i>Soil effects of humic acid</i> .....	6
1.3.1 <i>Complexing properties of humic acid in soil</i> .....	6
1.3.2 <i>Effect of humic acid on key indicators of soil health</i> .....	6
1.4 <i>Plant growth and nutrition effects of humic acid</i> .....	8
1.4.1 <i>Plant growth response to humic acid</i> .....	8
1.4.2 <i>Plant nutrition response to humic acid</i> .....	10
1.5 <i>Study context</i> .....	11
1.6 <i>Study aims</i> .....	13
1.7 <i>Thesis overview</i> .....	13
<b>2 Characterization of commercial Victorian lignite-derived products</b> .....	<b>17</b>
<i>Abstract</i> .....	17
2.1 <i>Introduction</i> .....	17
2.2 <i>Materials and method</i> .....	19
2.2.1 <i>Lignite-derived product descriptions and physicochemical characterization</i> .....	19
2.2.2 <sup>13</sup> C NMR.....	22
2.3 <i>Results</i> .....	22

2.3.1	Lignite-derived product physicochemical properties .....	22
2.3.2	<sup>13</sup> C NMR .....	25
2.4	<i>Discussion</i> .....	27
2.4.1	Lignite-derived product physicochemical properties .....	27
2.4.2	<sup>13</sup> C NMR .....	29
2.5	<i>Conclusion</i> .....	31
<b>3</b>	<b>Do lignite-derived organic amendments improve early-stage pasture growth and key soil biological and physicochemical properties?.....</b>	<b>33</b>
<b>4</b>	<b>Effect of application rate of lignite-derived soluble humate granules on the early-stage growth of lucerne, and key indicators of soil health. ....</b>	<b>47</b>
	<i>Abstract</i> .....	47
4.1	<i>Introduction</i> .....	47
4.2	<i>Materials and methods</i> .....	50
4.2.1	Characterization of lignite-derived products and soil .....	50
4.2.2	Glasshouse study: Lucerne growth and key indicators of soil health.....	51
4.2.3	Incubation study: Effect of soluble humate granules (SHG) on soil pH.....	53
<b>4.3</b>	<b><i>Results</i></b> .....	<b>55</b>
4.3.1	Glasshouse study: Lucerne growth and key indicators of soil health.....	55
4.3.2	Incubation study: Effect of soluble humate granules (SHG) on soil pH.....	62
<b>4.4</b>	<b><i>Discussion</i></b> .....	<b>62</b>
<b>4.5</b>	<b><i>Conclusion</i></b> .....	<b>67</b>
<b>5</b>	<b>Effect of soil amendment with lignite-derived potassium humate on pasture growth and nitrogen cycling in mesocosm conditions.....</b>	<b>69</b>
	<i>Abstract</i> .....	69
5.1	<i>Introduction</i> .....	69
5.2	<i>Materials and method</i> .....	72
5.2.1	Experimental layout and set-up .....	72

5.2.2	Sampling and analysis .....	79
5.2.3	Statistical analysis .....	83
5.3	<i>Results</i> .....	83
5.3.1	Soil nitrogen pools, losses and pH .....	83
5.3.2	Ryegrass and lucerne growth and nutrition.....	90
5.4	<i>Discussion</i> .....	96
5.4.1	Nitrogen pools and losses. ....	96
5.4.2	Ryegrass and lucerne shoot and root growth and nutrition.....	97
5.4.3	Limitations and further research .....	99
5.5	<i>Conclusion</i> .....	101
<b>6</b>	<b>Metagenomic and functional responses of soil microbial communities to humate addition</b>	<b>103</b>
	<i>Abstract</i> .....	105
6.1	<i>Introduction</i> .....	105
6.2	<i>Materials and methods</i> .....	108
6.2.1	Soil collection and characterization .....	108
6.2.2	Lignite-derived organic amendment.....	109
6.2.3	Microcosm set up and monitoring.....	110
6.2.4	Harvesting and soil analysis .....	111
6.2.5	DNA extraction and sequencing analysis .....	111
6.2.6	Bioinformatics .....	112
6.2.7	Calculations and statistical analysis. ....	112
6.3	<i>Results</i> .....	113
6.3.1	Soil physicochemical properties.....	113
6.3.2	Microbial activity.....	114
6.3.3	Soil bacterial activity, diversity and composition.....	115
6.3.4	Microbial community composition .....	116
6.4	<i>Discussion</i> .....	119

6.5	<i>Conclusion</i> .....	123
<b>7</b>	<b>General discussion, conclusion and future research</b> .....	<b>125</b>
7.1	<i>General discussion</i> .....	125
7.2	<i>Limitations</i> .....	128
7.3	<i>Conclusion</i> .....	129
7.4	<i>Further research</i> .....	131
	<b>Appendix 1 Chapter 3 supplementary table</b> .....	<b>133</b>
	<b>Appendix 2 Lucerne shoot nutrient, chlorosis and soil health data</b> .....	<b>135</b>
	<b>Appendix 3 Summary of project outputs</b> .....	<b>137</b>
	<b>Appendix 4 Meta-analysis and review paper</b> .....	<b>139</b>
<b>8</b>	<b>References</b> .....	<b>195</b>

## **Abstract**

Commercial lignite-derived products for use in agriculture are produced and used globally and a number of these products are manufactured from Victorian lignite coal. In marketing these products, manufacturers make claims of plant benefits including increased crop yield, enhanced root development and increased nutrient uptake and improvements in soil health by chelation of cations, pH buffering and promotion of beneficial soil bacteria and fungi. There is little evidence of published scientific studies that support these claims and of those that are available, few focus on those derived from Victorian lignite coal.

To investigate these claims, eight commercial products based on either raw-lignite or humate were sourced from three manufacturers. Raw lignite coal was also obtained directly from the mine. Chemical characterization of these products showed considerable variability in the humic acid (HA) concentration, ranging from 4 to 75% and in the inorganic components, particularly potassium, which ranged from 0.1 to 17%.

In a glasshouse study six lignite-derived products were applied at the manufacturer's recommended rate to two soil types. Shoot and root growth of two pasture species, lucerne (*Medicago sativa* L.) and ryegrass (*Lolium multiflorum* Lam.), shoot nutrient concentrations, soil pH, microbial biomass and mycorrhizal colonization analyses showed inconsistent results between the two soil types and the two pasture species. There was no clear evidence of a link between the HA and nutrient concentrations of the products, and pasture growth and soil health responses.

In a second glasshouse study, three lignite-derived products were applied at and in excess of the manufacturer's recommended rate to a mildly acidic, sandy soil. Raw lignite coal and a lignite-mineral blend did not promote a lucerne growth response however application of soluble humate granules (SHG) at rates up to 20 kg/ha

promoted shoot and root growth, with maximum benefit attained at 20 kg/ha; five-times the manufacturer's recommended rate. Application rates beyond 20 kg/ha did not provide additional growth benefits but importantly, resulted in a significant decrease in shoot nitrogen concentration with no concurrent loss in lucerne biomass. Application of SHG at 20 kg/ha also coincided with a delay in the appearance of chlorotic symptoms in lucerne shoots, and an increase in soil pH. A subsequent incubation study indicated that soil pH increases were driven by the pasture rather than product application.

To further investigate the effect on lucerne and ryegrass growth and to assess interactions with soil nitrogen species, SHG was applied at 20kg/ha to a sandy, low organic matter soil in outdoor mesocosms. Significantly, the product promoted early-stage shoot and root growth of ryegrass. Enhanced root growth was observed particularly at a lower soil depth which could enable the pasture to access moisture in drought conditions. The promotion of ryegrass growth coincided with the earlier provision and higher overall concentration of soil ammonium. At this application rate, in these conditions, a growth benefit was not identified for lucerne.

To investigate the manufacturer claims that commercial lignite-derived products promote beneficial soil bacteria and to further investigate the effect on soil nitrogen dynamics, the response of the soil bacterial community to the addition of SHG at 20 and 300 kg/ha was assessed by high-throughput 16S rRNA amplicon sequencing. The abundance of the nitrogen-cycling families *Rhodospirillaceae*, *Hyphomicrobiaceae*, *Bradyrhizobiaceae*, *Beijerinckiaceae*, *Burkholderiaceae* and *Nitrosomonadaceae* increased while *Nitrospiraceae* remained unchanged. Other bacterial families whose abundance increased significantly were *Xanthomonadaceae*, *Sinobacteraceae* and *Chitinophagaceae* while the abundance of *Gemmataceae*, *Streptomyetaceae* and *Ktedonobacteraceae* decreased. These results indicate that the addition of SHG changed the soil bacterial community structure.



The work presented in this study demonstrates that pasture growth and soil health benefits can be gained by the soil application of Victorian-lignite derived products. These benefits are highly dependent on the pasture species, soil type and product application rate, highlighting the need for farmers to evaluate these products within their individual environmental constraints and management systems. This work also indicates the need for manufacturers to reassess the product application rates that are recommended to farmers.



## **General Declaration**

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in peer reviewed journals and one unpublished paper currently under review. The core theme of the thesis is an assessment of commercial lignite-derived products for pasture growth and soil health. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, Karen Little, working within the School of Chemistry under the supervision of Associate Professor Tony Patti, Dr Timothy Cavagnaro and Professor William Roy Jackson.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of co-authored chapters three and six my contribution to the work involved the following:

<b>Thesis chapter</b>	<b>Publication title</b>	<b>Publication status</b>	<b>Nature and extent of candidate's contribution</b>
3	Do lignite-derived organic amendments improve early-stage pasture growth and key soil biological and physicochemical properties?	Published	85%
6	Metagenomic and functional responses of soil microbial communities to humate addition.	In review	75%

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

**Signed:** .....

**Date:** .....

## **Acknowledgments**

My PhD project has been an enjoyable learning experience largely due to the support that I have received from my supervisors Tim Cavagnaro, Tony Patti and William Roy Jackson. Thank you for your encouragement and patience. I feel very privileged have had the opportunity to work with and learn from you all.

Aside from my supervisors there are a number of other people who I am indebted to. Thank you to Mick Rose for his infectious enthusiasm, technical guidance and patience. Thank you also to Jess Drake who was an editor, cheerleader and counsellor and to Pree Johnson for her support in the laboratory. Thank you to all the members of the CavLab, it was great to be a part of such a supportive group of people.

For project funding, thank you to Brown Coal Innovation Australia (BCIA) who made it possible not only to complete the experimental work but also to attend conferences to share the research outcomes and learn from others.

This thesis represents not only my efforts but also those of my family. Thank you to Dad and Christine for being there for my family when I couldn't be. Thank you to my husband Stephen and children Declan and Rhianna, who have supported me every step of the way. From watering plants in the glasshouse to constructing wire cages to stop foxes stealing equipment from the mesocosms, without question, they have been there.



## Abbreviations

AM	Arbuscular mycorrhizae
ANOVA	Analysis of variance
CP-MAS	Cross polarization magic angle spinning
DAP	Di-ammonium phosphate
DAS	Days after seeding
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FA	Fulvic acid
GC-MS	Gas chromatography-mass spectroscopy
GHG	Greenhouse gas
HA	Humic acid
HS	Humic substance
HSC	Humate soil conditioner
HSD	Honestly significant difference
ICP	Inductively coupled plasma
IHSS	International Humic Substance Society
IR	Infrared
LC	Liquid chromatography
LDP	Lignite-derived product
LDPE	Low density polyethylene
LH	Liquid humate
LMB	Lignite-mineral blend
MBC	Microbial biomass carbon
MS	Mass spectrometry
nd	No date indicated
NMR	Nuclear magnetic resonance
OES	Optical emission spectroscopy
OM	Organic matter
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PCOA	Principle coordinates analysis
PLFA	Phospholipid fatty acid
PMN	Potentially mineralizable nitrogen
PP	Polypropylene
PVC	Polyvinyl chloride
QIIME	Quantitative insights into microbial ecology

qPCR	Quantitative polymerase chain reaction
RDW	Root dry weight
REML	Restricted maximum likelihood
RM	Repeated measures
ROM	Run of mine
rRNA	Ribosomal ribonucleic acid
SDW	Shoot dry weight
SHG	Soluble humate granules
SHP	Soluble humate powder
SOM	Soil organic matter
SR	Slow-release granulated humate
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TGA	Thermogravimetric analysis



# **1 Introduction**

## **1.1 Background**

The world's population is expected to reach 9.1 billion people by 2050, an increase from the current 7.2 billion (Food and Agriculture Organization of the United Nations, 2009, Worldometers, n.d.). With an increasing population there is unprecedented demand on arable land worldwide to produce more food, fibre and fuel to meet market needs (Erb et al., 2012, Bommarco et al., 2013, Robertson et al., 2014). Demand for cereals for both food and animal feed are projected to reach 3 billion tonnes annually by 2050, up from 2.1 billion tonnes in 2009 (Food and Agriculture Organization of the United Nations, 2009). In addition to food demands, the biofuel market which is growing in response to the need for fuel security and reduced greenhouse gas emissions, competes for arable land to produce feed stocks (Rathmann et al., 2010, Luckert, 2014).

There is a growing awareness that increased agricultural production needs to be achieved in an environmentally sustainable manner (Tilman et al., 2011, Godfray and Garnett, 2014). In recent decades, much of the increase in agricultural production has come from the use of inorganic fertilizers. In 2015, worldwide demand for fertilizers is predicted to be in excess of 190,000 thousand tonnes (nitrogen, phosphorus and potassium combined) with that of nitrogen fertilizer alone representing 113,000 thousand tonnes (Food and Agriculture Organization of the United Nations, 2011). Excessive or poorly-timed nitrogen fertilizer application can lead to a loss of nutrients from production with nitrate being leached into waterways or lost as the potent greenhouse gas nitrous oxide (Fageria, 2010, Chan, 2010, Hoben et al., 2011, Meng et al., 2005). Nitrate, the product of ammonium nitrification, is not well retained in soils and the combined effects of crop biomass removal and nitrate leaching can lead to the development of acidic soil conditions (Bolan et al., 1991) .

Worldwide it is estimated that 50% of arable land is acidic. Acidic soils limit agricultural productivity due to increased availability of aluminum which retards root growth, restricting access to water and nutrients (Bohn et al., 2002). Other plant essential nutrients including nitrogen, phosphorus, potassium and manganese become less available for plant uptake (Lucas and Davis, 1961). In these limiting conditions, a number of crop and pasture species will not grow successfully. Leguminous pasture species including lucerne and clover don't tolerate acidic conditions due to decreased rhizobia abundance and therefore nodulation, restricting the ability to fix nitrogen (Andrew, 1976). The net result of soil acidity is decreased plant growth, reduced profitability and increased requirement for fertilizers to sustain productive agriculture (Robson, 2012) .

Soil organic matter (SOM), of which carbon is the main component, is made up of plant and animal material in various stages of decay and is recognized as a key attribute of soil fertility. The SOM content of soils varies from 1% or less in sandy soils to nearly 100% in peat (Tipping, 2002). It affects the properties that influence soil productivity; physical (aggregation (Tisdall and Oades, 1982)), chemical (cation exchange capacity (Parfitt et al., 1995)) and biological (microbial activity (Schnürer et al., 1985)). Changes in SOM depend on the climate, soil texture, land management and inputs including organic amendments. Production of the soil greenhouse gases nitrous oxide and carbon dioxide can be controlled to some extent by SOM management (Merino et al., 2004).

Humic substances (HS) form the largest component of soils, generally in the range of 60-80% of the SOM (Stevenson, 1994) and play a pivotal role in soil productivity. They can improve soil aggregation and structure (Piccolo et al., 1997), increase water retention (Piccolo et al., 1996), improve the bioavailability of plant-essential nutrients (Chen et al., 2004a), bind heavy metals (Clemente and Bernal, 2006) and provide an energy source for the microbial community (MacCarthy, 2001). Whether by changing the soil properties or by direct action on the plant itself, the plant

benefits include stimulation of root and shoot growth and enhanced nutrient uptake (Chen and Aviad, 1990); however, the mechanisms have not been fully resolved.

Some agricultural practices implemented to increase productivity such as increased stocking rates and integrated crop-livestock systems can lower soil organic matter and HS levels (Hiltbrunner et al., 2012, Houlbrooke, 2011). While to some extent the SOM and HS content of soils can be maintained by land management, to reduce reliance on inorganic fertilizers and increase productivity, the use of organic amendments is growing in popularity (Jackson et al., 2008, Quilty and Cattle, 2011). Soil amendments containing high concentrations of humic substances extracted from a range of sources including vermicompost, urban and green wastes and lignite coal have been of interest for some time.

Humic substances can be separated into components by solubility in alkali and acid and can be extracted from a range of sources including soil (Hayes et al., 1975), vermicompost (Atiyeh et al., 2002), urban organic waste (Jindo et al., 2012) and lignite coal (Frost et al., 1959). A number of chemical methods can be used (Hayes et al., 1975), but typically and as defined by the International Humic Substances Society (IHSS) alkaline extraction of a substrate is performed with potassium hydroxide, with the resulting humic acid (HA) salt in solution referred to as a humate (International Humic Substances Society, 2008). The insoluble organic component is referred to as humin and is separated from the alkaline extract. The extract is then treated with acid and the pH adjusted to between pH 1-2. The precipitate obtained is defined as the HA component. The low molecular weight acids remaining in solution are referred to as the fulvic acid (FA) after removal of other water soluble polar compounds. Hence FA, is soluble in both alkalis and acids. To simplify terminology, humic substance (HS) will be used to refer to the naturally occurring component of SOM and humic acid (HA) will be used to refer to the product of alkaline extraction.

Lignite coal, also referred to as brown coal, is a particularly rich source of HS and is used for the commercial production of humic-based amendments that are available worldwide. Broadly, there are two product categories; those that contain raw lignite coal blended with plant essential nutrients and marketed as a raw soil conditioner or a lignite-mineral blend, and those that are comprised of the potassium salts of HA and FA, referred to as humates, derived from lignite coal. Fulvic acids have beneficial plant growth effects (Rauthan and Schnitzer, 1981, Mylonas and Mccants, 1980) however, in lignite they are present in much lower concentrations than the humic acid and so the product cost is higher. For this reason FA products are marketed only on a small-scale, and for specialized applications such as application to ornamental plants while humates and/or humic acid rich amendments are used for larger scale agricultural applications. Although processes between manufacturers differ, humates liberated from the coal by alkaline extraction are combined with plant essential nutrients and sold as a liquid, or dried to form soluble granules or powder, or slow-release pellets.

The soluble granules or powder and liquid forms can be applied directly to the soil or as a foliar spray, while the slow-release pellets are applied only to the soil. The products are generally supplied with a recommended application rate which can range from as little as 4 kilograms to in excess of 1 tonne per hectare depending on the form of product (e.g. liquid, solid, slow release pellets) and the application method used (e.g. fertigation, foliar spray, top-dressing). The use of lignite-derived products in conjunction with fertilizers is recommended by some manufacturers, particularly for products which are not formulated with plant essential nutrients. To avoid excessive supplementary inorganic fertilization, knowledge of the humate product composition is important.

Presented here is an overview of HS structure and chemistry and interactions with soil nutrients. The effects of HA on key indicators of soil health, plant growth and plant nutrition when applied to the soil as raw coal or humate are also discussed.

Humic acid from a number of sources will be explored but particular emphasis will be placed on that derived from lignite coal.

## **1.2 The structure and chemistry of humic substances**

The chemical structure of HS is complex and as such assigning a chemical definition has been an issue of some contention. There have been a number of hypothetical models proposed including polymeric assemblages (Swift, 1999, Schulten and Schnitzer, 1993, Stevenson, 1994, Schulten et al., 1991, Hayes et al., 1989) and more recently a supramolecular structure model in which lower molecular weight molecules are held together by hydrogen bonding and hydrophobic interactions (Piccolo, 2002). Since this model was proposed, to further characterize the small molecular components of the supramolecular HS, a 'Humeomics' approach was developed (Nebbioso and Piccolo, 2012, Nebbioso and Piccolo, 2011, Nebbioso et al., 2014). This chemical method reduces the complexity of the HS by sequentially separating smaller components from the bulk material without breaking the carbon backbone. Instead weak interactions, ester and ether bonds are targeted. The more simple products from each chemical fractionation step can then be characterized and to date this has been done by gas chromatography/mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy.

Spectroscopic methods including infrared (IR), fluorescence and NMR have enabled the identification of structural characteristics of HS. Aromatic and aliphatic structures dominate (Hatcher et al., 1981, Miano et al., 1988), and carbonyl, carboxyl, methoxyl and phenolic groups have been identified (Yonebayashi and Hattori, 1989, Senesi et al., 1991).

## **1.3 Soil effects of humic acid**

### *1.3.1 Complexing properties of humic acid in soil*

Humic acid forms complexes with metals and cationic soil nutrients by ionization at proton disassociating groups including carboxylic and phenolic groups. This increases plant-availability of soil nutrients from less soluble hydroxides, particularly iron, zinc and manganese (Chen et al., 2004a), potentially contributing to increased plant growth.

Organic amendments can minimise nutrient losses, particularly inorganic nitrogen (e.g. Suddick et al., 2011, Tuomisto et al., 2012, Knowles et al., 2011, Steiner et al., 2010). Research by Ahmed et al. (2006) demonstrated increased soil ammonium (approx. 30%) following HA application however the source of the HA was not identified and the application rate was not clearly stated. There is scope to investigate the capacity of humate to bind and retain plant-essential nutrients such as inorganic nitrogen species.

### *1.3.2 Effect of humic acid on key indicators of soil health*

The term 'soil health' is widely used to describe the general condition or quality of the soil resource. Biological (e.g. microbial activity), chemical (e.g. organic matter content) and physical (e.g. soil texture) attributes influence the productivity of the soil with in an agricultural context is the capacity of the soil to support the growth of crops or pasture. The effect of HA on key indicators of soil health such as pH and the soil microbial community have been less scrutinized than other areas. Often the focus of published studies is not on soil effects but rather plant shoot and root growth and nutrient uptake, and so changes in soil properties are not reported (e.g. Verlinden et al., 2009) or if the application rate was low, changes in soil properties may not be detectable. Also, studies may be conducted in nutrient solution (e.g. Adani et al., 1998, Ayuso et al., 1996) or in solid media other than soil (e.g. Abdel-

Mawgoud et al., 2007, Arancon et al., 2006) and so there is no opportunity to measure changes in soil health.

Acidic soils can substantially limit agricultural productivity and impact more widely on ecosystem function. It has been estimated that up to 50% of the world's arable land is acidic. Remediation techniques include addition of gypsum and/or lime, bioremediation and incorporation of amendments such as compost, manure and biochar (Smith et al., 1994, Qadir et al., 2007, Jalali and Ranjbar, 2009, van Zwieten et al., 2010a). Few studies focus on the use of lignite-derived humate to ameliorate acidic soil; however one study showed that addition of raw lignite coal increased soil solution pH by 1.5 units (Yazawa et al., 2000). This result was unexpected and unexplained by the authors, as the pH of the coal and the soil were the same, and so according to the model of amendment buffering capacity proposed by Wong et al. (1998), no proton transfer was expected. Given this result and the high cation exchange capacity of HA (Varanini and Pinton, 1995) further investigation of the pH buffering capacity of humate is warranted.

Soil microbes, including mycorrhizal fungi have an essential role in the turnover and acquisition of nutrients for plant growth (Smith and Read, 2010). The microbial community composition and abundance is sensitive to the application of organic soil amendments (Anderson et al., 2011, Paranychianakis et al., 2013, Valdrighi et al., 1996, Visser, 1985a). Microbial-driven changes in nutrient cycling not only effect plant growth but can also influence soil greenhouse gas emissions (van Zwieten et al., 2009).

Following application of soil-derived HA to two soil types, using selective growth media, Visser (1985b) identified increases in abundance of amylolytic, proteolytic and denitrifying microbes. In a similar study also using selective growth media, Visser (1985a) found increases in starch decomposers, aerobic cellulose decomposers and nitrifiers. Also in response to addition of soil-derived HA, Puglisi

et al. (2009) analysed the rhizosphere microbial community by denaturing gradient gel electrophoresis (DGGE) and showed that the composition of the microbial community changed in response to the addition of size-fractionated HA compared to the bulk material. Phospholipid fatty acids (PLFA) analysis showed increases in the total PLFA and those associated with bacteria and fungi in a low organic matter soil in response to application of a range of lignite-derived commercial humate products (Hartz, 2010).

Traditional culture techniques including the use of selective media rely on the growth of microbes within the supplied environment, and only a very small proportion of soil bacteria (1%) and fungi (17%) can be cultured (Kirk et al., 2004). Biochemical techniques such as enzyme and PLFA analysis, and molecular techniques, such as DGGE, while offering more comprehensive information have their own inadequacies (Steffan et al., 1988, Zhou et al., 1996, Kirk et al., 2004, Jumpponen, 2007, Muyzer and Smalla, 1998). Although DGGE can be highly reproducible (Puglisi et al., 2009), the methodological constraint of a limited number of wells in the gel prevents comparisons of a large number of samples. Newer molecular techniques such as next-generation sequencing can provide abundance information on microbes identified to the species level, as has been done for biochar amendments (Anderson et al., 2011). In combination with quantitative polymerase chain reaction (qPCR) analysis of specific gene copies such as *amoA* present in nitrifying bacteria, microbial community functional changes can be identified.

## **1.4 Plant growth and nutrition effects of humic acid**

### *1.4.1 Plant growth response to humic acid*

Soil amendment with HA from various sources has been demonstrated to stimulate shoot and root growth and yield of a variety of crops including grains and vegetables. (e.g. Eyheraguibel et al., 2008, Puglisi et al., 2009, Lee and Bartlett,



1976, Nardi et al., 2002, Arancon et al., 2006, Piccolo et al., 1993). The application of lignite-derived HA has also shown to benefit shoot and root growth of a range of crops including tomatoes (Adani et al., 1998), maize (Ertani et al., 2011, Olk et al., 2013), cucumber (Mora et al., 2010), potatoes (Verlinden et al., 2009) and wheat (Tahir et al., 2011). Studies on the effect of HA on pasture species are rarely reported however field studies of application of a commercial product to ryegrass and mixed species grasslands showed variable growth and nutrient uptake results across a range of soil types (Verlinden et al., 2010, Verlinden et al., 2009). Generally, high economic value is placed on pasture-based commodities, and so potential growth benefits from HA application would be of interest to farmers.

With growth benefits dependent on the origin of the HA, application rate, crop type and soil type (Rose et al., 2014), the mechanisms for improved plant growth in response to HA application have not been fully determined. There is evidence to indicate that HA has both direct and indirect effects on the plant. Humic acid is claimed to induce auxin- and gibberellin-like effects on plants (Nardi et al., 1994, Muscolo et al., 1999), and to increase production of the plasma membrane proton pump ( $H^+$ -ATPase) which both promotes cell elongation and enhances ion transport across the cell membrane (Nardi et al., 1991, Canellas et al., 2002). Despite these claims in the literature, compounds that could be identified with hormone-like behavior have not been isolated (Chen et al., 2004b). There is evidence for indirect effects which include improved soil properties such as aggregation and water holding capacity (Piccolo et al., 1997, Piccolo et al., 1996), and increased availability of plant essential nutrients (Chen et al., 2004b).

The rate at which HA is applied is important for optimal plant growth. Typically there is an upper limit at which maximal growth benefits are seen, with a depressive or lack of effect at rates that exceed this (Rose et al., 2014). The suggested reason for reduced shoot and root growth at high concentrations is the accumulation of HA at cell wall pores which restricts root water and nutrient uptake

(Asli and Neumann, 2010). Manufacturers of lignite-derived products recommend an application rate; however, previous studies have identified that these rates are generally too low to elicit an agronomic benefit (Feibert et al., 2003, Hartz, 2010, Duval et al., 1998).

#### *1.4.2 Plant nutrition response to humic acid*

The effect of HA on plant nutrient uptake is variable depending on the nutrient, the soil type, crop type and source of HA. To illustrate this variability, studies of lignite-derived HA showed decreased nitrogen uptake in canola grown in soil amended with milled leonardite (Akinremi et al., 2000), whereas sugarcane, okra, and cucumber in soil or hydroponic conditions increased shoot nitrogen uptake, ranging from 20 to 40% compared to the control (Govindasmy and Chandrasekaran, 1992, Kirn et al., 2010, Abad et al., 1991). Another study on tomatoes showed no change in shoot nitrogen (Adani et al., 1998), whereas soil-derived HA applied to corn resulted in an increase or decrease in shoot nitrogen depending on the applied rate (Tan and Nopamornbodi, 1979).

Phosphorus uptake can also be variable following application of HA. An increase in phosphorus was detected in tomato shoots (8% increase compared to the control) (Adani et al., 1998) and cucumber shoots (66% increase compared to the control) (Abad et al., 1991) but not in sugarcane shoots (Govindasmy and Chandrasekaran, 1992). Clearly mechanistic studies on the effect of HA on soil nutrient cycling are required.

In conclusion, the structure of soil humic substances is not fully known however significant contribution to understanding has been made using NMR techniques and more recently a “humeomics” approach. Complexes formed between HA and soil nutrients improve plant-availability and this same attribute could also decrease nutrient loss. Binding of soil inorganic nitrogen species such as ammonium by lignite-derived coal has not been well explored and the capacity for lignite-derived

HA to buffer pH has not been fully determined. While some progress has been made, effects on the soil microbial community are largely unknown, but advances in molecular analytical techniques could be used to gain further insights. Humic acid can improve plant growth through direct or indirect interactions and potential growth benefits for pasture species have not been well studied. The uptake of nutrients is not consistent between crops and mechanistic studies are needed to investigate effects on nutrient cycling.

## **1.5 Study context**

Australia has extensive lignite coal resources, estimated to be 20.5% of worldwide lignite reserves (Thielemann et al., 2007). In the state of Victoria, there are estimated to be 400 billion tonnes, with the largest deposit being in the Gippsland Basin area (Li, 2004). It is relatively inexpensive to mine due to a shallow overburden averaging only 10-20 m. In comparison with world standards, Victorian brown coal is of high quality, being low in nitrogen, sulphur, ash and heavy metals (Perry et al., 1984). Its high moisture content (Perry et al., 1984) increases transport costs and so at present the primary use is for electricity generation at power stations located close to the mine. With such an abundant, high quality, easily winnable resource, other applications are of interest.

A range of commercial lignite-derived products are manufactured from Victorian lignite and are marketed to farmers as having above- and below-ground benefits for a range of pasture and crop types. In marketing these products manufacturers make claims of agronomic benefits including:

- Increased crop yield;
- Improved soil structure;
- Enhanced root development;
- Increased nutrient uptake due to increased cell permeability;
- Promotion of beneficial soil bacteria and fungi;
- Chelation of cations;

- Faster seed germination;
- Suppression of some diseases ;
- Reduced plant uptake of some heavy metals including cadmium, lead, aluminum and fluoride; and
- pH buffering capacity to help neutralize the problems associated with pH extremes.

There is very little evidence of published complete scientific studies that support these claims. It is likely that the high chemical variability of commercial humate products and the complexities of assessing the interactions between the soil, plant and product contribute to this. Of the available studies that evaluate plant growth and soil health following amendment with commercial lignite-derived products, few focus on those produced from Victorian lignite.

In Australia, grazing livestock and livestock products have high economic value. In Victoria, milk, cattle and sheep are the top three agricultural commodities (National Farmers' Federation, 2011). The success of pasture growth is intrinsic to the value of these commodities. Growth benefits for ryegrass following amendment with HA have been previously identified. The HA derived from manure, compost, decomposed sawdust, straw and peat to ryegrass resulted in increases in shoot and root growth (Asenjo et al., 2000, Bidegain et al., 2000, Fortun et al., 1985) and a commercial product, Humifirst, derived from Canadian lignite also increased root and shoot growth however results were variable across soil types (Verlinden et al., 2010). Estimates suggest that 3 million hectares or 23% of Victoria's agricultural soils are affected by loss in productivity due to acidity with the most strongly acidic soils occurring in permanent pasture areas. There is scope to further investigate the growth response of pasture species to commercial Victorian lignite products in a range of soil types.

## **1.6 Study aims**

With a continual interest in amelioration of acid soils and increased crop yields there are a number of aspects in which the use of commercial Victorian lignite-derived products can be further investigated. The objectives of this study were to:

- Source a range of commercial Victorian lignite-derived products from a number of manufacturers and assess their physicochemical properties;
- Assess the shoot and root growth and nutrient uptake of selected pasture species relevant to farming practice in Victoria in response to soil application of commercial lignite-derived products; and
- Investigate the effect on key indicators of soil health including soil pH and the soil microbial community.

It is anticipated that research outcomes will help to better understand the effect of lignite-derived products on pasture growth and nutrition, and soil health. With an improved understanding of the fundamentals involved, it should be possible for manufacturers to develop more effective products and for farmers to make an informed decision of how these products could be integrated into their current management practices for improved crop production.

## **1.7 Thesis overview**

This thesis describes changes to key indicators of soil health and growth and nutrient uptake responses of selected pasture grasses to soil amendment with a range of Victorian lignite-derived products in the tightly controlled environment of the glasshouse, and then in semi-controlled conditions in outdoor plots. To further investigate potential mechanisms of pasture growth promotion, soil nitrogen cycling was also investigated in the outdoor plots. Finally, using molecular techniques, the microbial response to the addition of a humate product at a range of application rates was investigated in an incubation study.

The physicochemical characteristics of nine commercial Victorian lignite-derived products are presented in Chapter 2. The products were sourced from three manufacturers and were assessed by wet chemistry, solid-state  $^{13}\text{C}$  NMR and ICP (inductively coupled plasma) techniques.

The manufacturers of Victorian lignite-derived commercial products market the products with a recommended application rate. A glasshouse screening study was performed with a selection of products applied to two soil types into which ryegrass and lucerne were sown. While the primary focus of this study was on plant growth and nutrition, key measures of soil health were also monitored. Chapter 3 is a published paper describing this study and key findings.

Chapter 4 presents a second glasshouse study and an incubation study. In the glasshouse study a subset of products were applied at, and well in excess of, the manufacturers recommended rate. Again the focus for this study was pasture growth (lucerne) and nutrient uptake, with soil pH also measured. Significantly, lucerne nitrogen uptake was reduced in the presence of commercial soluble humate granules. Changes in soil pH were also detected and an incubation study without plants was conducted to identify if these changes were driven by the plant or as a result of humate product addition.

While glasshouse studies were important for initially identifying plant and soil responses and assessing parameters such as product application rate, an outdoor plot study was conducted to assess plant growth and soil health in an open environment. Results of the previous two glasshouse studies were used as a guide to select one humate product, one application rate and one soil type into which ryegrass and lucerne were sown. Over a 7 month period pasture growth and soil health were monitored. To identify potential mechanisms for improved pasture growth, soil mineral and inorganic nitrogen species, and nitrogen losses in soil

leachate and as nitrous oxide were also measured. This study is presented in Chapter 5.

To further investigate mechanisms for improved plant growth an incubation study was conducted to assess the response of the soil microbial community to a humate product applied at three rates. Abundance increases were detected in a number of bacterial families associated with nitrogen cycling. The molecular method used was next-generation sequencing which has not been previously applied to microbial studies with commercial humate products. This study is presented in Chapter 6 in the form of a manuscript which has been submitted for publication.

Chapter 7 brings together the key findings from the experiments conducted throughout the thesis and presents areas for future research.





## **2 Characterization of commercial Victorian lignite-derived products**

### **Abstract**

Commercial lignite-derived products (LDPs) reportedly promote growth of a range of crops, however, the mechanisms of stimulation are not known. The LDPs are generally of two types; raw-lignite or humate based. Plant essential nutrients may be added during formulation and so chemical variation between the products would be expected. Manufacturers do not disclose product formulation information and so chemical analysis is required. The physicochemical properties of nine LDPs were determined by humic acid extraction, pH, inductively coupled plasma-optical emission spectrometry (ICP-OES) and  $^{13}\text{C}$  solid state nuclear magnetic resonance ( $^{13}\text{C}$  NMR) spectroscopy. Based on the chemical and data acquired it was possible to identify the LDPs as being raw lignite –based or humate-based. Considerable variability was detected in the inorganic components and HA concentrations. This variability in chemical composition would be expected to result in varying effects on plant growth.

### **2.1 Introduction**

Commercial lignite-derived products are marketed to farmers as having above- and below-ground growth benefits for a range of pasture and crop types. These products are manufactured from lignite or leonardite. Leonardite is often found in association with lignite and is formed by the oxidation of lignite from prolonged exposure to air. From here on in, for the purpose of clarity, lignite and leonardite will be referred to collectively as lignite. Broadly, there are two commercial product categories; those that contain raw lignite and those that are comprised of the potassium salts of humic (HA) and fulvic (FA) acids (humates) derived from lignite coal. Crushed or milled lignite alone can be used as a soil amendment, or combined with plant essential nutrients and marketed as a raw soil conditioner or a lignite-

mineral blend. Humate products are generally manufactured by subjecting lignite to an alkaline extraction or digestion process, thereby extracting or 'liberating' the HA and FA. The processing often involves elevated temperature in generating the alkaline digest which causes some breakdown in the lignin structure (Durie, 1991). The complete separation of the HA and FA from the rest of the matrix adds considerably to the processing costs. In some cases, centrifugation or passive settling of suspended solids is used. While the product manufacturing process varies between manufacturers, the HA in the form of humate are generally combined with plant essential nutrients and sold as a liquid, or further processed to a dried state. The formulations available on the market are varied in composition and it is difficult to obtain information about them without undertaking extensive analysis.

Fulvic acids have been shown to have beneficial plant growth effects (Rauthan and Schnitzer, 1981, Mylonas and Mccants, 1980). The FA component can be separated from the HA by acidification, as they by definition are soluble in water whereas HA are not. However, the concentration of FA in lignite coal is much lower, and so this increases their product cost considerably. For this reason FA products are marketed for small-scale, specialized applications such as ornamental plants. Although the humate product manufacturing process has not been disclosed, it is unlikely that FA would be removed, and so these products would contain both HA and FA.

The component of LDPs considered to be the plant growth promoting ingredient is the HA (Rose et al., 2014). At present there is not a standardized method to quantify HA concentration in commercial humate products; however, progress has been made towards protocol development (Lamar et al., 2013). The plant essential nutrients added during product formulation may also have an impact on plant growth, and so it is important to identify the elements present and their concentrations. Soil pH is a critical factor in plant growth success (Lucas and Davis, 1961) and so the pH of an applied product should also be known.

This chapter provides an overview of the physicochemical properties of nine selected commercial LDPs as have been determined by wet chemistry methods including extraction of the HA from the products themselves, product pH, elemental analysis by ICP-OES and structural information about carbon forms and distribution by  $^{13}\text{C}$  NMR spectroscopy.

## **2.2 Materials and method**

### *2.2.1 Lignite-derived product descriptions and physicochemical characterization*

Seven solid and two liquid LDPs were sourced from three manufacturers; two water-soluble solid humate products (one granulated and one powdered), two lignite-mineral blends, one granulated slow-release humate product, one humate soil conditioner, two liquid humate products and raw lignite coal sourced directly from the mine (otherwise known as 'run-of-mine' coal (ROM)) in the Latrobe Valley, Victoria. These products were selected as they are claimed to contain raw or extracts of Victorian lignite, and are representative of the product types that are currently available on the market<sup>1</sup>. A description of each product and abbreviations by which each product will be referred to is included in Table 2.1.

---

<sup>1</sup> This was an independent study and no financial or in-kind support was received from companies who manufacture LDPs. Manufacturer details have been deliberately omitted however can be disclosed to thesis examiners if requested.

**Table 2.1** Lignite-derived product (LDP) descriptions and abbreviations.

LDP	Product description	Abbreviation	Manufacturer/ supplier	Lignite origin
ROM lignite coal	Raw coal	ROM	A	Morwell, Victoria
Lignite-mineral blend 1	Raw coal with mineral additives	LMB1	B	Victoria, Australia
Lignite-mineral blend 2	Raw coal with mineral additives	LMB2	B	Victoria, Australia
Humate soil conditioner	Raw coal with mineral additives	HSC	C	Victoria, Australia
Slow-release granulated humate	Granulated raw coal with mineral additives	SR	C	Victoria, Australia
Soluble humate granules	Dried humate	SHG	D	Victoria, Australia
Soluble humate powder	Dried humate	SHP	D	Victoria, Australia
Liquid humate 1	Liquid humate	LH1	D	Victoria, Australia
Liquid humate 2	Liquid humate	LH2	C	Victoria, Australia

### *Sample preparation prior to analysis*

The lignite coal (ROM) was crushed and sieved at <2 mm prior to analysis and the liquid products (LH1 and LH2) were oven-dried at 30°C until there was no moisture remaining. For elemental analysis by ICP-OES, carbon and nitrogen determination by dry combustion and carbon structural analysis by <sup>13</sup>C NMR, a sub-sample of each product was ground to a fine powder using a mortar and pestle. Humic acid concentration and pH were determined on each product in an 'as received' state.

### *Measurement of pH*

With the exception of the liquid products LH1 and LH2, pH was determined in 5 g subsamples suspended in deionized water (1:5 w/v), using a TPSWP-81 meter and probe (TPS Pty Ltd, Springwood, QLD). The pH of LH1 and LH2 was measured

directly on the undiluted product. Each measurement was in performed in triplicate.

#### *Moisture content*

With the exception of the liquid products LH1 and LH2, a 10 g sub-sample of each product was oven-dried at 105°C for 48 hours. The moisture content (%) was calculated by the weight difference before and after drying.

#### *Humic and fulvic acid concentrations*

A 5 g sub-sample was used to determine HA content by repeated alkaline extraction using a modification of the IHSS method (International Humic Substances Society, 2008), as follows. To each product, 0.1M HCl was added to give a 10:1 acid to LDP ratio (V/W). The slurry was then shaken at 120 rpm for 1 h and allowed to settle for 12 h. The supernatant was removed and retained for estimation of FA. Under an N<sub>2</sub> atmosphere, 0.1M NaOH was added to the solid residue at a ratio of 100:1 (v/w). The slurry pH was adjusted to 12.6 with 1M NaOH and shaken at 120 rpm for 4 h (Hayes et al., 2008). The pH of the slurry was reduced to 9 using 1M HCl, and the solids allowed to settle for 16 h. The supernatant was removed and retained, and alkaline extraction of the remaining solid repeated a further seven times until the supernatant was a pale brown color. The supernatants were pooled, and the HA precipitated by pH adjustment to 1-2 with 1M HCl. The HA was then dialyzed in cellulose membrane dialysis tubing MWCO 12000 (Sigma-Aldrich, St Louis, MO, USA) in deionized water until the conductivity of the surrounding water was less than 20 uS/m. The HA was then oven dried at 37°C and weighed. This was repeated in triplicate for each LDP. The ash content of the HA was determined by thermogravimetric analysis (TGA) with a sub-sample of each triplicate heated from ambient temperature to 550°C at a rate of 10°C/min. An estimation of FA content was made from analysis of the carbon in the retained supernatant by dichromate digestion (Cai et al., 2011), giving an upper limit for FA content.

### *Elemental analysis*

One subsample of each product was analysed for total carbon, hydrogen and nitrogen by dry combustion (by The Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand: <http://neon.otago.ac.nz/consulting/microlab/>; accessed April 2014). The second subsample was analysed for aluminium, calcium, iron, potassium, magnesium, manganese, phosphorus and zinc by ICP-OES (by Waite Analytical Services, University of Adelaide, Urrbrae, S. Aust.: <http://www.adelaide.edu.au/was/>; accessed April 2014).

### *2.2.2 <sup>13</sup>C NMR*

Cross-polarization magic angle spinning (CP-MAS) <sup>13</sup>C NMR spectra were generated on a Bruker Avance 400 (9.4 Tesla magnet) with a 4 mm multinuclear solid state probe operating at a <sup>13</sup>C frequency of 100.6 MHz. A sub-sample of each product was packed into 4mm ZrO<sub>2</sub> rotors with a Kel-F cap, and spun at 10000 Hz. Scans with 1500 data points were collected over an acquisition time of 25 ms, with a 3 sec delay and a 2 ms contact time. The spectra were collected and processed using Bruker's Topspin 2.1 program. Spectra were referenced to a glycine external reference. The spectra were area normalized and integrated to cover the following chemical shift ranges; 0-48 (aliphatic), 48-93 (C-O, C-N), 93-112 (anomeric), 112-162 (aromatic) and 162-188 ppm (carbonyls of ketones, quinines, aldehydes and carboxyls) (Canellas et al., 2010).

## **2.3 Results**

### *2.3.1 Lignite-derived product physicochemical properties*

The LDPs varied considerably in chemical and organic composition (Table 2.2). The pH of the raw lignite-based products ranged from 4.2 to 5.5 and of the humate products, 9.4 to 10.8. Of the raw lignite-based products, ROM had the highest moisture content (51.9% w/w), and LMB1 had the lowest (3.8% w/w). There were

clear differences between products in the HA concentration which ranged from 13.9 to 82.3%. Alkaline treatment of LMB2 did not result in HA extraction. As the focus of this thesis is commercial products derived from lignite, this product did not fit that criteria and will therefore not be discussed further. The estimated FA concentration was generally higher in the raw lignite-based (ROM, LMB1, HSC and SR) products (2.9 to 6.1%) than the humate (SHG, SHP, LH1 and LH2) products (0.1 to 2.9%).

Of the raw lignite-based products, ROM, SR and HSC were highest in carbon concentration at 66.4, 56.5 and 56.1% respectively, and LMB1 the lowest at 26%. Of the humate products, SHG and SHP had similar values of 47.5% and 42.1% respectively, with lower carbon concentration in the liquid products LH1 (38%) and LH2 (37.2%). The hydrogen concentrations ranged from 2.7% of LMB1 to 4.9% of HSC. The nitrogen concentrations were generally very low, as would be expected from Victorian lignitic coal-derived material and ranged from 0.5% of LH1 to 1.3% of SHG. Compared to the other LDPs, LMB1 had a considerably higher percentage of sulphur (1.7%).

There was also considerable variability in the inorganic components of the LDPs (Table 2.3). Whilst ROM and HSC had similar carbon and nitrogen concentrations, (Table 2.2), the levels of inorganic components were quite different. Product HSC had considerably higher concentrations of all elements measured, compared to ROM. The humate products SHG, SHP, LH1 and LH2 contained elevated potassium, and LMB1 contained high concentrations of calcium, phosphorus, manganese and zinc.

**Table 2.2** Physicochemical properties and organic composition of the LDPs.

LDP	pH (H <sub>2</sub> O)	Moisture (%)	HA <sup>#</sup> (%)	FA <sup>^</sup> (%)	Residue after HA extraction <sup>+</sup> (%)	C (%)	H (%)	N (%)	S (%)
ROM	4.5	51.9	67.4	6.1	67.5	66.4	4.8	0.7	0.2
LMB1	5.2	3.8	3.9	2.9	66.8	26.0	2.7	1.0	1.7
LMB2	5.5	4.3	*	*	n.a.	n.a.	n.a.	n.a.	n.a.
SR	4.2	6.2	71.4	5.8	17.1	56.5	3.5	1.2	0.4
HSC	4.8	39.5	23.0	4.3	25.3	56.1	4.9	0.6	0.6
SHG	9.4	4.5	66.3	2.9	25.7	47.5	4.2	1.3	0.3
SHP	10.4	9.1	75.4	1.0	0	42.1	4.5	0.7	0.2
LH1	10.6	n.a.	22.4	0.1	n.a.	38.0	3.8	0.5	0.3
LH2	10.8	n.a.	6.3	0.2	n.a.	37.2	3.2	0.9	0.5

\* No humic or fulvic acids extracted

# Ash-free content

n.a. not applicable

<sup>^</sup> Estimation of FA by carbon concentration

<sup>+</sup> Percentage of initial LDP mass

**Table 2.3** Inorganic components of the LDPs.

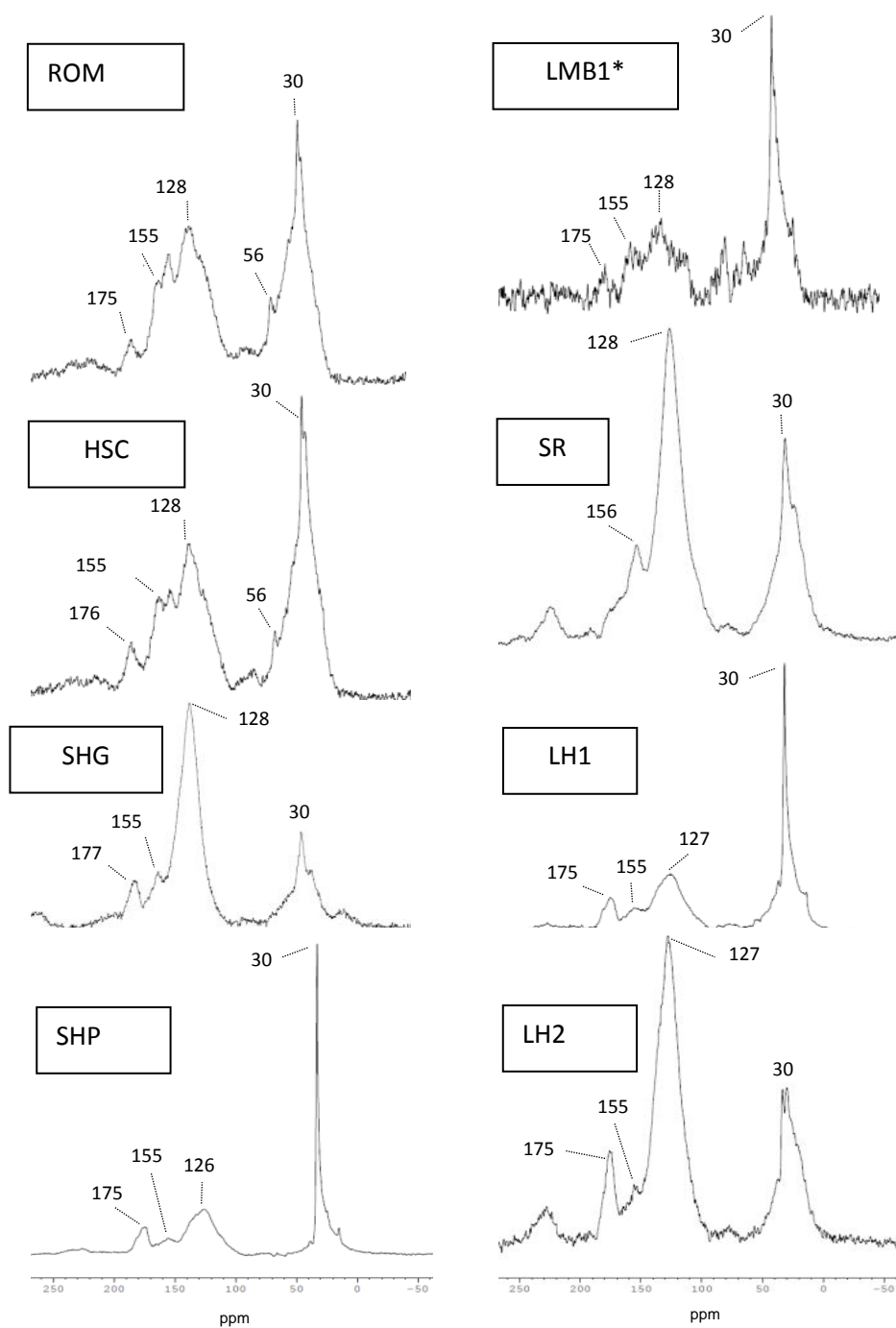
LDP	Al (%)	Ca (%)	Fe (%)	K (%)	Mg (%)	P (%)	Mn (mg/kg)	Zn (mg/kg)
ROM	<0.1	0.5	0.2	<0.1	0.2	<0.1	56	3
HSC	0.6	1.3	0.7	0.1	0.3	0.1	220	67
SR	0.6	0.3	0.4	0.1	0.1	<0.1	43	13
LMB1	1.1	15.7	1.7	0.2	0.8	5.3	550	510
SHG	1.0	1.0	1.0	8.5	0.1	<0.1	73	20
SHP	2.9	0.1	0.2	16.9	0.2	<0.1	5	5
LH1	1.8	0.1	0.3	15.7	0.3	<0.1	68	7
LH2	1.0	1.3	1.2	12.4	0.1	<0.1	85	32



### 2.3.2 $^{13}\text{C}$ NMR

All NMR spectra (Figure 2.1) were dominated by peaks in the chemical shift regions of 0-50 ppm and 110-160 ppm. The spectra of the raw lignite-based products ROM and HSC were similar in appearance, both with minor peaks at 56 and 155 ppm. There was the suggestion of peaks at the same positions for product LMB1 however this was difficult to determine due to excessive background noise. Spectra of products SR, SHG, SHP, LH1 and LH2 also had minor peaks at 155 and 175 ppm.

The  $^{13}\text{C}$  distribution in different regions of the NMR is given in Table 2.4. The relative carbon distribution across all chemical shift regions was similar in ROM and HSC. The spectra of LMB1 and LH1 were dominated by aliphatic carbon and SHG, SR and LH2 were dominated by aromatic carbon. There was a relatively even distribution of aliphatic and aromatic carbon in product SHP.



**Figure 2.1**  $^{13}\text{C}$  NMR spectra of LDPs. \* Peaks are not clearly defined due to excessive background noise.

**Table 2.4** Integrated carbon distribution (%) in <sup>13</sup>C NMR spectra.

	Chemical shift range (ppm)				
	0-48 aliphatic	48-93 C-O, C-N	93-112 anomeric	112-162 aromatic	162-188 aldehydes, carboxyls
	Normalized carbon distribution (%)				
ROM	34.9	12.6	7.7	39.6	5.1
LMB1	49.8	12.4	5.0	30.7	2.1
HSC	39.7	9.6	6.0	37.8	6.9
SR	28.5	5.0	7.2	53.2	6.2
SHG	21.2	4.7	4.6	60.6	9.0
SHP	44.3	1.3	4.0	38.9	11.4
LH1	44.5	6.8	4.4	34.3	10.0
LH2	25.1	3.2	5.3	57.2	9.2

## 2.4 Discussion

The considerable differences in chemical composition between LDPs may be attributed to the origin of the parent material, whether the product is raw lignite or potassium humate-based, extraction or digestion conditions (e.g. concentration of alkali used, time and temperature of reaction) and nutrient additions made during the product formulation process.

### 2.4.1 Lignite-derived product physicochemical properties

The extensive pH range of the LDPs was due to extraction conditions. Generally the pH of each product was within two distinct ranges; 4.2 to 5.5 (ROM, HSC, SR and LMB1) and 9.4 to 10.8 (SHG, SHP, LH1 and LH2). Products in the lower range were raw lignite-based, and those in the alkaline range were humate based with the high pH due to excess alkali carried over into the product from the extraction process.

Victorian lignite coal had a high moisture content, as was the case in both the raw lignite-based products ROM (51.9%), and HSC (39.5%). These two products had similar organic but differed in inorganic concentrations which verified the manufacturer's claim that HSC contained crushed lignite coal plus plant essential

nutrients. Product SR also contained lignite coal but its moisture content was substantially lower than would be expected for lignite coal. This product was in the form of a well-compressed pellet and so moisture may have been eliminated during manufacturing. The inorganic components were at similar concentrations to those in ROM, indicating the addition of little if any plant essential nutrients.

Product LMB1, a raw lignite-based product, contained a low concentration of lignite. This was indicated a low concentration of extracted HA (3.9%). The elevated calcium, iron, magnesium, phosphorus, manganese and zinc concentrations suggested the addition of plant essential nutrients, which reflects the labelling of this product as a lignite-mineral blend.

Products SHG and SHP contained dried humate, and so the moisture content would not be expected to be high. The source of elevated potassium concentration of both products is likely from the potassium hydroxide extraction process (Durie, 1991). Apart from potassium, phosphorus and nitrogen were at low concentrations and the additional nutrient value of these products would be minimal, particularly at the very low recommended application rates.

The large variation in HA concentration between the products was due to the amount of raw lignite or humate incorporated during product formulation. There does not appear to be any standard or industry guidelines that set lower or upper limits for the HA content of agricultural products. The relatively low estimated concentration of FA in each product is unlikely to make a significant contribution to plant growth (Rauthan and Schnitzer, 1981) due to the low manufacturer recommended application rates, which in the products here ranged between 4 kg/ha and 1 t/ha.

Overall, the concentrations of nutrients added during product formulation were low relative to straight chemical fertilizers. For example di-ammonium phosphate fertilizer generally contains 18% nitrogen and 20% phosphorus, which are

significantly higher concentrations than those of the LDPs studied here (0.5-1.3% nitrogen and <0.1 to 5.3% phosphorus). Taking into account the low manufacturer recommended application rates of LDPs and the requirement for application of conventional fertilizers in conjunction with LDPs, it is unlikely that these nutrients, if they are in fact plant-available, will make a significant contribution to plant growth.

#### 2.4.2 <sup>13</sup>C NMR

All the spectra were dominated by signals in the aliphatic (0-48 ppm), aromatic (112-162 ppm) and carboxyl (162-188 ppm) regions, as is typical of lignite, humic acid and commercial humic products (Pang et al., 1990, Canellas et al., 2010, Rumpel et al., 1998, Mao et al., 2013). While all spectra showed weak absorptions in the anomeric region (93-112 ppm), Victorian lignite does not contain any substantial carbohydrate residue, having long been lost in the coalification processes (Hedges et al., 1985, Wilson et al., 1987). Very weak signals in this region are likely to be caused by highly oxygen substituted carbon associated with lignin-type structures.

The spectra of the raw lignite-based products ROM and HSC appeared similar suggesting they were derived from the same lignite source. Both spectra had peaks in the chemical shift regions of 56 and 150 ppm, which are typically associated with the distribution of carbon atoms in lignin (Rumpel et al., 1998, Canellas et al., 2010, Hatcher, 1987, Kelemen et al., 2002), and methoxyl groups (56 ppm) on aromatic rings of lignin structures (Spaccini and Piccolo, 2007). The peaks in the LMB1 spectrum were in similar positions however the migration of the aliphatic peak towards lower chemical shift values indicated a higher concentration of shorter chain aliphatics (Kelemen et al., 2002) suggesting that the lignite may be derived from a different source. Peak positions in this spectrum were difficult to determine due to excessive background noise likely due to a relatively high Fe content. Interference by paramagnetic cations such as iron has been identified in other <sup>13</sup>C NMR studies of organic matter (Oades et al., 1987, Skjemstad et al., 1994). Product

SR, also raw lignite-based was likely derived from a different seam to the other raw lignite products as it had a higher concentration of aromatics.

Generally, spectra of the humate products showed a peak at 155 ppm which indicated carbon bonded to phenolic groups. As these products were manufactured by extraction in an alkaline environment, the presence of hydroxide groups would be expected (Tan et al., 1992).

The humate products SHP and LH1 were very similar in chemical structure and composition making it likely that SHP was a dried-down version of LH1. Product SHG was sourced from the same manufacturer and was reportedly a dried-down version of LH1 however this is unlikely given the differences in proportions of aromatic and aliphatic components. The spectra of product SHG is more similar to products SR and LH2 which were sourced from a different manufacturer. Similarities between the spectra of the humate product LH2 and SR suggest that the LH2 product was a potassium hydroxide extract of raw lignite derived from the same source.

It has been demonstrated that the proportions of aliphatic and aromatic carbons in a HA extract can differ from the parent material (Pang et al., 1990) however little evidence of this was seen here. In Pang et al. (1990), Loy Yang and Yallourn lignitic coals were treated with sodium hydroxide and the extracted HA had higher aromatic and lower aliphatic proportions compared to the respective parent materials. The authors hypothesized this to be due to the aliphatic microbial-derived components being more resilient to extraction. This does not explain the reverse trend for Bacchus Marsh lignite coal in which the extracted HA had lower aromatic and higher aliphatic proportions compared to the parent material. Regardless, assumptions regarding the parent material of humic acid extracts should be made cautiously.

## **2.5 Conclusion**

By comparing the chemical and structural composition data between products and with published studies, it has been possible to confirm the LDPs as being raw lignite –based (HSC, SR and LMB1) or humate-based (SHG, SHP, LH1 and LH2). Considerable variability was detected in the inorganic components and the HA concentrations. This variability in chemical composition would be expected to result in varying effects on plant growth. With the exact mechanism of plant promotion unknown, plant growth studies in glasshouse and field environments are required to demonstrate the efficacy of each product. These results will be important in selecting products for the following chapters.





### 3 Do lignite-derived organic amendments improve early-stage pasture growth and key soil biological and physicochemical properties?

This thesis chapter is published in the peer-reviewed journal *Crop and Pasture*. LITTLE, K. R., ROSE, M. T., JACKSON, W. R., CAVAGNARO, T. R. & PATTI, A. F. 2014. Do lignite-derived organic amendments improve early-stage pasture growth and key soil biological and physicochemical properties? *Crop and Pasture*, Advance online publication, doi: 10.1071/CP13433.

#### Declaration for thesis chapter 3.

Declaration by candidate.

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I performed 90% of the laboratory work and was the primary author of the manuscript.	85%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution for student co-authors only
Tim Cavagnaro	Contributed ideas to the work and co-authored the manuscript	
Tony Patti	Contributed ideas to the work and co-authored the manuscript	
W.Roy Jackson	Contributed ideas to the work and co-authored the manuscript	
Michael Rose	Contributed ideas to the work, co-authored the manuscript and performed 10% of the laboratory work.	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

**Candidate's  
Signature**

	<b>Date</b>
--	-------------

**Main  
Supervisor's  
Signature**

	<b>Date</b>
--	-------------

# Do lignite-derived organic amendments improve early-stage pasture growth and key soil biological and physicochemical properties?

Karen R. Little<sup>A,C</sup>, Michael T. Rose<sup>A</sup>, William R. Jackson<sup>A</sup>, Timothy R. Cavagnaro<sup>B</sup>, and Antonio F. Patti<sup>A</sup>

<sup>A</sup>School of Chemistry, Monash University, Clayton, Vic. 3800, Australia.

<sup>B</sup>School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, PMB1 Glen Osmond, SA, 5064, Australia.

<sup>C</sup>Corresponding author. Email: karen.little@monash.edu

**Abstract.** Commercial products derived from lignite (brown coal), sold mainly as humate preparations, are widely promoted as plant growth stimulants leading to higher crop yields. These products are also claimed to improve key indicators of soil health including soil pH and microbial biomass. In a glasshouse setting, we investigated the effect of six lignite-derived amendments applied at the manufacturer's recommended rate on the early-stage growth of two pasture species, lucerne (*Medicago sativa* L.) and ryegrass (*Lolium multiflorum* Lam.). We used two soil types common to south-eastern Australia, and following an 8-week growing period, assessed soil pH, microbial biomass carbon and mycorrhizal colonisation as key indicators of soil health. We hypothesised that humic acid (HA) and macronutrients derived from the products would positively influence pasture growth and soil health indicators. Although significant growth effects were observed in response to some products, the effects were inconsistent across pasture and soil types. Treatment effects on tissue nutrient accumulation were rare, with the exception of increased potassium in ryegrass in one soil amended with raw brown coal, and decreased nitrogen in lucerne in the same soil amended with a granulated, slow-release humate product. Further, we found no consistent trends in mycorrhizal colonisation or microbial biomass carbon in response to individual treatments. Given the variable responses of the plant species and soil types to the amendments used here, we emphasise the need for further mechanistic studies to help understand how these amendments can be used to greatest effect.

**Additional keywords:** agronomy, alfalfa, amendment, humic, legume, organic, ryegrass.

Received 9 December 2013, accepted 4 June 2014, published online 26 August 2014

## Introduction

There is increasing recognition of the need to produce more food on less land, with fewer external inputs (Kremen and Miles 2012). Much of the increase in food production in recent decades has come from the use of inorganic fertilisers. However, with global fertiliser resources dwindling, and increasing concerns about the energy-intensive nature of fertiliser production, there is a need to look to alternative methods to increase agricultural production in a sustainable manner (Tilman *et al.* 2002). Furthermore, excessive or poorly timed fertiliser application can lead to not only a loss of nutrients from production, but nutrients being leached into waterways or lost as the potent greenhouse gas nitrous oxide (N<sub>2</sub>O), and a deterioration of soil quality (Meng *et al.* 2005; Chan 2010; Fageria 2010; Hoben *et al.* 2011). There is a need to develop farming systems that maximise nutrient-use efficiency.

Healthy soils are the cornerstone of maintaining and enhancing agricultural productivity (Sparling *et al.* 2006). However, some agricultural practices that are implemented to

increase productivity, such as increased stocking rates and integrated crop–livestock systems, can, if inadequately managed, lead to reduced soil health via soil compaction, and a lowering of fertility and organic matter levels (Hiltbrunner *et al.* 2012; Houlbrooke 2011). Loss of organic matter is of particular concern, as organic matter is vital for maintaining the physical structure and stability of soils, as well as providing an energy source for soil microbial communities that drive key soil ecological processes. To help overcome impacts of agricultural intensification on soil health, there has been renewed interest in an agricultural paradigm that places greater reliance on soil organic amendments that improve fertiliser-use efficiency while increasing levels of soil organic matter (Jackson *et al.* 2008; Quilty and Cattle 2011).

Humic substances (HS) are naturally occurring, highly complex, organic mixtures predominantly formed by biochemical reactions that occur during the decay of plant, animal and microbial matter (MacCarthy 2001). They make up a significant component of soil organic matter and can improve

soil properties such as aggregation (Piccolo *et al.* 1997) and water-holding capacity, and act as a nutrient 'reservoir' by complexing macro- and micro-nutrients (Canarutto *et al.* 1996; Chen *et al.* 2004a; Imbufe *et al.* 2005; Ferreras *et al.* 2006; Alagöz and Yilmaz 2009). The application of HS to soil has been found to stimulate seed germination, and increase the growth and yields of a variety of important agricultural species (Lee and Bartlett 1976; Piccolo *et al.* 1993; Nardi *et al.* 2002; Arancon *et al.* 2006; Eyheraguibel *et al.* 2008; Puglisi *et al.* 2009). However, the effect of adding HS to plants and soils varies with the origin and concentration of the HS applied, and the species of plant and soil type to which it is applied (Rose *et al.* 2014). Consequently, it is difficult to generalise about the mechanisms by which HS affect plants and soils. Nevertheless, several mechanisms have been suggested, including 'hormone-like' effects (Muscolo *et al.* 1998, 2013; Chen *et al.* 2004a); however, this is the subject of ongoing debate, and there is a need for further detailed studies of a range of HS on more plant species and soil types (Rose *et al.* 2014).

Lignite (also known as brown coal) is widely used to manufacture a wide range of commercial HS products. Leonardite is often found in association with lignite and is formed by the oxidation of lignite from prolonged exposure to air. Lignite and leonardite are commonly marketed either in 'raw', 'run-of-mine' state or in the form of humic acid (HA) that has been extracted under alkaline conditions (Demirbas *et al.* 2006). Leonardite or lignite-derived product (LDP) can be formulated as soluble or slow-release granules and powders, or as liquids that are applied directly to the soil or as a foliar spray (Adani *et al.* 1998; Verlinden *et al.* 2010; Çelik *et al.* 2011; Seyedbagheri *et al.* 2012; Olk *et al.* 2013). Products vary in concentration of HA (generally 25–85%), and additional nutrients are commonly incorporated during product formulation. In many instances, these products are applied at the manufacturer's recommended rate, with little knowledge of optimal rates, timing and methods of application for a given plant–soil combination. This lack of informed application can lead to suboptimal outcomes, and highlights the need for direct investigation of the impacts of a range of HS on plants and soils.

Pastures support high-value animal-based production systems. Although there is an emerging trend towards the use of HS in the pasture sector in Australia and beyond, relatively few studies have investigated the effect(s) of LDP on the growth of pasture plant species. That said, some insights have been gained; for example, the shoot and root growth of ryegrass (*Lolium multiflorum* Lam.) has been found to be increased following application of HA derived from manure, compost, decomposed sawdust, straw and peat in both soil and hydroponic-based systems (Asenjo *et al.* 2000; Bidegain *et al.* 2000). Similarly, in a field study, there was an increase in the biomass of ryegrass plants following application of commercial LDPs; however, results were variable across soil types (Verlinden *et al.* 2010). Several other studies of impacts of LDP on a range of crop and pasture types have been reported, but most have been conducted in hydroponic or sand culture experiments rather than soil. Further, although it is often claimed that the addition of HS will improve soil health, to our

knowledge there have been no studies of the impacts of HS on common measures of soil health in pasture soil. For example, the effects of HS on the formation of arbuscular mycorrhizae (AM) appear not to have been assessed. Given that both AM and HS can affect plant growth and nutrition, this is an important knowledge gap. If LDPs are to become a viable strategy for pasture improvement, the recommendations provided to farmers must be sufficiently robust to return positive results under a wide variety of soil and management conditions.

Here we present results of a glasshouse study in which we sought to determine the effects of six commercial LDPs applied at the manufacturer's recommended rate on pasture-based systems. We hypothesised that higher applied rates of product-derived HA and macro-nutrients would result in positive plant growth and soil health effects. Effects measured were on: (i) the early-stage growth and nutrient contents of ryegrass and lucerne grown in two pasture soils; and (ii) soil pH, microbial biomass carbon and mycorrhizal colonisation as indicators of soil health.

## Materials and methods

### Characterisation of LDPs

We assessed six LDPs sourced from three manufacturers: two water-soluble solid humate products (A and B); one lignite-mineral blend (product C); one granulated, slow-release humate product (D); one humate soil conditioner (E); and brown coal sourced directly from the mine (otherwise known as 'run-of-mine' coal) (F) in the Latrobe Valley, Victoria. Key physicochemical properties of the products were quantified as follows. The pH was determined in 5-g subsamples suspended in deionised water (1 : 5 w/v), using a TPS WP-81 meter and probe (TPS Pty Ltd, Springwood, Qld). An additional 5-g subsample was used to determine HA content by repeated alkaline extraction using a modification of the IHSS method, as follows. To each product, 0.1 M HCl was added to give a 10 : 1 acid : LDP ratio (v/w). The slurry was then shaken at 120 rpm for 1 h and allowed to settle for 12 h. The supernatant was removed and discarded. Under N<sub>2</sub> atmosphere, 0.1 M NaOH was added to the solid residue at a ratio of 100 : 1 (v/w). The slurry pH was adjusted to 12.6 with 1 M NaOH and shaken at 120 rpm for 4 h (Hayes *et al.* 2008). The pH of the slurry was lowered to 9 using 1 M HCl, and solids were allowed to settle for 12–16 h. The supernatant was removed and retained, and alkaline extraction of the remaining solid repeated a further seven times until the supernatant was a pale brown colour. The supernatants were pooled, and HA precipitated by pH adjustment to 1–2 with 1 M HCl. The HA was then dialysed in cellulose membrane dialysis tubing MWCO 12000 (Sigma-Aldrich, St Louis, MO, USA) in deionised water until the conductivity of the surrounding water was <20  $\mu\text{S m}^{-1}$ . The HA was then oven-dried at 37°C and weighed. This was repeated in triplicate for each LDP. For each of the six products, a sample was ground to a fine powder using a mortar and pestle, homogenised and divided into two subsamples. The first subsample was analysed for total C, H and N by dry combustion (by The Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand: <http://neon.otago.ac.nz/consulting/microlab/>;

accessed October 2013). The second subsample was analysed for Al, Fe, K, Mn, P, S and Zn by radial view inductively coupled plasma-optical emission spectrometry (by Waite Analytical Services, University of Adelaide, Urrbrae, S. Aust.: www.adelaide.edu.au/was/; accessed October 2013). LDP composition is shown in Table 1.

### Soil collection and characterisation

Two soils were used in this study. The first, a Dermosol (Isbell 2002), was collected from grazed pasture near Stony Creek, Gippsland, in south-eastern Victoria (38°35'55"S, 146°3'7"E), and the second, a Podosol, from a vegetable farm recently converted from pasture in Cranbourne, Victoria (38°11'6"S, 145°18'50"E). These soils are referred to as Stony Creek (SC) and Cranbourne (CB) soils hereafter. The SC soil was acidic and had high organic matter content (11.3%), whereas the CB soil was mildly alkaline and had low organic matter content (2.4%). Both soils were collected in July 2011, from the 0–20 cm soil layer. Immediately following collection, the soils were air-dried and sieved to 2 mm. Subsamples (200 g) of each soil were then analysed for a range of key physicochemical properties (Table 2) (Environmental Analysis Laboratory, Southern Cross University, Lismore, NSW: http://scu.edu.au/eal/; accessed October 2013). Based on this soil analysis, it was decided to fertilise before use in the plant growth experiment; both soils received N, P and K at 100, 40 and 60 kg ha<sup>-1</sup>, respectively.

### Plant growth experiment

Plastic, free-draining pots (16 cm diameter) were filled with 800 g of SC or 1.1 kg CB soil. These masses were selected to match the field bulk densities for the two soils, which were 1.1 g cm<sup>-3</sup> (SC) and 1.3 g cm<sup>-3</sup> (CB). To each soil, the six LDPs were applied separately, following the manufacturer's recommended rate (Table 1). This approach was taken for two reasons. First, this best replicates the decision that is faced by farmers about when and how to apply these products. Second, the chemical composition of these products was highly variable (see Table 1) and so normalising application rates to a single property, e.g. % C or nutrient content, would necessitate the application of some products at unrealistic rates. The LDPs were mixed carefully into the top 1 cm of soil to simulate soil incorporation during pasture renovation or establishment, by topdressing before smudging, harrowing or aeration as per standard farming practice. The experiment also included a

control treatment, in which the soils were not amended with LDP. The pots were then left to equilibrate for 3 days before the sowing of seeds.

To five replicate pots of each treatment, 10 seeds of either lucerne (*Medicago sativa* L.) cv. Aurora or ryegrass (*Lolium multiflorum* Lam.) cv. Bealey were sown to ~2 mm below the soil surface. Thus, there were 140 pots in total. Although lucerne is a leguminous plant, N fertiliser was supplied to avoid any interactions between LDPs and rhizobial symbionts. The plants were then transferred to a glasshouse on the Monash University Clayton campus and grown from September to November 2011. The pots were arranged in a completely randomised design with their position rotated every 2 days. Conditions in the glasshouse were as follows: light levels maintained with supplemental lighting (16 h daylength), average  $228 \pm 14 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; temperature  $23.5 \pm 1.6^\circ\text{C}$  day and  $22.2 \pm 1.5^\circ\text{C}$  night. Plants were watered to field capacity determined following Asghari and Cavagnaro (2012) with tapwater as required, usually every 2 days. Seed emergence was determined as the number of seeds that emerged within 7 days post-seeding, and at this time, plants were thinned to two per pot.

### Plant harvesting and analysis

To examine the effects of LDPs on the early stages of growth of these pasture species, there was one destructive harvest at 8 weeks post-seeding. This was done to determine the efficacy of these products at the pasture establishment phase. The plants and soil were carefully removed from the pots. The soil was gently shaken from the roots, after which the shoots and roots separated. The roots were then thoroughly washed with water to remove any adhering soil, and rinsed with reverse osmosis water. The roots were then divided into two subsamples. The whole shoots and a subsample of the roots of each plant were oven-dried for 3 days at 55°C, following which shoot dry weight (SDW) and root dry weight (RDW) were determined. The dried plant material was then ground to a fine powder and nutrient concentrations were determined by radial view inductively coupled plasma-optical emission spectrometry (by Waite Analytical Services). The second subsample of roots was used to assess the percentage of root biomass colonised by mycorrhizae, using the gridline-intersect method (Giovannetti and Mosse 1980), after the roots were cleared in KOH (10% w/v) and stained with Trypan blue (omitting phenol from all reagents) (Phillips and Hayman 1970).

**Table 1. Description and composition (%) of lignite-derived products (LDPs)**

ROM, Run of mine; HA, humic acid. Application rates as recommended by the LDP manufacturer, except for product F, for which no recommended application was rate provided and rate was determined by an estimate of economic viability

Product	Application rate (ha <sup>-1</sup> )	pH	Moisture	HA	Element										
					C	H	N	S	P	K	S	Mg	Ca	Fe	Al
Soluble humate granules (A)	4 kg	9.4	4.5	50.2	47.5	4.19	1.32	0.33	0.02	8.50	2.70	0.14	0.95	1.04	1.00
Soluble humate powder (B)	10 L	10.4	9.1	82.3	42.1	4.45	0.73	0.23	0.001	16.90	0.26	0.20	0.10	0.23	2.90
Organic–mineral blend (C)	1 t	5.2	3.8	13.9	26.0	2.74	0.98	1.70	5.30	0.23	0.59	0.84	15.7	1.65	1.09
Slow-release granules (D)	50 kg	4.2	6.2	75.3	56.5	3.5	1.21	0.4	0.02	0.07	1.64	0.07	0.30	0.35	0.61
Humate soil conditioner (E)	1 t	4.8	39.5	26.1	56.1	4.95	0.55	0.61	0.10	0.08	0.20	0.31	1.29	0.71	0.56
ROM lignite coal (F)	5 t	4.5	51.9	68.4	66.4	4.84	0.66	0.21	0.0001	0.02	0.07	0.24	0.49	0.20	0.01

**Table 2. Physicochemical properties of the soils before the addition of lignite-derived products (LDPs)**

	Cranbourne	Stony Creek
pH (water)	8.0	5.2
Organic matter (%)	2.4	11.3
Carbon (%)	1.4	6.5
Nitrogen (%)	0.1	0.5
Calcium (mg kg <sup>-1</sup> )	1691	824
Iron (mg kg <sup>-1</sup> )	104	528
Potassium (mg kg)	157	87
Manganese (mg kg <sup>-1</sup> )	4	37
Phosphorus (Colwell) (mg kg <sup>-1</sup> )	86	49
Sulfur (mg kg <sup>-1</sup> )	52	20
Zinc (mg kg <sup>-1</sup> )	3.5	7
Texture	Sandy	Loam

### Soil analyses

Soils were refrigerated at  $-20^{\circ}\text{C}$  immediately following harvest. As-harvested soils were analysed for microbial biomass carbon (MBC) by chloroform fumigation (Vance *et al.* 1987). Subsamples of each soil (10 g) were fumigated with ethanol-free chloroform for 24 h, in a sealed desiccator in the dark. Non-fumigated subsamples (10 g) were also stored in a dark environment for this period. The following day, the desiccator was evacuated to remove chloroform from the soils. The fumigated and non-fumigated soils were extracted with 0.5 M K<sub>2</sub>SO<sub>4</sub> at a 1:3 (w/v) ratio and filtered. The carbon content of the filtered product was determined by TOC-V CPH/CPN total organic carbon analyser (Shimadzu Corp., Kyoto, Japan). Soil pH was determined by suspension of an air-dried soil subsample (5 g) suspended in deionised water (1:5 w/v), using a TPS WP-81 meter and probe.

### Calculations and data analyses

Because of differences in plant growth between soils, the plant biomass and MBC data were used to calculate plant responses relative to the control, following Eqn 1:

$$\% \text{ response} = \left( \frac{\text{mean} - \text{mean of control}}{\text{mean of control}} \right) \times 100 \quad (1)$$

Initially, all biomass, tissue nutrient and soil characterisation data were analysed by three-way analysis of variance (ANOVA); factors in the analysis were plant, soil type and product. Because of size asymmetry between the two species (i.e. large differences in plant size masking other effects), all data were then re-analysed by two-way ANOVA with factors in the analysis being soil type and product. Where significant differences were found, pairwise comparisons were made using Tukey's honestly significant difference (HSD) test. To explore further the responses to treatments, 95% confidence intervals (CIs) were calculated for plant biomass, mycorrhizal colonisation and MBC. These were then used to compare responses relative to zero; data with 95% confidence interval greater or lower than zero were considered significantly different. All data were analysed using JMP statistical software (JMP<sup>®</sup>, Version 10; SAS Institute Inc., Cary, NC, USA).

## Results

### LDP and soil characterisation

The LDPs varied considerably in chemical composition, reflecting differences in the source of lignite, extraction techniques and the addition of nutrients during formulation (Table 1). There were clear differences in the HA content of the products, ranging from 13.9% to 82.3% on a dry-weight basis (Table 1). Products A and B contained high levels of potassium and product C contained higher concentrations of sulfur, phosphorus and calcium than other products.

The two soils had differing physicochemical properties (Table 2). The CB soil had low levels of carbon and key plant nutrients. The SC soil was more acidic, with particularly high concentrations of iron and manganese.

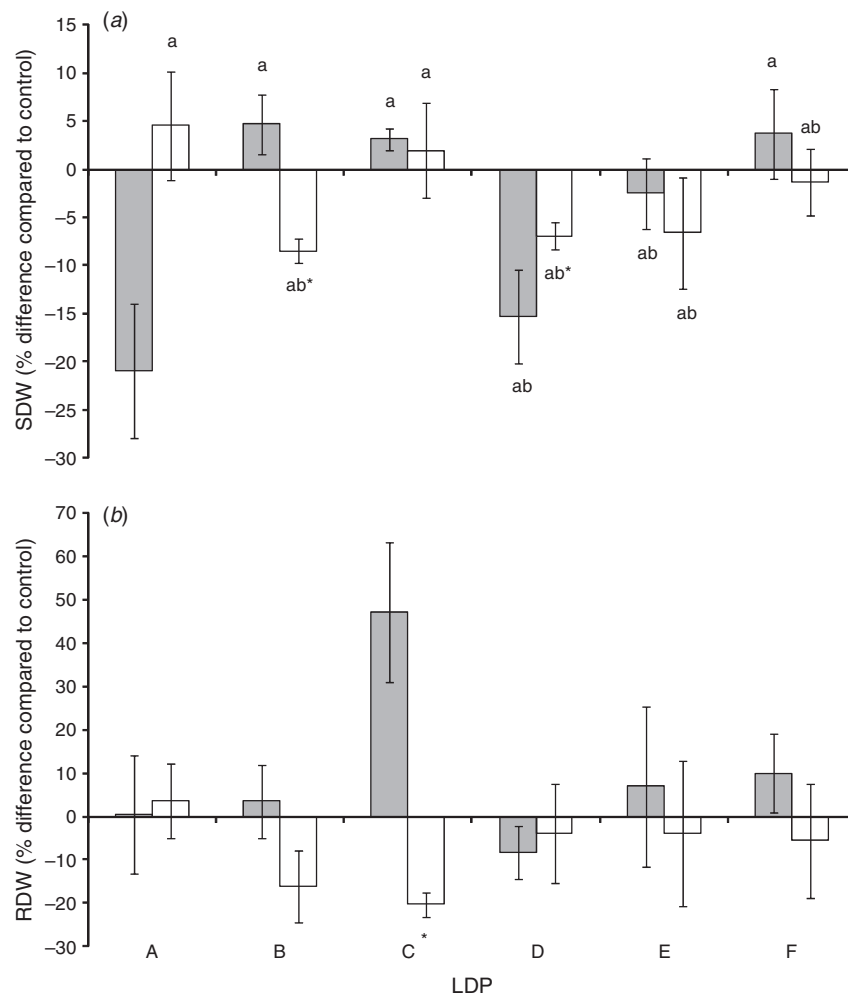
### Growth and nutrition

#### Ryegrass growth and nutrition

The effect of LDP on SDW varied considerably among the treatments and soil types (Fig. 1, Table 3). ANOVA indicated a significant interaction ( $P=0.04$ ) between soil type and product, with products A, B and F showing inconsistent growth effects in the two soils. Further, in the CB soil, treatment with product A resulted in significant (as indicated by 95% CI) shoot growth depression, but this was not the case in the SC soil. Products B and D had no significant effect (as indicated by 95% CI) on SDW in CB soil; however, they caused a negative growth response in SC soil. Overall, there were no strong positive ryegrass-shoot growth responses (as indicated by 95% CI) to any LDP in either soil.

The effects of LDP on RDW varied between the two soils (Fig. 1), and ANOVA (Table 3) indicated no significant main effects or interactions of LDP and soil type. Interestingly, analysis by 95% CI showed that product C gave a significantly positive root growth response in the CB soil, whereas the reverse was true in the SC soil. In addition, taking into account both root and shoot data (Fig. 1), product A in the CB soil caused a reduction in SDW, but there was no apparent effect on RDW.

The concentration of potassium in the shoots of the ryegrass plants was influenced by both LDP and soil type, as indicated by a significant interaction ( $P=0.005$ ) between these two factors (Table 3). Specifically, in CB soil, product F significantly increased shoot potassium concentration compared with product E and the control, whereas in SC soil there was no effect. Similarly, for shoot sulfur concentration, there was a significant interaction effect ( $P=0.02$ ). However, using Tukey's pairwise comparisons, we could not identify which treatment means differed significantly; this reflects the more conservative nature of the Tukey's test than the ANOVA. There was a significant main effect of LDP on shoot nitrogen concentrations. Product C increased tissue nitrogen concentration compared with product D; however, neither differed significantly from the control. Although there were no further effects of LDP on tissue nutrition, there were significant differences between the effects of each soil on plant nutrient uptake, especially in terms of manganese (higher uptake in SC) and aluminium (higher in CB) (Tables 3 and 4).



**Fig. 1.** Dry weight of (a) shoots (SDW) and (b) roots (RDW) of ryegrass grown for 56 days in soils amended with lignite-derived products (LDP). Letters along x-axis refer to individual LDPs as indicated in Table 1. Values are percentage change compared with the control value. Actual data are included in the Supplementary information (at journal website). Grey bars represent Cranbourne soil, white bars Stony Creek soil. Bars with the same lower case letter are not significantly different at  $P=0.05$  as assessed by Tukey's HSD. \*Indicates significant difference from zero as assessed by 95% confidence interval. Error bars are  $\pm$  s.e.

### Lucerne growth and nutrition

Similar to ryegrass, the effect of LDP on the SDW of lucerne varied considerably (Fig. 2). ANOVA indicated a significant main effect of soil type, with lucerne shoot growth higher in CB soil than SC soil. Products B and C gave a significant growth increase (indicated by 95% CI) in CB soil, whereas only product B had a positive effect in SC soil. Similarly, the RDW of lucerne was largely unaffected by LDP addition (Fig. 2). Only product C caused a positive root growth effect, but this occurred only in CB soil.

With regard to plant nutrition, ANOVA identified a significant interaction ( $P=0.05$ ) between LDP and soil type for shoot nitrogen concentration (Tables 3 and 4). Specifically, in CB soil, lucerne treated with product E contained lower concentrations of nitrogen than the control. For the other nutrients, there was no significant effect on shoot tissue

nutrients (Table 4); however, as for ryegrass, soil type did have an effect, particularly for aluminium, iron and potassium (higher uptake in CB soil) and manganese (higher in SC soil) (Tables 3 and 4).

### Soil biological and physiochemical properties

#### Ryegrass mycorrhizal colonisation

The effect of LDP on mycorrhizal colonisation was significant, yet variable, between soil types and among products (Fig. 3). ANOVA indicated a significant interaction ( $P=0.01$ ) between LDP and soil type. Colonisation was generally higher in SC soil; however, application of product A resulted in higher colonisation in CB soil. Comparing products in each soil type, application of product A resulted in a significant increase in colonisation in CB soil compared with products B, C,

**Table 3. ANOVA summary table for all response variables**

Factors in the analysis were soil and lignite-derived product (LDP) addition treatment. Both main effects and interaction terms are indicated. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., not significant ( $P > 0.05$ )

	Ryegrass			Lucerne		
	Soil	LDP	Soil × LDP	Soil	LDP	Soil × LDP
Shoot dry weight	***	*	**	***	n.s.	n.s.
Root dry weight	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Shoot Al concentration	**	n.s.	n.s.	**	n.s.	n.s.
Shoot Ca concentration	n.s.	n.s.	n.s.	**	n.s.	n.s.
Shoot Fe concentration	n.s.	n.s.	n.s.	***	n.s.	n.s.
Shoot K concentration	***	n.s.	*	***	n.s.	n.s.
Shoot Mg concentration	***	n.s.	n.s.	**	n.s.	n.s.
Shoot Mn concentration	***	n.s.	n.s.	***	n.s.	n.s.
Shoot P concentration	***	n.s.	n.s.	***	n.s.	n.s.
Shoot S concentration	***	n.s.	*	*	n.s.	n.s.
Shoot Zn concentration	***	n.s.	n.s.	***	n.s.	n.s.
Shoot N (%)	*	*	n.s.	**	*	*
Shoot C (%)	n.s.	n.s.	n.s.	*	n.s.	n.s.
Mycorrhizal colonisation	***	***	*	***	*	n.s.
Microbial biomass C	***	n.s.	n.s.	n.s.	n.s.	n.s.
Soil pH	***	n.s.	n.s.	***	n.s.	n.s.

E and F. In SC soil, there was no significant change compared with the control.

*Lucerne mycorrhizal colonisation*

The ANOVA indicated main effects of product ( $P = 0.01$ ) and soil type ( $P < 0.0001$ ) on mycorrhizal colonisation in lucerne (Table 3). Product A increased mycorrhizal colonisation in CB soil but not in SC soil (Fig. 3). Interestingly, application of LDP to SC soil had an overall negative effect on colonisation, with significant decreases on application of products B, C, D and F.

*Microbial biomass carbon*

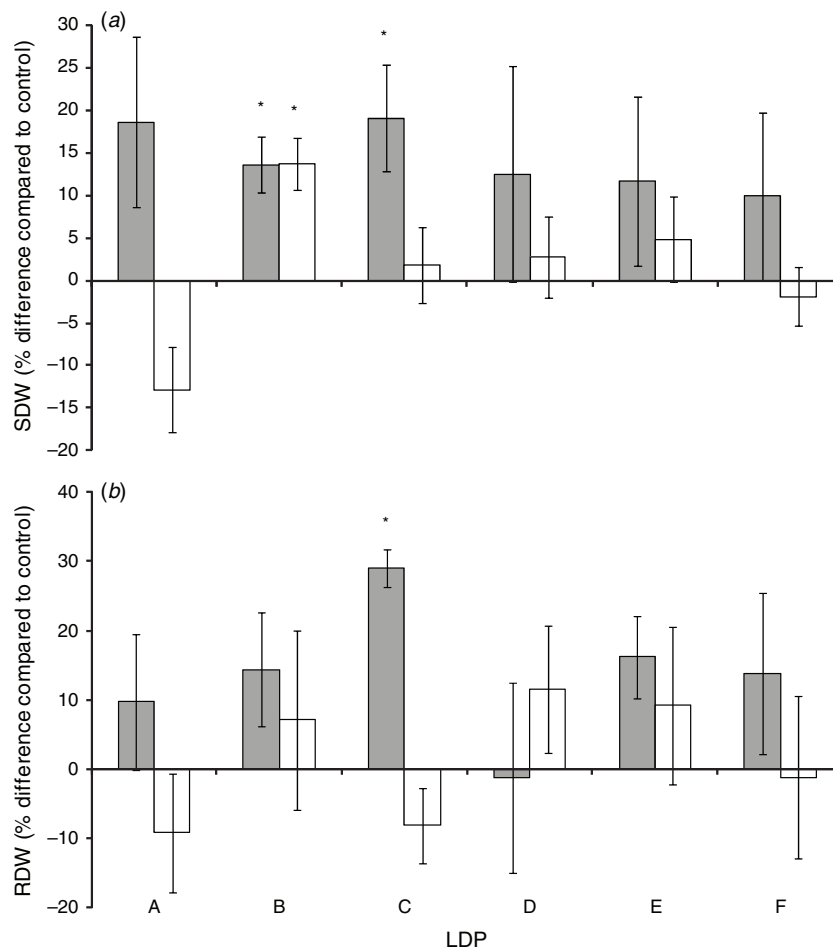
For ryegrass, ANOVA indicated a significant interaction ( $P < 0.001$ ) between LDP and soil type for MBC. In CB soil, the addition of LDP generally had a positive effect on microbial biomass (Fig. 4). In particular, products A, B, C, E and F promoted MBC significantly, as verified by 95% CI. By comparison, when LDP was applied to SC soil, there was no significant MBC response. For lucerne, there were no significant main or interactive effects (Table 3), and so regardless of soil type, the application of LDP did not significantly promote or depress MBC (Fig. 4).

**Table 4. Nutrient composition of ryegrass and lucerne shoot tissue harvested at 56 days post-seeding, grown in Cranbourne (CB) and Stony Creek (SC) soils amended with lignite-derived products (LDPs)**

Mean values are indicated ( $n = 5$ ) and values in parentheses are  $\pm$  s.e. Within columns, means followed by the same letter are not significantly different at  $P = 0.05$  as assessed by Tukey's HSD

Plant	Soil	LDP	Al (mg kg <sup>-1</sup> )	Fe (mg kg <sup>-1</sup> )	K (g kg <sup>-1</sup> )	Mn (mg kg <sup>-1</sup> )	P (g kg <sup>-1</sup> )	S (g kg <sup>-1</sup> )	Zn (mg kg <sup>-1</sup> )	%C	%N
Ryegrass	Cranbourne	A	90 (26)	90 (19)	21.8 (0.7)ab	26 (2)	3.0 (0.2)	1.9 (0.1)	38 (3)	40.4 (0.4)	0.7 (0.1)
		B	49 (10)	65 (6)	22.6 (1.4)ab	23 (1)	2.6 (0.1)	2.7 (0.2)	37 (1)	40.7 (1.0)	0.9 (0.1)
		C	69 (35)	84 (20)	24.0 (1.3)ab	24 (2)	2.9 (0.1)	2.3 (0.2)	38 (2)	39.8 (0.7)	1.0 (0.1)
		D	54 (15)	61 (8)	21.3 (1.5)ab	24 (3)	2.8 (0.1)	1.9 (0.2)	32 (2)	39.6 (0.5)	0.6 (0.1)
		E	44 (18)	65 (9)	19.8 (0.7)b	23 (3)	2.9 (0.1)	2.1 (0.2)	38 (3)	40.7 (1.1)	0.8 (0.1)
		F	37 (4)	58 (4)	25.6 (1.2)a	20 (2)	3.1 (0.1)	2.7 (0.3)	36 (2)	40.5 (0.5)	0.9 (0.1)
		Control	69 (33)	79 (19)	21.2 (0.4)b	24 (2)	2.8 (0.2)	2.4 (0.2)	36 (3)	39.6 (0.8)	0.8 (0.1)
	Stony Creek	A	26 (13)	69 (21)	7.9 (0.3)c	149 (9)	2.5 (0.1)	2.2 (0.1)	56 (4)	39.7 (0.9)	0.9 (0.1)
		B	22 (8)	66 (9)	7.3 (0.3)c	148 (7)	2.4 (0.1)	1.8 (0.1)	59 (4)	40.9 (0.3)	0.8 (0.1)
		C	31 (20)	75 (25)	7.9 (0.6)c	131 (13)	2.6 (0.1)	1.9 (0.1)	55 (2)	40.8 (0.7)	1.0 (0.1)
		D	26 (12)	74 (21)	6.9 (0.4)c	155 (8)	2.2 (0.1)	1.8 (0.2)	53 (5)	41.8 (0.9)	0.8 (0.1)
		E	16 (5)	68 (15)	7.3 (0.5)c	145 (11)	2.2 (0.1)	1.9 (0.1)	52 (5)	41.3 (0.7)	0.8 (0.1)
		F	23 (11)	67 (12)	6.3 (0.6)c	152 (11)	2.1 (0.1)	1.9 (0.1)	56 (2)	40.1 (1.1)	0.9 (0.1)
		Control	14 (2)	68 (9)	8.6 (0.7)c	152 (5)	2.6 (0.1)	2.0 (0.1)	63 (5)	40.3 (0.2)	1.1 (0.1)
Lucerne	Cranbourne	A	56 (27)	76 (17)	17.5 (1.0)	20 (2)	3.1 (0.1)	2.2 (0.1)	27 (2)	39.2 (0.6)	1.3 (0.1)abc
		B	33 (6)	79 (8)	18.4 (0.8)	20 (1)	2.8 (0.1)	2.1 (0.2)	26 (1)	40.8 (0.7)	1.4 (0.1)abc
		C	84 (52)	172 (105)	17.0 (0.5)	21 (1)	3.0 (0.1)	1.9 (0.1)	25 (2)	39.5 (0.9)	1.4 (0.1)abc
		D	58 (21)	103 (19)	16.9 (0.9)	24 (3)	2.9 (0.9)	2.4 (0.2)	26 (2)	40.0 (0.6)	1.2 (0.1)bc
		E	53 (10)	88 (9)	17.1 (1.3)	21 (2)	2.9 (0.2)	2.2 (0.1)	26 (1)	40.9 (0.5)	1.2 (0.1)c
		F	45 (17)	101 (21)	17.3 (0.7)	20 (2)	3.1 (0.1)	2.2 (0.2)	27 (1)	40.5 (0.9)	1.5 (0.1)abc
		Control	41 (11)	93 (14)	17.9 (0.6)	23 (2)	2.9 (0.6)	2.7 (0.4)	27 (1)	41.2 (0.8)	1.8 (0.1)a
	Stony Creek	A	11 (1)	49 (3)	9.8 (0.8)	124 (8)	2.2 (0.1)	1.8 (0.1)	34 (4)	41.1 (0.8)	1.4 (0.1)abc
		B	17 (5)	54 (8)	8.6 (0.4)	150 (19)	2.1 (0.2)	2.1 (0.2)	31 (4)	41.5 (0.6)	1.4 (0.1)abc
		C	9 (1)	47 (2)	10.6 (0.6)	155 (5)	2.5 (0.2)	2.1 (0.2)	35 (3)	42.3 (1.3)	1.6 (0.1)abc
		D	18 (3)	59 (11)	8.8 (0.3)	139 (12)	1.9 (0.2)	2.0 (0.1)	32 (3)	40.5 (0.3)	1.5 (0.1)abc
		E	11 (1)	55 (6)	8.9 (0.4)	163 (14)	2.0 (0.2)	2.0 (0.1)	37 (3)	41.1 (0.2)	1.6 (0.1)abc
		F	15 (3)	59 (9)	9.8 (0.4)	177 (22)	2.3 (0.1)	2.0 (0.1)	36 (3)	41.0 (0.4)	1.7 (0.1)ab
		Control	15 (2)	52 (3)	9.2 (0.4)	144 (11)	2.0 (0.1)	1.9 (0.2)	33 (3)	41.4 (0.3)	1.5 (0.1)abc





**Fig. 2.** Dry weight of (a) shoots (SDW) and (b) roots (RDW) of lucerne grown for 56 days in soils amended with lignite-derived products (LDP). Letters along x-axis refer to individual LDPs as indicated in Table 1. Values are percentage change compared with the control value. Actual data are included in the Supplementary information (at journal website). Grey bars represent Cranbourne soil, white bars Stony Creek soil. No significant difference was detected at  $P=0.05$  as assessed by Tukey's HSD. \*Indicates significant difference from zero as assessed by 95% confidence interval. Error bars are  $\pm$  s.e.

### Soil pH

The ANOVA indicated a significant main effect of soil type but not product on the post-harvest soil pH (Table 3). The pH of CB soil was higher than that of SC soil (Table 5). For both ryegrass and lucerne, application of LDP to both CB and SC soils did not have a significant effect on soil pH as shown by ANOVA and 95% CI.

### Discussion

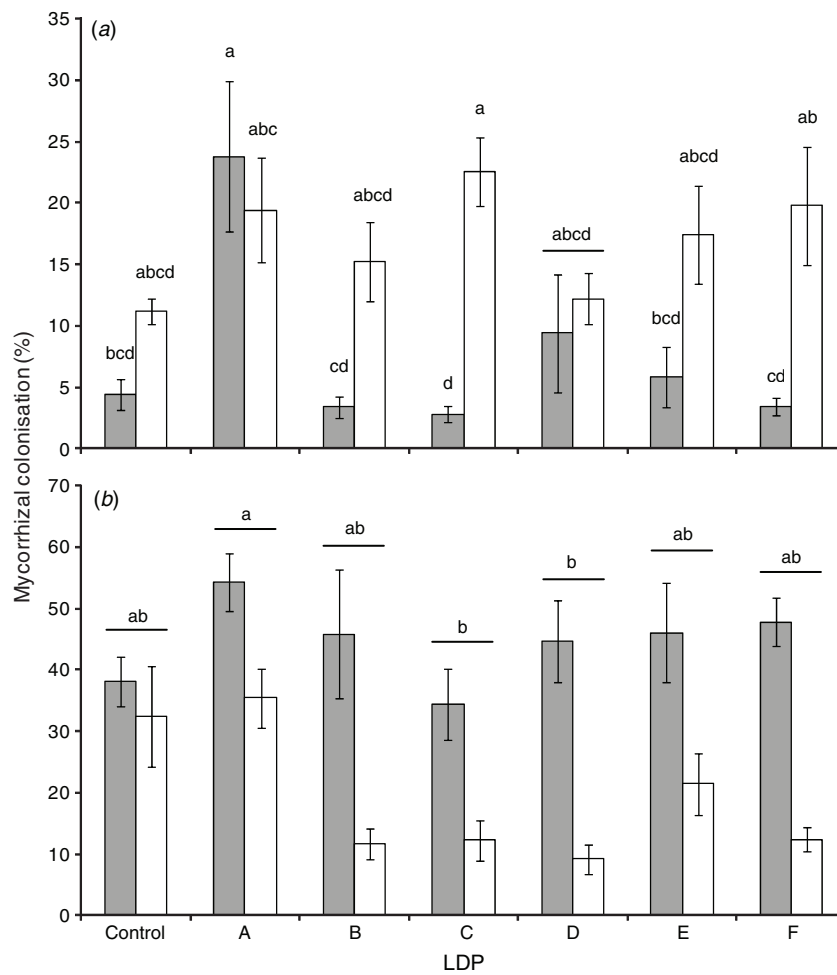
Variable responses in terms of both early-stage pasture growth and measures of soil health to the application of six commercially sourced LDPs to two different soils and pasture species were observed. This finding highlights the need for soil- and plant-specific optimisation when applying these amendments.

#### Soil and LDP characterisation

The considerable differences in chemical composition between LDPs may be attributed to the origin of the parent material, the

extraction technique, and additions made during the product formulation process. For example, the comparatively high concentrations of potassium in products A and B are likely an artefact of the extraction process. By contrast, the relatively elevated phosphorus and calcium contents of product C reflect the labelling of the product as a humate-fertiliser blend, in which minerals are probably added during the formulation process.

Numerous studies indicate the importance of HS application rate to plant nutrient availability and the magnitude of the plant growth response (Tan and Nopamornbodi 1979; Adani *et al.* 1998; Atiyeh *et al.* 2002; Albayrak and Camas 2005; Tahir *et al.* 2011). In this study, the HA content of the LDPs varied between 13.9% and 82.3%; therefore, using the manufacturer's recommended application rate resulted in a wide range of HA rates actually applied. Investigating the rate of application of HA in isolation is important, and is worthy of further consideration (Rose *et al.* 2014).



**Fig. 3.** Mycorrhizal colonisation in roots of (a) ryegrass and (b) lucerne grown for 56 days in soils amended with lignite-derived products (LDP). Letters along x-axis refer to individual LDPs as indicated in Table 1. Bars with the same lower case letter are not significantly different at  $P=0.05$  as assessed by Tukey's HSD. ANOVA showed a significant main effect of soil type irrespective of LDP application (see text for results). There was also a significant main effect of LDP application irrespective of soil type; these differences are indicated by letters above bars connected with a horizontal line to indicate where soil types should be pooled over LDP treatment. Grey bars represent Cranbourne soil, white bars Stony Creek soil. Error bars are  $\pm$  s.e.

#### Effect of LDPs on shoot growth

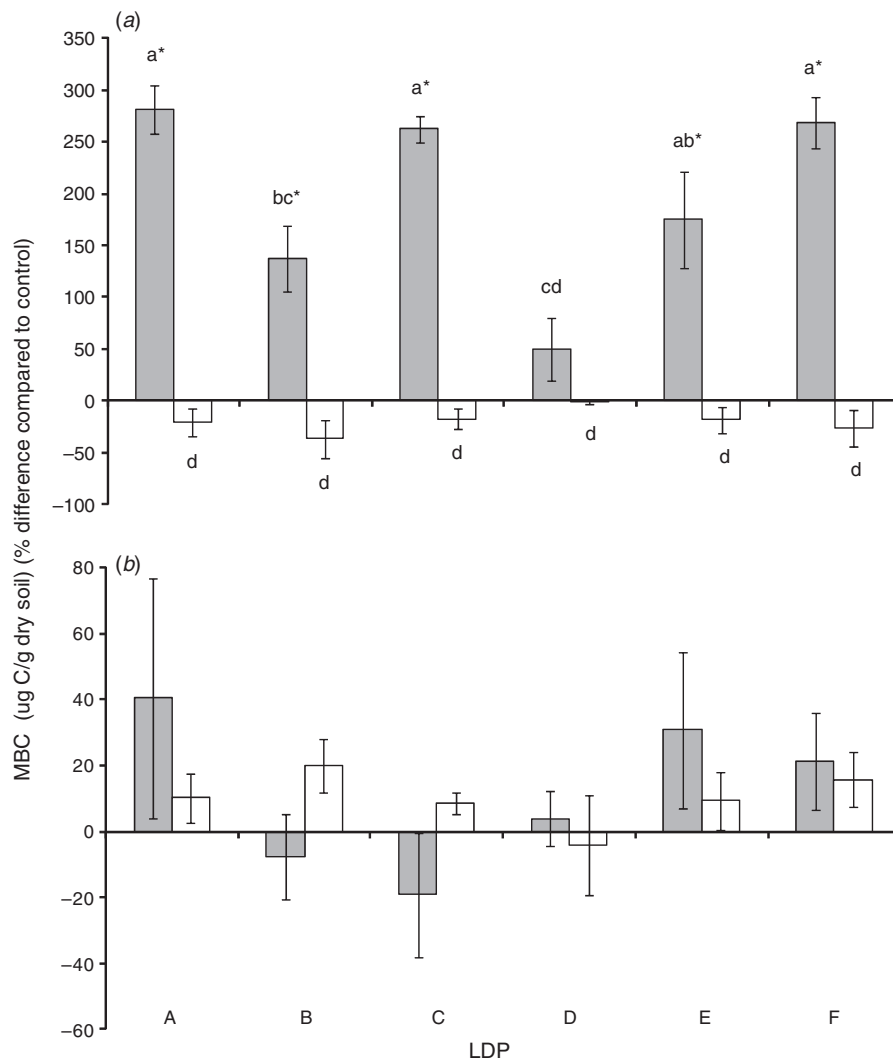
The effect of LDPs on the growth of ryegrass was not consistent between soils, with no observable trends on shoot and root dry-matter accumulation. Despite products A, B and F having relatively high HA contents (50.2, 82.3 and 68.4%, respectively), inconsistencies between the growth effects in each soil may suggest that soil type rather than HA content is an important factor in the performance of these products (Rose *et al.* 2014).

Product B was the only LDP that consistently improved lucerne shoot growth in both soil types. However, this result was not reflected in the ryegrass, suggesting that this effect may depend on plant species, as has been seen in other HA studies (Akinremi *et al.* 2000; Lodhi 2013). Product B is a soluble humate, recommended by the manufacturer to be applied as a liquid, and so for this plant type, it may have been more readily accessible. Similarly, Verlinden *et al.* (2009) reported higher

shoot yield of permanent grassland in field trials, and Italian ryegrass in pot trials, with a liquid humate product than the solid form. Thus, mode of application may be an important consideration.

#### Effect of LDPs on root growth

With respect to root growth, treatment of both lucerne and ryegrass in the sandy CB soil with product C increased biomass by 28% and 45%, respectively. Although this product is a mineral blend high in phosphorus and calcium, leaf-tissue nutrient analysis indicated that these elements were not elevated, and so this effect of increased biomass is unlikely to be due to increased uptake. Interestingly, this same product reduced the root growth of both pasture types in SC soil, indicating a soil-dependent effect. Previous studies indicate variable shoot and root growth effects in soils with differing organic matter content,



**Fig. 4.** Microbial biomass carbon (MBC) associated with (a) ryegrass and (b) lucerne grown for 56 days in soils amended with lignite-derived products (LDP). Letters along x-axis refer to individual LDPs as indicated in Table 1. Values are percentage change compared to the control value. Actual data are included in the Supplementary information (at journal website). Grey bars represent Cranbourne soil, white bars Stony Creek soil. Bars with the same lower case letter are not significantly different at  $P=0.05$  as assessed by Tukey's HSD. \*Indicates significant difference from zero as assessed by 95% confidence interval. Error bars are  $\pm$  s.e.

with more pronounced growth effects in soils with low organic matter content (Kunkel and Holstad 1968; Lee and Bartlett 1976; Fagbenro and Agboola 1993). The effects observed in this study may therefore be related to the difference in organic matter levels between the two soils; however, there were differences also in pH, texture and nutrient content. The precise mechanisms for root stimulation–inhibition remain unknown.

#### Effect of LDPs on ryegrass and lucerne nutrition

Previous studies have shown varied responses in the uptake of macro- and micro-nutrients, which could be related to crop type, growing media or source of HA (Tan and Nopamornbodi 1979; Ascaso *et al.* 1985; Fagbenro and Agboola 1993; Adani *et al.* 1998; Akinremi *et al.* 2000; Verlinden *et al.* 2009; Verlinden *et al.*

2010). Despite differences in chemical composition and application rates of LDPs, the only differences identified in nutrient uptake for this study were in potassium and nitrogen.

A significant increase in shoot tissue potassium was seen in ryegrass in CB soil with product F. Some studies have shown increases in potassium in a range of crop types (Verlinden *et al.* 2009; Yolcu *et al.* 2011; Tahir *et al.* 2011), whereas others have shown no change (Tan and Nopamornbodi 1979; Pinalan and Kaplan 2003; Liu *et al.* 1998). Product F did not have a high level of inherent potassium, so it was not the source. Despite elevated potassium in products A and B, no tissue accumulation was detected in ryegrass or lucerne treated with these products. This may be due to the low application rate or the availability of product-derived potassium for plant utilisation.

**Table 5. Post-harvest pH of soils following 56 days of ryegrass or lucerne growth**  
LDP, Lignite-derived product, as indicated in Table 1. Values in parentheses are  $\pm$  s.e.

Soil	LDP	Soil pH ryegrass	Soil pH lucerne
Cranbourne	A	7.57 (0.03)	7.49 (0.07)
	B	7.69 (0.04)	7.67 (0.03)
	C	7.62 (0.04)	7.59 (0.01)
	D	7.58 (0.05)	7.56 (0.05)
	E	7.61 (0.04)	7.64 (0.03)
	F	7.52 (0.03)	7.58 (0.04)
	Control	7.60 (0.05)	7.57 (0.04)
Stony Creek	A	4.75 (0.02)	4.66 (0.08)
	B	4.69 (0.02)	4.63 (0.07)
	C	4.73 (0.03)	4.72 (0.02)
	D	4.67 (0.02)	4.58 (0.04)
	E	4.70 (0.05)	4.69 (0.02)
	F	4.65 (0.02)	4.61 (0.03)
	Control	4.64 (0.03)	4.60 (0.03)

Application of LDP to both ryegrass and lucerne had an effect on shoot nitrogen concentration. In ryegrass, accumulation of nitrogen was significantly higher in ryegrass treated with product C than product D. Product D is a slow-release product and may not have had time to elicit an effect in the short growing period. In lucerne, a decrease in tissue nitrogen resulted from application of product E to CB soil compared with the control, yet this was not accompanied by a growth reduction. This result is in agreement with Akinremi *et al.* (2000), who found decreased nitrogen uptake in canola grown in soil amended with milled leonardite. Some studies have contrasting results, showing the application of HA to increase tissue nitrogen concentration in a range of crops (Çimrin *et al.* 2001; Verlinden *et al.* 2009; Verlinden *et al.* 2010) or showing increase or decrease depending on the application rate (Tan and Nopamombodi 1979). As has been demonstrated for plant growth (Rose *et al.* 2014), nutritional effects may also be dependent on the origin and rate of HA application, crop and/or soil type. More investigation into the effect of HA on nutrient cycling is required.

#### Indicators of soil health

Because of their complex chemical nature and high carbon content, HA-containing LDPs are likely to interact directly and indirectly with soil microorganisms. Research on these interactions is limited, despite extensive knowledge about the general role of soil microorganisms in plant health. The formation of AM between a specialised group of soil fungi and most plant species enables enhanced uptake of essential plant nutrients that can improve plant growth (Smith and Read 2010). It has been hypothesised that the presence of HA influences nutrient availability for plant uptake (Chen *et al.* 2004b).

Product A had a stimulatory effect on mycorrhizal colonisation of ryegrass in both soils, and of lucerne in CB soil. Although studies into the effect of HA on mycorrhizal colonisation are limited, a similar stimulatory effect was seen by Gryndler *et al.* (2005) in maize roots. For lucerne, products B,

C, D and F had a depressive effect on colonisation in SB soil. Vallini *et al.* (1993) reported a similar effect; however, that was at an HA concentration of 3000 mg kg<sup>-1</sup>, which is well above the concentrations used here. Levels of colonisation were similar to those previously reported for pasture (Ryan and Ash 1999), with a lower percentage of roots colonised in the ryegrass, which may be due to its fine, branched root structure, more easily able to access nutrients (Schweiger *et al.* 1995).

Interestingly, with the exception of product D, MBC was increased by LDPs with ryegrass in CB soil but there was no significant response with lucerne, or with either plant type in SC soil. MBC is a sensitive measure to monitor changes in soil organic matter status (Sparling 1992); hence, a lack of effect of the LDPs on MBC in the high-organic matter SC soil is not unexpected, because the soil already has high levels of HS. Limited studies have investigated the effect of HA on the soil microbial community; however, it has been found in nutrient culture medium that the structure of the humic product plays a role in promotion or suppression of populations within the soil microbial community (Visser 1985).

#### Conclusions

Application of a range of commercially available LDPs to lucerne or ryegrass in two contrasting soils gave variable results in terms of plant growth and soil health measures. There was no clear, consistent link between HA and nutrient content of the products and positive plant growth and soil health indicator effects. In agreement with others, it is possible that application rates were too low to elicit a significant agronomic response (Duval *et al.* 1998; Feibert *et al.* 2003; Chen *et al.* 2004b; Hartz 2010). Further investigation is needed into the mechanistic interaction between the LDP and the plant, and the impact on nutrient cycling. This, along with studies that include a wide range of application rates and longer term glasshouse and field studies, may enable the matching of each LDP with specific soil and plant types in specific environmental settings.

#### Acknowledgements

KRL gratefully acknowledges the provision of a Brown Coal Innovation Australia (BCIA) research scholarship and an Australian Postgraduate Award scholarship provided by Monash University. TRC gratefully acknowledges the Australian Research Council for supporting his research through the Future Fellowship program (FT120100463). The support of BCIA and partners (International Power, Clean Coal Victoria and Bass Coast Landcare) for a larger project on commercially available LDPs is gratefully acknowledged. The technical support of Dr Priscilla (Pree) Johnson is also gratefully acknowledged.

#### References

- Adani F, Genevini P, Zaccheo P, Zocchi G (1998) The effect of commercial humic acid on tomato plant growth and mineral nutrition. *Journal of Plant Nutrition* **21**, 561–575. doi:10.1080/01904169809365424
- Akinremi OO, Janzen HH, Lemke RL, Larney FJ (2000) Response of canola, wheat and green beans to leonardite additions. *Canadian Journal of Soil Science* **80**, 437–443. doi:10.4141/S99-058
- Alagöz Z, Yilmaz E (2009) Effects of different sources of organic matter on soil aggregate formation and stability: A laboratory study on a Lithic Rhodoxeralf from Turkey. *Soil & Tillage Research* **103**, 419–424. doi:10.1016/j.still.2008.12.006

- Albayrak S, Camas N (2005) Effects of difference levels and application times of humic acid on root and leaf yield and yield components of forage turnip (*Brassica rapa* L.). *Journal of Agronomy* **4**, 130–133. doi:10.3923/ja.2005.130.133
- Arancon NQ, Edwards CA, Lee S, Byrne R (2006) Effects of humic acids from vermicomposts on plant growth. *European Journal of Soil Biology* **42**, S65–S69. doi:10.1016/j.ejsobi.2006.06.004
- Ascaso C, Fortun C, Rapsch S (1985) Action of humic acid preparations on leaf development, mineral elements contents and chloroplast ultrastructure of ryegrass plants. *Photosynthetica* **19**, 294–299.
- Asenjo MCG, González JL, Maldonado JM (2000) Influence of humic extracts on germination and growth of ryegrass. *Communications in Soil Science and Plant Analysis* **31**, 101–114. doi:10.1080/00103620009370423
- Ashgari HR, Cavagnaro TR (2012) Arbuscular mycorrhizas reduce nitrogen loss via leaching. *PLoS ONE* **7**, e29825. doi:10.1371/journal.pone.0029825
- Atiyeh RM, Lee S, Edwards CA, Arancon NQ, Metzger JD (2002) The influence of humic acids derived from earthworm-processed organic wastes on plant growth. *Bioresource Technology* **84**, 7–14. doi:10.1016/S0960-8524(02)00017-2
- Bidegain RA, Kaemmerer M, Guirse M, Hafidi M, Rey F, Morard P, Revel JC (2000) Effects of humic substances from composted or chemically decomposed poplar sawdust on mineral nutrition of ryegrass. *The Journal of Agricultural Science* **134**, 259–267. doi:10.1017/S0021859699007492
- Canarutto S, Pera A, Lamarca M, Vallini G (1996) Effects of humic acids from compost-stabilized green waste or leonardite on soil shrinkage and microaggregation. *Compost Science & Utilization* **4**, 40–46. doi:10.1080/1065657X.1996.10701851
- Çelik H, Katkat AV, Aşık BB, Turan MA (2011) Effect of foliar-applied humic acid to dry weight and mineral nutrient uptake of maize under calcareous soil conditions. *Communications in Soil Science and Plant Analysis* **42**, 29–38. doi:10.1080/00103624.2011.528490
- Chan KY (2010) Assessing P fertiliser use in vegetable production: Agronomic and environmental implications. *Australian Journal of Soil Research* **48**, 674–681. doi:10.1071/SR10056
- Chen Y, Clapp CE, Magan H (2004a) Mechanisms of plant growth stimulation by humic substances: the role of organo-iron complexes. *Soil Science and Plant Nutrition* **50**, 1089–1095. doi:10.1080/00380768.2004.10408579
- Chen Y, De N, Maria, Aviad T (2004b) Stimulatory effects of humic substances on plant growth. In 'Soil organic matter in sustainable agriculture'. (CRC Press: Boca Raton, FL, USA)
- Çimrin K, Karaca D, Bozkurt M (2001) The effect of NPK and humic acid applications on growth and nutrition of corn plant (*Zea mays* L.). *Ankara University Journal of Agricultural Science* **7**, 95–100.
- Demirbas A, Kar Y, Deveci H (2006) Humic substances and nitrogen-containing compounds from low rank brown coals. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects* **28**, 341–351. doi:10.1080/009083190890111
- Duval JR, Dainello FJ, Haby VA, Earhart DR (1998) Evaluating leonardite as a crop growth enhancer for turnip and mustard greens. *HortTechnology* **8**, 564–567.
- Eyheraguibel B, Silvestre J, Morard P (2008) Effects of humic substances derived from organic waste enhancement on the growth and mineral nutrition of maize. *Bioresource Technology* **99**, 4206–4212. doi:10.1016/j.biortech.2007.08.082
- Fagbenro JA, Agboola AA (1993) Effect of different levels of humic acid on the growth and nutrient uptake of teak seedlings. *Journal of Plant Nutrition* **16**, 1465–1483. doi:10.1080/01904169309364627
- Fageria NK (2010) 'The use of nutrients in crop plants.' (Taylor & Francis: Oxford, UK)
- Feibert EB, Shock CC, Saunders LD (2003) Nonconventional additives leave onion yield and quality unchanged. *HortScience* **38**, 381–386.
- Ferreras L, Gomez E, Toresani S, Firpo I, Rotondo R (2006) Effect of organic amendments on some physical, chemical and biological properties in a horticultural soil. *Bioresource Technology* **97**, 635–640. doi:10.1016/j.biortech.2005.03.018
- Giovanetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytologist* **84**, 489–500. doi:10.1111/j.1469-8137.1980.tb04556.x
- Gryndler M, Hršelová H, Sudová R, Gryndlerová H, Řezáčová V, Merhautová V (2005) Hyphal growth and mycorrhiza formation by the arbuscular mycorrhizal fungus *Glomus claroideum* BEG 23 is stimulated by humic substances. *Mycorrhiza* **15**, 483–488. doi:10.1007/s00572-005-0352-7
- Hartz TK (2010) Humic substances generally ineffective in improving vegetable crop nutrient uptake or productivity. *HortScience* **45**, 906–910.
- Hayes TM, Hayes MHB, Skjemstad JO, Swift RS (2008) Compositional relationships between organic matter in a grassland soil and its drainage waters. *European Journal of Soil Science* **59**, 603–616. doi:10.1111/j.1365-2389.2007.01007.x
- Hiltbrunner D, Schulze S, Hagedorn F, Schmidt MWI, Zimmermann S (2012) Cattle trampling alters soil properties and changes soil microbial communities in a Swiss sub-alpine pasture. *Geoderma* **170**, 369–377. doi:10.1016/j.geoderma.2011.11.026
- Hoben JP, Gehl RJ, Millar N, Grace PR, Robertson GP (2011) Nonlinear nitrous oxide (N<sub>2</sub>O) response to nitrogen fertilizer in on-farm corn crops of the US Midwest. *Global Change Biology* **17**, 1140–1152. doi:10.1111/j.1365-2486.2010.02349.x
- Houlbrooke DJ (2011) Land-use intensification in New Zealand: Effects on soil properties and pasture production. *The Journal of Agricultural Science* **149**, 337–349. doi:10.1017/S0021859610000821
- Imbufe AU, Patti AF, Burrow D, Surapaneni A, Jackson WR, Milner AD (2005) Effects of potassium humate on aggregate stability of two soils from Victoria, Australia. *Geoderma* **125**, 321–330. doi:10.1016/j.geoderma.2004.09.006
- Isbell RF (2002) 'The Australian Soil Classification.' Revised edn (CSIRO Publishing: Melbourne)
- Jackson LE, Burger M, Cavagnaro TR (2008) Roots, Nitrogen Transformations, and Ecosystem Services. *Annual Review of Plant Biology* **59**, 341–363. doi:10.1146/annurev.arplant.59.032607.092932
- Kremen C, Miles A (2012) Ecosystem services in biologically diversified versus conventional farming systems: Benefits, externalities, and trade-offs. *Ecology and Society* **17**, doi:10.5751/ES-05035-170440
- Kunkel R, Holstad N (1968) Effects of adding humates to the fertilizer on the yield and quality of Russet Burbank potatoes. *American Potato Journal* **45**, 449–457. doi:10.1007/BF02850419
- Lee YS, Bartlett RJ (1976) Stimulation of plant growth by humic substances. *Soil Science Society of America Journal* **40**, 876–879. doi:10.2136/sssaj1976.03615995004000060023x
- Liu C, Cooper RJ, Bowman DC (1998) Humic acid application affects photosynthesis, root development, and nutrient content of creeping bentgrass. *HortScience* **33**, 1023–1025.
- Lodhi A (2013) Characterization of commercial humic acid samples and their impact on growth of fungi and plants. *Pakistan Journal of Soil Science* **32**, 63–70.
- MacCarthy P (2001) The principles of humic substances. *Soil Science* **166**, 738–751. doi:10.1097/00010694-200111000-00003
- Meng L, Ding W, Cai Z (2005) Long-term application of organic manure and nitrogen fertilizer on N<sub>2</sub>O emissions, soil quality and crop production in a sandy loam soil. *Soil Biology & Biochemistry* **37**, 2037–2045. doi:10.1016/j.soilbio.2005.03.007
- Muscolo A, Cutrupi S, Nardi S (1998) IAA detection in humic substances. *Soil Biology & Biochemistry* **30**, 1199–1201. doi:10.1016/S0038-0717(98)00005-4

- Muscolo A, Sidari M, Nardi S (2013) Humic substance: Relationship between structure and activity. Deeper information suggests univocal findings. *Journal of Geochemical Exploration* **129**, 57–63.
- Nardi S, Pizzeghello D, Muscolo A, Vianello A (2002) Physiological effects of humic substances on higher plants. *Soil Biology & Biochemistry* **34**, 1527–1536. doi:10.1016/S0038-0717(02)00174-8
- Olk D, Dinnes D, Callaway C, Raske M (2013) On-farm evaluation of a humic product in Iowa (US) maize production. In 'Functions of natural organic matter in changing environment'. (Eds J Xu, J Wu, Y He) (Springer: Berlin, Heidelberg)
- Phillips J, Hayman D (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* **55**, 158–161. doi:10.1016/S0007-1536(70)80110-3
- Piccolo A, Celano G, Pietramellara G (1993) Effects of fractions of coal-derived humic substances on seed germination and growth of seedlings. *Biology and Fertility of Soils* **16**, 11–15. doi:10.1007/BF00336508
- Piccolo A, Pietramellara G, Mbagwu JSC (1997) Use of humic substances as soil conditioners to increase aggregate stability. *Geoderma* **75**, 267–277. doi:10.1016/S0016-7061(96)00092-4
- Pilanal N, Kaplan M (2003) Investigation of effects on nutrient uptake of humic acid applications of different forms to strawberry plant. *Journal of Plant Nutrition* **26**, 835–843. doi:10.1081/PLN-120018568
- Puglisi E, Fragoulis G, Ricciuti P, Cappa F, Spaccini R, Piccolo A, Trevisan M, Crecchio C (2009) Effects of a humic acid and its size-fractions on the bacterial community of soil rhizosphere under maize (*Zea mays* L.). *Chemosphere* **77**, 829–837. doi:10.1016/j.chemosphere.2009.07.077
- Quilty JR, Cattle SR (2011) Use and understanding of organic amendments in Australian agriculture: a review. *Soil Research* **49**, 1–26. doi:10.1071/SR10059
- Rose MT, Patti AF, Little KR, Brown AL, Jackson WR, Cavagnaro TR (2014) A meta-analysis and review of plant-growth response to humic substances: Practical implications for agriculture. In 'Advances in agronomy'. Ch. 2. (Ed. LS Donald) (Academic Press: Waltham, MA, USA)
- Ryan M, Ash J (1999) Effects of phosphorus and nitrogen on growth of pasture plants and VAM fungi in SE Australian soils with contrasting fertiliser histories (conventional and biodynamic). *Agriculture, Ecosystems & Environment* **73**, 51–62. doi:10.1016/S0167-8809(99)00014-6
- Schweiger PF, Robson AD, Barrow NJ (1995) Root hair length determines beneficial effect of a *Glomus* species on shoot growth of some pasture species. *New Phytologist* **131**, 247–254. doi:10.1111/j.1469-8137.1995.tb05726.x
- Seyedbagheri M, He Z, Olk D (2012) Yields of potato and alternative crops impacted by humic product application. In 'Sustainable potato production: Global case studies'. (Eds Z He, R Larkin, W Honeycutt) (Springer: Berlin, Heidelberg)
- Smith SE, Read DJ (2010) 'Mycorrhizal symbiosis.' (Elsevier: Amsterdam) <http://books.google.com.au> (accessed November 2013)
- Sparling G (1992) Ratio of microbial biomass carbon to soil organic carbon as a sensitive indicator of changes in soil organic matter. *Soil Research* **30**, 195–207. doi:10.1071/SR9920195
- Sparling GP, Wheeler D, Vesely E-T, Schipper LA (2006) What is soil organic matter worth? *Journal of Environmental Quality* **35**, 548–557. doi:10.2134/jeq2005.0230
- Tahir MM, Khurshid M, Khan MZ, Abbasi MK, Kazmi MH (2011) Lignite-derived humic acid effect on growth of wheat plants in different soils. *Pedosphere* **21**, 124–131. doi:10.1016/S1002-0160(10)60087-2
- Tan KH, Nopamombodi V (1979) Effect of different levels of humic acids on nutrient content and growth of corn (*Zea mays* L.). *Plant and Soil* **51**, 283–287. doi:10.1007/BF02232891
- Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S (2002) Agricultural sustainability and intensive production practices. *Nature* **418**, 671–677. doi:10.1038/nature01014
- Vallini G, Pera A, Avio L, Valdrighi M, Giovannetti M (1993) Influence of humic acids on laurel growth, associated rhizospheric microorganisms, and mycorrhizal fungi. *Biology and Fertility of Soils* **16**, 1–4. doi:10.1007/BF00336506
- Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass C. *Soil Biology & Biochemistry* **19**, 703–707. doi:10.1016/0038-0717(87)90052-6
- Verlinden G, Pycke B, Mertens J, Debersaques F, Verheyen K, Baert G, Bries J, Haesaert G (2009) Application of humic substances results in consistent increases in crop yield and nutrient uptake. *Journal of Plant Nutrition* **32**, 1407–1426. doi:10.1080/01904160903092630
- Verlinden G, Coussens T, De Vlieghe A, Baert G, Haesaert G (2010) Effect of humic substances on nutrient uptake by herbage and on production and nutritive value of herbage from sown grass pastures. *Grass and Forage Science* **65**, 133–144. doi:10.1111/j.1365-2494.2009.00726.x
- Visser SA (1985) Effect of humic acids on numbers and activities of micro-organisms within physiological groups. *Organic Geochemistry* **8**, 81–85. doi:10.1016/0146-6380(85)90055-5
- Yolcu H, Seker H, Gullap MK, Lithourgidis A, Gunes A (2011) Application of cattle manure, zeolite and leonardite improves hay yield and quality of annual ryegrass (*Lolium multiflorum* Lam.) under semiarid conditions. *Australian Journal of Crop Science* **5**, 926–931.

## **4 Effect of application rate of lignite-derived soluble humate granules on the early-stage growth of lucerne, and key indicators of soil health.**

### **Abstract**

The application rate of humic acid (HA) is important to the plant growth response. Commercial lignite-derived products (LDP) are marketed with recommended application rates which may be based on the crop type but are more often than not a generic value. This chapter describes two studies; a glasshouse and an incubation study. In the glasshouse study, a lignite-mineral product and 'run of mine' lignite coal did not improve early-stage lucerne shoot or root growth despite application at a range of rates at, and in excess of, the manufacturer's recommendation. At an application rate of 20 kg/ha, five times that recommended by the manufacturer, soluble humate granules (SHG) produced a significant positive growth effect in both the lucerne shoots and roots, compared to the control. At this application rate, a significant delay in the appearance of chlorotic symptoms was observed along with an increase in soil pH which decreased availability of soil manganese and aluminium. Correlations between soil pH and calcium, phosphorus and zinc were also identified. Significantly, with increasing application rate of SHG, there was a concurrent decrease in plant tissue nitrogen with no loss of lucerne biomass. This result implies a potential reduction in nitrogen-based fertilizer usage when applied in conjunction with humate products. In the incubation study, investigation of pH changes identified initially in the glasshouse study showed that soil pH was driven by the plant rather than SHG application.

### **4.1 Introduction**

Humic acid addition to soil has been shown to improve root and shoot growth and yield of a range of crops including grains, vegetables, trees and flowers (Eyheraguibel et al., 2008, Puglisi et al., 2009, Nardi et al., 2002, Arancon et al.,

2006, Piccolo et al., 1993, Fagbenro and Agboola, 1993, Nikbakht et al., 2008). While the effect varies with the origin of the HA, species of plant treated and soil type, the concentration of HA added is also of importance. Studies conducted in soil or solid growth media indicate that the soil application rate of HA required to promote shoot and root growth ranges from 30 mg/kg to in excess of 4000 mg/kg (Khaled and Fawy, 2011, Tahir et al., 2011, Paksoy et al., 2013, Atiyeh et al., 2002, Sharif et al., 2002, Arancon et al., 2006). A trend commonly identified in these types of studies is an application rate at which a peak plant growth benefit is achieved, with application rates in excess of this providing no additional benefit (Rose et al., 2014).

A number of mechanisms have been proposed to account for the stimulatory effects of HA including a direct hormonal effect on the plant and increases in cell permeability (Canellas et al., 2002, Ertani et al., 2011, Nardi et al., 2007, Cacco and Dell'Agnola, 1984, Piccolo et al., 1992) and indirect effects including modification of soil nutrient availability and enhanced uptake of selected ions (Chen et al., 2004a, Chen and Aviad, 1990). The effect of HA on the uptake of specific ions is complex and it is difficult to identify consistent trends due to variability of the origin of the HA, the concentration applied and the type and pH of the soil or culture medium.

Manufacturers of commercial LDPs claim soil health improvements by buffering soil pH and promotion of beneficial soil bacteria and fungi. The formation of arbuscular mycorrhizas enables enhanced uptake of plant essential nutrients which can improve plant growth (Smith and Read, 2010) and the soil microbial community plays a significant role in nutrient cycling. Few studies focus on the effect of HA on these key indicators of soil health. Given that a hypothesis for plant growth promotion in response to HA application is increased nutrient availability for plant uptake (Chen et al., 2004b), further investigation of the effect of HA on soil pH and the soil microbial community is warranted.



This chapter describes two experiments; a glasshouse study and an incubation study. In the glasshouse study, as a continuation of the outcomes described in Chapter 3, three products were applied to a sandy, low organic matter soil at the manufacturer's recommended rate and at significantly higher rates. The LDPs selected were soluble humate granules (SHG), run of mine lignite coal (ROM) and lignite-mineral blend (LMB1) as being representative of the product types available on the market. The aims of this experiment were;

- To determine if applying an LDP above the manufacturer recommended rate promotes lucerne growth and to identify an optimal application rate;
- To assess lucerne shoot nutrition at higher LDP application rates; and
- To determine if higher LDP application rates have an impact on key indicators of soil health.

Lucerne growth promotion was assessed by shoot and root biomass measurements, and overall plant health assessed by shoot tissue nutrient analysis and the appearance of chlorotic symptoms at the leaf margin. The key indicators of soil health measured were soil pH, microbial biomass carbon (MBC) and mycorrhizal colonization.

As an extension of the glasshouse study, an incubation study was conducted in which the SHG product was applied at a range of rates to the same acidic, sandy soil as used in the glasshouse study and then incubated for six weeks. The aim of this experiment was to determine if addition of SHG had an impact on soil pH in the absence of lucerne.

## 4.2 Materials and methods

### 4.2.1 *Characterization of lignite-derived products and soil*

The three LDPs selected were 'run of mine lignite' (ROM), soluble humate granules (SHG) and lignite-mineral blend (LMB1). The analyses used to characterize the physicochemical properties of these products have been described in detail in Chapter 2, Section 2.2. The analyses conducted included pH, moisture content, HA concentration, organic and inorganic element concentrations and structural characterization of the organic constituents by solid state  $^{13}\text{C}$  NMR.

The soil used for this study was a Podsol, collected from a vegetable farm recently converted from pasture in Cranbourne, Victoria ( $38^{\circ}11'6''\text{S}$ ,  $145^{\circ}18'50''\text{E}$ ). The soil was collected in November 2011, air-dried and sieved to  $<2$  mm. A 200 g sub-sample was then analysed for a range of key physicochemical properties (Environmental Analysis Laboratory, Southern Cross University, Lismore, NSW: <http://scu.edu.au/eal/>; accessed July 2014). The soil was of sandy texture, mildly acidic (pH 5.8), with low organic matter content (1.9%) (Table 4.1). Based on this soil analysis nitrogen (urea), phosphorus (triple superphosphate) and potassium (sulphate of potash) were added at 100, 40 and 60 kg/ha respectively.

**Table 4.1** Soil physicochemical properties prior to fertilizer addition and amendment with LDPs.

<b>pH</b> (H <sub>2</sub> O)	5.8
<b>OM</b> (%)	1.9
<b>C</b> (%)	1.1
<b>N</b> (%)	0.1
<b>P</b> (Colwell)	62
<b>K</b> (mg/kg)	97
<b>Ca</b> (mg/kg)	839
<b>Fe</b> (mg/kg)	104
<b>Mn</b> (mg/kg)	5
<b>S</b> (mg/kg)	18.4
<b>Zn</b> (mg/kg)	2.1
<b>Texture</b>	Sandy

#### 4.2.2 Glasshouse study: Lucerne growth and key indicators of soil health

A glasshouse experiment was conducted from January to March, 2012 on the Monash University Clayton campus. Conditions in the glasshouse were controlled as follows: light levels maintained with supplemental lighting (16 h day length) averaged  $220 \pm 17 \mu\text{mol m}^{-2} \text{s}^{-1}$ , and the temperature was  $25.2 \pm 1.8^\circ\text{C}$  during the day and  $23.3 \pm 1.4^\circ\text{C}$  at night. Plastic pots (16 mm diameter) were filled with 800 g of soil. The appropriate amount of LDP (Table 4.2) was applied to the top 200 g of soil to simulate spreading of the product in an agricultural setting. Each amendment and rate was replicated five times, making a total of 70 pots. The soil was left to equilibrate for three days after which 10 lucerne (*Medicago sativa* L.) seeds were sown approximately 2 mm below the soil surface. Pots were arranged in a complete randomized design with their position rotated every two days. Plants were watered to field capacity according to Asghari and Cavagnaro (2012), with tap water as required, usually every two days. At seven days post-seeding, plants were thinned

to two per pot. During the growing period, plants were visually inspected for the appearance of chlorotic symptoms (yellowing at the leaf margin). Plants were defined as chlorotic if a minimum of five leaves were affected.

**Table 4.2** Application rates of soluble humate granules (SHG), 'run of mine' coal (ROM) and lignite-mineral blend (LMB1). The underlined value is the manufacturer's recommended application rate.

<b>LDP</b>	<b>Application rate</b>	<b>Actual mass of LDP added (g/kg soil)</b>
SHG (kg/ha)	<u>4</u>	0.004
	8	0.008
	20	0.02
	50	0.05
	100	0.1
ROM (t/ha)*	2	2
	5	5
	10	10
	25	25
LMB1 (t/ha)	<u>1</u>	1
	2	2
	5	5
	10	10

\*ROM has no manufacturer recommended application rate and so rates were based on economic and practical considerations.

#### *Plant harvesting and analysis*

To determine the effects of application rates of LDP at the early-stage growth phase, plants were destructively harvested six weeks post-seeding. The plant and soil were removed from the pots, soil gently shaken from the roots, and the shoots and roots separated. The roots were thoroughly washed with deionized water and divided into two sub-samples. The shoots and a sub-sample of the roots of each plant were oven dried for three days at 55°C following which the shoot dry weight

(SDW) and root dry weight (RDW) were determined. The dried shoot material was then ground to a fine powder and nutrient concentrations were determined by ICP-OES by Waite Analytical Services, University of Adelaide, Urrbrae, S. Aust.: <http://www.adelaide.edu.au/was/>; accessed April 2014). The second sub-sample of roots was used for assessment of mycorrhizal colonization, using the gridline intersect method (Giovannetti and Mosse, 1980), after the roots were cleared in KOH (10% w/v) and stained with trypan blue (omitting phenol from all reagents) (Phillips and Hayman, 1970).

#### *Post-harvest soil analysis*

Immediately following harvest, the soils were refrigerated at 4°C. Microbial biomass carbon (MBC) was quantified by 24 hour chloroform fumigation (Vance et al., 1987). Briefly, a 5 g sub-sample of each soil was fumigated with ethanol-free chloroform for 24 h in a sealed desiccator kept in the dark. An additional 5 g sub-sample of each soil to be used as a non-fumigated baseline was also stored in a dark environment for 24 h. The following day, the desiccator was evacuated to remove chloroform from the soils. The fumigated and non-fumigated soils were extracted for 1 h with 0.5 M K<sub>2</sub>SO<sub>4</sub> at a 1:3 (w/v) ratio and filtered with Whatman qualitative filter paper, grade 5. The total organic carbon of the filtered product was determined by dichromate digestion (Cai et al., 2011). The post-harvest soil pH was determined by suspension of an air-dried soil sub-sample (5 g) suspended in deionized water (1:5 w/v) using a TPS WP81 meter and probe (TPS Pty Ltd, Springwood, QLD).

#### *4.2.3 Incubation study: Effect of soluble humate granules (SHG) on soil pH*

An incubation study was conducted to further investigate the response in soil pH following amendment with SHG. The same soil was used as for Experiment 1 (air-dried and sieved to < 2mm), with the same fertilizer regime (nitrogen, phosphorus and potassium applied at 100, 40 and 60 kg/ha of respectively). The SHG was added to 100 g aliquots of soil at rates equivalent to 0, 4, 8, 20, 50 and 100 kg/ha in sterile 250 mL polypropylene microcosms and thoroughly mixed. Five replicates were

prepared of each application rate, giving a total of 30 microcosms. Following mixing, deionized water was sprayed onto the soil surface using a misting bottle, until a soil moisture content equal to 60% field capacity was achieved. The opening of each microcosm was loosely covered with a plastic cap to minimize moisture loss, while allowing gas exchange. The microcosms were then incubated at 25°C in the dark for six weeks during which time the soil moisture content was maintained at 60% of field capacity, as described above.

Following the six week incubation period, the microcosms were destructively harvested by removing the soil and mixing it (for each microcosm separately) thoroughly. Soil pH was determined in triplicate by suspension of an air-dried soil sub-sample (5 g) in deionized water (1:5 w/v) using a TPS WP81 meter and probe.

#### *Calculations and data analysis*

All data was analysed using JMP statistical software (JMP<sup>®</sup>, Version 10, SAS Institute Inc., Cary, NC). One-way ANOVA was used to investigate the effect of application rate on lucerne biomass, nutrient content of shoots, post-harvest soil pH, MBC and mycorrhizal colonization. Where significant differences were found, pairwise comparisons were made using Tukey's honestly significant difference (HSD). Correlations between the post-harvest soil pH and lucerne shoot nutrition were investigated using the Pearson product-moment correlation.

Where stated, shoot dry weight and shoot nitrogen concentrations were used to calculate plant responses relative to the control following equation 1 (Eq. 1).

$$\% \text{ response} = \left( \frac{\text{mean} - \text{mean of control}}{\text{mean of control}} \right) \times 100$$

(Eq. 1)

## 4.3 Results

### 4.3.1 Glasshouse study: Lucerne growth and key indicators of soil health

#### *Physicochemical properties of the lignite-derived products*

The physicochemical properties of the products ROM, SHG and LMB1 have been presented previously in Chapter 2 (Tables 2.2 and 2.3). Briefly, the LDPs varied considerably in chemical composition. There were large differences in the pH of the products, the most alkaline being SHG (pH 9.4) with ROM being moderately acidic (pH 4.5). The moisture content of ROM was highest of the three products (51.9%) with SHG and LMB1 being comparatively low in moisture at 4.5 and 3.8% respectively. There were differences in the HA content of the products, ranging from 3.9% for LMB1 to 67.4% for ROM. LMB1 had a significantly higher S content (1.7%) and higher concentrations of calcium, iron, magnesium, phosphorus, copper, manganese and zinc compared to the other products. SHG had a relatively high potassium content (8.5%).

#### *Lucerne root and shoot growth*

At the application rate of 20 kg/ha, SHG promoted lucerne shoot and root growth compared to the untreated control (Table 4.3). There was no further growth benefit at the higher application rates of 50 and 100 kg/ha. Lucerne grown in soil amended with ROM and LMB1 did not show a significant increase in shoot or root growth (Table 4.3). As these products did not have a significant impact on lucerne growth at these application rates, the inorganic and organic shoot nutrient data and soil health results (pH, MBC and mycorrhizal colonization) have been included in Appendix 2. No significant differences were identified in any of the data sets.

**Table 4.3** Dry weight of shoots (SDW) and roots (RDW) of lucerne grown in LDP amended soil. Mean values are indicated (n=5) and values in brackets are  $\pm$  s.e. Values allocated the same letter within each LDP type were not significantly different at the  $P < 0.05$  level as assessed by Tukey's HSD.

LDP	Application rate (kg/ha)	SDW (g)	RDW (g)
SHG	0	2.00 <sup>bc</sup> (0.16)	0.56 <sup>b</sup> (0.07)
	4	2.76 <sup>ab</sup> (0.30)	0.67 <sup>ab</sup> (0.03)
	8	2.43 <sup>abc</sup> (0.20)	0.62 <sup>ab</sup> (0.05)
	20	2.93 <sup>a</sup> (0.09)	0.81 <sup>a</sup> (0.06)
	50	1.86 <sup>c</sup> (0.13)	0.52 <sup>b</sup> (0.06)
	100	1.97 <sup>c</sup> (0.03)	0.69 <sup>ab</sup> (0.04)
	<b>Application rate (t/ha)</b>		
ROM	0	2.00 <sup>a</sup> (0.16)	0.56 <sup>a</sup> (0.07)
	2	2.39 <sup>a</sup> (0.12)	0.81 <sup>a</sup> (0.04)
	5	2.33 <sup>a</sup> (0.32)	0.74 <sup>a</sup> (0.17)
	10	2.05 <sup>a</sup> (0.19)	0.66 <sup>a</sup> (0.11)
	25	2.16 <sup>a</sup> (0.24)	0.62 <sup>a</sup> (0.11)
LMB1	0	2.00 <sup>a</sup> (0.16)	0.56 <sup>a</sup> (0.07)
	1	2.33 <sup>a</sup> (0.08)	0.82 <sup>a</sup> (0.05)
	2	2.43 <sup>a</sup> (0.18)	0.91 <sup>a</sup> (0.13)
	5	2.48 <sup>a</sup> (0.25)	0.88 <sup>a</sup> (0.15)
	10	2.57 <sup>a</sup> (0.12)	0.79 <sup>a</sup> (0.06)

#### *Lucerne nutrition*

The application rate of SHG had a significant effect on the lucerne shoot concentrations of aluminium, calcium, manganese, magnesium, potassium, phosphorus and sulphur (Table 4.4). Specifically, SHG applied at 20 kg/ha reduced the concentration of aluminium, manganese, magnesium and phosphorus compared to the control, and increased the concentration of calcium. Compared to untreated plants, significant decreases in the potassium and sulphur concentrations of the lucerne shoots were seen at the highest application rate of 100 kg/ha.



Analysis by ANOVA indicated a significant effect of application rate on iron concentration ( $P=0.04$ ); however, this was not identified by Tukeys HSD, which reflects the conservative nature of this type of statistical analysis.

**Table 4.4** Inorganic nutrient composition of lucerne shoot tissue. Mean values are presented (n=5) and values in parentheses are  $\pm$  s.e. Values allocated the same letter were not significantly different at the  $P<0.05$  level as assessed by Tukey's HSD.

SHG rate (kg/ha)	Al (mg/kg)	Ca (g/kg)	Fe (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	Mg (g/kg)	K (g/kg)	P (g/kg)	S (g/kg)
0	45 <sup>ab</sup> (6)	8.3 <sup>b</sup> (0.7)	109 (13)	182 <sup>a</sup> (18)	52 (4)	3.8 <sup>a</sup> (0.1)	25.2 <sup>a</sup> (0.6)	3.5 <sup>a</sup> (0.1)	30.2 <sup>a</sup> (0.1)
4	16 <sup>cd</sup> (3)	14.1 <sup>a</sup> (0.8)	133 (24)	92 <sup>bc</sup> (21)	43 (4)	3.4 <sup>ab</sup> (0.2)	24.2 <sup>a</sup> (0.9)	2.6 <sup>b</sup> (0.1)	28.6 <sup>ab</sup> (0.2)
8	33 <sup>bc</sup> (1)	9.4 <sup>b</sup> (0.4)	143 (12)	183 <sup>a</sup> (8)	47 (4)	4.0 <sup>a</sup> (0.2)	24.0 <sup>a</sup> (0.9)	3.4 <sup>a</sup> (0.1)	28.4 <sup>ab</sup> (0.2)
20	12 <sup>d</sup> (2)	12.3 <sup>a</sup> (0.6)	75 (10)	47 <sup>c</sup> (8)	36 (3)	2.9 <sup>b</sup> (0.2)	23.2 <sup>a</sup> (1.1)	2.5 <sup>b</sup> (0.1)	24.3 <sup>ab</sup> (0.2)
50	45 <sup>ab</sup> (5)	9.3 <sup>b</sup> (0.6)	126 (30)	156 <sup>ab</sup> (24)	49 (4)	3.5 <sup>ab</sup> (0.3)	25.0 <sup>a</sup> (0.9)	3.4 <sup>a</sup> (0.2)	26.2 <sup>ab</sup> (0.2)
100	52 <sup>a</sup> (6)	9.5 <sup>b</sup> (0.2)	75 (5)	140 <sup>ab</sup> (3)	44 (3)	3.6 <sup>ab</sup> (0.6)	19.4 <sup>b</sup> (0.7)	3.6 <sup>a</sup> (0.1)	21.4 <sup>b</sup> (0.1)

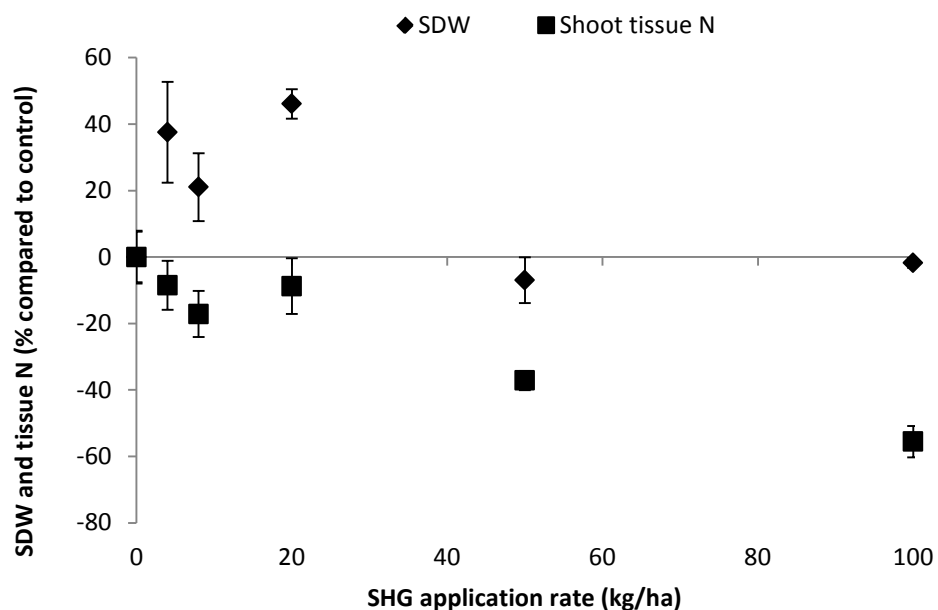
Analysis by ANOVA of the organic composition of the lucerne shoots indicated that SHG application rate had no significant effect on shoot carbon concentration (Table 4.5). There was a trend of decreasing nitrogen concentration of the shoots with increasing application rate of SHG, with 50 and 100 kg/ha significantly less than the control.

**Table 4.5** Organic composition of lucerne shoot tissue. Mean values are presented (n=5) and values in brackets are  $\pm$  s.e. Values allocated the same letter were not significantly different at the  $P<0.05$  level as assessed by Tukey's HSD.

<b>SHG application rate</b> (kg/ha)	<b>C</b> (%)	<b>N</b> (%)
0	41.7 (0.4)	3.0 (0.2) <sup>a</sup>
4	40.4 (0.4)	2.7(0.2) <sup>ab</sup>
8	41.8 (0.4)	2.5(0.2) <sup>ab</sup>
20	41.6 (0.3)	2.7(0.2) <sup>ab</sup>
50	40.2 (0.3)	1.9 (0.1) <sup>bc</sup>
100	40.7 (0.4)	1.3 (0.1) <sup>c</sup>

Analysis of the lucerne SDW together with the shoot tissue nitrogen, both expressed as a percentage difference compared to the control, indicated that with increasing application rate of SHG, the concurrent decrease in shoot tissue nitrogen at the higher application rates of 50 and 100 kg/ha was not accompanied by a loss in shoot biomass compared to the control (Figure 4.1).

Analysis by ANOVA indicated a significant difference in post-harvest soil pH with application rate. Further analysis by Tukeys HSD indicated significantly higher pH in soil amended with 4 and 20 kg/ha SHG, compared to 8, 50, 100 kg/ha and the unamended control (Table 4.6). There was a significant correlation between the post-harvest soil pH and SDW (Pearson  $r=0.68$ ,  $P<0.0001$ ).



**Figure 4.1** Shoot dry weight (SDW) and shoot tissue nitrogen concentration of lucerne with increasing application rate of SHG. Data is expressed as percentage compared to the control. Mean values are presented (n=5) and error bars represent  $\pm$  s.e.

**Table 4.6** Post-harvest pH of SHG amended soil. Mean values are presented (n=5) and values in brackets are  $\pm$  s.e. Values allocated the same letter were not significantly different at the  $P < 0.05$  level as assessed by Tukey's HSD.

SHG application rate (kg/ha)	Soil pH
0	4.5 <sup>b</sup> (0.1)
4	4.9 <sup>a</sup> (0.1)
8	4.6 <sup>b</sup> (0.1)
20	4.9 <sup>a</sup> (0.1)
50	4.5 <sup>b</sup> (0.1)
100	4.6 <sup>b</sup> (0.1)

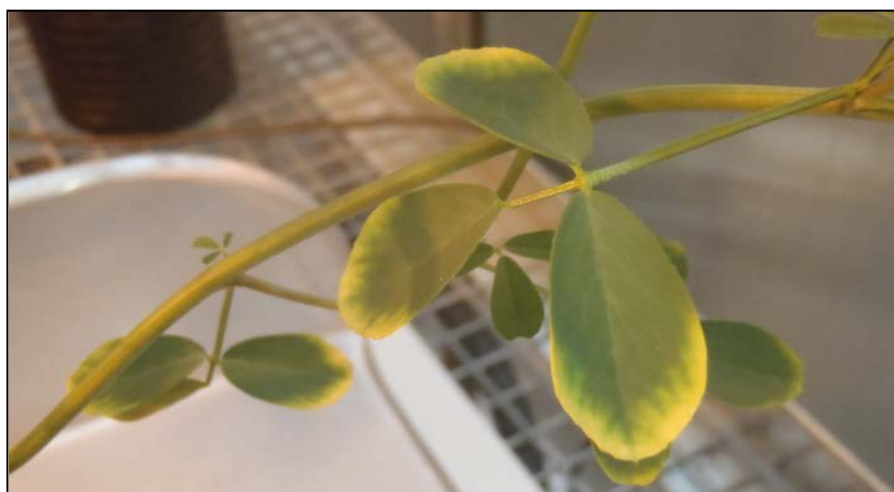
There were significant correlations between post-harvest soil pH and lucerne shoot nutrients (Table 4.7). Significant negative correlations were identified with soil pH and manganese, zinc, magnesium, phosphorus and aluminium and a positive correlation with calcium. There was no significant correlation of soil pH with potassium or sulphur.

**Table 4.7** Correlation (Pearson) coefficients of post-harvest soil pH and nutrient content of lucerne shoots grown in soil amended with SHG.

	Al	Ca	K	Mg	Mn	P	S	Zn
<b>Post-harvest soil pH</b>	-0.79 <sup>***</sup>	0.75 <sup>***</sup>	-0.0002	-0.57 <sup>**</sup>	-0.82 <sup>***</sup>	-0.88 <sup>***</sup>	-0.09	-0.49 <sup>*</sup>

Significant correlation at the\*0.01 level, \*\* 0.001 level and \*\*\*0.0001 level.

During the growing period, the lucerne leaves were evaluated for the appearance of chlorotic symptoms at the leaf margin (Figure 4.2). The maximum delay before the observation of symptoms (32 days) was associated with an application rate of 20 kg/ha of SHG (Table 4.8).



**Figure 4.2** Lucerne leaves displaying chlorotic symptoms (yellowing at the leaf margin).

**Table 4.8** Number of days post-seeding until the appearance of chlorotic symptoms. Values in brackets are  $\pm$  s.d.

SHG application rate (kg/ha)	0	4	8	20	50	100
Days until chlorotic symptom appearance	17 (1)	26 (4)	16 (1)	32 (1)	20 (2)	17 (2)

*Microbial biomass carbon (MBC) and mycorrhizal colonization*

There was no significant difference in MBC with SHG application rate (Table 4.9). There was a significant increase in mycorrhizal colonization of lucerne roots grown in soil amended with 20 kg/ha SHG.

**Table 4.9** Microbial biomass carbon (MBC) and mycorrhizal colonization of lucerne grown in soil amended with increasing application rates of SHG. Mean values are presented (n=5) and values in brackets are  $\pm$  s.e. Values allocated the same letter were not significantly different at the  $P < 0.05$  level as assessed by Tukey's HSD.

SHG application rate (kg/ha)	MBC (mg C/g dry soil)	Mycorrhizal colonization (%)
0	22.8 <sup>a</sup> (6.2)	2.0 <sup>b</sup> (1.3)
4	12.1 <sup>a</sup> (3.0)	8.8 <sup>b</sup> (3.8)
8	21.2 <sup>a</sup> (2.5)	0.8 <sup>b</sup> (0.5)
20	7.6 <sup>a</sup> (2.9)	24.8 <sup>a</sup> (7.6)
50	14.9 <sup>a</sup> (3.6)	1.2 <sup>b</sup> (0.7)
100	16.3 <sup>a</sup> (3.4)	2.6 <sup>b</sup> (1.9)

#### 4.3.2 Incubation study: Effect of soluble humate granules (SHG) on soil pH

Following a six week incubation period, no significant difference was detected in the pH of soils treated with increasing application rates of SHG (Table 4.10).

**Table 4.10** Soil pH following six weeks incubation with SHG. Mean values are presented (n=5) and values in brackets are  $\pm$  s.e.

SHG application rate (kg/ha)	Soil pH
0	4.1 (0.1)
4	4.2 (0.1)
8	4.2 (0.1)
20	4.2 (0.1)
50	4.1 (0.1)
100	4.2 (0.1)
Pre-incubation pH	4.6 (0.1)

## 4.4 Discussion

Variation in chemical composition between products SHG, ROM and LMB1 may be attributed to the origin of the parent material, the source and form of HA and plant-essential nutrients added during product formulation (see also Chapter 2). The comparatively high concentration of potassium in SHG is most likely a consequence of the HA extraction process involving alkaline treatment of the lignite. The relatively elevated phosphorus and calcium concentrations of LMB1 reflect the labelling of the product as a lignite-fertilizer blend, in which minerals are incorporated during formulation.

A low organic matter soil was selected for this study because published precedents and the product screening study described in Chapter 3 indicate that the growth-promoting ability of HA can be masked in high organic matter soils (Lee and Bartlett, 1976, Duplessis and MacKenzie, 1983). Although lucerne is an nitrogen-fixing leguminous pasture species, nitrogen-fertilizer was added to suppress any

involvement of rhizobial symbiosis as this was not a focus of the study and interactions with HA could have confounded interpretation of results.

Despite product ROM having the highest HA content (67.4%), a lack of growth benefit even at the higher application rates could be a result of recalcitrance of the product and minimal bioavailability of the HA. Product LMB1 also contained raw lignite, albeit at a low concentration (indicated by 3.9% HA) and similarly the HA may not have been in a plant/soil/microbial accessible form. Application of SHG resulted in a positive growth response up to an application rate of 20 kg/ha and as this product was soluble, the HA would have been immediately available and thus had a positive impact on the plant, soil and/or microbial activity.

Although product LMB1 had high concentrations of plant-essential nutrients, it did not induce a significant growth response in lucerne shoots or roots. At a product application rate of 10 t/ha, the calculated phosphorus application was 500 kg/ha. Lucerne has limited phosphorus uptake efficiency that will result in shoot growth promotion, and the applied rate here was well in excess of that (Gourley et al., 1993). Prior to LMB1 application, the phosphorus concentration in the soil was optimal for lucerne growth, and so the product-derived addition gave no further benefit. Further study to assess this product on plant growth under phosphorus-deficient conditions, in comparison with conventional phosphorus-fertilizers would be worthwhile.

The identification of a peak SHG application rate (20 kg/ha) at which an optimal growth response was identified has been observed in other studies (Rose et al., 2014). It has been hypothesized that growth promotion at high HA concentrations cannot occur due to an accumulation of HA at the root cell wall surface which narrows or blocks cell wall pores, thereby restricting plant access to water and nutrients (Asli and Neumann, 2010).

While the carbon concentration of the lucerne shoots remained constant, the nitrogen concentration decreased significantly with increasing application rate of SHG. With no associated loss in biomass at application rates of 50 and 100 kg/ha this finding would be of interest to farmers as it suggests nitrogen fertilizer savings. A similar decrease in barley tissue nitrogen with increasing application rate of HA derived from a range of sources was identified by Ayuso et al. (1996). The trend was more subtle than the 60% decrease in nitrogen concentration observed here but the application rates used were comparatively lower. As has been demonstrated by binding of plant essential nutrients such as iron and zinc to HA (Chen et al., 2004a), it is possible that the binding of positively-charged inorganic nitrogen species such as ammonium onto the cation exchange sites on the HA may limit availability. This proposed mechanism requires further investigation. Also, further analysis of the amino acid and protein contents of the lucerne shoots would be required to determine if a lower nitrogen content pasture would meet the nutritional requirements of livestock.

There was a strong correlation between lucerne shoot growth and post-harvest soil pH. The SHG product, in solution, had a pH of 9.4 but at low application rates addition of this product would not be expected to directly alter the soil pH due to the soil's buffering capacity. Instead, the soil pH changes were driven by the lucerne rather than SHG product application as was confirmed in the incubation study. Possible contributors to the increase in soil pH identified in the glasshouse study could include root exudates which are composed of a range of organic molecules including those that contain carboxylate groups (Dakora and Phillips, 2002) and utilization of hydrogen ions associated with nitrogen-fixing in the lucerne nodules. The application of nitrogen fertilizer to the soil prior to planting should have suppressed nodulation however this was not assessed in the study. In the incubation study, a decrease in soil pH from the pre-incubated soil compared to the final post-harvest soil was likely due to the activity of the soil microbial community



who produce organic acids to perform mineralization functions (Arshad and Frankenberger Jr, 1991, Landeweert et al., 2001).

With the exception of potassium and sulphur, the significant differences identified in the aluminium, calcium and manganese nutrient concentrations of the lucerne shoots were not correlated with application rate. It has been well established that the availability of soil nutrients for plant uptake is dependent on soil pH (Lucas and Davis, 1961). The increase in soil pH identified at 20 kg/ha SHG would have decreased the availability of aluminium, a well-known inhibitor of root elongation resulting in depressed crop yields (Munns, 1965), resulting in increased growth.

Lucerne, like other pasture species can display chlorotic symptoms when grown in compromised soils. Chlorosis, as indicated by yellowing at the leaf margin, can indicate toxicity or deficiency of plant essential nutrients. The extension of time before chlorotic symptoms were identified in plants grown in soil amended with 20 kg/ha of SHG is likely due to decreased availability of manganese and aluminium due to the increase in soil pH at this application rate. This is verified by reduced amounts of these nutrients in the lucerne shoot tissue.

Magnesium is generally expected to behave in a similar way to calcium in plant tissue (Lucas and Davis, 1961); however, in this study there was decreased uptake at 20 kg/ha compared to the control. This could be accounted for by findings from a nutritional study on another type of legume, soybeans, which demonstrated that when calcium is available for plant uptake a competitive uptake mechanism may limit the uptake of magnesium, resulting in a lower than expected magnesium concentration in the shoot tissue (Leggett and Gilbert, 1969). The negative correlation between soil pH and phosphorus has not been identified previously in this type of study. It is generally accepted that in acidic soil, as pH increases, phosphorus becomes more available due to release from complexes with aluminum and iron. Humic acid has been shown to increase the uptake of phosphorus (Adani

et al., 1998) however in this case there is the added complexity of soil pH differences at the different application rates.

The significant differences in potassium and sulphur content of the lucerne shoot are related to a product application rate effect rather than associated with soil pH changes. Sulphur is required for production of the amino acids cysteine and methionine. With decreased nitrogen uptake at the higher application rates, there may be less requirement for sulphur. Amino acid analysis of the lucerne shoots would be required to investigate this further. As for sulphur, potassium also decreased with increasing application rate which has been identified previously (Paksoy et al., 2013) but not accounted for. The complexity of interactions between the plant, microbial community and amendment highlights the requirement for further nutrient cycling studies.

In the study described in Chapter 3 there was no significant MBC response to the soil addition of SHG at the manufacturer's recommended rate, a similar result to that observed by Albiach et al. (2000). As demonstrated here, increasing the application rate did not change this. For comparison, studies of the impact of a range of application rates of commercial HA products on MBC were not able to be located. An increase in MBC could be expected in response to supplying additional carbon to the soil (Schnürer et al., 1985) and variability in the MBC data may have masked a significant response. Verification of the soil microbial community response using an alternative method such as a molecular-based technique is warranted.

At an application rate of 20 kg/ha, SHG had a stimulatory effect on mycorrhizal colonization of lucerne compared to the control but there was no correlation with product application rate. While studies of the effect of HA on mycorrhizal colonization are limited, a similar stimulatory effect was seen by Gryndler *et al.* (2005) in maize roots. It has been demonstrated that mycorrhizal associations form

in low phosphorus soils conditions in an attempt to improve phosphorus acquisition (Watts-Williams and Cavagnaro, 2012). There was a negative correlation between phosphorus content of the lucerne shoots and mycorrhizal colonisation ( $r=-0.59$ ,  $P=0.0007$ ) and so it is likely that the change in mycorrhizal colonization was due to the soil nutritional status rather than the application of SHG.

#### **4.5 Conclusion**

Application up to 20 kg/ha of SHG had beneficial growth effects on lucerne shoot and roots with maximum growth benefit obtained at 20 kg/ha. This application rate coincided with a delay in the appearance of chlorotic symptoms in lucerne which further correlated with an increase in soil pH and inversely with plant tissue aluminium and manganese concentrations. Increasing the SHG application rate above 20 kg/ha resulted in decreased shoot nitrogen with no loss in biomass. Soil microbial biomass was not influenced by SHG and changes in mycorrhizal colonisation were more likely driven by the soil nutrient status than SHG application. Further investigation is needed into the mechanistic interactions between the soil nutrients (particularly nitrogen), the LDP and the plant.



## **5 Effect of soil amendment with lignite-derived potassium humate on pasture growth and nitrogen cycling in mesocosm conditions**

### **Abstract**

The environmental impacts of nitrogen losses from agricultural systems are significant. The impact of commercial lignite-derived products on soil nitrogen pools and mitigation of losses has not been extensively explored. A mesocosm study was undertaken for a period of seven months to investigate the effect of soluble humate granules (SHG) on soil organic and inorganic nitrogen pools and nitrogen losses through leaching and nitrous oxide emissions. In conjunction, the growth and nutrient status of lucerne and ryegrass were investigated to determine if the 20 kg/ha SHG application rate identified in the glasshouse as being optimal for lucerne shoot and root growth (Chapter 4) was effective in natural environmental conditions. An earlier provision and higher overall concentration of soil ammonium was identified in soil amended with SHG and this coincided with a significant early-stage shoot growth benefit for ryegrass along with promotion of roots particularly at a lower soil depth.

### **5.1 Introduction**

Nitrogen fertilizer use, primarily in the form of ammonium, has increased dramatically over the past 20 years as farmers strive to increase productivity to meet the demands of a growing population. Since 1990, in Australia alone, yearly usage of nitrogen fertilizers has increased from 440 kt (elemental nitrogen equivalent) per year to 900 kt in 2009 (Australian Bureau of Agricultural and Resource Economics and Sciences, 2013). In 2012, widespread use across all Australian agricultural commodities resulted in nitrogen-based fertilizers being applied to a total of 32.3 million hectares of land, with ammonium phosphate application covering the largest proportion at 12.4 million hectares (Australian Bureau of Statistics, 2013). Determining ways in which we can use nitrogen fertilizer

more efficiently through the reduction of environmental losses would be beneficial environmentally and financially.

Nitrogen losses, through leaching and nitrous oxide emission, as a result of excessive or poorly-timed fertilizer application can have detrimental effects on the environment. When applied to soil in the ammonium form, nitrogen is relatively immobile. When fertilizer ammonium is converted to nitrate, it will readily move with the soil water, and losses by leaching can be significant. This can result in the water quality of rivers, estuaries and reservoirs being compromised resulting in eutrophication, algal bloom and fish poisoning (Di and Cameron, 2002). Drinking water contaminated with high concentrations of nitrate can be toxic to humans and livestock (e.g. Gulis et al., 2002, Weng et al., 2011, Costagliola et al., 2014). Nitrous oxide is produced in soils during nitrification (Bremner and Blackmer, 1978); the aerobic conversion of ammonium to nitrate, and denitrification; the anaerobic conversion of nitrate to nitrous oxide and dinitrogen, which dominates during and after periods of heavy rainfall (Wijler and Delwiche, 1954). Nitrous oxide is a greenhouse gas and contributes to the depletion of the ozone layer (Ravishankara et al., 2009). While dinitrogen is not a greenhouse gas, its loss from the soil means less fertilizer nitrogen is available for plant-uptake. It has been estimate that up to 50% of applied nitrogen can be lost due to leaching and gaseous emissions (Smith et al., 1990).

A number of strategies can be used to reduce nitrogen losses from soil, with organic amendment application being one. Some strategies that have been previously used to reduce nitrogen requirements include: growing nitrogen-fixing legume cover crops such as clover or lucerne (Smith et al., 1987), strategic fertilizer application based on seasonal or grazing regimes (Cameron et al., 2013), and application of nitrification inhibitors to mitigate nitrous oxide emissions (e.g. Zaman and Blennerhassett, 2010, Luo et al., 2013). The use of organic amendments has also been shown to minimise nitrogen losses (e.g. Suddick et al., 2011, Tuomisto et al.,

2012, Knowles et al., 2011, Steiner et al., 2010). As an example, biochar can mitigate nitrate leaching by adsorbing ammonium and slowing its oxidation to nitrate (Clough et al., 2013). The humic acid (HA) in commercial lignite-derived products (LDPs) has similar cation binding properties to biochar, and may have similar attributes in regulating nitrate and ammonium in soils. There has been no published data on nitrogen fertilization regulation with LDP. However, research by Ahmed et al. (2006) found an increase in soil ammonium with humic acid application. In addition, the research presented in Chapter 4 showed a significant decrease in nitrogen concentration in lucerne shoot tissue (up to 56% less compared to the control with no depression in shoot or root growth). This suggests that the LDP was able to reduce ammonium and/or nitrate availability, thereby reducing environmental losses. Irrespective of this research, the capacity for LDPs to affect soil nitrogen pools and losses has not been well explored.

This study was designed to investigate the effect of an LDP on soil nitrogen pools, nitrogen losses and growth and nutritional responses of ryegrass and lucerne. A mesocosm approach was undertaken to enable the control of variables such as soil type and inputs, while exposing the study to natural environmental conditions. Soil nitrogen pools were assessed by measuring total and mineralizable nitrogen, ammonium and nitrate, and nitrogen losses were assessed by measuring ammonium and nitrate in soil leachate, and nitrous oxide emissions. Soil pH was also assessed as it can impact nitrogen cycling. Repeated sampling from the mesocosms occurred at regular intervals for a period of seven months.

The specific research questions this study addressed were;

- Does the application of SHG have an effect on nitrogen pools and losses over time or irrespective of time? and
- Does the application of SHG have an effect on lucerne or ryegrass growth responses over time or irrespective of time?

The research from this study will demonstrate whether an LDP has the capacity to bind nitrogen in the form of ammonium and/or nitrate in the soil, thereby reducing losses by leaching and nitrous oxide emissions.

## **5.2 Materials and method**

### *5.2.1 Experimental layout and set-up*

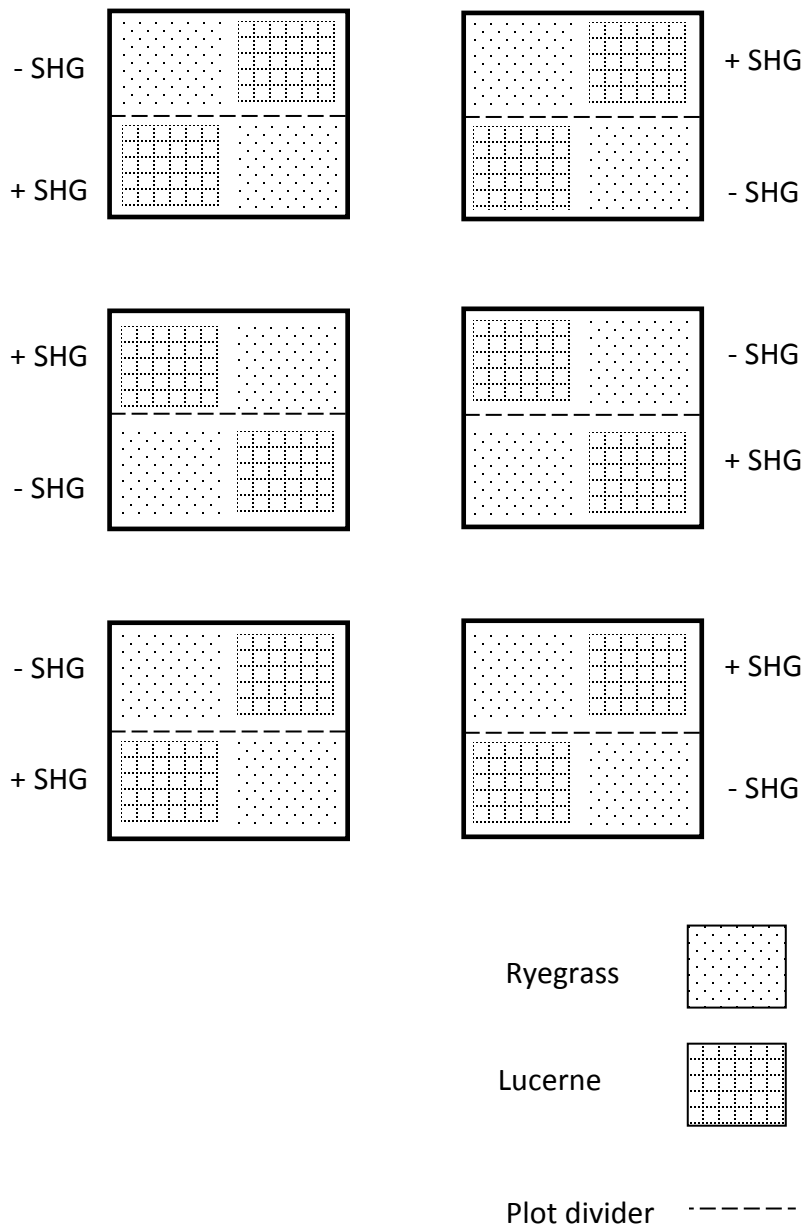
The LDP selected for this study was in the form of commercially-available soluble humate granules (SHG). The analyses used to characterise the physiochemical properties of this product have been described in detail in Chapter 2, Section 2.2 and its attributes are available in Chapter 2, Tables 2.2 and 2.3. The analyses conducted included pH, moisture content, HA content, organic and inorganic element concentrations and structural characterisation by  $^{13}\text{C}$  NMR. Briefly, the product was alkaline (pH 9.4), the moisture content was 4.5%, the HA content was 50.2% and the potassium content relatively high at 8.5%.

The study was conducted at the Monash University, Clayton, Victoria campus in the grounds of the glasshouse facility (Figure 5.1). It was set out in six square mesocosms constructed of wood; measuring 2.2 x 2.2 x 1 m. The treatments used in this study were plus or minus SHG, with either lucerne or ryegrass seeds sown into the surface making a total of two treatments with two different pasture types. Each plot had each of these treatments, whereby each plot was divided in half with plastic-coated board that was impervious to moisture transfer. They were then divided again with string to delineate plant types. Study conditions were replicated in each of the six plots, in a randomized complete block design, (Figure 5.2), making a total of 24 individual plots as quarters in each of the six mesocosms.





**Figure 5.1** Positioning of the six replicated mesocosms within the grounds of the glasshouse facility at Monash University, Clayton campus.



**Figure 5.2** Allocation of SHG application and pasture species to the six replicated mesocosms.

The soil used in the trial was a Podsol (Isbell, 2002), sourced from a vegetable farm recently converted from pasture in Cranbourne, Victoria (38°11'6"S, 145°18'50"E). Due to the sandy nature of the soil and the absence of large aggregates or pieces of organic debris, it was used in an as-received condition. A 200 g composite sub-sample, taken from the six mesocosms, was analysed for a range of key physicochemical properties (Environmental Analysis Laboratory, Southern Cross University, Lismore, NSW: <http://scu.edu.au/eal/>; accessed July 2014). These are listed in Table 5.1.

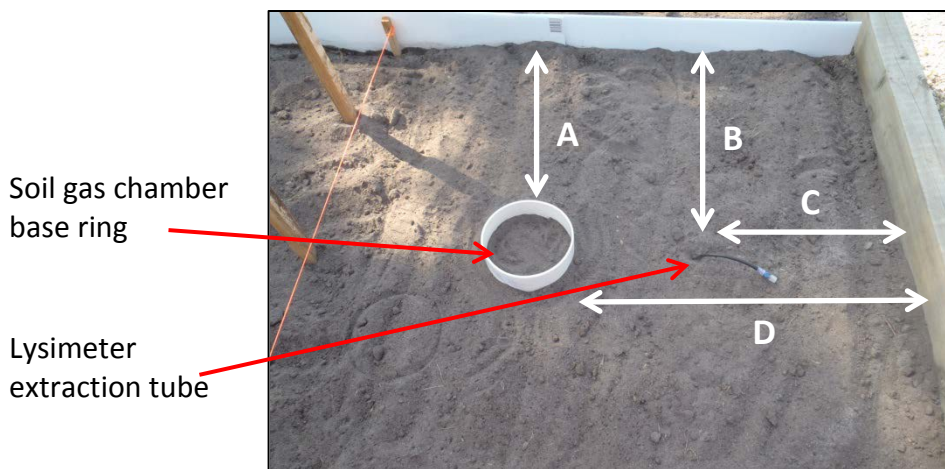
**Table 5.1** Soil physicochemical properties prior to amendment.

<b>pH</b> (H <sub>2</sub> O)	<b>OM</b> (%)	<b>C</b> (%)	<b>N</b> (%)	<b>Fe</b> (mg/kg)	<b>K</b> (mg/kg)	<b>Mn</b> (mg/kg)	<b>P (Colwell)</b> (mg/kg)	<b>Zn</b> (mg/kg)	<b>Texture</b>
5.84	1.9	1.1	0.1	104	97	5	62	2.1	Sandy

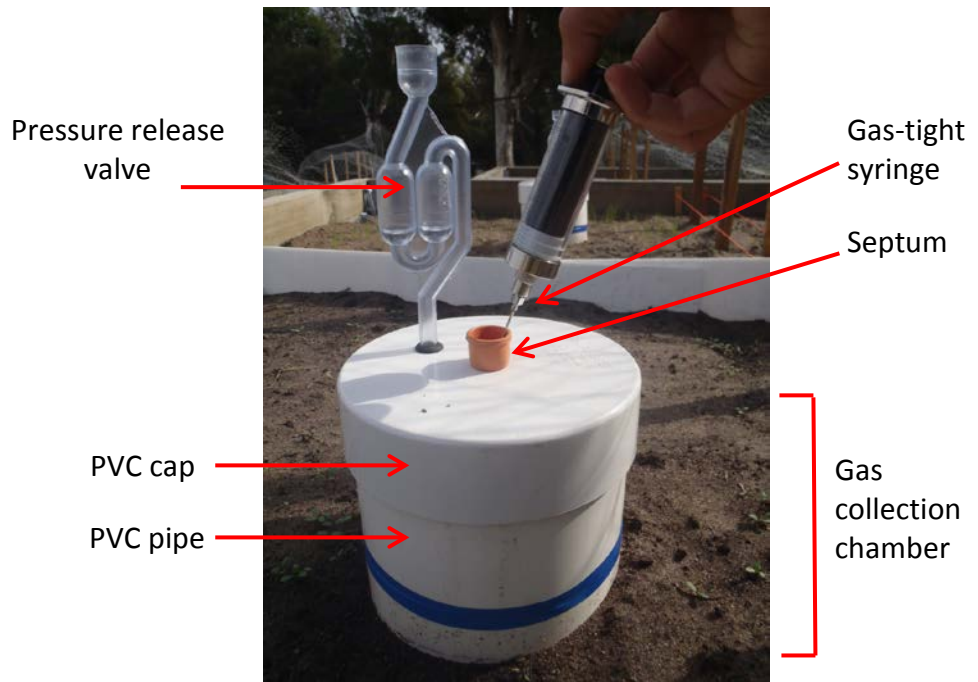
Prior to application of the treatments, sampling devices were installed to enable the measurement of gas flux from the soil. In each quarter plot, a ring of high density polyvinyl chloride (PVC) pipe (200 mm diam and 70 mm high) was pressed into the soil with the top 4 cm protruding. The exact location within each quarter plot is shown in Figure 5.3. The PVC ring remained in the soil for the duration of the study and provided a base onto which a gas collection chamber could be attached at each sampling time point. No pasture was allowed to grow within the ring area, though sub-soil root growth below the base was possible. The gas collection chamber was constructed from PVC pipe (200 mm diam and 150 mm high) which was enclosed at one end with a PVC cap. A rubber septum (20 mm diam.) and plastic pressure valve was inserted into the cap and sealed with gas-tight tape (Figure 5.4).

Lysimeters were installed to collect soil leachate. Construction was based on Deery et al. (2006). Briefly, PVC pipe (40 mm diam) was cut to 150 mm lengths. A ceramic tensiometer tip (Hi-flow, size 4, Coinda Ceramics, Bayswater, Victoria), was glued with Araldite to one end, with care taken to ensure that the glue did not run into

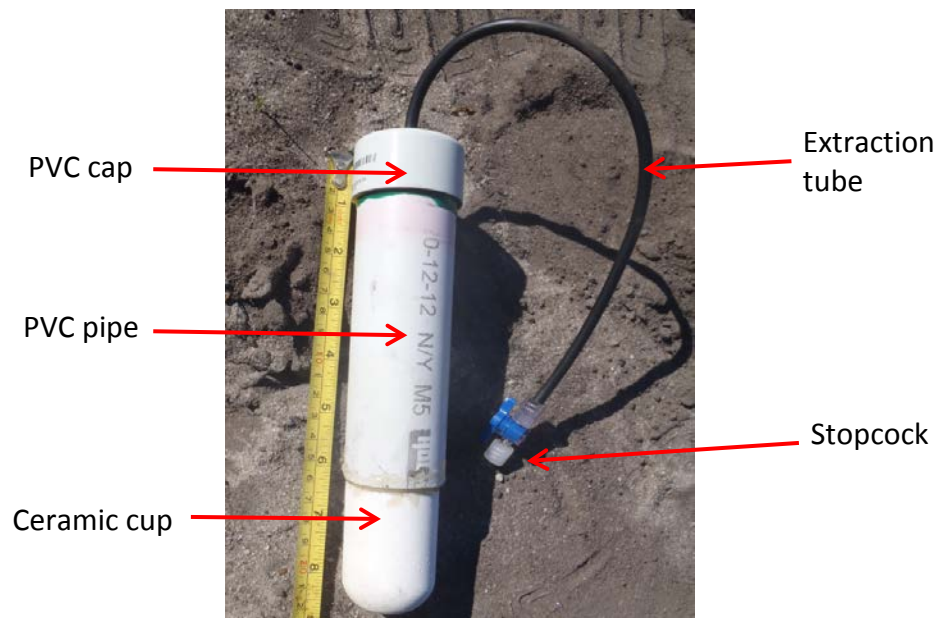
the ceramic cup, thereby reducing the sampling surface area. A PVC cap (40 mm diam) with a hole drilled in the center to accommodate an low-density polyethylene (LDPE) extraction tube (5mm diam), was fixed to the other end of the pipe. To ensure good adhesion, the PVC pipe surface was first treated with Protek Priming Fluid followed by Protek PVC Cement For Pressure Pipes. The LDPE extraction tube was cut to 600 mm lengths, with one end cut at a 45° angle to prevent vacuum adhesion to the bottom of the ceramic cup. This end was fed through the drilled hole in the PVC cap and pushed through the length of the pipe until it was positioned in the bottom of the ceramic cup. Where the LDPE extraction tube entered the cap, Loctite Super Glue was applied to prevent moisture entry. A 1-way stopcock with luer connection (Cole Palmer, part number EW-30600-05) was pushed into the extraction tube to facilitate soil solution retrieval. A constructed lysimeter is shown in Figure 5.5. One lysimeter was installed into each plot quarter (total of 24) at a soil depth of 400 mm. The location within each plot quarter is shown in Figure 5.3.



**Figure 5.3** Positioning of the soil gas chamber base ring and lysimeter within each mesocosm quarter. This was replicated in all 24 plots. Distance A = 1 m, B = 1.1 m, C = 0.4 m and D = 0.6 m.



**Figure 5.4** A soil gas collection chamber fixed to the base ring, showing gas sampling via syringe through the septum.

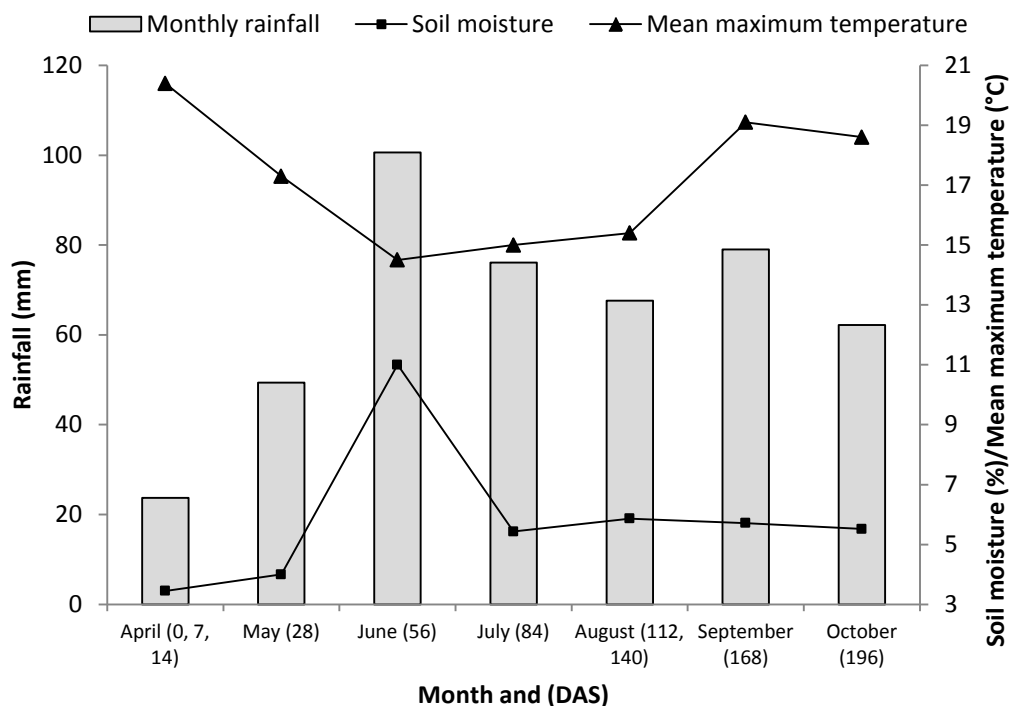


**Figure 5.5** A constructed lysimeter for collecting soil leachate showing the PVC cap and pipe, ceramic cup, LDPE extraction tube and stopcock components.

After installation of the sampling tools, diammonium phosphate fertilizer (DAP) was uniformly applied to the surface of each plot at a rate of 50 kg/ha, as is recommended for pasture establishment (N.S.W. Department of Primary Industries, 1997). To one half of each mesocosm, 20 kg/ha of SHG was applied to the soil surface as solid granules (Figure 5.2). The DAP and SHG were then manually incorporated into the top 2 cm of soil. Then, using wooden pegs and stringline divides for plant species, ryegrass (*Lolium multiflorum* Lam. cv. Bealey) and lucerne (*Medicago sativa* L. cv. Aurora) seeds were then sown at rates of 30 kg/ha and 20 kg/ha respectively into randomly assigned quarter sections of each mesocosm (Figure 5.2). The seeds were gently incorporated into the top 1 cm of soil.

#### *Weather monitoring*

Rainfall and daily maximum temperature readings were recorded by monitoring equipment permanently installed at the study site. Over the study period (April 2013 until October 2013) the maximum daily temperature averaged over each month was highest in April (20.4°C) and lowest in June (14.5°C). The sampling time point at 56 days after seeding (DAS) (June) was preceded by two weeks of above-average rainfall, resulting in a higher soil moisture content (11%) (Figure 5.6).



**Figure 5.6** Total monthly rainfall, soil moisture content and mean monthly maximum air temperature for the duration of the study. Numbers in brackets refer to days after seeding (DAS).

## 5.2.2 Sampling and analysis

### *Sampling overview*

From each mesocosm quarter (Figure 5.2), soil gas emissions and soil leachate were sampled at the time of seed sowing (0 DAS) and then 7, 14, 28, 56, 84, 112, 140, 168 and 196 DAS. The ryegrass and lucerne shoots were first cut and collected at 112 DAS and this was repeated at 140, 168 and 196 DAS. Ryegrass and lucerne root biomass was assessed at 112 and 196 DAS. Details of collection and analysis follow.

### *Soil sampling and analysis*

Soil was collected at each sampling time point (0, 7, 14, 28, 56, 84, 112, 140, 168 and 196 DAS), using a 25 mm diameter soil auger. In each mesocosm quarter, five soil cores were collected at a depth of 0-10 cm, and then combined to form a composite sample representative of that quarter. Immediately following collection, each soil sample was passed through a 2 mm sieve and refrigerated at 4°C for analysis the following day.

A sub-sample of the fresh 2mm sieved soil was used for moisture content determination by drying overnight at 105°C, after which the moisture content was determined by mass difference before and after drying. A second sub-sample of the fresh 2mm sieved soil was used for determination of ammonium and nitrate concentrations, and rates of potentially mineralizable N (PMN) by anaerobic incubation as follows. Inorganic nitrogen was extracted from 7 g of soil with 2M KCl and then measured colorimetrically using a modified method as described in (Forster, 1995) for ammonium and in Miranda et al. (2001) for nitrate. For PMN determination, 10 mL of deionized water was added to 7 g of soil in a 50 mL centrifuge tube, following which the headspace of the tube was filled with N<sub>2</sub>. The soil was incubated for 7 days at 37°C, after which 10 mL of 4M KCl was added and the ammonium concentration was analysed as described previously. The rate of PMN of the soil was expressed as the difference between the ammonium extracted from the fresh soil and the ammonium extracted following the 7 day incubation.

The remaining soil was then allowed to air-dry. The soil pH was determined by suspension of an air-dried soil sub-sample (5 g) suspended in deionized water (1:5 w/v) using a TPS WP81 meter and probe. A second sub-sample of air-dried soil was ground to a fine powder and then total carbon and total nitrogen measured by dry combustion in an ANCA GSL 2 elemental analyzer (Sercon Ltd., UK).



### *Soil gas collection and analysis*

At each sampling time point, a gas collection chamber was fixed onto each base ring with gas tight tape. Immediately following attachment of the gas chamber, a sample of soil gas was collected using a 25 mL gas-tight syringe (SGE, 25MDR-LL-GT) to penetrate the rubber septum (Figure 5.4). The gas sample was drawn up into the syringe barrel and the sample then introduced into a pre-evacuated 12 mL Exetainer vial with grey silicon septum (Labco, UK). This process was then repeated at 20, 40 and 60 min intervals. Gas samples were analysed for nitrous oxide using an Agilent 7890A gas chromatograph (GC) fitted with a Gilson (GX271) autosampler. Linear interpolation of the gas concentrations collected at 0, 20, 40 and 60 min were used to calculate the final CO<sub>2</sub> and N<sub>2</sub>O flux. The nitrous oxide flux was calculated according to the method and equations detailed in Van Zwieten et al. (2012).

### *Soil leachate collection and analysis*

At each sampling time point, negative pressure was applied to each lysimeter by attaching a clean 50 mL polypropylene (PP) syringe to the stopcock and removing as much air as possible. The lysimeters were left under negative pressure for 6 hours after which, via the stopcock, a clean 50 mL PP syringe was used to withdraw the soil solution that had accumulated in the ceramic cup (Figure 5.7). Following filtration through a 0.45 µm syringe filter (LabTek, L7196), the leachate was analysed for ammonium and nitrate using the methods described previously for the soil.



**Figure 5.7** Retrieving the soil leachate through the LDPE extraction tube from the installed lysimeter.

#### *Ryegrass and lucerne shoot and root sampling*

At 112 DAS, the ryegrass and lucerne had grown sufficiently for grazing. For ryegrass, this was to the 3-leaf stage (Fulkerson et al., 1997, Fulkerson and Donaghy, 2001) and for lucerne to approximately 15-20 cm in height (Fraser et al., 2004). To simulate grazing, the ryegrass and lucerne shoots were cut at 5 cm from the soil surface, and the cut biomass collected. The sampling was also repeated at 196 DAS. At both times, the shoots were oven-dried at 55°C and weighed to determine the shoot dry weight (SDW). The dried shoot material was then ground to a fine powder and nutrient concentrations (iron, potassium, manganese, phosphorus and zinc) were determined by inductively coupled plasma-optical emission spectrometry by (Waite Analytical Services, Urrbrae, S. Aust.: <http://www.adelaide.edu.au/was/>; accessed April 2014).

To assess root biomass, at 112 and 196 DAS, using a 25mm diameter auger, five soil cores were taken from each mesocosm quarter. Before being combined to form a composite sample, each core was divided into two parts; 0-10 and 10-30 cm soil depths (48 samples in total). Samples were weighed and a small sub-sample was removed for moisture content determination by mass difference before and after

drying overnight at 105°C. The remainder of the sample was washed through a 2mm screen, with the roots retained on the screen. The roots were then dried at 55°C and weighed. The RDW was expressed as the mass of dry roots per mass of dry soil.

### 5.2.3 *Statistical analysis*

Data was analysed using JMP statistical software (JMP<sup>®</sup>, Version 10, SAS Institute Inc., Cary, NC) and SPSS Statistics 20 (IBM SPSS Statistics for Windows, Version 20, IBM Corp., Armonk, NY). To understand the differences between the products over time, all pasture and soil data were analysed by two-way ANOVA; factors in the analysis were *time* and *product*, with plant types analysed separately. Each mesocosm was included in the statistical model as an individual block. Where significant differences were found, pairwise comparisons were made using Tukey's honestly significant difference (HSD) or Student's t-test (root dry weight data). Repeated measures restricted maximum likelihood (RM REML) was used to determine if the application of SHG had an effect on pasture and soil attributes irrespective of time. For the model the fixed factor was *product* (SHG amended or unamended), with time as the repeated measure (covariate).

## 5.3 Results

### 5.3.1 *Soil nitrogen pools, losses and pH*

#### *Nitrogen pools over time - total and mineralizable nitrogen (PMN), ammonium and nitrate*

There was no significant ( $P>0.05$ ) interaction between time and SHG treatment on soil PMN, ammonium or nitrate concentrations, or total nitrogen for either the ryegrass or the lucerne plots (Table 5.2, 5.3 and 5.4). Although not significant, in the ryegrass plots (Table 5.3) a peak concentration of ammonium in the SHG amended soil occurred at 7 DAS (4.11 µg/g dry soil) compared to 28 DAS (4.03 µg/g dry soil) in the unamended soil.

**Table 5.2** Total soil nitrogen concentration (%) in the ryegrass and lucerne plots. Mean values are presented (n=6). Values in brackets are  $\pm$  s.e. No significant differences were identified. DAS = days after seeding

DAS	Soil N (%)			
	Ryegrass		Lucerne	
	With SHG	Without SHG	With SHG	Without SHG
0	0.04 (0.01)	0.05 (0.01)	0.05(0.01)	0.07(0.01)
7	0.05 (0.01)	0.07 (0.01)	0.06(0.01)	0.06(0.01)
14	0.07 (0.01)	0.05 (0.01)	0.04(0.01)	0.05(0.01)
28	0.06 (0.01)	0.05 (0.01)	0.05(0.01)	0.06(0.01)
56	0.05 (0.01)	0.05 (0.01)	0.05(0.01)	0.05(0.01)
84	0.04 (0.01)	0.04 (0.01)	0.06(0.01)	0.07(0.01)
112	0.05 (0.01)	0.04 (0.01)	0.06(0.01)	0.08(0.01)
140	0.05 (0.01)	0.07 (0.01)	0.04(0.01)	0.05(0.01)
168	0.05 (0.01)	0.04 (0.01)	0.05(0.01)	0.04(0.01)
196	0.07 (0.01)	0.08 (0.01)	0.07(0.01)	0.08(0.01)

**Table 5.3** Soil potentially mineralizable nitrogen (PMN), ammonium ( $\text{NH}_4^+$ -N) and nitrate ( $\text{NO}_3^-$ -N) concentrations in the ryegrass plots. Mean values are shown (n=6). Values in brackets are  $\pm$  s.e. No significant differences were identified.

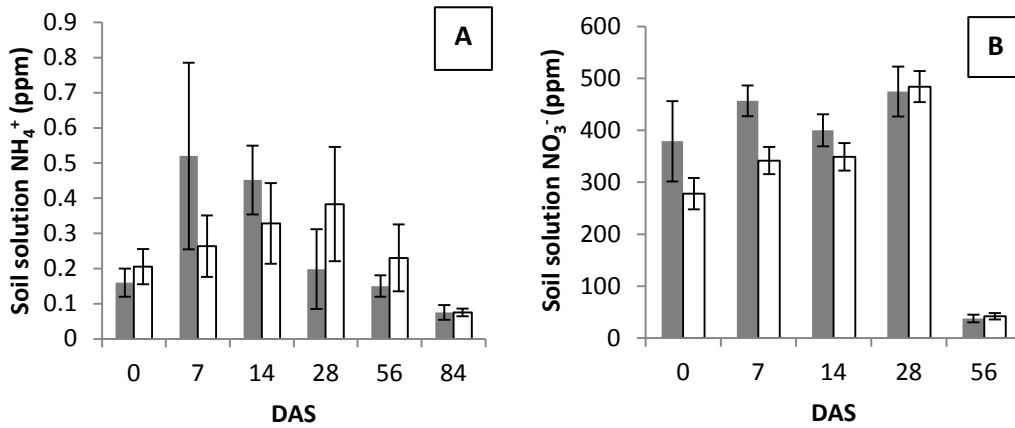
DAS	PMN ( $\mu\text{g/g}$ dry soil)		$\text{NH}_4^+$ -N ( $\mu\text{g/g}$ dry soil)		$\text{NO}_3^-$ -N ( $\mu\text{g/g}$ dry soil)	
	With SHG	Without SHG	With SHG	Without SHG	With SHG	Without SHG
	0	2.06 (0.70)	1.93 (0.50)	2.24 (0.51)	2.24 (0.51)	9.81 (0.71)
7	1.51 (0.99)	2.86 (0.90)	4.11 (0.58)	2.55 (0.65)	12.15 (2.19)	10.59 (1.78)
14	1.82 (0.71)	0.67 (0.25)	2.45 (0.88)	2.72 (0.92)	13.04 (1.95)	12.19 (2.31)
28	2.60 (1.04)	1.79 (0.63)	2.83 (0.56)	4.03 (0.83)	17.89 (3.93)	13.65 (1.99)
56	0.96 (0.71)	2.25 (0.55)	2.89 (0.42)	2.33 (0.34)	1.62 (0.70)	0.33 (0.18)
84	2.09 (0.72)	4.09 (1.10)	2.22 (0.28)	1.45 (0.39)	0.03 (0.03)	0.32 (0.32)
112	5.17 (0.42)	4.96 (0.42)	1.27 (0.16)	1.38 (0.14)	0.02 (0.01)	0.27 (0.27)
140	0.99 (0.19)	1.13 (0.17)	1.66 (0.18)	1.17 (0.13)	0.02 (0.02)	0.67 (0.67)
168	3.01 (0.43)	3.35 (0.33)	0.02 (0.24)	0.02 (0.13)	0.24 (0.16)	0.46 (0.19)
196	0.82 (0.41)	0.33 (0.11)	1.20 (0.24)	0.76 (0.21)	0.87 (0.87)	0.00 (0.00)

**Table 5.4** Soil potentially mineralizable nitrogen (PMN), soil ammonium (NH<sub>4</sub><sup>+</sup>-N) and nitrate (NO<sub>3</sub><sup>-</sup>-N) concentrations in the lucerne plots. Mean values are shown (n=6). Values in brackets are ± s.e. No significant differences were identified.

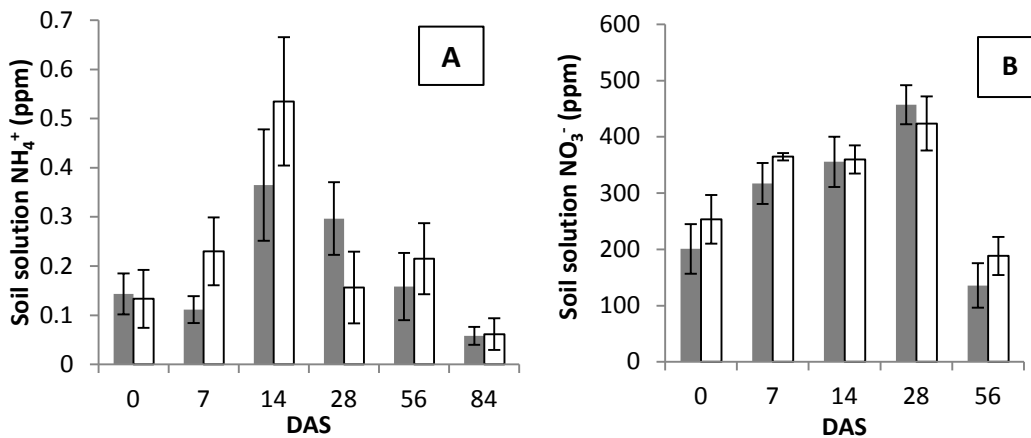
DAS	PMN (µg/g dry soil)		NH <sub>4</sub> <sup>+</sup> -N (µg/g dry soil)		NO <sub>3</sub> <sup>-</sup> -N (µg/g dry soil)	
	With SHG	Without SHG	With SHG	Without SHG	With SHG	Without SHG
0	1.50 (0.76)	1.05 (0.27)	3.01 (0.99)	5.08 (1.53)	12.73 (2.52)	11.59 (2.34)
7	1.90 (0.77)	1.35 (0.40)	3.75 (1.08)	3.46 (0.63)	10.30 (0.65)	10.77 (1.41)
14	1.42 (0.80)	2.10 (0.74)	6.37 (3.55)	4.48 (1.29)	12.83 (1.39)	10.87 (1.10)
28	1.90 (0.77)	2.34 (0.65)	3.20 (0.89)	2.48 (0.45)	11.98 (1.19)	15.43 (2.99)
56	1.64 (0.82)	1.05 (0.64)	3.03 (0.48)	4.17 (0.98)	2.97 (1.98)	1.90 (0.76)
84	2.97 (1.05)	3.01 (0.86)	1.74 (0.16)	1.51 (0.35)	0.14 (0.09)	0.58 (0.46)
112	5.24 (0.62)	4.69 (0.29)	1.19 (0.14)	1.16 (0.18)	0.00 (0.00)	0.00 (0.00)
140	0.82 (0.31)	0.70 (0.20)	1.47 (0.35)	1.26 (0.15)	0.10 (0.09)	0.08 (0.07)
168	2.85 (0.51)	3.22 (0.78)	0.03 (0.03)	0.11 (0.07)	0.18 (0.05)	0.15 (0.04)
196	0.73 (0.26)	0.82 (0.25)	1.03 (0.15)	0.98 (0.11)	0.11 (0.06)	0.10 (0.04)

*Nitrogen losses over time – leachate and nitrous oxide emissions.*

There was a significant difference in nitrate concentration in the leachate at 7 DAS ( $P=0.02$ ), with 457 ppm in the SHG amended soil compared to 342 ppm in the unamended soil in the ryegrass plots. For both ryegrass and lucerne, amendment with SHG did not significantly change the ammonium concentration leached from the soil at any of the sampling time points (Figure 5.8 and Figure 5.9). The nitrate concentrations in the soil leachate collected at 84 DAS and both the nitrate and ammonium concentrations collected at 112, 140, 168, 196 DAS were at the very lower detection limit of the assay and so are not included in Figure 5.8 and Figure 5.9.

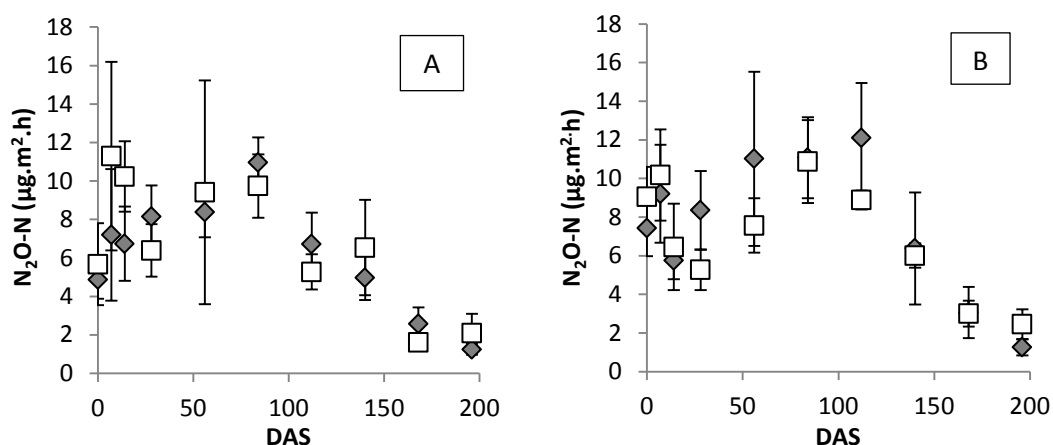


**Figure 5.8** Ammonium (NH<sub>4</sub><sup>+</sup>-N) (A) and nitrate (NO<sub>3</sub><sup>-</sup>-N) (B) concentrations in the soil leachate from the ryegrass plots. Grey bars represent SHG amended soil, white bars are unamended. Mean values are presented (n=6). Error bars are ± s.e.



**Figure 5.9** Ammonium (NH<sub>4</sub><sup>+</sup>-N) (A) and nitrate (NO<sub>3</sub><sup>-</sup>-N) (B) concentration in soil leachate from lucerne plots. Grey bars represent SHG amended soil, white bars are unamended. Mean values are presented (n=6). Error bars are ± s.e.

At each sampling time point, amendment with SHG had no significant effect on the soil emissions of nitrous oxide from either the ryegrass or lucerne plots (Figure 5.10). From 112 DAS for ryegrass and 140 DAS for lucerne there was a trend of decreasing emissions at each time point.



**Figure 5.10** Nitrous oxide (N<sub>2</sub>O-N) soil emissions **(A)** ryegrass **(B)** lucerne from SHG amended and unamended soils. Grey diamonds represent SHG amended soil, white squares are unamended. Mean values are presented (n=6). Error bars are ± s.e.

#### *Soil pH over time*

Amendment with SHG did not have an impact on the soil pH measured at any of the sampling time points from the ryegrass or lucerne plots (Table 5.5).

**Table 5.5** Soil pH of the ryegrass and lucerne plots. No significant differences were identified. Mean values are presented (n=6). Values in brackets are ± s.e. No significant differences were identified.

DAS	Soil pH			
	Ryegrass		Lucerne	
	With SHG	Without SHG	With SHG	Without SHG
0	5.29 (0.10)	5.38 (0.09)	5.35(0.11)	5.20(0.10)
7	5.31(0.08)	5.36 (0.08)	5.29(0.10)	5.39(0.09)
14	5.29 (0.10)	5.41 (0.10)	5.42(0.13)	5.29(0.06)
28	5.29 (0.10)	5.43 (0.07)	5.34(0.12)	5.30(0.09)
56	5.31 (0.09)	5.47 (0.07)	5.40(0.12)	5.35(0.10)
84	5.62 (0.14)	5.77 (0.08)	5.77(0.12)	5.62(0.08)
112	5.26 (0.12)	5.30 (0.10)	5.20(0.14)	5.23(0.11)
140	5.34 (0.12)	5.33 (0.10)	5.29(0.11)	5.26(0.08)
168	5.37 (0.12)	5.48 (0.10)	5.35(0.15)	5.36(0.09)
196	5.47 (0.11)	5.49 (0.12)	5.42(0.15)	5.41(0.15)

*Soil nitrogen pools over all time - total and mineralisable nitrogen (PMN), ammonium and nitrate.*

Overall, where time was a covariate in the REML model, there was no significant effect of SHG amendment on the soil nitrogen concentration in either the ryegrass or lucerne plots (Table 5.6). In the ryegrass plots there was a significantly higher concentration of ammonium in those that had been amended with SHG (Table 5.7). No such trend was observed for the lucerne plots and no other significant differences were identified in PMN, ammonium or nitrate for either pasture species (Table 5.7). Irrespective of time, in the ryegrass plots, amendment with SHG resulted in a higher nitrate concentration in the soil leachate (Table 5.8). This was not observed in the leachate from the lucerne plots and there were no significant differences in the leachate ammonium concentration from both the ryegrass and lucerne plots (Table 5.8). The application of SHG had no effect on the overall nitrous oxide emissions from either the ryegrass or lucerne plots (Table 5.9). Irrespective of time, the application of SHG did not significantly change soil pH in either the ryegrass or lucerne plots (Table 5.10).

**Table 5.6** Soil nitrogen concentration in the ryegrass and lucerne plots, irrespective of sampling time. Mean values are presented (n=60). Values in brackets are  $\pm$  s.e. The abbreviation 'n.s.' indicates  $P>0.05$ .

Treatment	Soil N (%)	
	Ryegrass	Lucerne
With SHG	0.05 (0.01)	0.05 (0.01)
Without SHG	0.05 (0.01)	0.05 (0.01)
<i>P</i> value	n.s.	n.s.



**Table 5.7** Soil potentially mineralizable nitrogen (PMN), ammonium (NH<sub>4</sub><sup>+</sup>-N) and nitrate (NO<sub>3</sub><sup>-</sup>-N) concentrations in the ryegrass and lucerne plots, irrespective of sampling time. Mean values are shown (n=60). Values in brackets are ± s.e. The abbreviation 'n.s.' indicates *P*>0.05.

	PMN (µg/g dry soil)	NH <sub>4</sub> <sup>+</sup> -N (µg/g dry soil)	NO <sub>3</sub> <sup>-</sup> -N (µg/g dry soil)
<b>Ryegrass</b>			
With SHG	2.10 (0.26)	2.18 (0.20)	5.77 (1.00)
Without SHG	2.34 (0.25)	1.78 (0.27)	4.63 (0.78)
<i>P</i> value	n.s.	0.03	n.s.
<b>Lucerne</b>			
With SHG	2.10 (0.26)	2.48 (0.43)	5.29 (0.80)
Without SHG	2.03 (0.23)	2.47 (0.31)	5.19 (0.86)
<i>P</i> value	n.s.	n.s.	n.s.

**Table 5.8** Soil leachate ammonium (NH<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) concentrations collected from the ryegrass and lucerne plots, irrespective of sampling time. Mean values are presented (n=24). Values in brackets are ± s.e. The abbreviation 'n.s.' indicates *P*>0.05.

	Ryegrass		Lucerne	
	NH <sub>4</sub> <sup>+</sup> -N (ppm)	NO <sub>3</sub> <sup>-</sup> -N (ppm)	NH <sub>4</sub> <sup>+</sup> -N (ppm)	NO <sub>3</sub> <sup>-</sup> -N (ppm)
With SHG	0.37 (0.09)	358.13 (33.63)	0.21 (0.03)	293.33 (26.87)
Without SHG	0.25 (0.04)	282.93 (30.37)	0.22 (0.04)	317.93 (21.36)
<i>P</i> value	n.s.	0.01	n.s.	n.s.

**Table 5.9** Nitrous oxide (N<sub>2</sub>O-N) emissions from the ryegrass and lucerne plots. Mean values are presented (n=60). Values in brackets are ± s.e. The abbreviation 'n.s.' indicates *P*>0.05.

	N <sub>2</sub> O-N emissions (µg/m <sup>2</sup> .h)	
	Ryegrass	Lucerne
With SHG	6.38 (0.61)	7.56 (0.82)
Without SHG	6.82 (0.91)	6.98 (0.56)
<i>P</i> value	n.s.	n.s.

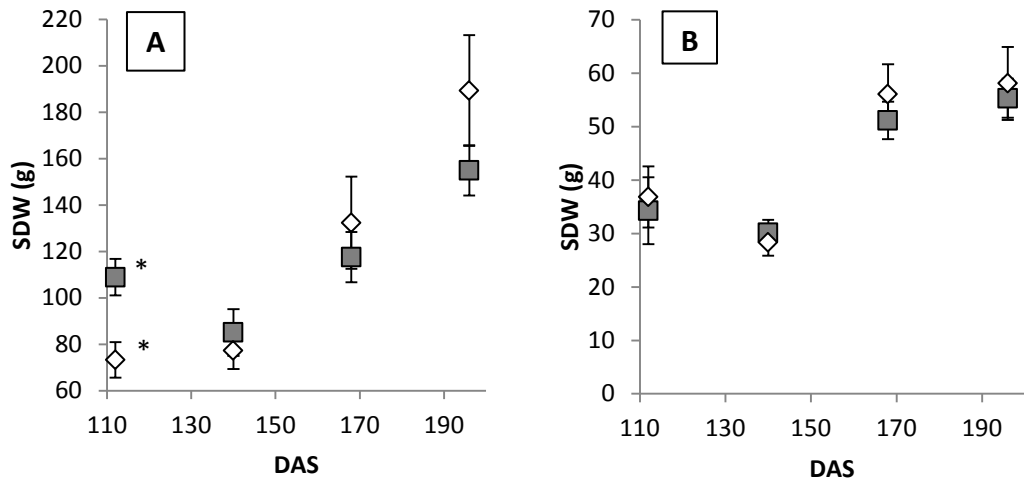
**Table 5.10** Soil pH of ryegrass and lucerne plots, irrespective of sampling time. Mean values are presented (n=60). Values in brackets are  $\pm$  s.e. The abbreviation 'n.s.' indicates  $P>0.05$ .

	Soil pH	
	Ryegrass	Lucerne
With SHG	5.35 (0.03)	5.37 (0.03)
Without SHG	5.41 (0.03)	5.37 (0.03)
<i>P</i> value	n.s.	n.s.

### 5.3.2 Ryegrass and lucerne growth and nutrition

#### *Ryegrass and lucerne growth and nutrition over time*

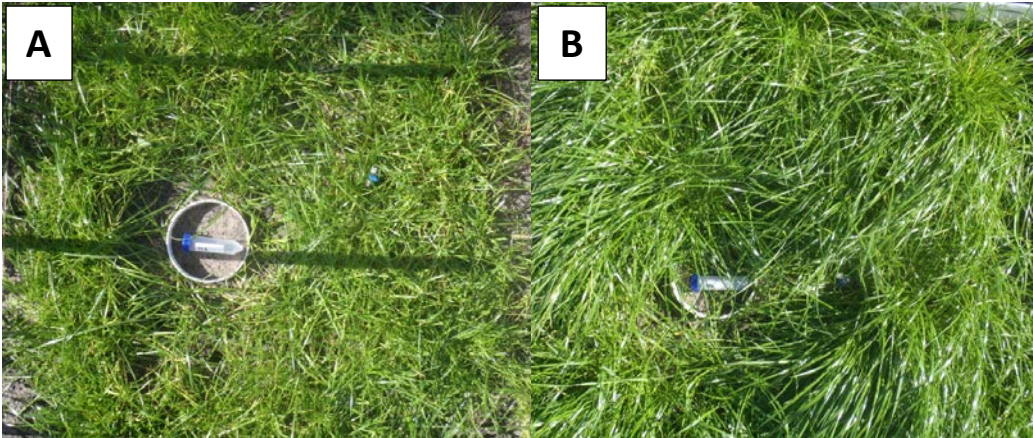
At 112 DAS there was a significant increase in the SDW of ryegrass grown in soil amended with SHG compared to unamended soil ( $P=0.001$ ) (Figures 5.11, 5.12 and 5.13), equivalent to a growth increase of approximately 48%. This growth benefit was not evident at 140 and 168 DAS, with no significant difference between the ryegrass SDW in amended soil compared to unamended soil. Although not significant, at 196 DAS, there was a trend of more shoot growth in the unamended soil. The SDW of lucerne grown in soil amended with SHG was not significantly different at any of the sampling time points. At 112 DAS there was a significant increase in ryegrass root dry weight (RDW) in response to SHG amendment at both 0-10 ( $P=0.04$ ) and 10-30 cm ( $P=0.02$ ) soil depths (Table 5.11 and Figure 5.14). The root biomass of the SHG-amended ryegrass at 10-30 cm depth was more than double that of ryegrass that had not received SHG. There was no significant difference in lucerne root biomass at either soil depth (Table 5.11). Application of SHG did not result in a response in the concentration of any of the analysed nutrients in the ryegrass or lucerne shoots (Tables 5.12 and 5.13).



**Figure 5.11** Shoot dry weight (SDW) of ryegrass **(A)** and lucerne **(B)** in soil amended with 20 kg/ha of SHG compared to unamended soil. Grey diamonds represent SHG amended soil, white squares are unamended. Mean values are presented (n=6). Error bars are  $\pm$  s.e. \* indicates a significant difference at that sampling time point.



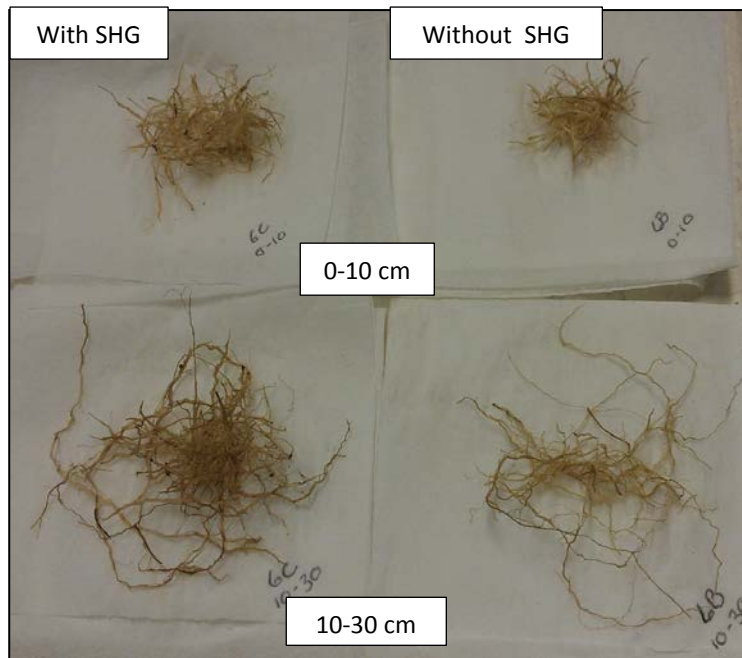
**Figure 5.12** One of the mesocosms at 112 DAS, just prior to ryegrass and lucerne shoot sampling. The front half of the plot was amended with 20 kg/ha of SHG and shows an increase in the shoot growth of ryegrass (front right corner) compared to the unamended soil (back left corner).



**Figure 5.13** Top view of ryegrass plots at 112 DAS growing in unamended (A) and SHG amended soil (B).

**Table 5.11** Root dry weight (RDW) of ryegrass and lucerne at 0-10 and 10-30 cm soil depths. Mean values are presented (n=6). Values in brackets are  $\pm$  s.e. \* indicates a significant difference at that soil depth.

Pasture species and sampling depth (cm)	RDW (g/g dry soil) With SHG		RDW (g/g dry soil) Without SHG	
	DAS		DAS	
	112	196	112	196
Ryegrass 0-10	0.20*	0.61	0.14*	0.73
	(0.03)	(0.03)	(0.03)	(0.10)
Ryegrass 10-30	0.13*	0.34	0.06*	0.32
	(0.04)	(0.04)	(0.01)	(0.02)
Lucerne 0-10	0.07	0.22	0.09	0.26
	(0.01)	(0.04)	(0.04)	(0.05)
Lucerne 10-30	0.03	0.14	0.05	0.13
	(0.01)	(0.02)	(0.02)	(0.04)



**Figure 5.14** Root biomass of ryegrass with and without SHG at 112 DAS at 0-10 and 10-30 cm soil depths.

**Table 5.12** Ryegrass shoot nutrient concentrations at 112, 140, 168 and 196 DAS. Mean values are presented (n=6). Values in brackets are  $\pm$  s.e. No significant differences were identified.

	Days after seeding (DAS)							
	112		140		168		196	
	With SHG	Without SHG	With SHG	Without SHG	With SHG	Without SHG	With SHG	Without SHG
<b>C (%)</b>	38.6	40.1	38.2	40.7	41.5	41.0	40.8	42.1
	(1.5)	(1.3)	(1.5)	(0.6)	(0.8)	(0.2)	(0.6)	(0.5)
<b>N (%)</b>	2.9	2.9	2.9	2.9	3.0	3.2	2.9	2.7
	(0.2)	(0.2)	(0.2)	(0.2)	(0.2)	(0.2)	(0.2)	(0.2)
<b>Fe (mg/kg)</b>	104.3	104.3	111.0	106.8	104.5	96.3	102.3	94.8
	(4.2)	(5.1)	(1.6)	(5.7)	(7.1)	(2.8)	(4.5)	(3.5)
<b>K (g/kg)</b>	33.2	32.0	36.0	36.7	31.7	34.5	30.0	33.5
	(1.9)	(2.0)	(2.1)	(2.4)	(1.4)	(1.2)	(1.5)	(1.9)
<b>Mn (mg/kg)</b>	93.7	99.2	135.8	121.3	137.5	133.7	120.2	103.0
	(14.2)	(16.6)	(23.2)	(18.6)	(24.0)	(27.9)	(19.1)	(18.6)
<b>P (g/kg)</b>	4.8	4.5	4.9	4.8	3.9	4.2	3.5	3.7
	(0.3)	(0.1)	(0.1)	(0.2)	(0.1)	(0.2)	(0.3)	(0.2)
<b>Zn (mg/kg)</b>	53.2	52.5	46.3	40.5	40.7	45.3	46.5	41.3
	(4.1)	(5.0)	(2.0)	(1.7)	(1.3)	(1.4)	(4.9)	(2.2)

**Table 5.13** Lucerne shoot nutrient concentrations at 112, 140, 168 and 196 DAS. Mean values are presented (n=6). Values in brackets are  $\pm$  s.e. No significant differences were identified.

	Days after seeding (DAS)							
	112		140		168		196	
	With SHG	Without SHG	With SHG	Without SHG	With SHG	Without SHG	With SHG	Without SHG
<b>C (%)</b>	39.3	40.2	40.2	42.1	43.5	42.1	43.9	42.9
	(1.1)	(1.5)	(1.4)	(0.7)	(0.7)	(0.5)	(0.6)	(0.5)
<b>N (%)</b>	3.9	3.9	3.4	3.6	4.3	4.3	5.0	4.9
	(0.3)	(0.3)	(0.2)	(0.2)	(0.1)	(0.2)	(0.1)	(0.2)
<b>Fe</b>	122.3	126.0	116.5	118.8	135.0	138.7	120.7	131.2
(mg/kg)	(4.6)	(4.2)	(5.8)	(6.5)	(4.6)	(7.7)	(3.8)	(9.5)
<b>K</b>	28.8	28.2	31.7	30.5	33.0	33.0	31.8	33.2
(g/kg)	(1.6)	(0.8)	(1.5)	(0.4)	(2.6)	(1.4)	(1.4)	(0.9)
<b>Mn</b>	101.8	104.5	96.0	92.3	99.7	100.2	73.8	82.7
(mg/kg)	(26.7)	(26.8)	(27.5)	(19.6)	(22.8)	(15.8)	(11.8)	(15.4)
<b>P</b>	4.4	4.1	4.4	4.3	4.8	4.7	4.9	4.8
(g/kg)	(0.2)	(0.1)	(0.2)	(0.1)	(0.2)	(0.2)	(0.3)	(0.2)
<b>Zn</b>	74.3	73.3	77.7	79.2	99.2	98.0	90.0	92.8
(mg/kg)	(3.6)	(2.1)	(3.7)	(5.7)	(5.6)	(7.8)	(5.9)	(6.9)

#### *Ryegrass and lucerne growth and nutrition overall*

Overall, where time was a covariate in the REML model, there was no significant effect of SHG amendment on the shoot dry weight of the ryegrass or lucerne (Table 5.14). There was a ryegrass root growth benefit ( $P=0.003$ ) at 10-30cm soil depth, with 0.24 g/g dry soil with SHG amendment compared to 0.19 g/g dry soil without (Table 5.15). There were no significant differences in the shoot nutrient concentrations of the ryegrass or the lucerne (Table 5.16).

**Table 5.14** Shoot dry weight (SDW) of ryegrass and lucerne, irrespective of sampling time. Mean values are presented (n=24). Values in brackets are  $\pm$  s.e. The abbreviation 'n.s.' indicates  $P>0.05$ .

Treatment	Ryegrass SDW (g)	Lucerne SDW (g)
With SHG	116.65 (4.30)	42.72 (1.88)
Without SHG	118.07 (7.91)	44.84 (2.30)
<i>P</i> value	n.s.	n.s.

**Table 5.15** Root dry weight (RDW) of ryegrass and lucerne grown, irrespective of sampling time. Mean values are presented (n=12). Values in brackets are  $\pm$  s.e. The abbreviation 'n.s.' indicates  $P>0.05$ .

Treatment	Pasture species and sampling depth (cm)			
	Ryegrass RDW (g/g dry soil)		Lucerne RDW (g/g dry soil)	
	0-10	10-30	0-10	10-30
With SHG	0.40 (0.07)	0.24 (0.04)	0.15 (0.03)	0.08 (0.02)
Without SHG	0.43 (0.10)	0.19 (0.04)	0.18 (0.04)	0.09 (0.02)
<i>P</i> value	n.s.	0.003	n.s.	n.s.

**Table 5.16** Ryegrass and lucerne shoot nutrient concentration irrespective of time. Mean values are presented (n=12). Values in brackets are  $\pm$  s.e. No significant differences were identified.

	Ryegrass		Lucerne	
	With SHG	Without SHG	With SHG	Without SHG
<b>C</b> (%)	39.8 (0.6)	40.9 (0.4)	41.7 (0.6)	41.8 (0.1)
<b>N</b> (%)	2.9 (0.1)	2.9 (0.1)	4.1 (0.2)	4.2 (0.1)
<b>Fe</b> (mg/kg)	105.5 (2.3)	100.6 (2.3)	123.6 (2.6)	128.7 (3.7)
<b>K</b> (g/kg)	32.7 (0.9)	34.0 (1.0)	31.3 (0.9)	31.2 (0.6)
<b>Mn</b> (mg/kg)	121.8 (10.2)	114.3 (10.2)	92.8 (11.0)	94.9 (9.5)
<b>P</b> (g/kg)	4.3 (0.2)	4.3 (0.1)	4.6 (0.1)	4.5 (0.1)
<b>Zn</b> (mg/kg)	46.7 (1.8)	44.9 (1.7)	85.3 (3.1)	85.8 (3.5)

## 5.4 Discussion

### 5.4.1 Nitrogen pools and losses.

The application of SHG increased the pool of soil ammonium but not nitrate. The loss of nitrate in the leachate was increased from the soil amended with SHG, and there was no effect on the soil nitrous oxide emissions.

The peak identified in the soil ammonium concentration at 7 DAS in the SHG amended soil compared to 28 DAS in the unamended soil suggests earlier availability for plant uptake. The possible mechanisms by which this earlier peak occurred could be of a chemical or microbial nature. Chemically, the addition of potassium inherent in the SHG product (8.5% potassium), may have displaced ammonium from the surface of the soil particles (Martin et al., 1946). This mechanism is the basis for the widely accepted soil ammonium analysis technique in which potassium chloride is used to displace the ammonium from the soil (Forster, 1995). An alternative or complimentary mechanism may be an increase in the transformation rate of organic nitrogen to ammonium due to stimulation of decomposer soil microbes in response to addition of the carbon in the product. Further information would be gained from molecular analysis of the soil microbial community to determine the abundance of decomposer microorganisms.

Overall, there was an increase in the soil ammonium concentration. Similar increases were identified in an incubation study with HA of unknown origin (Ahmed et al., 2006) and a glasshouse study in which HA derived from peat was applied to maize (Kasim et al., 2009). Accumulation of ammonium is likely due to the cation exchange capacity of HA at the carboxylic and phenolic hydroxyl groups (Schnitzer and Skinner, 1965). The application of SHG made no difference to the overall soil nitrate concentration which is consistent with the findings of Ahmed et al. (2006) in which peat-derived HA was applied to soil in an incubation study. However, in a glasshouse study conducted by Ahmed et al. (2012) in which palm oil mill effluent-



derived HA was applied to maize there was a decrease in soil nitrate, the reasoning being that the HA bound the ammonium making it unavailable for transformation to nitrate. The HA application rate was approximately 10 times that used here and so a higher concentration of ammonium bound to the HA would be expected.

The overall higher concentration of nitrate in the soil leachate from the SHG amended ryegrass plots was unexpected. Being an anion, nitrate is unlikely to be retained by binding to the HA, but it is not clear why nitrate loss would be promoted. It is possible that the higher ammonium concentration in the soil under HA treatments also stimulated nitrification, but the additional nitrate was leached rather than accumulating in the topsoil. There is no published precedent for this observation and the controlled conditions of a column leaching study are necessary for further investigation.

The application of SHG had no significant effect on nitrous oxide emissions which is in agreement with findings by Mukherjeenet al. (2014) who applied commercial coal-derived HA at 7.5 t/ha in a field setting. Although that study showed a trend towards lower emissions, high variability in the data reduced the likelihood of identifying significant differences. In this study, as would be expected, lower nitrous oxide emissions were measured towards the end of the study when the soil was becoming depleted of ammonium and nitrate resulting in reduced rates of nitrification and denitrification (Dalal et al., 2003).

#### *5.4.2 Ryegrass and lucerne shoot and root growth and nutrition*

The application of SHG promoted shoot and root growth in ryegrass but not lucerne. There was no difference in shoot nutrition for either pasture type. The growth response identified in the ryegrass may be due to the earlier availability of ammonium in the soil. Ryegrass responds well to ammonium and will preferentially take it up over nitrate (Griffith and Streeter, 1994). Lucerne, a nitrogen-fixer, was not affected because it is able to self-produce its own nitrogen requirements.

There are other factors that may have affected lucerne growth. In the glasshouse study described in Chapter 5, lucerne shoot and root growth was promoted (a 46.5% increase in shoots and a 30.9% increase in roots) by the SHG product applied at the same 20 kg/ha rate as here. The glasshouse study was conducted in optimal, tightly-controlled environmental conditions whereas this study was conducted in outdoor plots subject to environmental variation. Pasture-sowing was in April (Autumn) which provides ideal conditions for ryegrass but not for lucerne which ideally would be established in Spring. In addition, the pH of the soil (pH 5.3) was at the very lower limit of the tolerable range for lucerne growth (pH 5.3 to 8.0) (Staley et al., 1989, Latta et al., 2002) . During the pasture-establishment phase, lucerne is sensitive to acidic conditions due to the increased availability of inorganic elements such as aluminium (Wheeler and Dodd, 1995), and decreased survival rates of nitrogen-fixing microbes that are associated with nodulation (Pijnenborg et al., 1990).

In the early-stages of ryegrass establishment, the application of SHG had a positive effect on shoot growth however this benefit was not sustained. A similar result was observed in a field study in which commercial humic products were applied to grasslands (no species details given) and a positive growth effect was seen in the first cut, but not in the second (Verlinden et al., 2009). In agreement with the conclusion reached by the authors, this could be due to depletion of water-soluble carbohydrates by the initial burst of growth, leaving less reserves for regrowth (Fulkerson and Donaghy, 2001).

Early-stage promotion of ryegrass root growth, particularly at the lower soil depth, may improve pasture survival rates during drought when extensive exploration by roots could draw on water reserves deep in the soil profile (Jupp and Newman, 1987). The promotion of root growth in response to the application of HA from a range of sources has been identified in numerous studies (e.g. Canellas et al., 2002, Atiyeh et al., 2002, Adani et al., 1998). With the demonstrated ability of HA to bind

nutrients (Chen et al., 2004a) it is possible that the roots needed to explore further to find adequate nutrition for growth.

The lack of uptake response in the ryegrass shoot nutrients is in partial agreement with Adani et al., (1998) who observed no change in nitrogen, potassium and iron concentrations in tomato plants following application of a commercial HA product. In the same study, an increase in phosphorus concentration was identified but this was not observed here. Studies conducted by Hartz (2010) and Atiyeh et al., (2002) also observed no change in shoot nitrogen concentrations upon application of a commercial HA formulation to lettuce, and vermicompost-derived HA to tomatoes. Other studies show contrasting results in nitrogen concentration, with it increased in some crops (Verlinden et al., 2009, Verlinden et al., 2010, Çimrin et al., 2001), decreased in others (Akinremi et al., 2000) and increased or decreased depending on the application rate (Tan and Nopamornbodi, 1979). There is little consistency in nutrient concentrations and as has been demonstrated for plant growth (Rose et al., 2014), nutritional effects may also be dependent on the origin and rate of HA application, crop and/or soil type.

#### *5.4.3 Limitations and further research*

The lack of significant findings in soil organic nitrogen and inorganic nitrogen concentrations may be due to high variation in the data set. In field conditions, environmental factors such as temperature, moisture and spatial variability contribute to high analytical variation (Ros et al., 2011, Bhogal et al., 1999). An increase in the number of replicates would assist in reducing variability but with the time and labour intensive nature of these assays, it is not always practical. These assays are better suited to glasshouse and incubation studies in which spatial and temporal variability are reduced by pre-treatment of soils by sieving and homogenising, and controlled temperature and moisture (Ros et al., 2011, Smith and Li, 1993).

The use of suction lysimeters comes with very high spatial variability (Weihermüller et al., 2007, Keeney and Olson, 1986). Only the soil solution in the immediate vicinity is collected and so variables such as root density around the lysimeter can influence data variation. Strategies to overcome this could include installation of additional lysimeters to increase the number of replicates, use of a ceramic plate rather than a cup to cover more surface area or the use of resin bags that adsorb cations and anions (Keeney and Olson, 1986).

The periodic nature of gas sampling may result in missing data capture, resulting in a lack of significant trends. Static flux chambers are cheap and easy to use and it's possible to miss a flux event because of the periodic nature of the sampling. Although automated chambers are expensive, they provide near-continuous measurements of gas fluxes so that such events are not missed.

Further research could include additional field and laboratory studies. In the field, reapplication of the SHG product and DAP would show if further surges in ryegrass growth are possible. The application of SHG at higher rates would identify if the soil ammonium pool is further increased and the identification of abundance and functional changes in the soil microbes associated with nitrogen cycling could provide information about the mechanisms involved. In the laboratory, a column leaching study would verify the finding of higher nitrate in leachate, which is important when considering the environmental impact of SHG.

## **5.5 Conclusion**

The earlier provision and higher overall concentration of soil ammonium in soil amended with SHG and the increase in shoot and root growth of ryegrass are likely related. Investigation of the soil microbial community could assist in identifying the mechanisms involved. The early-stage promotion of ryegrass roots particularly at the lower soil depth could be beneficial in circumstances of low water availability. The promotion of nitrate loss from SHG amended soil could be verified in a column leaching experiment.



## 6 Metagenomic and functional responses of soil microbial communities to humate addition

This thesis chapter has been submitted to The ISME Journal and is currently under review.

### Declaration for thesis chapter 6.

Declaration by candidate.

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

<b>Nature of contribution</b>	<b>Extent of contribution (%)</b>
I designed the study, performed 50% of the laboratory work and was the primary author of the manuscript.	75%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

<b>Name</b>	<b>Nature of contribution</b>	<b>Extent of contribution (%) for student co-authors only</b>
Han Gan Ming	Performed 30% of the laboratory work	
Tim Cavagnaro	Contributed ideas to the work and co-authored the manuscript	
Tony Patti	Contributed ideas to the work and co-authored the manuscript	
W. Roy Jackson	Contributed ideas to the work and co-authored the manuscript	
Michael Rose	Contributed ideas to the work and co-authored the manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

**Candidate's  
Signature**

	<b>Date</b>
--	-------------

**Main  
Supervisor's  
Signature**

	<b>Date</b>
--	-------------



## Abstract

Commercial lignite-derived humate products for agriculture are promoted as plant growth stimulants and are also claimed to promote beneficial soil bacteria. Humic acid derived from sources other than lignite has been shown to increase the abundance (plate counts) of nitrogen-cycling bacteria but to our knowledge humate from lignite has not been investigated in this way. In an incubation study we investigated the effect of commercial soluble humate granules on soil bacteria using a metagenomic approach. We hypothesised that the addition and application rate of this product would alter microbial activity and influence the abundance of families associated with nitrogen-cycling. Investigation of bacterial diversity by high-throughput 16S rRNA amplicon sequencing showed that increasing the humate application rate increased microbial activity and had an effect on bacterial phyla, with *Proteobacteria* promoted at the expense of *Chloroflexi*. The abundance of the nitrogen-cycling families *Rhodospirillaceae*, *Hyphomicrobiaceae*, *Bradyrhizobiaceae*, *Beijerinckiaceae*, *Burkholderiaceae* and *Nitrosomonadaceae* increased while *Nitrospiraceae* remained unchanged. Other bacterial families whose abundance increased significantly were *Xanthomonadaceae*, *Sinobacteraceae* and *Chitinophagaceae* while the abundance of *Gemmataceae*, *Streptomycetaceae* and *Ktedonobacteraceae* decreased. These results suggest altered soil nitrogen-cycling which could contribute to the growth promotion of crops and pasture commonly associated with humate application.

## 6.1 Introduction

Global demand for food, fibre and fuel is growing, and this trend is expected to continue as the global human population increases in number (Erb et al., 2012, Bommarco et al., 2013, Robertson et al., 2014). These demands necessitate an increase in global agricultural production however these increases must be made in a time of significant environmental change and in a sustainable manner (Tilman et al., 2011, Godfray and Garnett, 2014). Management practices that involve the use

of organic-based fertilizers and biostimulants, including humic substances, are being promoted as one means to achieve this goal (Quilty and Cattle, 2011, Calvo et al., 2014).

There is substantial evidence demonstrating that humic substances can increase plant growth and crop yields (Rose et al., 2014, Olk et al., 2013) through a variety of mechanisms (Trevisan et al., 2010). For example, Chen et al. (2004a) proposed that one of the major mechanisms by which humic substances promote plant growth is via increased micronutrient availability, and that this results in larger root systems through nutritional feedback. Alternative hypotheses suggest that humic substances possess hormone-like activities that can directly stimulate root branching and enhanced nutrient uptake (Trevisan et al., 2010, Mora et al., 2010). It has also been suggested that humic addition may have an impact on soil microbial communities, which may have consequences for soil nutrient cycling (Pascual et al., 1999, Visser, 1985b, Visser, 1985a).

Humic substances can interact with soil and fertilizer nitrogen through both abiotic and biotic mechanisms, with significant implications for plant growth. For example Al-Kanani et al. (1990) showed that humic substances reduced urea hydrolysis from urea–ammonium nitrate and also retarded the formation of nitrate in soil, implying urease- and nitrification inhibition activity. Reduced urea hydrolysis in soils treated with humics has been linked to biological buffering properties of the humic substances on microbial communities and enzyme activities (Dong et al., 2009). Similarly, by formulating a nitrogen/phosphorus/potassium (NPK) fertilizer with humic substances, Erro et al. (2007) were able to reduce ammonia volatilization and nitrogen leaching with respect to an NPK control fertilizer, leading to increased plant growth. Humic substances applied together with nitrogen-fixing bacteria were also shown to promote root colonization by the bacteria and subsequently improved plant growth (Canellas et al., 2013). Given the central role that microbes

play in the soil nitrogen cycle, changes in nitrogen cycling with humic addition may be due to impacts upon soil microbes.

Despite knowledge that microorganisms play a key role in regulating nitrogen transformations, the availability of other macro and micronutrients, and plant growth, there is little information about the direct impacts of humic substances on soil microbial communities (Bünemann et al., 2006). Of the few studies available, some show that humic substances increase the growth of some microbial groups (Vallini et al., 1993, Valdrighi et al., 1996), whilst other studies show minimal (Kim et al., 1997, Albiach et al., 2000) or even negative (Whiteley and Pettit, 1994) impacts on microbial communities and activity. Even less information is known about the impacts on specific microbial functional groups involved in mediating nitrogen transformations, and most of this information comes from culture-dependant studies (Valdrighi et al., 1996, Visser, 1985a).

While culture-dependant studies are important in investigating microbial communities in changed environments they are biased in their evaluation of microbial diversity by selecting specific populations and are limited with only a small proportion of microorganisms being culturable (Hill et al., 2000). Recent advances in sequencing technologies provide a considerable amount of taxonomic information and have changed our understanding of microbial diversity in the environment. A high-throughput 16S rRNA amplicon sequencing technique has been used to study microbial communities in grassland and forest soils (Roesch et al., 2007, Lauber et al., 2009, Nacke et al., 2011), activated sludge (Sanapareddy et al., 2009, Yu and Zhang, 2012), and freshwater sediments (Röske et al., 2012, Wang et al., 2012). These studies demonstrate that the method can be reliably applied to a variety of matrices and can detect changes in microbial groups that represent as little as 0.1% of the population.

Here we present the results of a study in which we sought to determine the effect of a commercial lignite-derived potassium humate product on the soil microbial community. Lignite-derived humate was used because it is widely available and represents the most common form marketed to farmers. We hypothesised that the addition of soluble humate granules (SHG) would alter the activity and composition, and influence the abundance of families associated with soil nitrogen cycling. We also hypothesised any effect of SHG would be depend on application rate, as has been shown in numerous plant-based studies. These hypotheses were tested in a microcosm-based experiment in which soil respiration, inorganic nitrogen pools and bacterial diversity were determined by a high-throughput 16S rRNA amplicon sequencing technique. To our knowledge this is the first study of the metagenomic responses of soil communities to SHG application.

## **6.2 Materials and methods**

### *6.2.1 Soil collection and characterization*

The soil used for this study was a Podosol, collected from a vegetable farm recently converted from pasture in Cranbourne, Victoria (38°11'6"S, 145°18'50"E). The soil was collected from the 0-10 cm soil layer, air-dried and sieved to < 2 mm. The soil was analysed for organic matter (OM), carbon and nitrogen by dry combustion, pH in 1:5 water, Colwell phosphorus by bicarbonate extraction, potassium by ammonium acetate extraction, and ammonium and nitrate by KCl extraction (by The Environmental Analysis Laboratory (EAL), <http://scu.edu.au/eal/>) and are presented in Table 6.1. Based on this analysis, we added N, P and K at 100, 40 and 60 kg/ha respectively to match typical fertilization practice for pasture-based systems.

**Table 6.1** Physicochemical properties of the soil prior to the addition of nitrogen, phosphorus and potassium fertilizers and soluble humate granules (SHG).

pH (H <sub>2</sub> O)	OM (%)	C (%)	N (%)	P (Colwell) (mg/kg)	K (mg/kg)	NH <sub>4</sub> <sup>+</sup> (mg/kg)	NO <sub>3</sub> <sup>-</sup> (mg/kg)	Texture
6.0	1.6	0.9	0.1	31.0	81.0	8.3	4.7	Sandy

### 6.2.2 Lignite-derived organic amendment

The lignite-derived amendment was a commercially-available granulated soluble potassium humate (SHG) product. Key physicochemical properties of the product were quantified as follows: pH was determined in triplicate 5 g sub-samples suspended in deionized water (1:5 w/v) using a TPS WP81 meter and probe. An additional 5 g sub-sample was used for humic acid (HA) content determination by repeated alkaline extraction using a modification of the International Humic Substance Society (IHSS) method (Swift, 1996), as follows. To each product, 0.1M HCl was added to give a 10:1 acid to SHG ratio (v/w). The slurry was then shaken at 120 rpm for 1 h and allowed to settle for 12 h. The supernatant was removed and discarded. Under an N<sub>2</sub> atmosphere, 0.1M NaOH was added to the solid residue at a ratio of 100:1 (V/W). The slurry pH was adjusted to 12.6 with 1M NaOH and shaken at 120 rpm for 4 h (Hayes et al., 2008). The pH of the slurry was reduced to 9 using 1M HCl, and the solids were allowed to settle for 12 h. The supernatant was removed and retained and alkaline extraction of the remaining solid was repeated a further seven times until the supernatant was a pale brown colour. The supernatants were pooled and HA precipitated by adjustment to pH 2 with 1M HCl. The humic acid was then dialysed in cellulose membrane dialysis tubing MWCO 12000 (Sigma-Aldrich, St Louis, MO, USA) in deionized water until the conductivity of the surrounding water was less than 20 uS/m. The HA was then oven dried at 37°C and weighed. This procedure was undertaken in triplicate. A sub-sample was ground to a fine powder using a mortar and pestle, homogenised and divided into two. The first sub-sample was analysed for total carbon, hydrogen and nitrogen by

dry combustion (by The Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand: <http://neon.otago.ac.nz/consulting/microlab/>; accessed April 2014). The second sub-sample was analysed for phosphorus and potassium by inductively coupled plasma-optical emission spectrometry (by Waite Analytical Services, University of Adelaide, Urrbrae, S. Aust.: <http://www.adelaide.edu.au/was/>; accessed April 2014). The product composition is shown in Table 6.2.

**Table 6.2** Physicochemical properties of the soluble humate granulated (SHG) product.

<b>pH</b> (H <sub>2</sub> O)	<b>Moisture</b> (%)	<b>HA</b> (%)	<b>C</b> (%)	<b>N</b> (%)	<b>Ca</b> (%)	<b>Fe</b> (%)	<b>K</b> (%)	<b>P</b> (%)	<b>S</b> (%)	<b>Al</b> (%)
9.4	4.5	50.2	47.5	1.3	8.3	4.7	8.5	0.1	0.3	1.0

### 6.2.3 *Microcosm set up and monitoring*

The SHG was added to the soil at rates of 0, 20 and 300 kg/ha. These rates span the range of additions typically used by farmers (Rose et al., 2014). The treatments were applied by thoroughly mixing 0, 2 and 30 mg of SHG granules in 100 g of soil in sterile 250 mL polypropylene microcosms. Five replicates were prepared of each application rate, giving a total of 15 microcosms. Following mixing, deionized water was sprayed onto the soil surface using a misting bottle, until a soil moisture content equal to 60% field capacity was achieved. The opening of each microcosm was loosely covered with a plastic cap to minimise moisture loss, while allowing gas exchange. The microcosms were then incubated at 25°C in the dark for six weeks during which time the soil moisture content was maintained at 60% of field capacity, as described above.

Soil respiration in the microcosms was measured at 0.08, 1, 4, 7, 14, 21, 28 and 42 days after the start of the experiment as follows. With the lid loosely covering the microcosm opening, an initial gas sample was taken from the headspace. Prior to

taking the sample, the air in the headspace was mixed by filling and emptying a 5 mL gas-tight syringe (SGE, 25MDR-LL-GT) back into the microcosm. Then, a 2 mL gas sample was taken and injected into a CO<sub>2</sub> gas analyser (LICOR, LI-820, USA). The microcosm was sealed immediately with the lid and left for 90 min at room temperature (21 ± 1°C) before taking the next gas sample through a central rubber septum in the lid of the microcosm, again mixing the air before taking the sample (Smith et al., 2012). Prior measurements of this soil had established a linear CO<sub>2</sub> flux over this time period (data not shown). Measured CO<sub>2</sub> concentrations were converted to mg/m<sup>2</sup>/h according to van Zwieten et al. (2010b) .

#### *6.2.4 Harvesting and soil analysis*

At the conclusion of the incubation period (42 days), the microcosms were destructively harvested by removing the soil and mixing it (for each microcosm separately) thoroughly. The soil from each microcosm was then divided into four sub-samples. The first sub-sample was frozen at -20°C immediately for DNA metagenomic analysis (see below). The second sub-sample (10 g) was weighed, dried at 105°C (until constant mass was achieved), weighed again, and gravimetric moisture content calculated. The third subsample (7 g) was shaken with 2M KCl for 30 mins at 200 rpm, centrifuged at 3000 rpm for 10 mins, and the supernatant analysed for ammonium and nitrate (plus nitrite) using the methods of Forster (1995) and Miranda et al. (2001). The fourth sub-sample was air-dried and used for pH determination in triplicate 5 g sub-samples suspended in deionized water (1:5 W/V) using a TPS WP81 meter and probe.

#### *6.2.5 DNA extraction and sequencing analysis*

Soil genomic DNA was extracted using the PowerSoil® DNA Isolation Kit (MoBio Laboratories), following the manufacturer's procedure with the only exception being a double wash with Solution C5 on the spin filter prior to DNA elution. Yields and purity of the DNA were determined by NanoDrop (Thermo Scientific, MA, USA) at 260 and 280 nm. High fidelity PCR was performed on each genomic DNA

template (normalized input of 20 ng) using NEBNext high fidelity PCR mastermix and Illumina-compatible primers targeting the V3-V4 region of microbial 16S rDNA (Klindworth et al., 2012, Bartram et al., 2011). The PCR conditions involved an initial denaturation step at 98° C for 30 sec followed by 30 cycles of 98° C for 10 sec, 60° C for 30 sec and 72° C for 30 sec and ended with an extension step at 72° C for 1 min. The PCR products were separated on a 2% TAE agarose gel and the band corresponding to the size of approximately 600 bp was excised and purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany). The gel purified products were quantified using KAPA library quantification kit (KAPA Biosystems, Cape Town, South Africa), normalized and pooled in equal molar amounts. The final pooled library was denatured and subsequently sequenced on the MiSeq (2 x 250 bp paired-end run).

#### *6.2.6 Bioinformatics*

Each pair of fastq files generated for each sample were overlapped and trimmed (4 bp from 5' end) using PandaSeq (Masella et al., 2012). The generated output was subsequently dereplicated, clustered at 97% identity cutoff and chimera-filtered using UPARSE (Edgar, 2013) with a slight modification e.g. for uchime\_ref, the default gold.fa reference was replaced with the more comprehensive Greengene OTU database (gg\_otu97). Taxonomy assignment, abundance estimation, diversity metric calculation and PCOA plot construction were performed using QIIME 1.8 (Caporaso et al., 2010).

#### *6.2.7 Calculations and statistical analysis.*

All data were analysed using JMP statistical software (JMP®, Version 10, SAS Institute Inc., Cary, NC). All microbial data is expressed as relative abundance (%). Prior to analysis, microbial abundance data was arcsin transformed however untransformed data is presented for ease of interpretation. All microbial abundance and soil characterisation data were analysed by one way ANOVA and where significant differences were found, pairwise comparisons were made using Tukey's



honestly significant difference (HSD). The post-incubation ammonium data was highly variable and so the Students t-test was used to identify significant differences between treatments.

## 6.3 Results

### 6.3.1 Soil physicochemical properties

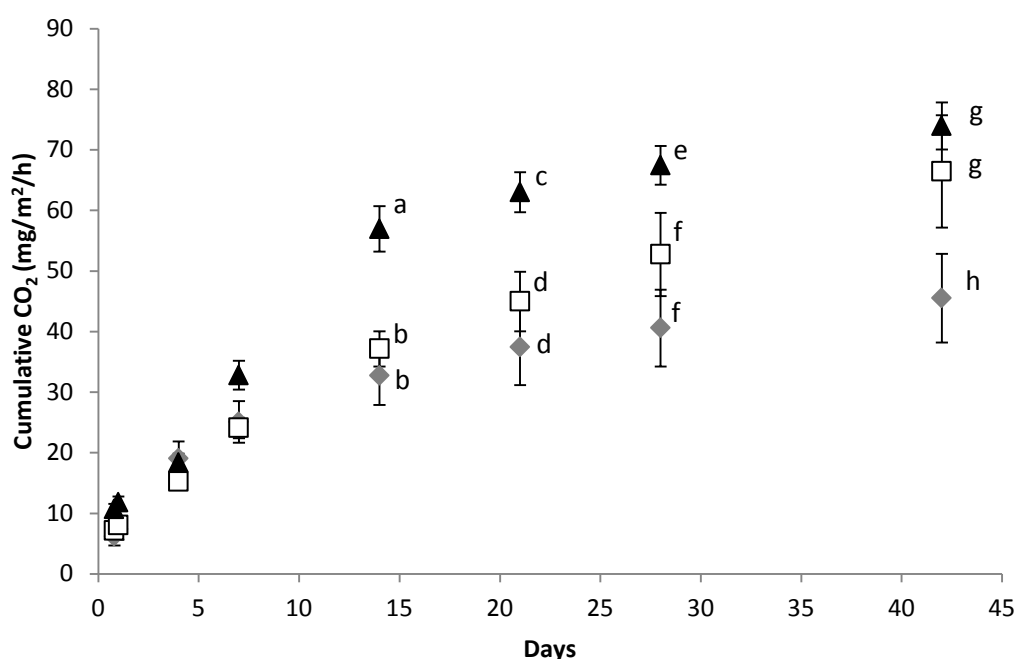
The addition of SHG did not have a significant ( $P>0.05$ ) impact on soil pH, ammonium or nitrate concentrations (Table 6.3). Although there were also no significant differences in soil ammonium concentrations identified in the ANOVA, there was a trend towards a decrease in ammonium concentration with SHG addition. Further analysis of soil ammonium concentration data using targeted Students t-tests, revealed that concentrations were significantly ( $P=0.05$ ) lower in the 300 kg/ha addition treatment compared to the unamended control. No other differences were revealed in this targeted analysis.

**Table 6.3** Post-incubation ammonium ( $\text{NH}_4^+\text{-N}$ ), nitrate ( $\text{NO}_3^-\text{-N}$ ) and pH of soil amended with SHG at 0, 20 or 300 kg/ha. No significant differences were identified by ANOVA analysis but post-incubation  $\text{NH}_4^+\text{-N}$  concentration in unamended soils compared to soil amended with 300 kg/ha of SHG were significantly different ( $P=0.05$ ) by Students t-test\*.

SHG application rate (kg/ha)	$\text{NH}_4^+\text{-N}$ ( $\mu\text{g/g}$ dry soil)	$\text{NO}_3^-\text{-N}$ ( $\mu\text{g/g}$ dry soil)	Soil pH
0	6.64* ( $\pm 1.39$ )	291.2 ( $\pm 23.9$ )	4.12 ( $\pm 0.03$ )
20	6.35 ( $\pm 0.79$ )	275.5 ( $\pm 8.1$ )	4.17 ( $\pm 0.01$ )
300	3.51* ( $\pm 0.30$ )	274.3 ( $\pm 11.7$ )	4.14 ( $\pm 0.02$ )

### 6.3.2 Microbial activity

The cumulative CO<sub>2</sub> respired (Figure 6.1) was significantly higher for SHG amended soils. This increase in cumulative respiration was evident at 14 days in the soil receiving 300 kg/ha and only at the end of the experiment in soil amended with SHG at a rate of 20 kg/ha.



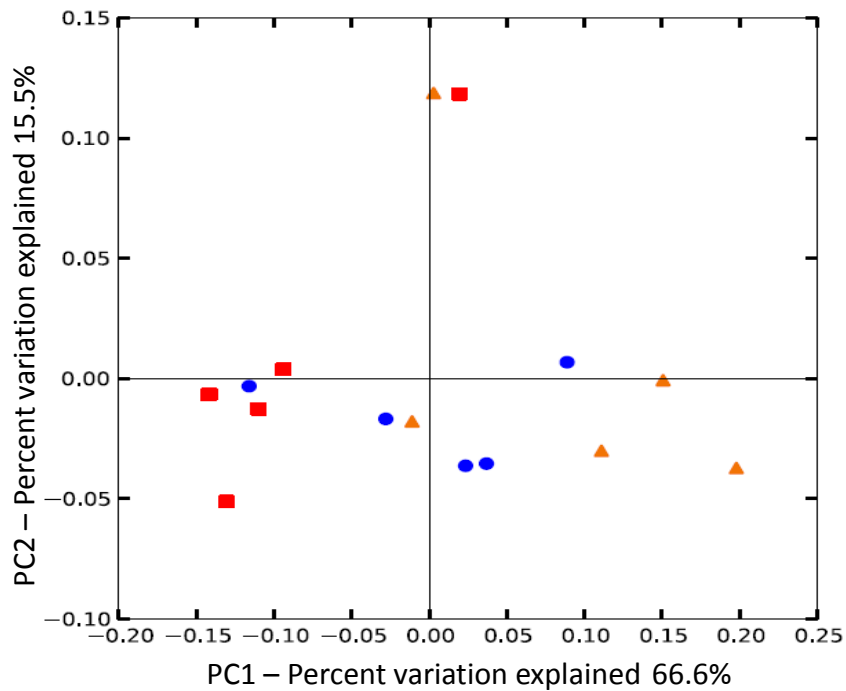
**Figure 6.1** Cumulative CO<sub>2</sub> calculated from CO<sub>2</sub> flux measured at 0.8, 1, 4, 7, 14, 21, 28 and 42 days during the incubation period from unamended and soils amended with 20 and 300 kg/ha of SHG.  $\blacklozenge$  represents 0 kg/ha,  $\blacktriangle$  represents 20 kg/ha and  $\square$  represents 300 kg/ha amendment with SHG. Values allocated different letters are significantly different at the  $P < 0.05$  level as assessed by Tukey's HSD. Values without allocated letters were not significantly different at the  $P < 0.05$  level. Mean values are presented ( $n=5$ ) and error bars represent  $\pm$  s.e.

### 6.3.3 Soil bacterial activity, diversity and composition

Levels of bacterial diversity (Shannon) and evenness (Simpson) (Table 6.4) were generally high (Chau et al., 2011, Fierer and Jackson, 2006) and richness (Chao1) moderate (Morales et al., 2009). Although there were no differences in these indices among SHG addition treatments, there were distinct differences in the microbial community structure of each of the treatments. Analysis of the soil bacterial community structure by PCOA showed general clustering patterns between unamended and SHG amended soils (Figure 6.2). With the exception of two outliers, the unamended soil was well separated from the soil amended with 300 kg/ha of SHG. PC1 explained 66.6% of the variability, and PC2 explained 15.5% of the variability.

**Table 6.4** Indices of Shannon diversity, richness and Simpson evenness. Mean values are presented (n=5) and values in brackets are  $\pm$  s.e.

SHG application rate kg/ha	Richness Chao1	Diversity Shannon	Evenness Simpson
0	1976 ( $\pm$ 47)	8.7 ( $\pm$ 0.1)	0.994 ( $\pm$ 0.0002)
20	1954 ( $\pm$ 35)	8.5 ( $\pm$ 0.1)	0.993 ( $\pm$ 0.0003)
300	1980 ( $\pm$ 42)	8.5 ( $\pm$ 0.1)	0.993 ( $\pm$ 0.0007)
<i>P</i> value	0.89	0.16	0.08



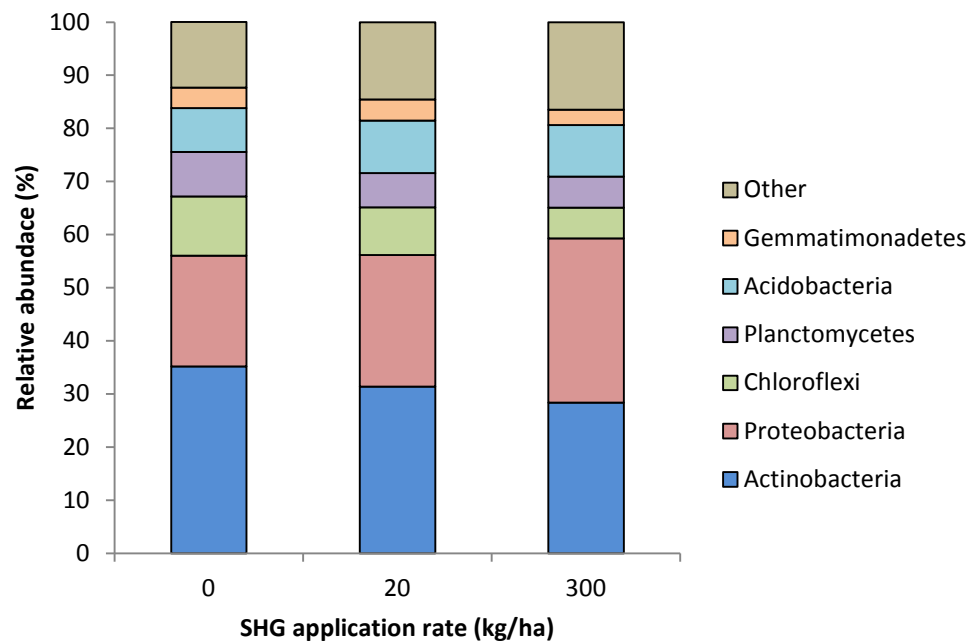
**Figure 6.2** PCOA analysis of the soil bacterial community in soils amended with 0, 20 and 300 kg/ha of SHG. Soils amended with 300 kg/ha of SHG are clustered towards the left of the plot and unamended soils clustered towards the right of the plot. ▲ represents 0 kg/ha, ● represents 20 kg/ha and ■ represents 300 kg/ha.

### 6.3.4 Microbial community composition

#### *Bacterial phyla abundance*

Sequences belonging to 25 different bacterial phyla were identified (Figure 6.3). The dominant phyla detected were *Actinobacteria* (28.4-35.2% across all samples), *Proteobacteria* (20.8-30.9%), *Chloroflexi* (5.8-11.1%), *Planctomycetes* (5.8-8.4%), *Acidobacteria* (8.3-9.9) and *Gemmatimonadetes* (3.8-4.0%). Phyla representing less than 2.5% of the microbial community, including unclassified sequences (0.94-1.17% across all samples) were classified as Other (12.3-16.5% across all samples). The application of SHG had an effect on the proportions of phyla in each soil microbial community. The application of SHG significantly promoted the abundance

of *Proteobacteria* ( $P=0.008$ ) which represented 21% of the total bacterial diversity in unamended soil and 31% in soil amended with 300 kg/ha of SHG. The application of SHG significantly reduced the abundance of *Chloroflexi* ( $P=0.01$ ) which represented 11% in unamended soil and 6% in soil amended with 300 kg/ha. The abundance of phyla *Actinobacteria* ( $P=0.13$ ), *Planctomycetes* ( $P=0.12$ ), *Acidobacteria* ( $P=0.07$ ) and *Gemmatimonadetes* ( $P=0.13$ ) were not significantly altered by the application of SHG.



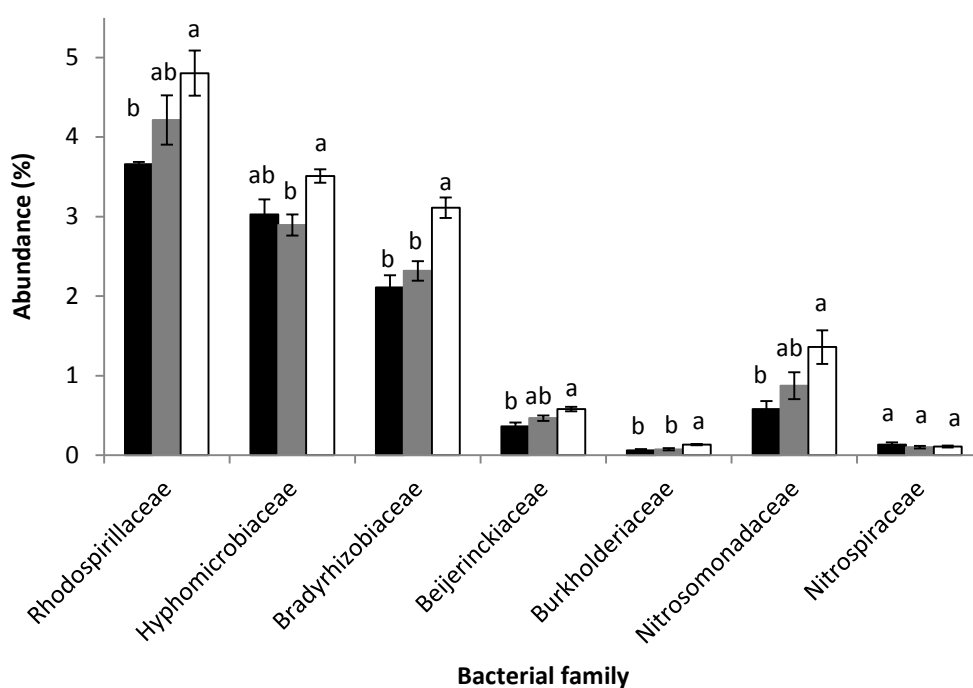
**Figure 6.3** Relative abundance of bacteria phyla with application rate of SHG. Mean values are presented ( $n=5$ ). Phyla representing less than 2.5% of the microbial community in each soil were classified as 'Other'.

### *Bacterial family abundance*

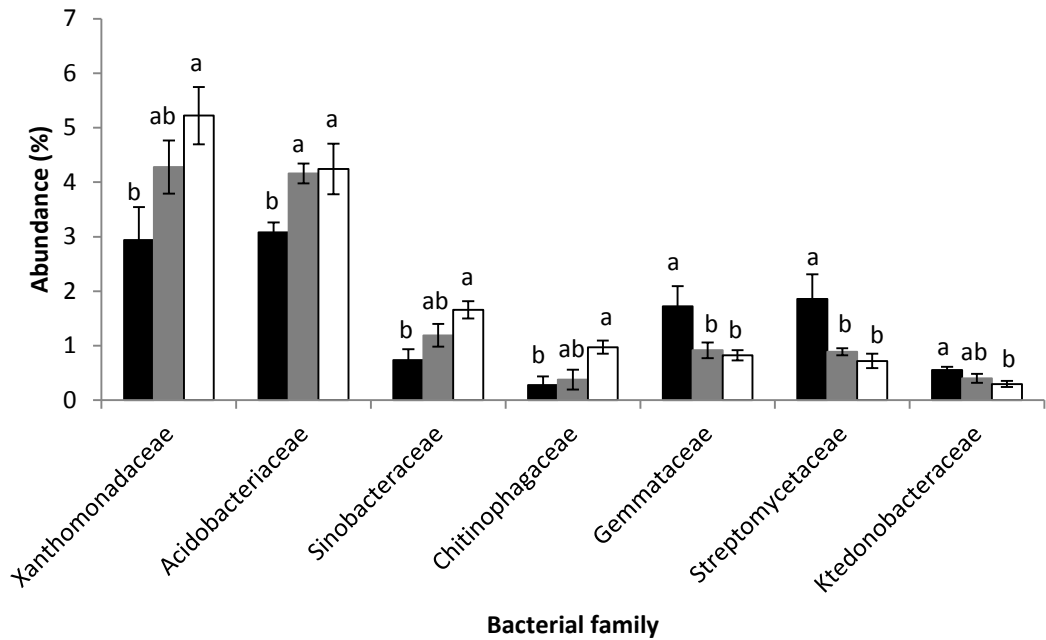
Bacterial families with a role in soil nitrogen cycling responded differently to SHG application. The abundance of *Rhodospirillaceae* ( $P=0.02$ ), *Hyphomicrobiaceae*

( $P=0.027$ ), *Bradyrhizobiaceae* ( $P=0.0006$ ), *Beijerinckiaceae* ( $P=0.005$ ), *Burkholderiaceae* ( $P=0.0076$ ) and *Nitrosomonadaceae* ( $P=0.020$ ) increased significantly with SHG addition. The exception to this was *Nitrospiraceae* ( $P=0.54$ ), which did not respond to SHG addition (Figure 6.4).

There were a number of other bacterial families whose abundance changed significantly with SHG addition. Specifically *Xanthomonadaceae* ( $p=0.03$ ), *Sinobacteraceae* ( $P=0.017$ ) and *Chitinophagaceae* ( $P=0.02$ ) increased and *Gemmataceae* ( $P=0.026$ ), *Streptomycetaceae* ( $P=0.0099$ ) and *Ktedonobacteraceae* ( $P=0.037$ ) decreased with increasing SHG application rate (Figure 6.5).



**Figure 6.4** Abundance of soil bacterial families associated with nitrogen cycling in response to amendment with SHG. Black bars represent unamended soil, grey bars amendment with 20 kg/ha SHG and white bars 300 kg/ha SHG. Mean values are presented ( $n=5$ ) and error bars represent  $\pm$  s.e. Within each bacterial family, values allocated the same letter were not significantly different at the  $P<0.05$  level as assessed by Tukey's HSD.



**Figure 6.5** Abundance of other soil bacterial families in response to SHG amendment. Black bars represent unamended soil, grey bars amendment with 20 kg/ha SHG and white bars 300 kg/ha SHG. Mean values are presented (n=5) and error bars represent  $\pm$  s.e. Within each bacterial family, values allocated the same letter were not significantly different at the  $P < 0.05$  level as assessed by Tukey's HSD.

## 6.4 Discussion

The application of SHG stimulated microbial activity and altered the community composition. Cumulative respiration was greater with the addition of SHG in both the 20 and 300 kg/ha treatments likely due to stimulation of microbial activity by the addition of a carbon source, in this case the SHG product. While microbial community richness and diversity remained the same, there was a trend of evenness decreasing with application of the product. Multivariate analysis revealed that the composition of the soil microbial community was strongly influenced by

application of the SHG product, with clustering of the unamended soil well separated from that amended with 300 kg/ha of SHG.

In addition to changes in the diversity and activity of the soil microbial community there were also changes at the level of bacteria phyla. We detected 25 bacterial phyla across all three amendment conditions (0, 20 and 300 kg/ha) with approximately 85% belonging to a narrow variety which were *Actinobacteria*, *Proteoacteria*, *Chloroflexi*, *Planctomycetes*, *Acidobacteria* and *Gemmatimonadetes*; phyla that are commonly dominant in soil (Janssen, 2006, Fierer et al., 2007), some which are important in soil nitrogen cycling. In particular, in response to SHG application an increase in abundance of *Proteobacteria* (Philippot et al., 2007), largely at the expense of the *Chloroflexi* phylum.

Microbial community compositional changes were also detected in bacterial families. With the application of SHG, the abundance increase of the symbiotic dinitrogen fixers *Hyphomicrobiaceae*, *Bradyrhizobiaceae*, *Beijerinckiaceae*, and *Burkholderiaceae* and the free-living dinitrogen-fixers *Rhodospirillaceae* (Madigan et al., 1984) suggests an increased rate or capacity for microbial contribution to plant-available ammonium. Increases in soil ammonium have been identified in response to the addition of humic acid in previous studies (Ahmed et al., 2006, Kasim et al., 2009). In this study, while there was no accumulation of ammonium in the amended soil, given the aerobic conditions and increase in abundance of the nitrifying bacteria *Nitrosomandaceae*, we could speculate that nitrification was also occurring.

There was no accumulation of soil nitrate which would be expected in light of the increases in abundance of *Burkholderia*, *Nitrosomanas*, *Bradyrhizobium*, *Hyphomicrobium* and *Rhizobium* which can both fix dinitrogen and denitrify (Philippot et al., 2007). These results complement those of a culture-dependant study in which HA isolated from a loam soil to selective media stimulated activity of



denitrifying bacteria (Visser, 1985a). Biochar amendment also stimulates denitrifying bacteria (Nielsen et al., 2014) and reduces nitrous oxide emissions as shown by increases in gene copy numbers of nirK, nirS and nosZ (Wang et al., 2013). With HA stimulation of denitrifying bacteria as shown in this study, there are grounds to investigate gene copy numbers and denitrification rates through isotopic-nitrogen studies (Meijide et al., 2010, Baggs, 2008) to determine possible changes to nitrous oxide emissions.

Amendment with SHG and the resulting increased abundance of nitrogen cycling bacteria could be beneficial for farmers, particularly if nitrous oxide emissions are reduced. Potentially, the increased availability of inorganic nitrogen species due to the increased microbial activity could contribute to the growth promotion of crops and pasture commonly associated with HA application (e.g. Fernández-Escobar et al., 1996, Adani et al., 1998, Atiyeh et al., 2002, Rose et al., 2014). This may also explain the increased nitrogen concentration in shoot tissue as has been identified in a number of studies (Verlinden et al., 2010, Verlinden et al., 2009, Çimrin et al., 2001, Little et al., 2014).

With application of SHG there were significant changes in the abundance of other bacterial families. *Acidobacteriaceae* increased in abundance with increasing SHG application rate. These bacteria are found in a wide range of environments and constitute a large component of the soil bacteria. They grow more prevalently in soil that is less than pH 6 which explains their prevalence here (Sait et al., 2006). Their function in the soil has not been fully resolved but there is suggestion that they play a role in biogeochemical cycling of carbon and are capable of nitrification (Ward et al., 2009, Quaiser et al., 2003).

Members of *Xanthomonadaceae* vary widely in function including pathogenic species and those that produce compounds that can suppress the growth of pathogens (Kyselkova and Moenne-Loccoz, 2012). *Sinobacteraceae* are not well

described but are of the same order as *Xanthomonadaceae*. There is the suggestion that they could also play a role in the inhibition of plant pathogens (Naushad and Gupta, 2013). As there were no specific species of these families identified, the implications of increased abundance with SHG application are not clear. Similar increases in abundance were identified in response to biochar addition (Anderson et al., 2011). *Chitinophagaceae* which are characterized as being free-living and saprophytic (Kämpfer, 2011) with the ability to produce chitinases to degrade chitin (Del Rio et al., 2010). The increase in abundance of this family with application of SHG suggests the potential to increase the rate of liberation of nutrients from soil organic matter.

Generally the presence of *Streptomycetaceae* in soil is considered to be of benefit due to their role in mineralisation of plant and animal matter (Schrempf, 2008) however there are pathogenic strains such as *Streptomyces puniscabiei* (Loria et al., 2006). In this study, *Streptomyces* represented 57% of the sequences however as there were no specific species of these families identified, the implications of their decreased abundance is not clear.

*Gemmataceae*, a member of the *Planctomycetes* phylum has been isolated from a diverse range of environments (Fuerst and Sagulenko, 2011, Wang et al., 2002). It is known that reproduction is by budding, and that this family is unique in that no peptidoglycan is found in the cell walls (König et al., 1984) but investigation of the role that this bacteria plays in soil is in its infancy. Similarly, *Ktedonobacteriaceae*, a member of the *Chloroflexi* phylum has not been well characterized, with a pure culture of a strain from this family only recently achieved (Cavaletti et al., 2006). Consequently the impact of decreased abundance of these families in response to the addition of SHG is not able to be determined.

## **6.5 Conclusion**

Soil amendment with SHG increased microbial activity and altered the microbial community composition, with the higher application rate having the most impact. The increased abundance of families of dinitrogen-fixing, nitrifying and denitrifying bacteria implies a greater potential for nitrogen-flux through the soil system. Further quantification of associated functional genes (*nif*, *amoA*, *nirS*, *nirK* and *nosZ*) combined with biochemical measurements of process rates are needed to confirm the extent of any alterations to nitrogen cycling.



## **7 General discussion, conclusion and future research**

### **7.1 General discussion**

This thesis describes the findings of a cross-disciplinary study to characterize a range of Victorian lignite-derived products, the growth response of pasture species and effects on key indicators of soil health. As is common in research conducted in plant/soil systems, there were challenges such as variability in the chemical composition of the products, sampling from highly heterogeneous environments and inherent differences between glasshouse and field studies. As well as the challenges, working within this biological system has presented the opportunity to apply advancements in molecular biology tools to study the soil bacterial community response to humate amendment.

Scientific studies were needed of the commercial lignite-derived products currently available to farmers. The products studied here were chemically complex. The source of the lignite coal, method of humate extraction, identity and concentration of nutrient additives were not disclosed by the manufacturers. Often humic acid (HA) extracts are prepared by the researchers themselves and so the source and extraction method are known and the nutrient additions are controlled. Due to the high variability of nutrient concentrations between products, the application rates used in each study could not be normalized to a single property, e.g. % HA as this would have resulted in the application of some products at unrealistic rates and potentially excessive additions of nutrients. The approach taken was to initially screen the products for pasture growth improvements using the manufacturer's recommended application rate (Chapter 3). These results were then used to define the range of application rates for subsequent experiments (Chapters 4, 5 and 6).

In the glasshouse study presented in Chapter 4, the effect of application rate of soluble humate granules (SHG) on the growth of lucerne at an application rate of 20

kg/ha resulted in an increase in lucerne shoot and root growth. In the outdoor mesocosms (Chapter 5) despite the same soil type, product and application rate, a shoot or root growth benefit for lucerne was not observed. As other studies have also shown, extrapolation of results from glasshouse trials to field trials is not always possible (Bell et al., 2000, Zeller et al., 2010, Birch et al., 2007). The likely reason for the differences observed here is that the glasshouse conditions are tightly controlled and so seasonal changes had no impact on lucerne growth. This along with a regular watering regime resulted in the maintenance of optimal conditions for pasture growth for the duration of the experiment which was not the case in the outdoor mesocosms.

The addition of HA to soil reportedly enhances the plant uptake of nutrients (Chen et al., 2004a) however little evidence of this was seen here. For example, increased uptake of potassium was detected in ryegrass in a low organic matter soil (Chapter 3) but this result was not replicated in ryegrass grown in a similar soil in the mesocosms (Chapter 5). In each case, soil amendment was with a different product type (run-of-mine (crushed) lignitic coal and soluble humate granules (SHG) respectively) suggesting that nutrient effects could depend on the product type or form. The most interesting result was that of lucerne nitrogen uptake. Both the application of humate soil conditioner (Chapter 3) and SHG (Chapter 4) decreased nitrogen uptake and for SHG application rate was an important factor, with decreases seen only at the higher rates. In the mesocosm study in which SHG was applied at 20 kg/ha (Chapter 5), no change in lucerne shoot nitrogen concentration was detected which was consistent with the findings in Chapter 4, with shoot nitrogen decreases only occurring at the higher application rates of 50 and 100 kg/ha. These results suggest that the SHG product binds nitrogen species in the soil, which may have an impact on nitrogen cycling.

Further evidence of interaction of the SHG product with soil nitrogen species were identified in both the mesocosm (Chapter 5) and the metagenomic studies (Chapter

6). In the mesocosm study, in the amended soils, the increase in soil ammonium indicated that the product may bind ammonium thereby increasing the soil concentration. In the metagenomic study in which next-generation sequencing was applied to DNA extracted from SHG amended soils, a wide range of bacterial species were present; however, significant abundance increases occurred mainly in those associated with nitrogen cycling. The evidence here of an interaction of SHG with soil nitrogen species provides a basis for further research into nitrogen cycling and plant-availability and mechanistic studies to identify if pasture growth increases can be attributed to product induced changes in soil nitrogen status.

When a landholder is considering the use of lignite-derived products, the financial cost for maximum agronomic benefit must be taken into account. For example, the application of SHG resulted in significantly less nitrogen in lucerne shoots (Chapter 5) and while this may suggest nitrogen fertilizer savings, the observation was only made at the higher application rates of 50 and 100 kg/ha, which is approximately 12 and 25 times respectively the manufacturer recommended application rate. Generally the application rate recommended by manufacturers is low, likely for economic viability (Hartz, 2010, Jones et al., 2007). It is recommended that farmers conduct their own trials or seek to consult an agronomist to ascertain if the products can provide benefits within their specific soil and pasture system.

The responses of key indicators of soil health including soil pH, mycorrhizal colonization and microbial biomass carbon (MBC) to the application of commercial lignite derived products were varied with no consistent changes detected. Soil pH was measured in product amended soils from glasshouse (Chapters 3 and 4), incubation (Chapter 4) and mesocosm (Chapter 5) studies and across all conditions there was no evidence that any of the products influenced or buffered soil pH in acidic soils as is claimed by manufacturers. Mycorrhizal colonization, which enables the enhanced uptake of nutrients, was stimulated or depressed depending on the applied product or soil type (Chapter 3) and the application rate was also shown to

have an effect (Chapter 4), with SHG applied at 20 kg/ha only stimulating colonization. This was linked to the soil phosphorus status rather than product application. Product application had little effect on soil MBC (Chapters 3 and 4). Increases were detected in the lower organic matter soil as would be expected when a carbon source is added to a low carbon background (Chapter 3) but interestingly increasing the SHG application rate did not further increase the MBC (Chapter 4). While there were no strong trends identified, the addition of these products was not detrimental to soil health from any of the aspects considered here.

## **7.2 Limitations**

Soil is a complex and highly heterogeneous environment and as such, issues of representative sampling arise. Studies showing significant spatial variability particularly of gas emissions and nitrogen transformations in soil are numerous (e.g. Laverman et al., 2000, Savage and Davidson, 2003, Fang et al., 1998). At the conclusion of pot and incubation studies, the soil can be thoroughly mixed before sampling to decrease analytical variability (Ros et al., 2011, Smith and Li, 1993). In field conditions, one way to account for high spatial variability is to increase the number of sampling replicates. This is not always practical due to the time and labour intensive nature of the assays. Alternatively taking a number of soil cores and combining them to form a composite sample can be effective (Ros et al., 2011, Bhogal et al., 1999, Laverman et al., 2000) and it was this approach that was taken when soil sampling the mesocosms (Chapter 5).

The sequencing of DNA fragments by next generation sequencing is followed by bioinformatics-based analysis to pre-process, cluster, database match and classify sequences. The software to complete these tasks is compiled into a processing 'pipeline'. There are a number of published pipelines (Patel and Jain, 2012, Scholz et al., 2012, Giongo et al., 2010) or a skilled operator can integrate bioinformatics software to form a custom pipeline. The choice of software included in the



processing pipeline can influence the interpretation of the microbial community data (Schloss et al., 2011). The software chosen is usually specific for the sample type and for this reason Greengenes (used in Chapter 6) was selected as it is a database specifically for matching extracted DNA sequences with known sequences from environmental samples (DeSantis et al., 2006).

There are sources of bias and error in metagenomic studies and one of these is chimera formation during PCR amplification. This occurs when an incomplete PCR product acts as a primer and binds to other sites in the genome which are then amplified. It has been estimated that the rate of chimerism is 5-45% of sequences (Haas et al., 2011). There is bioinformatic software that can identify chimeras and remove them from the analysis. These include ChimeraSlayer (Haas et al., 2011), Perseus (Quince et al., 2011), Uchime (Edgar et al., 2011) and UPARSE (Edgar, 2013). The UPARSE program has been previously applied to sequences derived from soil (Wagner et al., 2014, Deng et al., 2014) and was included here in the processing pipeline (Chapter 6).

### **7.3 Conclusion**

The overall aim of this study was to chemically characterize and assess the impacts of commercial Victorian lignite-derived products on the growth of selected pasture species and key indicators of soil health. There was no clear evidence of a consistent link between the HA and nutrient concentrations of the products and pasture growth and soil health responses.

Soil amendment with a selection of products applied at the manufacturer's recommended rate resulted in inconsistent lucerne and ryegrass growth effects. It is likely that the application rates used were too low to elicit a significant growth response. The SHG product application rate was shown to be important to lucerne growth with a maximum growth benefit observed at 20 kg/ha with application beyond this rate providing no additional benefit. In the mesocosms, the SHG

product applied at 20 kg/ha significantly promoted the early-stage shoot and root growth of ryegrass making this product ideal for conditions of low water availability during which the quick establishment of roots at depth can improve the chances of pasture survival. Similar growth benefit in the same conditions was not replicated in lucerne, suggesting the possibility that this product is more effective on non-leguminous pasture.

Generally there were no consistent effects of the lignite-derived products on pasture nutrient uptake with differences in shoot nutrient concentrations attributed to soil pH changes. These pH changes were driven by the pasture rather than application of the SHG product as demonstrated in the incubation study. Increasing the SHG application rate to 50 and 100 kg/ha decreased lucerne shoot nitrogen concentrations with no loss in biomass production. This could represent fertilizer savings for farmers but with the high product application rate, the cost would need to be taken into consideration.

In mesocosm conditions an effect of the SHG product on soil nitrogen species was evident with application of SHG at 20kg/ha resulting in the earlier provision and higher overall concentration of soil ammonium. This was accompanied by an increased loss of soil nitrate while soil nitrous oxide emissions were unaffected. Further evidence of altered nitrogen cycling was also observed by increases in the abundance of dinitrogen-fixing, nitrifying and denitrifying bacteria families with increasing SHG application rate. The implications on nitrogen cycling are not fully resolved and further research is required.

Soil application of the selected lignite-derived products both at the manufacturers recommended rates and for SHG at high application rates, did not have an effect on soil pH or MBC. While mycorrhizal colonization was not negatively affected by the application of products, increases detected were likely due to the nutritional status of the soil.

It is clear from the research presented in this thesis that pasture growth and soil health benefits that may be gained from the use of commercial lignite-derived products studied are strongly dependent on the soil type, pasture species and product application rate. Further mechanistic studies are needed to give insights into why these products are effective in some conditions but not others.

#### **7.4 Further research**

Given the complex chemical nature of commercial humic products and the difficulties in deciphering plant, soil and product interactions, the development of further mechanistic studies requires a standardized protocol for quantifying the HA and FA in the products. While advancements have been made in this area (Lamar et al., 2013), further work is required to develop a protocol that is effective across the wide range of product types available.

In this thesis the response of the two pasture species, ryegrass and lucerne, to lignite-derived amendment was assessed as two individual species. This provided important information, particularly highlighting the different responses of a leguminous species compared to a non-leguminous species but the agronomic benefit to an individual species cannot be extended to a mixed pasture system (Sanderson et al., 2004). The pasture composition affects a number of ecosystem functions including nutrient cycling (Sanderson et al., 2004). One of the most studied systems is of nitrogen fixation and cycling in mixed pasture comprised of both leguminous and non-leguminous species (Haynes and Williams, 1993). Previous studies on commercial lignite-derived products applied to mixed grasslands have reported shoot growth benefits however soil and nutrient cycling effects were not considered (Verlinden et al., 2010, Verlinden et al., 2009). There is scope to study the effects of Victorian lignite-derived products on the mixed pasture as would be found in realistic grazing conditions, and assess nutrient cycling and soil health effects.

Given the inconsistencies in pasture growth effects between soil types and pasture species identified here, it would be of benefit to farmers and product manufacturers to conduct further glasshouse and longer term field studies using a range of soil types. This may enable the matching of each commercial product with specific environmental settings. Also of interest would be the effect of product reapplication on pasture growth and soil health during the growing season.

Further investigation of the impact of lignite-derived products on nitrogen cycling could lead to further understanding of the mechanisms by which these products improve pasture growth. For farmers there is the potential for nitrogen-based fertilizer savings and for manufacturers, identification of a market for blended lignite-fertilizer products. As shown here, the increased abundance of soil nitrogen cycling bacteria suggests an alteration in soil nitrogen cycling; however, quantification of the nitrogen functional genes *nif*, *amoA*, *nirS*, *nirK* and *nosZ* by quantitative polymerase chain reaction (qPCR) would be required to confirm functional changes. The response of the soil fungal and archaea communities to humate addition was not been explored and to my knowledge there are currently no published precedents. Next-generation sequencing using fungal- and archaea-specific primers would give insights.

In conclusion, while the work presented here provides previously lacking information of the agronomic performance of Victorian lignite-derived products there is clearly more research to be done in this area. This work would assist farmers to decide if these products could be of benefit within their pasture and soil system and could encourage manufactures to assess usage guidelines and recommended application rates, and produce more effective products.

## Appendix 1 Chapter 3 supplementary table

**Table A1.1** Mean shoot (SDW) and root dry weight (RDW), and microbial biomass carbon (MBC) following 56 days of ryegrass or lucerne growth. LDP refers to lignite-derived product as indicated in Table 1. Values in parenthesis are +/- s.e.

Soil	LDP	SDW ryegrass (g)	SDW lucerne (g)	RDW ryegrass (g)	RDW lucerne (g)	MBC ryegrass (ug C/g dry soil)	MBC lucerne (ug C/g dry soil)
Cranbourne	A	5.89(0.52)	6.33(0.53)	3.15(0.43)	2.44(0.22)	0.12(0.01)	0.15(0.04)
	B	7.79(0.23)	6.06(0.17)	3.24(0.27)	2.54(0.18)	0.08(0.01)	0.10(0.01)
	C	7.68(0.09)	6.35(0.33)	4.61(0.51)	2.87(0.06)	0.12(0.00)	0.08(0.02)
	D	6.30(0.36)	6.01(0.68)	2.87(0.19)	2.20(0.30)	0.05(0.01)	0.11(0.01)
	E	7.26(0.27)	5.96(0.53)	3.35(0.58)	2.58(0.13)	0.09(0.01)	0.14(0.02)
	F	7.72(0.35)	5.86(0.52)	3.44(0.29)	2.53(0.26)	0.12(0.01)	0.13(0.02)
	Control	7.44(0.33)	5.34(0.57)	3.13(0.37)	2.22(0.23)	0.03(0.01)	0.10(0.01)
Stony Creek	A	9.62(0.52)	6.36(0.36)	3.80(0.32)	2.21(0.21)	0.47(0.08)	0.71(0.05)
	B	8.43(0.12)	8.30(0.22)	3.08(0.31)	2.61(0.32)	0.37(0.11)	0.78(0.05)
	C	9.39(0.46)	7.43(0.33)	2.93(0.10)	2.23(0.13)	0.49(0.06)	0.70(0.02)
	D	8.57(0.13)	7.50(0.35)	3.53(0.42)	2.72(0.22)	0.58(0.01)	0.62(0.10)
	E	8.60(0.53)	7.65(0.37)	3.53(0.62)	2.66(0.28)	0.48(0.08)	0.71(0.06)
	F	9.08(0.32)	7.16(0.26)	3.47(0.48)	2.41(0.29)	0.43(0.11)	0.75(0.05)
	Control	9.20(0.38)	7.30(0.36)	3.67(0.20)	2.44(0.22)	0.59(0.03)	0.65(0.09)



## Appendix 2 Lucerne shoot nutrient, chlorosis and soil health data

**Table A2.1** Nutrient composition of lucerne shoot tissue. Mean values are indicated (n=5) and values in parentheses are  $\pm$ s.e. No significant differences at  $P<0.05$  were identified.

LDP	Application rate (t/ha)	Al (mg/kg)	Ca (g/kg)	Fe (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	Mg (g/kg)	K (g/kg)	P (g/kg)	S (g/kg)
ROM	0	45 (6)	8.3 (0.8)	108 (13)	182 (18)	51 (4)	3.8 (0.1)	25.2 (0.6)	3.5 (0.1)	3.0 (0.1)
	2	32 (3)	10.2 (0.5)	138 (30)	205 (30)	51 (5)	4.2 (0.2)	24.8 (2.1)	3.9 (0.1)	3.3 (0.2)
	5	35 (6)	9.5 (0.4)	174 (48)	192 (16)	55 (3)	4.4 (0.2)	26.6 (2.4)	3.5 (0.1)	3.1 (0.2)
	10	50 (4)	9.7 (0.4)	126 (16)	224 (7)	64 (5)	4.4 (0.4)	28.2 (2.1)	3.7 (0.5)	3.1 (0.1)
	25	49 (6)	8.5 (0.4)	131 (23)	226 (36)	58 (7)	4.5 (0.5)	25.6 (1.4)	3.3 (0.1)	3.5 (0.4)
	LMB1	0	45 (6)	8.3 (0.7)	108 (13)	182 (18)	51 (4)	3.8 (0.1)	25.2 (0.6)	3.5 (0.1)
LMB1	1	39 (5)	9.1 (0.6)	116 (22)	183 (13)	51 (2)	3.9 (0.2)	22.8 (1.0)	3.0 (0.1)	2.7 (0.1)
	2	49 (5)	9.7 (0.2)	122 (20)	187 (14)	55 (5)	4.4 (0.2)	23.2 (1.0)	3.5 (0.2)	2.9 (0.1)
	5	37 (7)	10.1 (0.5)	115 (11)	185 (13)	55 (4)	4.5 (0.2)	22.6 (1.0)	3.2 (0.1)	2.8 (0.1)
	10	37 (3)	9.4 (0.5)	159 (48)	219 (25)	60 (2)	4.2 (0.2)	25.2 (1.1)	3.5 (0.1)	3.1 (0.1)

**Table A2.2** Organic composition of lucerne shoot tissue. Mean values are presented (n=5) and values in brackets are  $\pm$  s.e. No significant differences at  $P<0.05$  were identified.

LDP	Application rate (t/ha)	C (%)	N (%)
ROM	0	41.7 (0.4)	3.0 (0.2)
	2	42.0 (0.3)	2.8 (0.2)
	5	40.2 (0.2)	2.8 (0.3)
	10	40.3 (0.2)	3.3 (0.2)
	25	41.0 (1.0)	2.9 (1.3)
LMB1	0	41.7 (0.4)	3.0 (0.2)
	1	41.6 (0.5)	2.9 (0.2)
	2	41.7 (0.9)	2.7 (0.2)
	5	40.9 (0.7)	2.7 (0.1)
	10	41.4 (0.8)	3.0 (0.1)

**Table A2.3** Number of days post-seeding until the appearance of chlorotic symptoms. Values in brackets are  $\pm$ s.d. No significant differences were identified.

<b>LDP</b>	<b>Application rate (t/ha)</b>	<b>Days until chlorotic symptom appearance</b>
ROM	0	17 (1)
	2	17 (5)
	5	23 (4)
	10	18 (4)
	25	15 (2)
LMB1	0	17 (1)
	1	15 (2)
	2	16 (3)
	5	17 (3)
	10	17 (3)

**Table A2.4** Microbial biomass C (MBC) and mycorrhizal colonization of lucerne. Mean values are presented (n=5) and values in brackets are  $\pm$  s.e. No significant differences at  $P<0.05$  were identified.

<b>LDP</b>	<b>Application rate (t/ha)</b>	<b>MBC (mg C/g dry soil)</b>	<b>Mycorrhizal colonization (%)</b>	<b>Soil pH</b>
ROM	0	22.8 (6.2)	2.0 (1.3)	4.5 (0.1)
	2	20.6 (1.3)	1.2 (0.6)	4.6 (0.1)
	5	19.7 (1.9)	1.2 (0.6)	4.6 (0.1)
	10	20.6 (4.9)	0.6 (0.4)	4.5 (0.1)
	25	15.0 (3.1)	1.8 (0.7)	4.5 (0.1)
LMB1	0	22.8 (6.2)	2.0 (1.3)	4.5 (0.1)
	1	29.3 (1.6)	1.6 (0.7)	4.6 (0.1)
	2	14.0 (4.0)	2.2 (0.7)	4.5 (0.1)
	5	13.2 (3.8)	2.0 (1.1)	4.6 (0.1)
	10	14.8 (5.8)	1.2 (0.6)	4.6 (0.1)



## Appendix 3 Summary of project outputs

### Papers

#### *Published*

LITTLE, K. R., ROSE, M. T., JACKSON, W. R., CAVAGNARO, T. R. & PATTI, A. F. 2014. Do lignite-derived organic amendments improve early-stage pasture growth and key soil biological and physiochemical properties? *Crop and Pasture*, Advance online publication, doi: 10.1071/CP13433.

ROSE, M. T., PATTI, A. F., LITTLE, K. R., BROWN, A. L., JACKSON, W. R. & CAVAGNARO, T. R. 2014. A meta-analysis and review of plant-growth response to humic substances: practical implications for agriculture. *Advances in Agronomy*, 124, 37-89. (Appendix 4).

#### *In review*

LITTLE, K. R., PATTI, A. F., ROSE, M. T., JACKSON, W. R., & CAVAGNARO, T. R. 2014. Metagenomic and functional responses of the soil microbial community to humate addition.

### Conference posters and presentations

*A commercial humic product stimulates bacterial families involved in soil nitrogen cycling.*

17<sup>th</sup> Meeting of the International Humic Substances Society, Ioannina, Greece, September 2012. September 2014. Poster presented by Tony Patti.

*Effect of application rate of commercial lignite coal-derived amendments on early-stage growth of Medicago sativa and soil health, in acidic soil conditions.*

3<sup>rd</sup> International Low Rank Coal Industry Symposium, Melbourne, April 2014. Poster presentation.

*Brown coal-derived amendment improves plant growth and fertilizer efficiency.*

10<sup>th</sup> Australian Coal Science Conference, Brisbane, November 2013. Oral presentation.

*Effect of application rate of commercial lignite-derived amendments on early-stage growth of Medicago sativa and soil health, in acidic soil conditions.*

5th Joint Australian and New Zealand Soil Science Conference, Hobart, December 2012. Poster presentation.

*Effect of application rate of commercial lignite coal-derived amendments on early-stage growth of Medicago sativa and soil health, in acidic soil conditions.*

16<sup>th</sup> Meeting of the International Humic Substances Society, Hangzhou, China, September 2012. Poster presentation.

*Effect of lignite coal-derived amendments on vegetative growth and nutrition of pasture species in acidic soils.*

Eurosoil, Bari, Italy, July 2012. Poster presentation.

## **Appendix 4 Meta-analysis and review paper**

The full reference for this paper is:

ROSE, M. T., PATTI, A. F., LITTLE, K. R., BROWN, A. L., JACKSON, W. R. & CAVAGNARO, T. R. 2014. A meta-analysis and review of plant-growth response to humic substances: practical implications for agriculture. *Advances in Agronomy*, 124, 37-89.





# A Meta-Analysis and Review of Plant-Growth Response to Humic Substances: Practical Implications for Agriculture

Michael T. Rose<sup>\*,†</sup>, Antonio F. Patti<sup>\*</sup>, Karen R. Little<sup>\*,†,‡</sup>,  
Alicia L. Brown<sup>†</sup>, W. Roy Jackson<sup>\*</sup>, Timothy R. Cavagnaro<sup>†,§</sup>

<sup>\*</sup>School of Chemistry, Monash University, Clayton, Victoria, Australia

<sup>†</sup>School of Biological Sciences, Monash University, Clayton, Victoria, Australia

<sup>‡</sup>School of Applied Sciences and Engineering, Monash University, Churchill, Victoria, Australia

<sup>§</sup>School of Agriculture, Food and Wine, The University of Adelaide, Waite Campus, PMB1 Glen Osmond, SA, Australia

## Contents

1. Introduction	38
2. Meta-Analysis	41
2.1 Methods	41
2.2 Results	45
3. Plant-Growth Response to HS: Moderating Factors	51
3.1 General plant-growth response	51
3.2 Application rate	52
3.3 HS properties	54
3.4 Environmental conditions	55
3.5 Plant type	56
4. Practical Use of HS in Agriculture	57
4.1 Direct application	57
4.2 Application as synergists	59
5. Knowledge Gaps and Research Needs	62
6. Conclusions	63
Acknowledgments	63
Appendix A. Study References for SDW Data used in the Meta-Analysis	64
Appendix B. Study References for RDW Data used in the Meta-Analysis	77
References	85

## Abstract

The breakdown products of plant and animal remains, extracted in an alkaline solution, are commonly referred to as humic substances (HS). They can be extracted from a wide

variety of sources, including subbituminous coals, lignites (brown coals), peat, soil, composts, and raw organic wastes. The application of HS to plants has the potential to improve plant growth, but the extent of plant-growth promotion is inconsistent and relatively unpredictable when compared to inorganic fertilizers. The goal of this review was to determine the magnitude and likelihood of plant-growth response to HS and to rank the factors contributing to positive growth promotion. These factors included the source of the HS, the environmental growing conditions, the type of plant being treated, and the manner of HS application. Literature reports of exogenously applied HS–plant interactions were collated and quantitatively analyzed using meta-analytic and regression tree techniques. Overall, random-effects meta-analysis estimated shoot dry weight increases of  $22 \pm 4\%$  and root dry weight increases of  $21 \pm 6\%$  in response to HS application. Nevertheless, actual responses varied considerably and were mainly influenced by the source of the HS applied, the rate of HS application, and to a lesser extent, plant type and growing conditions. HS from compost sources significantly outperformed lignite and peat-derived HS in terms of growth promotion, while HS application rate nonlinearly moderated the growth response under different circumstances. Our results demonstrate the difficulty in generalizing recommendations for the use of HS in agriculture; however, some specific suggestions for maximizing the efficacy of HS under certain conditions are offered. We also outline some recent developments in the use of HS as synergists for improving fertilizer use efficiency and the activity of microbial inoculants. Finally, we identify a number of research gaps, which, when addressed, should clarify how, when, and where HS can be best applied for the greatest benefit.



## 1. INTRODUCTION

Humic substances (HS) are a category of naturally occurring organic compounds that arise from the decomposition and transformation of plant, animal, and microbial residues (MacCarthy, 2001). They are a natural component of practically all soils, but levels vary and there is considerable evidence that modern agriculture involving practices such as soil tillage has resulted in their decline (Novotny et al., 1999; Shepherd et al., 2001). The loss of humic material, together with overall reductions in soil organic matter, is of concern because they play important roles in maintaining key soil functions and plant productivity (Lal, 2004; Sparling et al., 2006). Consequently, there is interest in the application of HS-based amendments to agricultural systems in order to reverse this trend (Piccolo and Mbagwu, 1997; Quilty and Cattle, 2011).

HS are chemically complex with no clearly defined chemical structure, although generalized models have been proposed (Brucocoleri et al., 2001). While traditionally viewed as complex macromolecules, they have more recently been described as mixtures of smaller molecules, containing

aromatic rings, aliphatic chains, and ionizable functional groups that interact with each other to form aggregated colloids (Piccolo, 2001; Pinton et al., 2009; Sutton and Sposito, 2005). There is significant evidence that the exogenous application of HS can help improve soil fertility, primarily through their complex chemistry which facilitates interactions with a variety of mineral and nonmineral organic soil components. Some of the documented benefits of soil amendment with HS include improved soil aggregation and structure, increased pH buffering and cation exchange capacity, increased water retention capacity, increased bioavailability of immobile nutrients (such as P, Fe, and Zn), and decreased toxicity of aluminum and heavy metals (Chen et al., 2004a; Imbufe et al., 2005; Peiris et al., 2002; Piccolo and Mbagwu, 1989; Piccolo et al., 1997; Tan and Binger, 1986).

As well as indirectly influencing plant productivity through modification of soil characteristics, HS can also directly impact on physical and metabolic plant processes. A recent review by Muscolo et al. (2013) reviews evidence for the hormone-like effects of HS and how these relate to the chemical structural features of these materials. The authors highlight a predominance of auxin-like effects and that nonlignin structures are the principal contributors. These effects can be elicited through an interaction with either roots or shoots. For example, hormonal-like responses on plant roots were demonstrated by Trevisan et al. (2010) and HS may also stimulate  $H^+$ -ATPase and ion transporter activity in the root plasma membrane (Mora et al., 2010; Pinton et al., 1997, 2009). Both these effects can enhance nutrient acquisition, the former through increased soil exploration, and the latter by accelerating nutrient uptake. These effects appear to be especially prominent for cases involving HS derived from compost and vermicomposts, which may contain auxin-related compounds (Muscolo et al., 1999; Quaggiotti et al., 2004), including indole-acetic acid derivatives and other low molecular weight organic acids (Russell et al., 2006). In contrast, effects on leaf function have been less well documented and appear somewhat contradictory (Nardi et al., 2002). Foliar application of HS may increase leaf chlorophyll concentration (Sladký, 1959), but it is also recognized that HS contain a range of functional groups which are able to interfere with photosynthesis (Pflugmacher et al., 2006). Foliar applications have also been shown to influence transpiration, though the mechanism is unclear and both increases and decreases in water loss and leaf gas exchange have been observed.

Despite numerous publications on the potential positive effects of HS on plant growth and productivity over more than five decades (Billingham,

2012; Chen et al., 2004b; Quilty and Cattle, 2011) and substantial interest in their potential for improving nutrient-use efficiency and contributing to C sequestration in the soil, the use of commercial products containing HS in agriculture varies and there is scepticism about their effectiveness (Billingham, 2012). Part of the reason for this is no doubt related to the wide range in physicochemical properties of HS, which vary according to the method of extraction and the environmental matrix from which they are sourced. HS are formed under a variety of environmental conditions and are, therefore, highly heterogeneous and structurally difficult to define (Senesi, 1994). Commercial products often contain mixtures of humic materials and added plant nutrients; hence, the cause of any observed beneficial effect cannot be easily attributed to the HS themselves. In addition, the recommended rates of application of commercial products are generally very small in relation to the natural levels of HS present in the soil. As a consequence, the effect of an HS product is substantially less predictable than other plant or soil amendments of a known chemical structure, such as inorganic fertilizers or synthetic organics including pesticides and growth regulators. Moreover, because of the multiple chemical functional groups of HS, a particular HS product may behave completely differently under different environmental conditions, or when applied to different plant species. Finally, as with many chemical fertilizers, the timing, location, and rate of application will play a crucial role in determining whether beneficial or harmful effects will evolve and whether or not any beneficial effects are economically worthwhile. This is particularly important because recent publications have pointed out potential negative effects and have questioned the economic viability of applying HS for improved crop production (Asli and Neumann, 2010; de Santiago et al., 2010; Hartz and Bottoms, 2010).

In light of the potential benefits of HS, together with their inconsistent performance under field conditions, we sought to improve the understanding of the effects of HS on plant growth by conducting a meta-analysis of the published literature. More specifically, our objectives were (i) to quantify the magnitude and likelihood of plant-growth promotion, in terms of shoot and root biomass, resulting from HS application; (ii) to determine the influence of environmental conditions, plant type, HS properties, and the manner of application on plant-growth response to HS; (iii) to identify gaps in our understanding of the interaction of HS with plants; and (iv) to provide some general recommendations for the practical use of HS in agronomic systems and suggestions for future work.





## **2. META-ANALYSIS**

### **2.1. Methods**

#### ***2.1.1 Literature search and refinement***

We conducted a search of the databases Scopus and ISI Web of Science using a combination of search terms including “humic” AND “plant” AND “effect” AND “growth OR yield.” This search was designed to provide an unbiased selection of potential studies, rather than act as an exhaustive search for all studies in this area. The search yielded 390 papers, the abstracts of which were screened in the first instance to determine if the experiments conducted involved the application of HS to plants. Unsuitable abstracts (no plants grown or no HS applied: 185 papers), nonresearch articles (6 papers), and publications in languages other than English (19 papers) were not reviewed further. The full text of all remaining papers were sought and scrutinized to determine if a measure of plant shoot (SDW) or root dry weight (RDW) was reported for both an HS treatment and a suitable untreated control. Papers not fulfilling this minimum requirement were also excluded from our analysis (99 papers). The full reference list including rejected papers is available on request or on our Web site ([soilecology.org](http://soilecology.org)), and a list of accepted papers is given in [Appendices A and B](#). From a total of 390 papers originally found, 81 were retained for the meta-analysis; with 57 studies presenting data on SDW and 39 studies reporting RDW. This provided over 700 data points on which to base our analysis, which can be updated and expanded in future as research progresses. It is important to note that few of the retrieved studies report results from statistically rigorous field trials testing plant-growth responses to HS through to crop maturity. We would like to emphasize that our meta-analysis therefore reflects this limitation, but nevertheless provides important information about trends in plant-growth response and how they might be manipulated for maximum agronomic benefit.

#### ***2.1.2 Response and moderator (explanatory) variables***

The focus of many investigations is on either shoot or root responses to HS, but not both; consequently, we assessed SDW and RDW as separate response variables. We were also interested to examine if HS affect both root and shoot biomass in a similar manner, or whether growth effects are biased toward either plant organ under different circumstances. In order to test our hypotheses, we used the data available in the papers included in our analysis

to identify a set of continuous and categorical groups that we predicted would influence the responsiveness of plants to HS applications. These groups fell under four broad areas: environmental conditions, plant type, HS properties, and the method of HS application.

#### 2.1.2.1 Environmental conditions

Originally, we attempted to populate a data matrix containing quantitative data of experimental growth conditions, including pH, EC, nutrient availability, and temperature; however, full data sets were rare, and we did not further pursue this avenue of investigation. Instead, we created two proxy categories based on data that were routinely reported: growth media and stress conditions. Growth media contained three levels: hydroponic culture, soil culture, or hybrid culture. Hybrid culture entailed the growth of plants on a solid, but relatively inert media (e.g., sand, vermiculite, perlite, or peat) and regular fertilization with nutrient solution. The stress conditions category was also designated into one of three levels: no stress, moderate stress, or high stress. No stress included studies that did not explicitly state stress as an investigation factor, or did not include treatments (other than HS application) that reduced growth to less than 90% of nontreated controls. Moderate and high stress involved treatments (additional to HS application) that reduced growth by 10–50% or >50% as compared with nonstress controls, respectively.

#### 2.1.2.2 Plant type

The plant species used in each study was recorded and subsequently categorized into three levels: monocotyledonous plants, dicotyledonous herbs, and woody perennials.

#### 2.1.2.3 HS properties

To characterize the HS used, we initially tried to obtain quantitative chemical data on the composition of HS used in each study, such as percentage C, H, N, and O; molecular weight range distribution; and carbon functional group composition as analyzed by nuclear magnetic resonance (NMR) spectroscopy. Unfortunately, such data were sparse. As an alternative, we created a subcategory based on the source of the humic acids, which included brown coal (BC), peat, soil, compost (green waste), compost (manure), and unreported. The level “brown coal” included HS extracted from lignite, leonardite, and subbituminous coals. Although many papers

used commercial HS, these were usually identified by trade name or manufacturer and could therefore be traced to the original source. Composts included both vermicomposts and traditional composts.

#### 2.1.2.4 Method of application

Two subcategories were created to characterize the method of HS application. The first included the “site” of application as foliar-applied, root-applied, combined foliar–root application or soil application. Root application generally involved addition of the HS into the growing medium. HS applied to seed were designated as “combined” application, our rationale being that both the roots and shoots come into contact with the HS on germination. The second moderator within this category was a continuous variable that specified the rate of HS application. All rates were converted into milligram of HS per kilogram of growing medium. In the case where rates were reported as mass of HS per volume of growing medium and bulk density was not given, a bulk density of  $1.0 \text{ g cm}^{-3}$  was assumed. In the case where rates were reported as mass of HS per unit area, we assumed passive incorporation to a depth of 10 mm, and again, a bulk density of 1.0 if not otherwise reported.

#### 2.1.3 Statistical analyses

Response ratios to HS treatment were calculated for SDW and RDW, such that

$$L = \ln(DW_{\text{HS}}/DW_{\text{C}})$$

where  $DW_{\text{HS}}$  is the dry weight of shoot or root biomass of plants treated with HS, and  $DW_{\text{C}}$  is the dry weight of the nontreated control grown under the same conditions. The variance of the response ratio was calculated according to [Hedges et al. \(1999\)](#) using the standard error and number of replicates reported for each individual study. Where standard errors were not presented or could not be calculated, we assumed a standard error of 10% of the mean ([Gattinger et al., 2012](#); [Luo et al., 2006](#)). Response ratios were analyzed using the “metafor” package ([Viechtbauer, 2010](#)) within the statistical program R ([R Development Core Team, 2005](#)). The “metafor” package provides functions for fitting both fixed- and random-effects models to observed outcome measures, with or without the inclusion of moderator variables (study-level covariates). For our purpose, the SDW

or RDW response was taken as the observed outcome measure, and the variables growth media, stress condition, plant type, HS source, application site, and application rate were designated as moderators. The overall heterogeneity was initially assessed by excluding all moderator variables, and each moderator was subsequently tested one by one as a sole covariate, in order to ascertain its individual power to explain the observed heterogeneity. All models were run using the restricted maximum-likelihood estimator function. Publication bias was assessed by creating funnel plots (Egger et al., 1997) and assessing asymmetry in the data by conducting a meta-analytic regression test using variance as a predictor in the “metafor” function `regtest.rma` (Viechtbauer, 2010).

Although mixed-effect modeling in this framework is useful for combining multiple studies and estimating aggregate effects of covariates, it lacks the capability to model nonlinear functions and is not very efficient for modeling interactions between variables, both of which are common occurrences in environmental systems. In order to further explore the complex nature of HS effects on plant growth, we therefore also conducted classification and regression tree (CART) modeling (De’ath and Fabricius, 2000). This nonparametric approach repeatedly splits heterogeneous data into increasingly homogeneous subsets. It is commonly used to establish prediction criteria based on a number of explanatory variables, but can also be used to rank the importance of these explanatory variables in describing the overall heterogeneity of a data set (De’ath and Fabricius, 2000). Depending on splitting criteria, a number of different CARTs can be produced from the same data set, with differing bias and predictive power. To overcome the issues inherent in constructing a singular CART, we performed a boosted regression tree (BRT) using the R package “gbm” (Ridgeway, 2013) combined with the “rt” vignette (Elith et al., 2008). Boosting grows the suite of trees by sequentially modeling the residuals throughout all parts of the data space, including those for atypical observations that depart from the dominant patterns explained by the initial trees (Elith et al., 2008). Weakly predictive trees are aggregated to create an improved model, thereby reducing both bias (through forward stagewise fitting) and variance (through model averaging).

Using the output from the regression tree analysis, we partitioned the data sets according to the two most influential explanatory factors, and plotted the growth response against the rate of HS application. Inferences were made by fitting linear models to log-transformed data.

## 2.2. Results

### 2.2.1 Data quality and aggregate effect of HS on plant growth

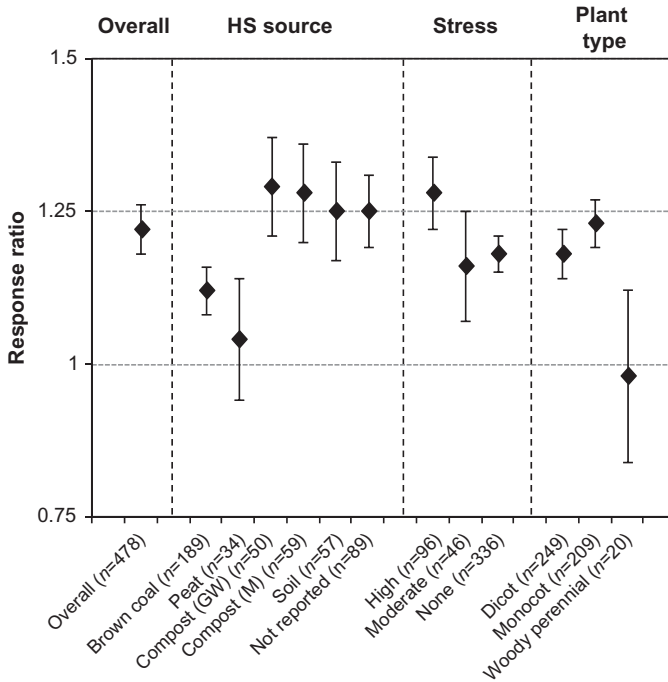
Case diagnostics performed using the “influence” function of the metafor package identified 12 outlier data points in the SDW data set and four outliers in the RDW data set that exerted considerable influence on the random-effects model fit; these data points were therefore excluded from the model. The revised random-effects model predicted that HS application significantly (Table 2.1) increases both SDW and RDW by  $19 \pm 3\%$  and  $20 \pm 4\%$ , respectively. Publication bias was not detected by regression tests of funnel plot asymmetry for either the SDW ( $p=0.96$ ) or the RDW ( $p=0.51$ ) data set.

Subsequent inclusion of moderator variables into the model showed that the shoot growth response was not significantly influenced by the growth media or the application site, but was significantly affected by the source of HS used, stressful growing conditions, the type of plant being treated, and the rate of HS applied (Table 2.1). Of the HS source categories, only peat-derived HS did not significantly affect shoot biomass accumulation ( $4 \pm 12\%$ ) (Fig. 2.1). BC-derived HS increased SDW response ( $12 \pm 4\%$ ), but was less effective than HS extracted from green waste compost ( $29 \pm 8\%$ ), manure compost ( $28 \pm 8\%$ ), and soil ( $25 \pm 8\%$ ). Plants were significantly more likely to increase shoot growth in response to HS application under highly stressful conditions ( $28 \pm 6\%$ ) than nonstressful conditions

**Table 2.1** Significance ( $p$ -value) of models containing a single moderator, according to plant shoot or root dry weight response to HS application

Moderator	Shoot growth	Root growth
None <sup>a</sup>	<0.001	<0.001
Media	0.336	<b>0.016</b>
Stress	<b>0.015</b>	0.144
Plant type	<b>0.026</b>	<b>0.031</b>
Application location	0.380	0.063
Application rate	<b>0.002</b>	0.261
Source	<0.001	<0.001

<sup>a</sup>The overall random-effect model without any moderators; significance indicates statistical difference between HS-treated plants and control (no HS treatment) plants. Significant models are shown in bold ( $p < 0.05$ ).



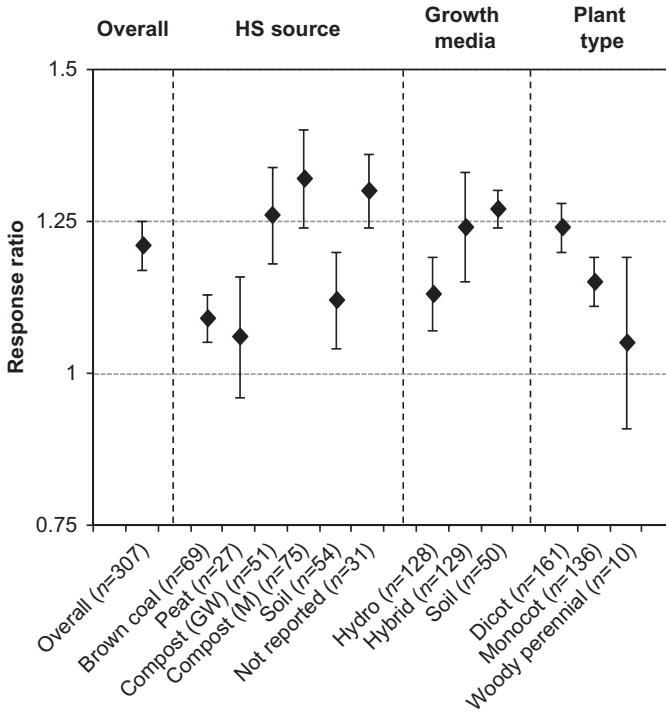
**Figure 2.1** Estimated shoot growth response (weighted mean  $\pm$  95% confidence level) of plants to HS application for three significant explanatory moderators. Ratios  $> 1$  indicate growth promotion and  $< 1$  indicate growth suppression. The number of data points in each group is given in parentheses.

( $18 \pm 3\%$ ). Also, woody perennials did not show any significant shoot growth promotion in response to HS application.

In contrast to shoot growth, the effect of HS on root growth was not significantly influenced by stress or application rate, but the source of the HS still moderated the growth response in a similar fashion to that of shoots (Table 2.1). Under these circumstances, both peat- and BC-derived HS did not affect root growth, but all other HS promoted plant root growth by 12–40% (Fig. 2.2). The root growth of woody perennials was similarly not significantly affected by HS application, and although the growth media moderated the root growth response (Table 2.1), there were no significant differences between the different growth media (Fig. 2.2).

### 2.2.2 Factors influencing HS efficacy

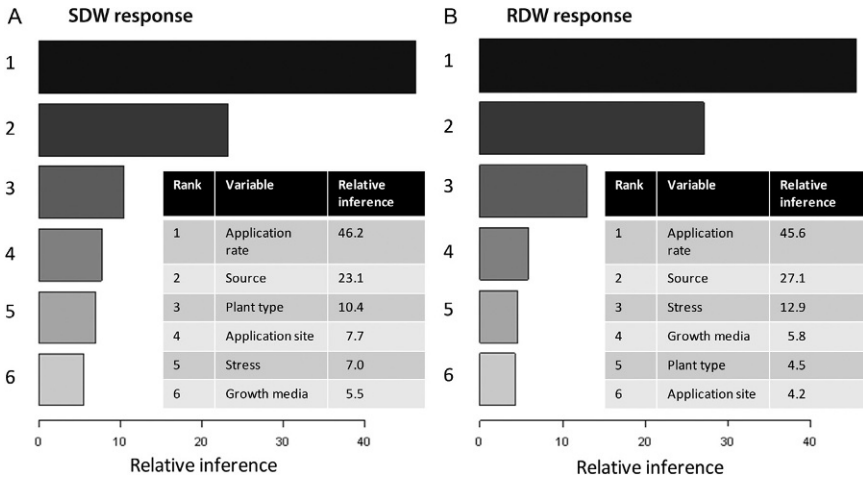
To further investigate the source for variability in plant-growth response to HS, a BRT model was constructed and analyzed. The optimized BRT was



**Figure 2.2** Estimated root growth response (weighted mean  $\pm$  95% confidence level) of plants to HS application for three significant explanatory moderators. Ratios  $>1$  indicate growth promotion and  $<1$  indicate growth suppression. The number of data points in each group is given in parentheses.

superior to the mixed-model meta-analysis in terms of model fit to both SDW and RDW. The BRT revealed that application rate, HS source, and plant type were the most important factors regulating HS impact on shoot and root growth; this was in agreement with the results of the mixed-effect model (Fig. 2.3). In comparison, application rate, HS source, and stress conditions were most important for root growth. The growth media used and the location of application played less of a role in influencing HS efficacy than the other variables.

The distributions of the modeled data emphasized the variability in response of plant growth to HS application (Figs. 2.4 and 2.5). As in the mixed model, BC- and peat-derived HS did not promote plant growth as strongly as other HS, and woody perennials generally responded negatively to HS application. Trends in other explanatory variables were not readily apparent, suggesting more complex interactions between variables were



**Figure 2.3** Relative contribution of explanatory variables to the optimum boosted regression tree model for A. SDW response and B. RDW response to HS application.

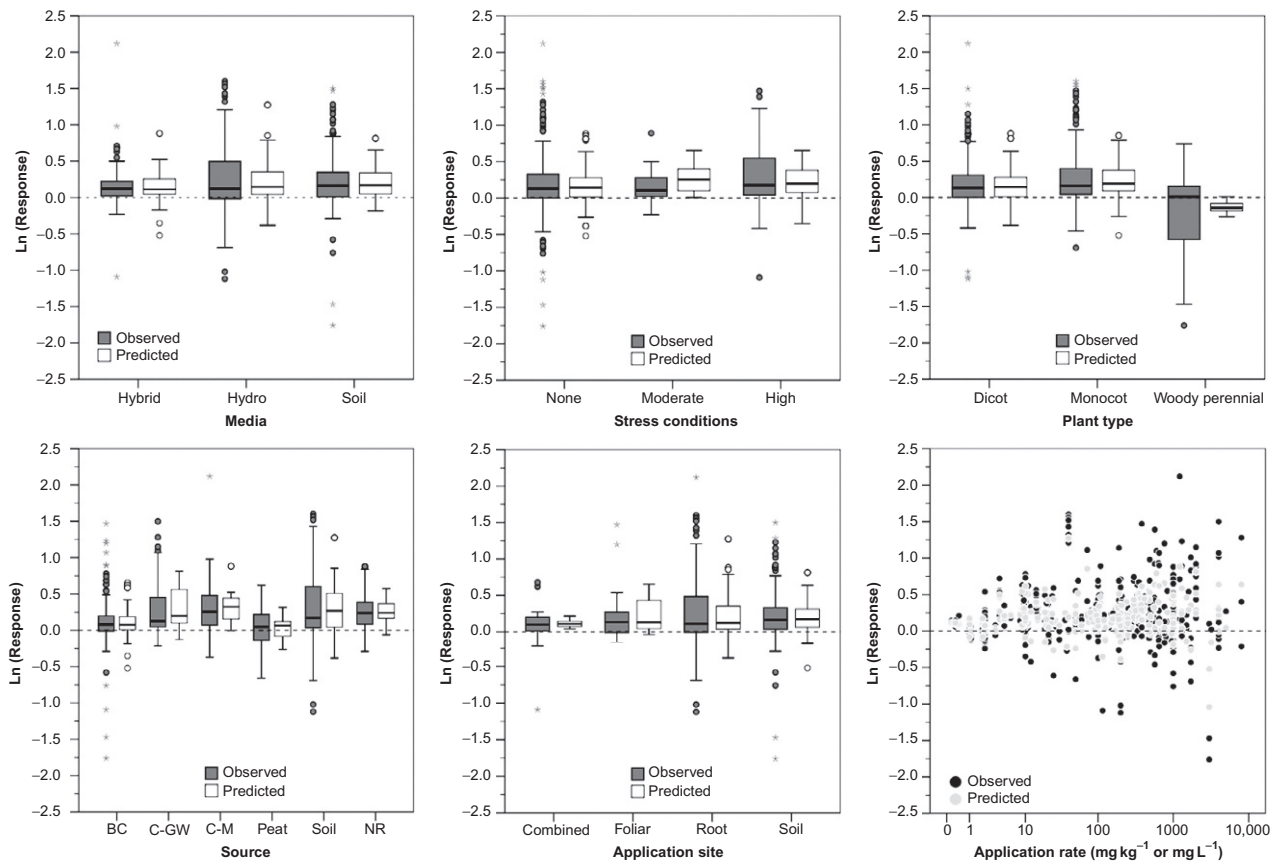
responsible for explaining the observed heterogeneity of shoot and root growth response.

### 2.2.3 Factor interactions

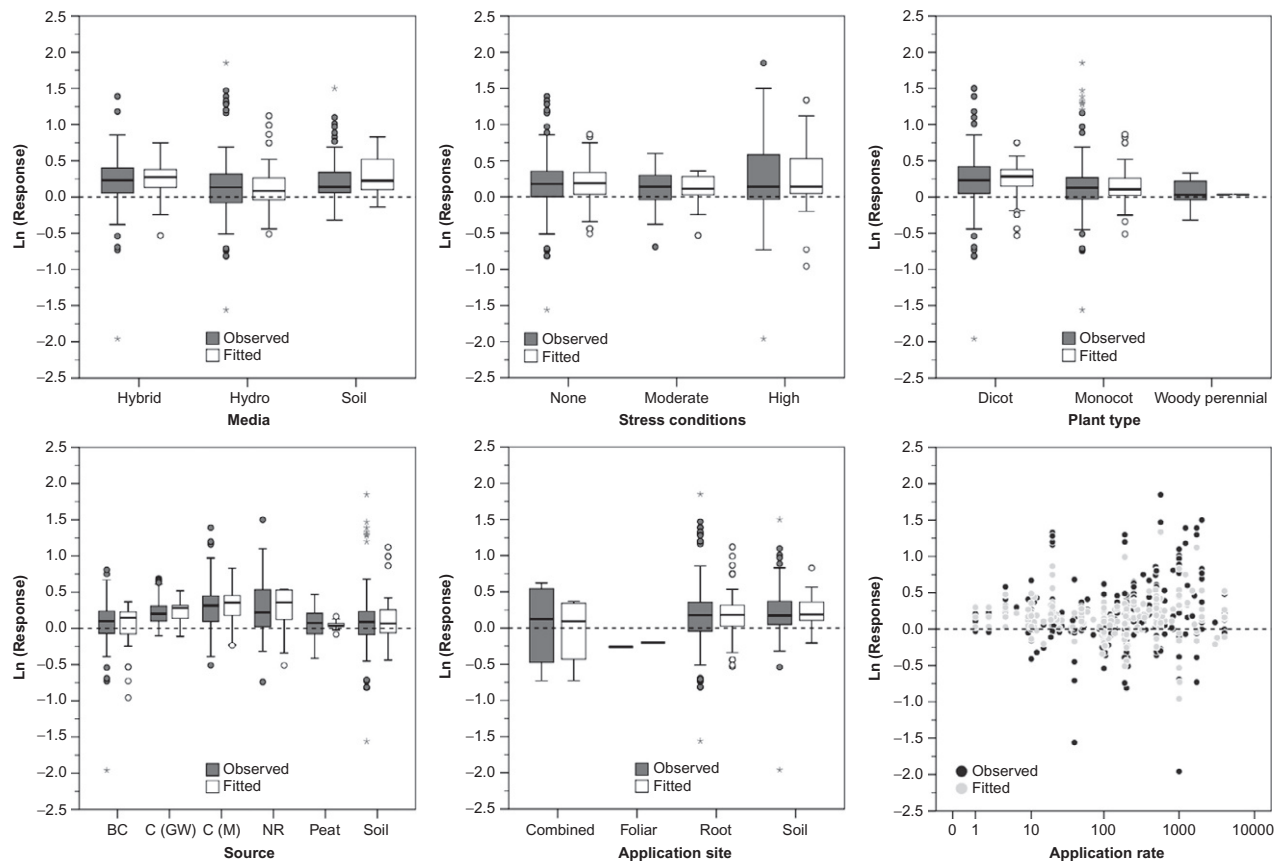
Interactions between HS source and application rate were found to be important in explaining the variation in both shoot and root growth response to HS. An interaction between plant type and application rate was also apparent for shoot growth, while an interaction between HS source and stress conditions was the most important pairwise interaction involved in root growth response (Table 2.2).

To further investigate the interactive effects revealed in our analysis, we replotted both sets of response data according to the interactions between the three most important explanatory variables: in the case of shoots this was application rate, HS source, and plant type; and for roots this was application rate, HS source, and stress conditions. Whereas increasing rates of green waste compost HS application to both monocots and dicots was positively related to shoot growth over untreated control plants, the application of soil-derived HS appeared to stimulate plant shoot growth more effectively at lower application rates (Fig. 2.6). Furthermore, higher rates of BC- and peat-derived HS appeared to inhibit shoot growth in woody perennials relative to untreated controls, but the application rate of these HS did not affect the shoot growth of monocots and dicots in any consistent fashion.





**Figure 2.4** Modeled distribution of SDW response, grouped by explanatory predictor. Boxplots show median values (solid bold horizontal lines), 25th–75th quartiles (box), 1.5 times the interquartile range (whiskers), outliers (circle points), and extreme outliers (star points). Abbreviations for the HS source are brown coal (BC), green waste compost (CGW), manure compost (CM), and not reported (NR).



**Figure 2.5** Modeled distribution of RDW response, grouped by explanatory predictor. Boxplots show median values (solid bold horizontal lines), 25th–75th quartiles (box), 1.5 times the interquartile range (whiskers), outliers (circle points), and extreme outliers (star points). Abbreviations for the HS source are brown coal (BC), green waste compost (CGW), manure compost (CM), and not reported (NR).

**Table 2.2** Pairwise interactions between explanatory variables in the optimized BRT model

	Media	Stress	Plant	HS source	Application site	Application rate
Media		2.38	0.03	0.82	0.03	2.72
Stress	0.39		1.23	<b>7.89</b>	0.86	1.73
Plant	0.05	2.93		0.94	0.27	0.65
HS source	0.41	0.3	0.96		0.57	<b>5.38</b>
Application site	0.37	0.24	0.18	0.63		0.76
Application rate	0.11	0.24	<b>4.93</b>	<b>7.18</b>	0.77	

Higher numbers indicate increased importance of the interaction for predicting a growth response. Gray cells are interactions contributing to RDW response; white cells are interactions contributing to SDW response. Numbers in bold show the two most important interactions.

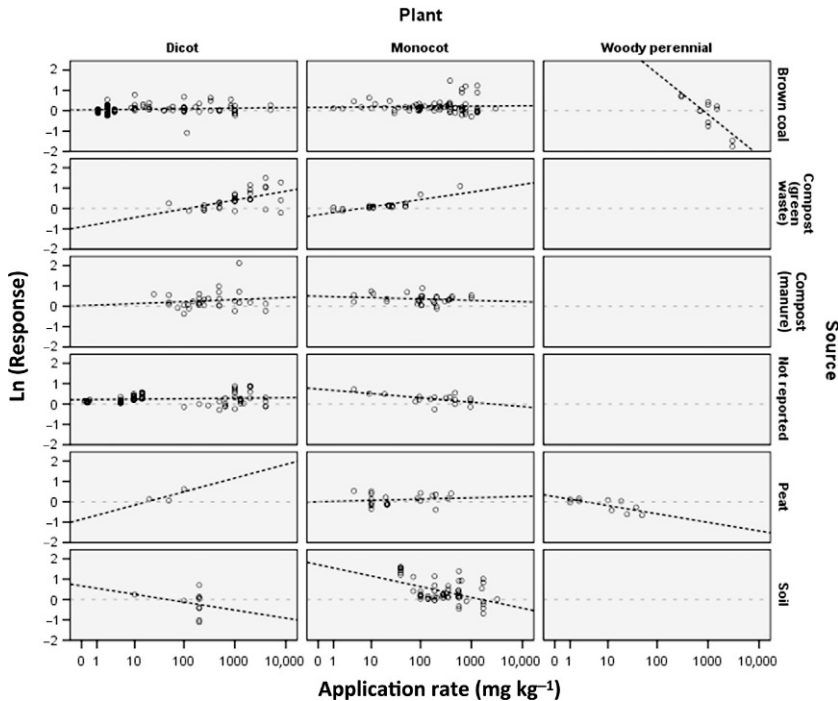
With regard to root biomass response (Fig. 2.7), increasing rates of BC-derived HS were negatively related to root growth under conditions of stress, but did not consistently affect growth under nonstress conditions. The opposite occurred with soil-derived HS, with a positive root growth response to increased application rates under high stress conditions. However, as with BC-derived HS, inconsistent effects were observed under low and nonstress conditions.



### 3. PLANT-GROWTH RESPONSE TO HS: MODERATING FACTORS

#### 3.1. General plant-growth response

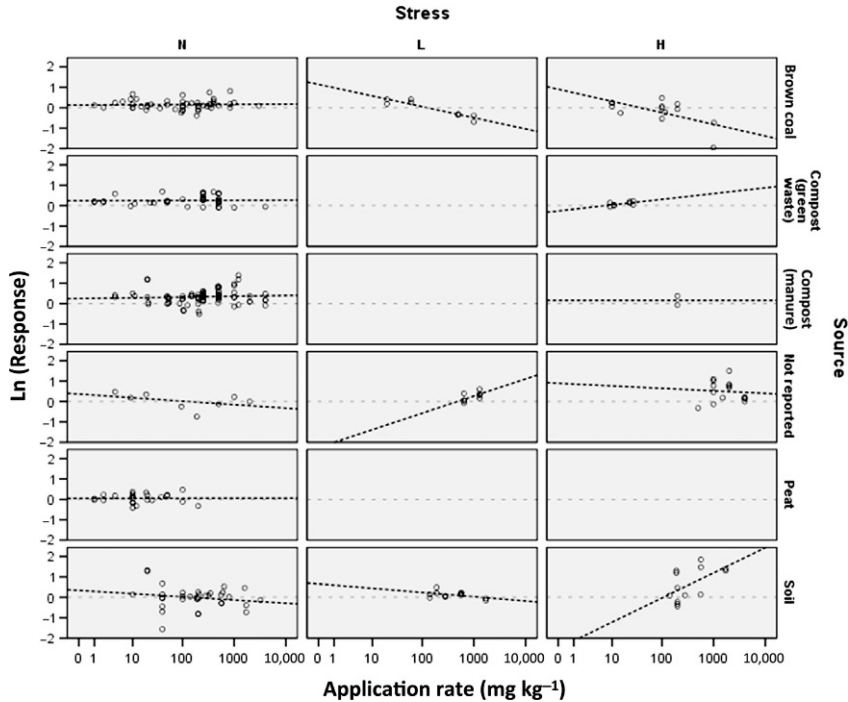
HS are becoming increasingly available as commercial supplements for crop improvement, but growth effects can be positive or negative and difficult to predict (Quilty and Cattle, 2011). In considering a wide range of published studies, we found that HS generally increase shoot and root growth by 15–25%, but high variation increases risk to farmers. For example, approximately half of the studies on SDW response and one-third of RDW studies failed to increase growth by more than 5%, which we consider to be agronomically significant. Thus, there is a strong need to improve consistency and predictability of the growth response.



**Figure 2.6** Effect of application rate on SDW response under different scenarios of HS source and plant type. The black dashed lines show linear fits to the data and have been superimposed to aid interpretation. Data above the gray dashed line indicate shoot growth promotion by HS; data below indicate shoot growth suppression.

### 3.2. Application rate

Regression tree-modeling showed the rate of HS application and its interaction with other factors as important predictors of growth promotion by HS. This is in contrast to the *linear* mixed-effect modeling, which suggested only a minor significance to shoot dry weight. The contradiction between the two models implies that although the application rate has a strong influence on the growth of plants receiving HS, the response is nonlinear. Biological responses to increasing concentrations of HS are often best described by quadratic functions, whereby an optimum concentration is identified after which the response declines or inhibition occurs (Chen and Aviad, 1990; Dobbss et al., 2010; Liu et al., 1998; Schluckebier and Martin, 1997). Although quadratic functions may hold over concentration ranges covering an order or two of magnitude, there is some evidence that other functions (e.g., cubic) can sometimes be more appropriate (Liu et al., 1998).



**Figure 2.7** Effect of application rate on RDW response under different scenarios of HS source and environmental stress. Stress levels are given as: no stress (N), moderate stress (L), and high stress (H). The black dashed lines show linear fits to the data and have been superimposed to aid interpretation. Data above the gray dashed line indicate shoot growth promotion by HS; data below indicate shoot growth suppression.

In these situations, where positive–negative–positive responses are observed, we speculate HS operate through different mechanisms that only become pronounced at particular concentrations. [Chen et al. \(2004a\)](#) suggest that in hydroponic studies, this occurs because of increasing then decreasing bio-availability of micronutrients brought about by HS–micronutrient complex stability. In soil environments, similar chemical and biological processes may occur at lower rates, while physical effects might begin to dominate at higher rates. Importantly, interactions between the application rate and other factors means that a particular response curve generated under one condition is unlikely to be transferable to other conditions. This was illustrated by [Dobbss et al. \(2010\)](#), who used a quadratic function to describe plant root branching stimulated by different concentrations of HS derived from vermicompost, but found that the optimal dose varied between the HS used and also the plants to which they were applied.

### 3.3. HS properties

Plant-growth responses are strongly affected by the type of HS applied. The importance of the source of HS was discussed by [Chen et al. \(2004a\)](#), who attributed the variability in plant-growth response to the variability in HS used before the introduction of standardized extraction procedures by the International Humic Substances Society. Our study and analysis of the literature suggests that compost- and soil-derived HS have a greater positive effect than BC- and peat-derived HS. Such an effect is likely to be related to the chemical structure of HS derived from each source, and possibly also related to co-extracted mineral nutrients remaining in HS formulations.

With regard to the chemical structure of HS, we hypothesize that the N content of the compost- and soil-derived HS, which is generally higher than that found in BC- and peat-derived HS (e.g., [Simpson et al., 2003](#)), could be a strong driver of growth via a number of mechanisms. First, mineralization of HS can liberate plant-available N (and possibly other nutrients, such as P) to stimulate plant growth ([Alvarez and Steinbach, 2011](#); [Valdrighi et al., 1996](#)). Amide (N-containing) functional groups of HS become quickly depleted in soils ([Tatzber et al., 2009a](#)), with initial decomposition half-lives of HS derived from fresh animal manures being as rapid as 2–4 months ([Tatzber et al., 2009b](#)). Because compost- and soil-derived HS are generally at a lower stage of humification than BC and peat HS, their decomposition by biological activity is likely to be faster. The fact that green waste compost HS application leads to increased plant productivity in a dose-dependent manner ([Fig. 2.6](#)) supports a direct N-fertilization hypothesis: for example, 50% mineralization of compost HS containing 5% N applied at  $1000 \text{ mg kg}^{-1}$  would provide approximately 25 kg mineral N per ha in a 0.01 m layer. Indeed, in the study conducted by [Valdrighi et al. \(1996\)](#), compost-derived HS only significantly improved plant growth at rates  $\geq 1000 \text{ mg kg}^{-1}$ . In comparison, more biologically stable BC-derived HS, containing only 1–2% N and usually applied at lower rates, is unlikely to contribute to the nitrogen nutrition of plants to any appreciable extent.

Although this explanation is appropriate for the case for high-N-containing HS applied at high rates, it does not account for their performance when applied at lower rates. Examination of the rate-dependent effects of soil-derived HS ([Fig. 2.6](#)) shows a positive growth response between approximately 25 and  $750 \text{ mg kg}^{-1}$  that declines at higher rates. Low rates of most HS should not contribute enough N to explain observed

growth increases. An alternative, or complementary, mechanism may involve direct stimulation of plant growth through hormone-like activity at lower HS concentrations. Hormone-like activity of HS has been linked to N-containing compounds, including indoles such as auxins (Nardi et al., 2000) and polyamines (Young and Chen, 1997). More recently, Canellas et al. (2012) showed that the induction of lateral roots in plants by HS is positively related to the hydrophilicity of the HS, especially the O-alkyl and methoxyl/N-alkyl chemical functional groups identified by NMR. Less-humified HS that contain more polar N- and carboxyl-functional groups also exhibit a greater ability to chelate micronutrient elements, such as Zn, Cu, and Fe (Chen et al., 2004a), which may contribute to improved plant growth under some conditions (Garcia-Mina et al., 2004). As an example, Azcona et al. (2011) found superior growth promotion and more rapid maturation of peppers by a compost-derived HS compared with a leonardite-derived HS. Although the compost HS had a higher N content (7.1%) than the peat-derived HS (1.3%), the authors ruled out nutrient supply effects (including N) by ensuring adequate chemical fertilization, and concluded that the growth effects were probably a result of the structural organic characteristics of the HS.

It is important to note that the true role of HS in plant signaling is still being strongly debated (Chen et al., 2004a; Trevisan et al., 2010) and the contribution of N-containing residues to plant-growth stimulation has not been directly investigated. In addition, HS derived from younger organic material, such as those derived from compost and soils, usually contain a lower molecular weight distribution of molecules/aggregates, which has also been implicated in initiating plant-growth responses. Regardless, what is agreed is that there is a need for detailed chemical and spectroscopic characterization of HS to ensure that suitable comparisons can be made between different studies (Canellas et al., 2012; Trevisan et al., 2010). In this respect, it is difficult to make a concrete conclusion on the cause for the rate-response dynamics of plants to soil-derived HS observed here.

### 3.4. Environmental conditions

Environmentally stressful conditions, such as salinity, heavy metal toxicity, or nutrient deficiency, rather than the plant type, played a more prominent role in shaping the root growth response to HS. This finding is especially relevant to the agronomic use of HS, because soil degradation, climate change, and diminishing water and nutrient resources are becoming

increasingly important constraints to agricultural production, and recommendations for using HS are often directed at alleviating these stresses (Billingham, 2012). Although application rates greater than 100–200 mg kg<sup>-1</sup> of BC-derived HS generally inhibited root growth under stressful conditions, the stress condition under which these types of HS were applied was limited to micronutrient deficiency. In comparison, higher application rates of soil-derived HS actually improved root growth; but, the stress conditions under which these HS were applied did not include micronutrient deficiency, instead involving salinity or heavy metal toxicity. Both these effects can be accounted for by the high cation exchange capacity of HS. On the one hand, high rates of HS (regardless of source) could easily aggravate micronutrient deficiency by depleting the available pool for plant uptake, as highlighted by reduced Zn uptake in the hydroponic studies of Vaughan and Macdonald (1976). Conversely, high rates of HS would alleviate heavy metal or salinity stress by binding excess cations. Taking this into consideration, the type of stress, rather than a stress-by-HS source interaction, would therefore be a more important factor in HS efficacy at high rates.

Unfortunately, our capacity to draw further conclusions is limited by the paucity of studies available that characterize plant growth under stressful conditions when treated with low application rates of HS. There is evidence to suggest that under micronutrient-deficient conditions, low rates of HS can actually assist in mobilizing micronutrients, while maintaining a capacity to reduce plant uptake of micronutrients at high or toxic levels (Chen et al., 2004a; Garcia-Mina et al., 2004; Stevenson, 1994). More recent studies also show that low rates (<100 mg kg<sup>-1</sup>) of compost HS can also reduce the severity of plant stress directly, by stimulating an antioxidant stress response in roots that effectively primes the plant to resist other stresses (Garcia et al., 2012). Overall, the actual consequence of the interaction between HS source, application rate, and stress conditions is likely to arise from both indirect and direct mechanisms. More research is therefore needed in order to quantify and predict the conditions under which specific mechanisms will dominate.

### 3.5. Plant type

The effect of HS on shoot biomass was not only dependent on the source and rate of application but also the plant type. Such an interaction is not altogether surprising and has been previously emphasized (Vaughan and Malcolm, 1985). In our synthesis, not only did HS treatment inhibit the



shoot growth of woody perennial plants as compared with herbaceous plant species, but it also resulted in significantly lower biomass than nontreated controls. Partitioning of the data set showed that only BC- and peat-derived HS were applied to woody perennials, rather than compost- or soil-derived HS, which may have inflated the difference between plant types. Furthermore, the fact that the number of studies examining woody perennials was low ( $n_s=3$ ) and the rates of BC-derived HS used on this plant type were relatively high ( $>300$  mg kg), means that these results are not fully representative of HS interaction with woody perennials. Nevertheless, in each of these studies, a dose-response trend was observed (Kelting et al., 1998; Marino et al., 2008; Vallini et al., 1993), implying a causal relationship and again emphasizing the importance of application rate in determining the growth response.

With regards to broad-acre cropping, a more useful distinction would concern differences between monocotyledonous and dicotyledonous plant species. Although our results only suggest marginal differences between monocots and dicots, there is some evidence in the literature showing clear differences between plant types. It is possible that some differences between plant types are related to the inherent susceptibilities to particular soil conditions, especially micronutrient availability. For example, Garcia-Mina et al. (2004) found that the effects of a Zn-HS complex on the SDW and RDW of alfalfa under Zn-deficient conditions were significantly positive, but not so in wheat. They speculated that the results reflected the greater sensitivity of alfalfa to Zn deficiency. In contrast, Dobbss et al. (2010) found that the optimum concentration of HS required to stimulate root branching in maize was approximately half that required for maximum stimulation of the dicots tomato and Arabidopsis, suggesting a greater efficacy toward monocots.



---

## 4. PRACTICAL USE OF HS IN AGRICULTURE

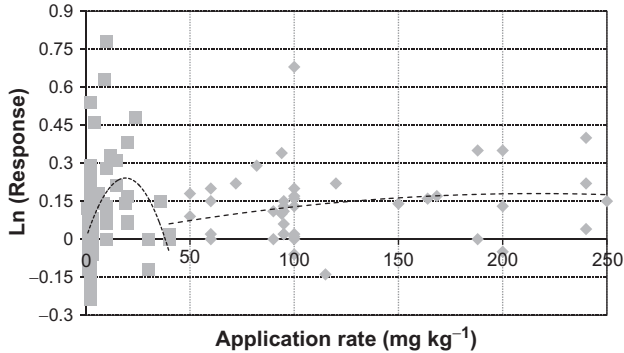
### 4.1. Direct application

A key decision for a farmer or land holder in applying any soil or plant amendment is the rate at which it should be applied in for maximum efficacy at minimum cost. According to Quilty and Cattle (2011), the cost of HS is in the range of \$40–800  $\text{ton}^{-1}$ . At application rates of  $100$  mg  $\text{kg}^{-1}$ , approximately equivalent to  $100$  kg  $\text{ha}^{-1}$  in topsoil (10 cm incorporation), this translates to costs in the range of \$4–80  $\text{ha}^{-1}$ . In comparison, the cost of N fertilizer is approximately \$1000  $\text{ton}^{-1}$  (per unit of N) (USDA, 2013), such that  $100$  kg N  $\text{ha}^{-1}$  translates to a cost of approximately \$100  $\text{ha}^{-1}$ .

Considering that the yield response of crops to N fertilizer is consistent and profitable (Liu et al., 2006), the use of HS at rates higher than  $100 \text{ mg kg}^{-1}$  for the sole purpose of short-term increases in biomass productivity in noncompromized soils is unlikely to be competitive with conventional fertilizer practices at current prices. Compost-derived HS, which significantly increase plant-growth response at high rates, may be cost-effective if the waste is produced locally and is available at little or no cost. Even so, the additional step of isolating HS from the compost would likely be more of a hindrance than the alternative option of spreading solid compost directly. The results of our study also caution against using high rates of HS on woody perennials because of potential growth inhibition, although more research is needed for this recommendation to be conclusive.

Despite the lack of incentive for applying high rates of HS under satisfactory growth conditions, it may be justified in certain instances where environmental conditions are a constraint to plant growth. Indeed, our synthesis indicates that HS may be most efficacious under stress conditions. Amelioration of saline soils or soils contaminated with heavy metals with HS appears to have positive growth effects on plants, and could assist in reclaiming marginal lands with these characteristics. The effectiveness of HS for assisting plants to tolerate or overcome stress could also extend to conditions of drought (Zhang and Schmidt, 2000) or pathogen control (Loffredo et al., 2008), but these possibilities could not be addressed by the data available in our study. In any case, care should be taken to identify the environmental constraint and ensure that the stress is not exacerbated; for example, HS application greater than  $100 \text{ mg kg}^{-1}$  to micronutrient-deficient soils may actually further inhibit, rather than stimulate, plant growth.

The question then remains: Are low rates ( $<100 \text{ mg kg}^{-1}$ ) of HS application efficacious and if so, economically and practically worthwhile? To answer this question, we focussed on HS derived from BCs, as these usually form the basis of commercial products. We found that the growth response to low rates of BC-derived HS is nonlinear and is more appropriately described by the sum of two quadratic functions rather than a single quadratic or higher polynomial (Fig. 2.8). An initial sharp peak in growth response is observed between 5 and  $40 \text{ mg kg}^{-1}$  with a maximum around  $20 \text{ mg kg}^{-1}$ , followed by a more gradual growth increase from 40 to  $200 \text{ mg kg}^{-1}$ . There appears to be a greater opportunity to maximize plant-growth promotion by applying BC-derived HS in the lower range ( $5\text{--}40 \text{ mg kg}^{-1}$ ) of the initial peak, which would also be more economically rational. Based on the extrapolation of a number of studies, Chen et al.



**Figure 2.8** Effect of application rate of brown-coal-derived HS on SDW. Dashed lines show quadratic fits to rates less than  $50 \text{ mg kg}^{-1}$  (short dash, square points) and rates greater than or equal to  $50 \text{ mg kg}^{-1}$  (long dash, diamond points).

(2004b) calculated the amount of HS required for an effective soil application to be  $22.5 \text{ mg kg}^{-1}$ , equivalent to  $75 \text{ mg L}^{-1}$  of HS dispersed in a soil at a moisture content of 30%. This value is very close to the peak of the low-range quadratic response calculated here.

Unfortunately, the reasons for growth promotion at these low rates cannot be directly determined through meta-analysis of the data collated here. As outlined earlier, the interaction of HS with plant essential elements, including N, P, and micronutrients, is known to improve nutrient availability and may be one reason for growth promotion at low rates. If this is correct, a substantial agronomic opportunity therefore exists to improve the efficiency of fertilizer nutrient use, rather than enhancing growth *per se*. There is also evidence that HS can positively interact with beneficial microorganisms, offering the possibility of additional productivity gains if harnessed appropriately.

## 4.2. Application as synergists

The chemical properties of HS, including hydrophilic and hydrophobic domains and zwitterionic features, facilitate interactions with a wide variety of soil constituents. Theoretically, these properties act to buffer biological susceptibility to nutritional extremes, such that high activities of salts, metals, and protons in the soil solution can be reduced, while low activities of nutrients are mobilized into plant-available forms. Recent research has demonstrated the potential for exploiting these properties of HS to design slow- or controlled-release fertilizers that better match the availability of nutrients to the plant lifecycle (Davidson and Gu, 2012).

Nitrogen fertilizers coated with humic acids are commercially available and are reported to increase fertilizer use efficiency (Chen et al., 2008), probably through a number of mechanisms. First, HS have been shown to significantly reduce urea hydrolysis from urea–ammonium nitrate and also retard the formation of  $\text{NO}_3^-$ , implying urease- and nitrification-inhibition activity (Alkanani et al., 1990). Reduced urea hydrolysis in HS-treated soils has been linked to biological buffering of the HS on microbial populations and enzyme activities (Dong et al., 2009). However, GarciaSerna et al. (1996) also showed that humic acids (1%, w/w) sprayed onto the surface of urea or Nitrophoska granules slow nitrogen release through physicochemical mechanisms, but probably not solely by acting as a physical coating since the release curve was observed to be convex, not concave. Aside from slowing the formation and release of ammonium ( $\text{NH}_4^+$ ) from urea, HS can also reduce the volatilization of ammonia without significantly altering pH, which is one of the main drivers of  $\text{NH}_3$  emission (Kasim et al., 2009). Erro et al. (2007) formulated a compound HS-NPK fertilizer and observed reduced ammonia volatilization, reduced N-leaching, and increased plant growth with respect to an NPK control fertilizer. However, Kiran et al. (2010) found that humic-coated urea did not improve N-use efficiency in rice paddy systems, whereas a number of other controlled-release fertilizer formulations were effective.

There is also evidence that the association of soluble phosphate with HS reduces its binding and precipitation in soil, allowing for greater plant uptake (Alvarez et al., 2004; Hua et al., 2008; Schefe et al., 2008). Indeed, Gerke (2010) suggests that the majority of bicarbonate-extractable P (e.g., Olsen-P, Colwell-P) actually exists in soil as humic-metal-P, rather than free or sorbed orthophosphate, but becomes liberated by acidification steps during analysis. On the basis of these reports, Erro et al. (2009, 2012) developed and characterized the performance of several “organic complexed superphosphate (CSP)” fertilizers. The CSPs were produced by introducing HS during the chemical synthesis of single-super phosphate (SSP). Glasshouse experiments showed that CSPs consistently enhanced P-accumulation in wheat grown in both acid and alkaline P-fixing soils when compared against an SSP control treatment. The authors suggested that greater P-uptake efficiency afforded by the CSPs is related to the formation of stable monocalcium–phosphate–humic complexes during CSP preparation.

Together, these results suggest a role for the use of HS in improving N- and P-use efficiency in cropping systems, but more work is needed in developing effective formulations. The potential for using HS to improve micronutrient availability and absorption is also well recognized (Chen

et al., 2004a; Garcia-Mina et al., 2004), but there is a noticeable lack of experimental studies reporting the efficacy of micronutrient-HS fertilizer formulations. In fact, one recent study showed that HS-Fe complexes were ineffective at supplying iron to Fe-deficient soybean, either as a foliar spray or through root absorption, whereas synthetic chelates (e.g., EDTA) were effective at delivering Fe (Rodriguez-Lucena et al., 2010). Because there are an ever increasing number of humic-coated or humic-containing fertilizers on the market, further research and validation of such products is urgently needed to provide farmers with reliable information for making agronomic decisions.

Another strategy gaining popularity in sustainable agronomy is the use of microbial inoculants in agriculture as plant-growth promoters (PGPs). These PGPs can assist in nutrient acquisition, stress tolerance, and pathogen suppression through diverse biological functions. Although substantial work has been done in this area, little is known about the interactions of PGPs with indigenous or exogenous HS. To our knowledge, only one study has directly examined the potential to use HS in conjunction with plant-growth promoting rhizobacteria (PGPR) for stimulating plant growth (Canellas et al., 2013). These authors speculated that the auxin-like action of HS could improve the colonization ability of PGPR via root-branching nodes, as has been previously observed with the synthetic auxin 2,4-D (Katupitiya et al., 1995). They found that coinoculation of *Herbaspirillum seropedicae* with 20 mg HS C L<sup>-1</sup> improved colonization of maize roots, but that this effect was dose dependent and that colonization was inhibited at a higher HS concentration. Validation of this effect in the field confirmed a synergistic effect, with maize yield increasing an additional 45–48% when HS and *H. seropedicae* were applied in combination, compared with sole treatments of *H. seropedicae* or HS, respectively. The authors caution against generalizing this result until further studies can be performed, but their results clearly warrant more research in this area with other PGPR strains.

It would also be interesting to extend this research to the effects of HS on plant-microbial symbioses, such as rhizobial and mycorrhizal associations. These cooperative plant-microbial associations are critical components of nutrient cycling in agro-ecosystems and enhance plant nutrient acquisition (Peoples and Craswell, 1992; Smith and Read, 2008; Zhu et al., 2001). Although Vallini et al. (1993) reported a depression in the mycorrhizal colonization of laurel roots and hyphal length in the presence of high concentrations of HS (>800 mg kg<sup>-1</sup>), a more recent study by Gryndler et al. (2005) found that HS applied in hydroponics at a rate of approximately 800 mg L<sup>-1</sup> stimulated maize root colonization and production of extraradical mycelium

by the mycorrhizal fungus *Glomus claroideum* BEG 23. In a similar fashion, Gaur and Bhardwaj (1971) observed a greater nodule formation in the legume *Sesbania aculeata* by native rhizobia when sodium humate was amended into soil at a rate of 600 mg kg<sup>-1</sup>. Unfortunately, as Gryndler et al. (2005) acknowledged, experiments on the effects of HS on plant–microbial symbioses are rare and it is difficult to make any consistent conclusions.



## 5. KNOWLEDGE GAPS AND RESEARCH NEEDS

Through the process of meta-analysis, a number of knowledge gaps have also been identified. First, the majority of papers reporting experiments on HS lack information about the organic structure, molecular nature and size, and mineral concentrations of the HS amendments. Considering the importance of HS source shown by our analysis, provision of this kind of information in future studies will be necessary in order to increase our understanding of how particular HS improve plant growth. Such knowledge may subsequently open the door to tailoring HS products for specific purpose. In conjunction with HS characteristics, more complete metadata about the environmental conditions under which the HS are applied are needed. Data about the soil, such as the nature of native organic matter, pH, EC, texture, and mineral nutrient concentrations are required to increase our understanding of HS–soil–plant interactions. The focus of most studies conducted in soil is on the HS applied and few, if any, have attempted to reconcile the possible interactions between HS already in the soil (where HS levels would be much higher) and those applied. The complexity of scientifically addressing this research question is possibly why data are lacking. In addition, there is the recognized fact that the extraction processes, generally involving alkaline treatments, are likely to have chemically modified the original organic materials (Swift et al., 1996).

In terms of agronomic management, HS application rate is a critical decision that cannot only affect plant growth but also economic margins. More work is needed involving valid, scientifically designed field trials with crops grown to harvest, in order to define application rate windows that will maximize growth while minimizing the risk of economic loss. Interestingly, the majority of studies reviewed here only measured growth responses for less than 3 months during early vegetative growth. It remains unknown if the trends observed in the early stages of plant growth will be maintained for the duration of the plant life cycle and therefore translates into yield gains at harvest. This knowledge gap is currently being further pursued by the authors.

The use of HS in conjunction with inorganic fertilizers is of direct relevance here, as HS may improve nutrient recovery by plants without enhancing growth *per se*, leading to reduced fertilizer input costs. There is also a real need to systematically determine the effects of adding HS on soil microbes and related carbon and nutrient cycling. Much of the work focused on the effect of HS on nutrient acquisition by plants has been conducted in hydroponic systems and may therefore overlook the importance on plant–microbial associations in the rhizosphere.

Finally, there is a paucity of data surrounding the long-term effects of HS, or of repeated HS application. The majority of studies reported here had durations of less than 6 months, and in many cases were only observed over daily or weekly timeframes. Any improvements to soil quality are likely to occur over longer periods and effects on crop growth may not be quantifiable in the short term, in accordance with long-term studies of other “organic” agronomy practices (Clark *et al.*, 1998; Gosling and Shepherd, 2005).



---

## 6. CONCLUSIONS

This meta-analysis has shown that the growth response of plants to HS, although generally positive, is influenced by a number of environmental and management factors. Our findings indicate that the source of the HS, in particular, will have a strong impact on whether or not plant growth is significantly improved. Plant type and stress conditions also influence the plant-growth response to HS, but to a lesser extent. Interactions between each of these factors and the HS application rate also moderate the plant-growth response, emphasizing the complexity of obtaining predictable responses. More research is needed to characterize the structure–activity relations of HS, and how these can be exploited either through direct application or application as synergists with chemical or biological fertilizers. We conclude by reiterating that the prospects for using HS as plant-growth stimulants in agricultural systems are theoretically strong, but continued research and extension is needed to realize their full potential under diverse environmental conditions.

## ACKNOWLEDGMENTS

The authors would like to thank Brown Coal Innovation Australia, Clean Coal Victoria, and International Power for financial support. T. R. C. gratefully acknowledges the Australian Research Council for supporting his research.



## APPENDIX A. STUDY REFERENCES FOR SDW DATA USED IN THE META-ANALYSIS

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
1	Azcona, I., Pascual, I., Aguirreolea, J., Fuentes, M., Garcia-Mina, J., Sanchez-Diaz, M.	Growth and development of pepper are affected by humic substances derived from composted sludge	2011	Journal of Plant Nutrition and Soil Science	174	6	916	924
2	Ertani, A., Francioso, O., Tugnoli, V., Righi, V., Nardi, S.	Effect of commercial lignosulfonate-humate on Zea mays L. metabolism	2011	Journal of Agricultural and Food Chemistry	59	22	11940	11948
3	Yarkova, T.A.	Chemical modification of humic acids by the introduction of indole-containing fragments	2011	Solid Fuel Chemistry	45	4	261	266
4	Saruhan, V., Kusvuran, A., Kokten, K.	The effect of different replications of humic acid fertilization on yield performances of common vetch ( <i>Vicia sativa</i> L.)	2011	African Journal of Biotechnology	10	29	5587	5592



5	Tahir, M.M., Khurshid, M., Khan, M.Z., Abbasi, M.K., Kazmi, M.H.	Lignite-derived humic acid effect on growth of wheat plants in different soils	2011	Pedosphere	21	1	124	131
6	Obsuwan, K., Namchote, S., Sanmanee, N., Panishkan, K., Dharmvanij, S.	Effect of various concentrations of humic acid on growth and development of eggplant seedlings in tissue cultures at low nutrient level	2011	Proceedings of World Academy of Science, Engineering and Technology	80		276	278
7	Khaled, H., Fawy, H.	Effect of different Levels of humic acids on the nutrient content, plant growth, and soil properties under conditions of salinity	2011	Soil and Water Research	6	1	21	29
8	Cordeiro, F.C., Santa-Catarina, C., Silveira, V., De Souza, S.R.	Humic acid effect on catalase activity and the generation of reactive oxygen species in corn ( <i>Zea mays</i> )	2011	Bioscience, Biotechnology and Biochemistry	75	1	70	74
9	Morard, P., Eyheraguibel, B., Morard, M., Silvestre, J.	Direct effects of Humic-Like substance on growth, water, and mineral nutrition of various species	2011	Journal of Plant Nutrition	34	1	46	59

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
10	Celik, H., Katkat, A.V., Asik, B.B., Turan, M.A.	Effect of foliar-applied humic acid to dry weight and mineral nutrient uptake of maize under calcareous soil conditions	2011	Communications in Soil Science and Plant Analysis	42	1	29	38
11	Cimrin, K.M., Turkmen, O., Turan, M., Tuncer, B.	Phosphorus and humic acid application alleviate salinity stress of pepper seedling	2010	African Journal of Biotechnology	9	36	5845	5851
12	Baldotto, L.E.B., Baldotto, M.A., Canellas, L.P., Bressan-Smith, R., Olivares, F.L.	Growth promotion of pineapple "Vitoria" by humic acids and burkholderia spp. during acclimatization	2010	Revista Brasileira de Ciencia do Solo	34	5	1593	1600
13	Gulser, F., Sonmez, F., Boysan, S.	Effects of calcium nitrate and humic acid on pepper seedling growth under saline condition	2010	Journal of Environmental Biology	31	5 Suppl.	873	876
14	Paksoy, M., Turkmen, O., Dursun, A.	Effects of potassium and humic acid on emergence, growth and nutrient contents of okra ( <i>Abelmoschus esculentus</i> L.) seedling under saline soil conditions	2010	African Journal of Biotechnology	9	33	5343	5346

15	Petrus, A.C., Ahmed, O.H., Muhamad, A.M.N., Nasir, H.M., Jiwan, M.	Effect of K-N-humates on dry matter production and nutrient use efficiency of maize in Sarawak, Malaysia	2010	The Scientific World Journal	10		1282	1292
16	Asli, S., Neumann, P.M.	Rhizosphere humic acid interacts with root cell walls to reduce hydraulic conductivity and plant development	2010	Plant and Soil	336	1	313	322
17	Hartz, T.K., Bottoms, T.G.	Humic substances generally ineffective in improving vegetable crop nutrient uptake or productivity	2010	HortScience	45	6	906	910
18	Mora, V., Bacaicoa, E., Zamarreno, A.-M., Aguirre, E., Garnica, M., Fuentes, M., Garcia-Mina, J.-M.	Action of humic acid on promotion of cucumber shoot growth involves nitrate-related changes associated with the root-to-shoot distribution of cytokinins, polyamines and mineral nutrients	2010	Journal of Plant Physiology	167	8	633	642

*Continued*

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
19	Kim, A., Kashif, S.R., Yaseen, M.	Using indigenous humic acid from lignite to increase growth and yield of okra ( <i>Abelmoschus esculentus</i> L.)	2010	Soil and Environment	29	2	187	191
20	El-Hefny, E.M.	Effect of saline irrigation water and humic acid application on growth and productivity of two cultivars of cowpea ( <i>Vigna unguiculata</i> L. Walp)	2010	Journal of Applied Sciences Research	6	12	6154	6168
21	Bandiera, M., Mosca, G., Vamerli, T.	Humic acids affect root characteristics of fodder radish ( <i>Raphanus sativus</i> L. var. <i>oleiformis</i> Pers.) in metal-polluted wastes	2009	Desalination	246	1-3	78	91
22	Asik, B.B., Turan, M.A., Celik, H., Katkat, A.V.	Effects of humic substances on plant growth and mineral nutrients uptake of wheat ( <i>Triticum durum</i> cv. Salihli) under conditions of salinity	2009	Asian Journal of Crop Science	1	2	87	95

23	Kasim, S., Ahmed, O.H., Majid, N.M.A., Yusop, M.K., Jalloh, M.B.	Effect of organic based N fertilizer on dry matter (Zea mays L.), Ammonium and nitrate recovery in an acid soil of Sarawak, Malaysia	2009	American Journal of Applied Sciences	6	7	1289	1294
24	Nikbakht, A., Kafi, M., Babalar, M., Xia, Y.P., Luo, A., Etemadi, N.-A.	Effect of humic acid on plant growth, nutrient uptake, and postharvest life of gerbera	2008	Journal of Plant Nutrition	31	12	2155	2167
25	Eyheraguibel, B., Silvestre, J., Morard, P.	Effects of humic substances derived from organic waste enhancement on the growth and mineral nutrition of maize	2008	Bioresource Technology	99	10	4206	4212
26	De Santiago, A., Quintero, J.M., Carmona, E., Delgado, A.	Humic substances increase the effectiveness of iron sulfate and Vivianite preventing iron chlorosis in white lupin	2008	Biology and Fertility of Soils	44	6	875	883
27	Clapp, C.E., Shenker, M., Hayes, M.H.B., Liu, R., Cline, V.W., Palazzo, A.J., Chen, Y.	Microsystems for rapid evaluation of plant growth response to organic amendments	2008	Soil Science	173	5	342	349

*Continued*

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
28	Willis, J.M., Hester, M.W.	Evaluation of enhanced Panicum amarum establishment through fragment plantings and humic acid amendment	2008	Journal of Coastal Research	24	2 Suppl. B	263	268
29	Marino, G., Francioso, O., Carletti, P., Nardi, S., Gessa, C.	Mineral content and root respiration of in vitro grown kiwifruit plantlets treated with two humic fractions	2008	Journal of Plant Nutrition	31	6	1074	1090
30	Celik, H., Katkat, A.V., Asik, B.B., Turan, M.A.	Effects of soil applied humic substances to dry weight and mineral nutrients uptake of maize under calcareous soil conditions	2008	Archives of Agronomy and Soil Science	54	6	605	614
31	Canellas, L.P., Zandonadi, D.B., Busato, J.G., Baldotto, M.A., Simoes, M.L., Martin-Neto, L., Facanha, A.R., Spaccini, R., Piccolo, A.	Bioactivity and chemical characteristics of humic acids from tropical soils sequence	2008	Soil Science	173	9	624	637

32	Khan, S., Cao, Q., Chen, B.-D., Zhu, Y.-G.	Humic acids increase the phytoavailability of Cd and Pb to wheat plants cultivated in freshly spiked, contaminated soil	2006	Journal of Soils and Sediments	6	4	236	242
33	Gryndler, M., Hrselova, H., Sudova, R., Gryndlerova, H., Rezacova, V., Merhautova, V.	Hyphal growth and mycorrhiza formation by the arbuscular mycorrhizal fungus <i>Glomus claroideum</i> BEG 23 is stimulated by humic substances	2005	Mycorrhiza	15	7	483	488
34	Turkmen, O., Dursun, A., Turan, M., Erdinc, C.	Calcium and humic acid affect seed germination, growth, and nutrient content of tomato ( <i>Lycopersicon esculentum</i> L.) seedlings under saline soil conditions	2004	Acta Agriculturae Scandinavica Section B: Soil and Plant Science	54	3	168	174
35	Sharif, M., Khattak, R.A., Sarir, M.S.	Effect of different levels of lignitic coal derived humic acid on growth of maize plants	2002	Communications in Soil Science and Plant Analysis	33	19–20	3567	3580

*Continued*

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
36	Badora, A.	Bioaccumulation of Al, Mn, Zn and Cd in Pea Plants ( <i>Pisum sativum</i> L.) Against a Background of Unconventional Binding Agents	2002	Polish Journal of Environmental Studies	11	2	109	116
37	Atiyeh, R.M., Lee, S., Edwards, C.A., Arancon, N.Q., Metzger, J.D.	The influence of humic acids derived from earthworm-processed organic wastes on plant growth	2002	Bioresource Technology	84	1	7	14
38	Masciandaro, G., Ceccanti, B., Ronchi, V., Benedicto, S., Howard, L.	Humic substances to reduce salt effect on plant germination and growth	2002	Communications in Soil Science and Plant Analysis	33	3-4	365	378
39	Bidegain, R.A., Kaemmerer, M., Guiresse, M., Hafidi, M., Rey, F., Morard, P., Revel, J.C.	Effects of humic substances from composted or chemically decomposed poplar sawdust on mineral nutrition of ryegrass	2000	Journal of Agricultural Science	134	3	259	267



40	Adani, F., Genevini, P., Zaccheo, P., Zocchi, G.	The effect of commercial humic acid on tomato plant growth and mineral nutrition	1998	Journal of Plant Nutrition	21	3	561	575
41	Kelting, M., Harris, J.R., Fanelli, J., Appleton, B.	Biostimulants and soil amendments affect two-year posttransplant growth of red maple and Washington hawthorn	1998	HortScience	33	5	819	822
42	Loffredo, E., Senesi, N., D'Orazio, V.	Effects of humic acids and herbicides, and their combinations on the growth of tomato seedlings in hydroponics	1997	Journal of Plant Nutrition and Soil Science	160	5	455	461
43	Ayuso, M., Moreno, J.L., Hernandez, T., Garcia, C.	Characterization and evaluation of humic acids extracted from urban waste as liquid fertilizers	1997	Journal of the Science of Food and Agriculture	75	4	481	488
44	Ayuso, M., Hernandez, T., Garcia, C., Pascual, J.A.	Stimulation of barley growth and nutrient absorption by humic substances originating from various organic materials	1996	Bioresource Technology	57	3	251	257

*Continued*

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
45	Gonet, S.S., Dziamski, A., Gonet, E.	Application of humus preparations from oxyhumolite in crop production	1996	Environment International	22	5	559	562
46	Ayuso, M., Hernandez, T., Garcia, C., Pascual, J.A.	A comparative study of the effect on barley growth of humic substances extracted from municipal wastes and from traditional organic materials	1996	Journal of the Science of Food and Agriculture	72	4	493	500
47	Valdrighi, M.M., Pera, A., Agnolucci, M., Frassinetti, S., Lunardi, D., Vallini, G.	Effects of compost-derived humic acids on vegetable biomass production and microbial growth within a plant (Cichorium intybus)-soil system: A comparative study	1996	Agriculture, Ecosystems and Environment	58	2-3	133	144
48	Vallini, G., Pera, A., Avio, L., Valdrighi, M., Giovannetti, M.	Influence of humic acids on laurel growth, associated rhizospheric microorganisms, and mycorrhizal fungi	1993	Biology and Fertility of Soils	16	1	1	4

49	Piccolo, A., Celano, G., Pietramellara, G.	Effects of fractions of coal-derived humic substances on seed germination and growth of seedlings ( <i>Lactuca sativa</i> and <i>Lycopersicum esculentum</i> )	1993	Biology and Fertility of Soils	16	1	11	15
50	Lobartini, J.C., Tan, K.H., Rema, J.A., Gingle, A.R., Pape, C., Himmelsbach, D.S.	The geochemical nature and agricultural importance of commercial humic matter	1992	Science of the Total Environment	113	1-2	1	15
51	Senesi, N., Loffredo, E., Padovano, G.	Effects of humic acid-herbicide interactions on the growth of <i>Pisum sativum</i> in nutrient solution	1990	Plant and Soil	127	1	41	47
52	Cabrera, D., Young, S.D., Rowell, D.L.	The toxicity of cadmium to barley plants as affected by complex formation with humic acid	1988	Plant and Soil	105	2	195	204
53	Tan, K.H., Binger, A.	Effect of humic acid on aluminum toxicity in corn plants	1986	Soil Science	141	1	20	25

*Continued*

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
54	Tan, K.H., Nopamornbodi, V.	Effect of different levels of humic acids on nutrient content and growth of corn ( <i>Zea mays</i> L.)	1979	Plant and Soil	51	2	283	287
55	Vaughan, D., Linehan, D.J.	The growth of wheat plants in humic acid solutions under axenic conditions	1976	Plant and Soil	44	2	445	449
56	Gaur, A.C., Bhardwaj, K.K.R.	Influence of sodium humate on the crop plants inoculated with bacteria of agricultural importance	1971	Plant and Soil	35	1	613	621



## APPENDIX B. STUDY REFERENCES FOR RDW DATA USED IN THE META-ANALYSIS

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
1	Mora, V., Baigorri, R., Bacaicoa, E., Zamarreno, A.M., Garcia-Mina, J.M.	The humic acid-induced changes in the root concentration of nitric oxide, IAA and ethylene do not explain the changes in root architecture caused by humic acid in cucumber	2012	Environmental and Experimental Botany	76		24	32
2	Azcona, I., Pascual, I., Aguirreolea, J., Fuentes, M., Garcia-Mina, J., Sanchez-Diaz, M.	Growth and development of pepper are affected by humic substances derived from composted sludge	2011	Journal of Plant Nutrition and Soil Science	174	6	916	924
3	Ertani, A., Francioso, O., Tugnoli, V., Righi, V., Nardi, S.	Effect of commercial lignosulfonate-humate on Zea mays L. metabolism	2011	Journal of Agricultural and Food Chemistry	59	22	11940	11948
4	Jindo, K., Martim, S.A., Navarro, E.C., Perez-Alfocea, F., Hernandez, T., Garcia, C., Aguiar, N.O., Canellas, L.P.	Root growth promotion by humic acids from composted and non-composted urban organic wastes	2011	Plant and Soil			1	12

*Continued*

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
5	Yarkova, T.A.	Chemical modification of humic acids by the introduction of indole-containing fragments	2011	Solid Fuel Chemistry	45	4	261	266
6	Kirn, A., Kashif, S.R., Yaseen, M.	Using indigenous humic acid from lignite to increase growth and yield of okra ( <i>Abelmoschus esculentus</i> L.)	2010	Soil and Environment	29	2	187	191
7	Cimrin, K.M., Turkmen, O., Turan, M., Tuncer, B.	Phosphorus and humic acid application alleviate salinity stress of pepper seedling	2010	African Journal of Biotechnology	9	36	5845	5851
8	Baldotto, L.E.B., Baldotto, M.A., Canellas, L.P., Bressan-Smith, R., Olivares, F.L.	Growth promotion of pineapple "Vitoria" by humic acids and burkholderia spp. during acclimatization	2010	Revista Brasileira de Ciencia do Solo	34	5	1593	1600
9	Gulser, F., Sonmez, F., Boysan, S.	Effects of calcium nitrate and humic acid on pepper seedling growth under saline condition	2010	Journal of Environmental Biology	31	5 Suppl.	873	876
10	Paksoy, M., Turkmen, O., Dursun, A.	Effects of potassium and humic acid on emergence, growth and nutrient contents of okra ( <i>Abelmoschus esculentus</i> L.) seedling under saline soil conditions	2010	African Journal of Biotechnology	9	33	5343	5346

11	Petrus, A.C., Ahmed, O.H., Muhamad, A.M.N., Nasir, H.M., Jiwana, M.	Effect of K-N-humates on dry matter production and nutrient use efficiency of maize in Sarawak, Malaysia	2010	The Scientific World Journal	10		1282	1292
12	Busato, J.G., Zandonadi, D.B., Dobbss, L.B., Facanha, A.R., Canellas, L.P.	Humic substances isolated from residues of sugar cane industry as root growth promoter	2010	Scientia Agricola	67	2	206	212
13	Canellas, L.P., Spaccini, R., Piccolo, A., Dobbss, L.B., Okorokova-Facanha, A.L., Santos, G.D.A., Olivares, F.L., Facanha, A.R.	Relationships between chemical characteristics and root growth promotion of humic acids isolated from Brazilian oxisols	2009	Soil Science	174	11	611	620
14	Bandiera, M., Mosca, G., Vamerali, T.	Humic acids affect root characteristics of fodder radish ( <i>Raphanus sativus</i> L. var. <i>oleiformis</i> Pers.) in metal-polluted wastes	2009	Desalination	246	1-3	78	91
15	Nikbakht, A., Kafi, M., Babalar, M., Xia, Y.P., Luo, A., Etemadi, N.-A.	Effect of humic acid on plant growth, nutrient uptake, and postharvest life of gerbera	2008	Journal of Plant Nutrition	31	12	2155	2167

*Continued*

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
16	Eyheraguibel, B., Silvestre, J., Morard, P.	Effects of humic substances derived from organic waste enhancement on the growth and mineral nutrition of maize	2008	Bioresource Technology	99	10	4206	4212
17	De Santiago, A., Quintero, J.M., Carmona, E., Delgado, A.	Humic substances increase the effectiveness of iron sulfate and Vivianite preventing iron chlorosis in white lupin	2008	Biology and Fertility of Soils	44	6	875	883
18	Marino, G., Francioso, O., Carletti, P., Nardi, S., Gessa, C.	Mineral content and root respiration of in vitro grown kiwifruit plantlets treated with two humic fractions	2008	Journal of Plant Nutrition	31	6	1074	1090
19	Clapp, C.E., Shenker, M., Hayes, M.H.B., Liu, R., Cline, V.W., Palazzo, A.J., Chen, Y.	Microsystems for rapid evaluation of plant growth response to organic amendments	2008	Soil Science	173	5	342	349
20	Willis, J.M., Hester, M.W.	Evaluation of enhanced <i>Panicum amarum</i> establishment through fragment plantings and humic acid amendment	2008	Journal of Coastal Research	24	2 Suppl. B	263	268



21	Zandonadi, D.B., Canellas, L.P., Facanha, A.R.	Indolacetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H <sup>+</sup> pumps activation	2007	Planta	225	6	1583	1595
22	Arancon, N.Q., Edwards, Clive.A., Lee, S., Byrne, R.	Effects of humic acids from vermicomposts on plant growth	2006	European Journal of Soil Biology	42	Suppl. 1	65	69
23	Khan, S., Cao, Q., Chen, B.-D., Zhu, Y.-G.	Humic acids increase the phytoavailability of Cd and Pb to wheat plants cultivated in freshly spiked, contaminated soil	2006	Journal of Soils and Sediments	6	4	236	242
24	Gryndler, M., Hrselova, H., Sudova, R., Gryndlerovaj, H., Rezacova, V., Merhautova, V.	Hyphal growth and mycorrhiza formation by the arbuscular mycorrhizal fungus <i>Glomus claroideum</i> BEG 23 is stimulated by humic substances	2005	Mycorrhiza	15	7	483	488
25	Turkmen, O., Dursun, A., Turan, M., Erdinc, C.	Calcium and humic acid affect seed germination, growth, and nutrient content of tomato ( <i>Lycopersicon esculentum</i> L.) seedlings under saline soil conditions	2004	Acta Agriculturae Scandinavica Section B: Soil and Plant Science	54	3	168	174

*Continued*

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
26	Arancon, N.Q., Lee, S., Edwards, C.A., Atiyeh, R.	Effects of humic acids derived from cattle, food and paper-waste vermicomposts on growth of greenhouse plants	2003	Pedobiologia	47	5-6	741	744
27	Sharif, M., Khattak, R.A., Sarir, M.S.	Effect of different levels of lignitic coal derived humic acid on growth of maize plants	2002	Communications in Soil Science and Plant Analysis	33	19-20	3567	3580
28	Badora, A.	Bioaccumulation of Al, Mn, Zn and Cd in Pea Plants ( <i>Pisum sativum</i> L.) Against a Background of Unconventional Binding Agents	2002	Polish Journal of Environmental Studies	11	2	109	116
29	Atiyeh, R.M., Lee, S., Edwards, C.A., Arancon, N.Q., Metzger, J.D.	The influence of humic acids derived from earthworm-processed organic wastes on plant growth	2002	Bioresource Technology	84	1	7	14
30	Bidegain, R.A., Kaemmerer, M., Guisresse, M., Hafidi, M., Rey, F., Morard, P., Revel, J.C.	Effects of humic substances from composted or chemically decomposed poplar sawdust on mineral nutrition of ryegrass	2000	Journal of Agricultural Science	134	3	259	267

31	Liu, C., Cooper, R.J., Bowman, D.C.	Humic acid application affects photosynthesis, root development, and nutrient content of creeping bentgrass	1998	HortScience	33	6	1023	1025
32	Adani, F., Genevini, P., Zaccheo, P., Zocchi, G.	The effect of commercial humic acid on tomato plant growth and mineral nutrition	1998	Journal of Plant Nutrition	21	3	561	575
33	Loffredo, E., Senesi, N., D'Orazio, V.	Effects of humic acids and herbicides, and their combinations on the growth of tomato seedlings in hydroponics	1997	Journal of Plant Nutrition and Soil Science	160	5	455	461
34	Ayuso, M., Hernandez, T., Garcia, C., Pascual, J.A.	Stimulation of barley growth and nutrient absorption by humic substances originating from various organic materials	1996	Bioresource Technology	57	3	251	257
35	Lobartini, J.C., Tan, K.H., Rema, J.A., Gingle, A.R., Pape, C., Himmelsbach, D.S.	The geochemical nature and agricultural importance of commercial humic matter	1992	Science of the Total Environment	113	1-2	1	15
36	Senesi, N., Loffredo, E., Padovano, G.	Effects of humic acid-herbicide interactions on the growth of <i>Pisum sativum</i> in nutrient solution	1990	Plant and Soil	127	1	41	47

*Continued*

No.	Authors	Title	Year	Source title	Vol.	Iss.	Page start	Page end
37	Cabrera, D., Young, S.D., Rowell, D.L.	The toxicity of cadmium to barley plants as affected by complex formation with humic acid	1988	Plant and Soil	105	2	195	204
38	Tan, K.H., Nopamornbodi, V.	Effect of different levels of humic acids on nutrient content and growth of corn ( <i>Zea mays</i> L.)	1979	Plant and Soil	51	2	283	287
39	Vaughan, D., Linehan, D.J.	The growth of wheat plants in humic acid solutions under axenic conditions	1976	Plant and Soil	44	2	445	449

## REFERENCES

- Alkanani, T., Mackenzie, A.F., Blenkhorn, H., 1990. Volatilization of ammonia from urea-ammonium nitrate solutions as influenced by organic and inorganic additives. *Fertil. Res.* 23, 113–119.
- Alvarez, R., Steinbach, H.S., 2011. Modeling apparent nitrogen mineralization under field conditions using regressions and artificial neural networks. *Agron. J.* 103, 1159–1168.
- Alvarez, R., Evans, L.A., Milham, P.J., Wilson, M.A., 2004. Effects of humic material on the precipitation of calcium phosphate. *Geoderma* 118, 245–260.
- Asli, S., Neumann, P.M., 2010. Rhizosphere humic acid interacts with root cell walls to reduce hydraulic conductivity and plant development. *Plant Soil* 336, 313–322.
- Azcona, I., Pascual, I., Aguirreolea, J., Fuentes, M., Garcia-Mina, J.M., Sanchez-Diaz, M., 2011. Growth and development of pepper are affected by humic substances derived from composted sludge. *J. Plant Nutr. Soil Sci.* 174, 916–924.
- Billingham, K., 2012. Humic Products—Potential or Presumption for Agriculture. NSW Department of Primary Industries, Orange, NSW, Australia.
- Bruccoleri, A., Sorenson, B., Langford, C., 2001. Molecular modeling of humic structures. In: Ghabbour, E.A., Davis, G. (Eds.), *Humic Substances—Structures, Models and Functions*. Royal Society of Chemistry, Cambridge, pp. 193–208.
- Canellas, L.P., Dobbss, L.B., Oliveira, A.L., Chagas, J.G., Aguiar, N.O., Rumjanek, V.M., Novotny, E.H., Olivares, F.L., Spaccini, R., Piccolo, A., 2012. Chemical properties of humic matter as related to induction of plant lateral roots. *Eur. J. Soil Sci.* 63, 315–324.
- Canellas, L.P., Balmori, D.M., Medici, L.O., Aguiar, N.O., Campostrini, E., Rosa, R.C.C., Facanha, A.R., Olivares, F.L., 2013. A combination of humic substances and *Herbaspirillum seropedicae* inoculation enhances the growth of maize (*Zea mays* L.). *Plant Soil* 366, 119–132. <http://dx.doi.org/10.1007/s11104-012-1382-5>.
- Chen, Y., Aviad, T., 1990. Effects of humic substances on plant growth. In: MacCarthy, P., Clapp, C.E., Malcolm, R.L., Bloom, P.R. (Eds.), *Humic Substances in Soil and Crop Sciences: Selected Readings*. American Society of Agronomy, Madison, WI.
- Chen, Y., Clapp, C.E., Magen, H., 2004a. Mechanisms of plant growth stimulation by humic substances: the role of organo-iron complexes. *Soil Sci. Plant Nutr.* 50, 1089–1095.
- Chen, Y., De Nobili, M., Aviad, T., 2004b. Stimulatory effects of humic substances on plant growth. In: Magdoff, F., Weil, R.R. (Eds.), *Soil Organic Matter in Sustainable Agriculture*. CRC Press, Boca Raton, FL.
- Chen, D., Suter, H., Islam, A., Edis, R., Freney, J.R., Walker, C.N., 2008. Prospects of improving efficiency of fertiliser nitrogen in Australian agriculture: a review of enhanced efficiency fertilisers. *Aust. J. Soil Res.* 46, 289–301.
- Clark, M.S., Horwath, W.R., Shennan, C., Scow, K.M., 1998. Changes in soil chemical properties resulting from organic and low-input farming practices. *Agron. J.* 90, 662–671.
- Davidson, D., Gu, F.X., 2012. Materials for sustained and controlled release of nutrients and molecules to support plant growth. *J. Agric. Food Chem.* 60, 870–876.
- de Santiago, A., Exposito, A., Quintero, J.M., Carmona, E., Delgado, A., 2010. Adverse effects of humic substances from different origin on lupin as related to iron sources. *J. Plant Nutr.* 33, 143–156.
- De'ath, G., Fabricius, K.E., 2000. Classification and regression trees: a powerful yet simple technique for ecological data analysis. *Ecology* 81, 3178–3192.
- Dobbss, L.B., Canellas, L.P., Olivares, F.L., Aguiar, N.O., Peres, L.E.P., Azevedo, M., Spaccini, R., Piccolo, A., Facanha, A.R., 2010. Bioactivity of chemically transformed humic matter from vermicompost on plant root growth. *J. Agric. Food Chem.* 58, 3681–3688.

- Dong, L., Cordova-Kreylos, A.L., Yang, J., Yuan, H., Scow, K.M., 2009. Humic acids buffer the effects of urea on soil ammonia oxidizers and potential nitrification. *Soil Biol. Biochem.* 41, 1612–1621.
- Egger, M., Smith, G.D., Schneider, M., Minder, C., 1997. Bias in meta-analysis detected by a simple, graphical test. *Br. Med. J.* 315, 629–634.
- Elith, J., Leathwick, J.R., Hastie, T., 2008. A working guide to boosted regression trees. *J. Anim. Ecol.* 77, 802–813.
- Erro, J., Urrutia, O., Francisco, S.S., Garcia-Mina, J.M., 2007. Development and agronomical validation of new fertilizer compositions of high bioavailability and reduced potential nutrient losses. *J. Agric. Food Chem.* 55, 7831–7839.
- Erro, J., Zamarreno, A.M., Garcia-Mina, J.M., Yvin, J.C., 2009. Comparison of different phosphorus-fertiliser matrices to induce the recovery of phosphorus-deficient maize plants. *J. Sci. Food Agric.* 89, 927–934.
- Erro, J., Urrutia, O., Baigorri, R., Aparicio-Tejo, P., Irigoyen, I., Torino, F., Mandado, M., Yvin, J.C., Garcia-Mina, J.M., 2012. Organic complexed superphosphates (CSP): physicochemical characterization and agronomical properties. *J. Agric. Food Chem.* 60, 2008–2017.
- Garcia, A.C., Santos, L.A., Izquierdo, F.G., Sperandio, M.V.L., Castro, R.N., Berbara, R.L.L., 2012. Vermicompost humic acids as an ecological pathway to protect rice plant against oxidative stress. *Ecol. Eng.* 47, 203–208.
- Garcia-Mina, J.M., Antolin, M.C., Sanchez-Diaz, M., 2004. Metal-humic complexes and plant micronutrient uptake: a study based on different plant species cultivated in diverse soil types. *Plant Soil* 258, 57–68.
- GarciaSerna, J., Juarez, M., Jorda, J., SanchezAndreu, J., 1996. Influence of organic compounds on nitrogen-fertilizer solubilization. *Commun. Soil Sci. Plant Anal.* 27, 2485–2491.
- Gattinger, A., Muller, A., Haeni, M., Skinner, C., Fließbach, A., Buchmann, N., Mader, P., Stolze, M., Smith, P., Scialabba, N.E.H., Niggli, U., 2012. Enhanced top soil carbon stocks under organic farming. *Proc. Natl. Acad. Sci. U. S. A.* 109, 18226–18231.
- Gaur, A.C., Bhardwaj, K.K.R., 1971. Influence of sodium humate on the crop plants inoculated with bacteria of agricultural importance. *Plant Soil* 35, 613–621.
- Gerke, J., 2010. Humic (organic matter)-Al(Fe)-phosphate complexes: an underestimated phosphate form in soils and source of plant-available phosphate. *Soil Sci.* 175, 417–425.
- Gosling, P., Shepherd, M., 2005. Long-term changes in soil fertility in organic arable farming systems in England, with particular reference to phosphorus and potassium. *Agric. Ecosyst. Environ.* 105, 425–432.
- Gryndler, M., Hřelová, H., Sudová, R., Gryndlerová, H., Řezáčová, V., Merhautová, V., 2005. Hyphal growth and mycorrhiza formation by the arbuscular mycorrhizal fungus *Glomus claroideum* BEG 23 is stimulated by humic substances. *Mycorrhiza* 15, 483–488.
- Hartz, T.K., Bottoms, T.G., 2010. Humic substances generally ineffective in improving vegetable crop nutrient uptake or productivity. *Hortscience* 45, 906–910.
- Hedges, L.V., Gurevitch, J., Curtis, P.S., 1999. The meta-analysis of response ratios in experimental ecology. *Ecology* 80, 1150–1156.
- Hua, Q.X., Li, J.Y., Zhou, J.M., Wang, H.Y., Du, C.W., Chen, X.Q., 2008. Enhancement of phosphorus solubility by humic substances in ferrosols. *Pedosphere* 18, 533–538.
- Imbufe, A.U., Patti, A.F., Burrow, D., Surapaneni, A., Jackson, W.R., Milner, A.D., 2005. Effects of potassium humate on aggregate stability of two soils from Victoria, Australia. *Geoderma* 125, 321–330.
- Kasim, S., Ahmed, O.H., Majid, N.M.A., Yusop, M.K., Jalloh, M.B., 2009. Reduction of ammonia loss by mixing urea with liquid humic and fulvic acids isolated from tropical peat soil. *Am. J. Agric. Biol. Sci.* 4, 18–23.

- Katupitiya, S., New, P.B., Elmerich, C., Kennedy, I.R., 1995. Improved N-2 fixation in 2,4-D treated wheat roots associated with *Azospirillum lipoferum*—studies of colonization using reporter genes. *Soil Biol. Biochem.* 27, 447–452.
- Kelting, M., Harris, J.R., Fanelli, J., Appleton, B., 1998. Biostimulants and soil amendments affect two-year posttransplant growth of red maple and Washington hawthorn. *Hortscience* 33, 819–822.
- Kiran, J.K., Khanif, Y.M., Amminuddin, H., Anuar, A.R., 2010. Effects of controlled release urea on the yield and nitrogen nutrition of flooded rice. *Commun. Soil Sci. Plant Anal.* 41, 811–819.
- Lal, R., 2004. Soil carbon sequestration impacts on global climate change and food security. *Science* 304, 1623–1627.
- Liu, C.H., Cooper, R.J., Bowman, D.C., 1998. Humic acid application affects photosynthesis, root development, and nutrient content of creeping bentgrass. *Hortscience* 33, 1023–1025.
- Liu, Y.Y., Swinton, S.M., Miller, N.R., 2006. Is site-specific yield response consistent over time? Does it pay? *Am. J. Agric. Econ.* 88, 471–483.
- Loffredo, E., Berloco, M., Senesi, N., 2008. The role of humic fractions from soil and compost in controlling the growth in vitro of phytopathogenic and antagonistic soil-borne fungi. *Ecotoxicol. Environ. Saf.* 69, 350–357.
- Luo, Y.Q., Hui, D.F., Zhang, D.Q., 2006. Elevated CO<sub>2</sub> stimulates net accumulations of carbon and nitrogen in land ecosystems: a meta-analysis. *Ecology* 87, 53–63.
- MacCarthy, P., 2001. The principles of humic substances: an introduction to the first principle. In: Ghabbour, E.A., Davis, G. (Eds.), *Humic Substances—Structures, Models and Functions*. Royal Society of Chemistry, Cambridge, pp. 19–30.
- Marino, G., Francioso, O., Carletti, P., Nardi, S., Gessa, C., 2008. Mineral content and root respiration of in vitro grown kiwifruit plantlets treated with two humic fractions. *J. Plant Nutr.* 31, 1074–1090.
- Mora, V., Bacaicoa, E., Zamarreno, A.M., Aguirre, E., Garnica, M., Fuentes, M., Garcia-Mina, J.M., 2010. Action of humic acid on promotion of cucumber shoot growth involves nitrate-related changes associated with the root-to-shoot distribution of cytokinins, polyamines and mineral nutrients. *J. Plant Physiol.* 167, 633–642.
- Muscolo, A., Bovalo, F., Gionfriddo, F., Nardi, S., 1999. Earthworm humic matter produces auxin-like effects on *Daucus carota* cell growth and nitrate metabolism. *Soil Biol. Biochem.* 31, 1303–1311.
- Muscolo, A., Sidari, M., Nardi, S., 2013. Humic substance: relationship between structure and activity. Deeper information suggests univocal findings. *J. Geochem. Explor.* 129, 57–63.
- Nardi, S., Pizzeghello, D., Gessa, C., Ferrarese, L., Trainotti, L., Casadoro, G., 2000. A low molecular weight humic fraction on nitrate uptake and protein synthesis in maize seedlings. *Soil Biol. Biochem.* 32, 415–419.
- Nardi, S., Pizzeghello, D., Muscolo, A., Vianello, A., 2002. Physiological effects of humic substances on higher plants. *Soil Biol. Biochem.* 34, 1527–1536.
- Novotny, E.H., Blum, W.E.H., Gerzabek, M.H., Mangrich, A.S., 1999. Soil management system effects on size fractionated humic substances. *Geoderma* 92, 87–109.
- Peiris, D., Patti, A.F., Jackson, W.R., Marshall, M., Smith, C.J., 2002. The use of Ca-modified, brown-coal-derived humates and fulvates for treatment of soil acidity. *Aust. J. Soil Res.* 40, 1171–1186.
- Peoples, M.B., Craswell, E.T., 1992. Biological nitrogen-fixation—investments, expectations and actual contributions to agriculture. *Plant Soil* 141, 13–39.
- Pflugmacher, S., Pietsch, C., Rieger, W., Steinberg, C.E.W., 2006. Dissolved natural organic matter (NOM) impacts photosynthetic oxygen production and electron transport in coontail *Ceratophyllum demersum*. *Sci. Total Environ.* 357, 169–175.
- Piccolo, A., 2001. The supramolecular structure of humic substances. *Soil Sci.* 166, 810–832.

- Piccolo, A., Mbagwu, J.S.C., 1989. Effects of humic substances and surfactants on the stability of soil aggregates. *Soil Sci.* 147, 47–54.
- Piccolo, A., Mbagwu, J.S.C., 1997. Exogenous humic substances as conditioners for the rehabilitation of degraded soils. *Agro Food Ind. Hi Tech* 8, 2–4.
- Piccolo, A., Pietramellara, G., Mbagwu, J.S.C., 1997. Use of humic substances as soil conditioners to increase aggregate stability. *Geoderma* 75, 267–277.
- Pinton, R., Cesco, S., Santi, S., Varanini, Z., 1997. Soil humic substances stimulate proton release by intact oat seedling roots. *J. Plant Nutr.* 20, 857–869.
- Pinton, R., Cesco, S., Varanini, Z., 2009. Role of humic substances on the rhizosphere. In: Senesi, N., Xing, B., Huang, P.M. (Eds.), *Biophysico-Chemical Processes Involving Natural Nonliving Organic Matter in Environmental Systems*. John Wiley & Sons, Hoboken, NJ, USA, pp. 341–359.
- Quaggiotti, S., Ruperti, B., Pizzeghello, D., Francioso, O., Tugnoli, V., Nardi, S., 2004. Effect of low molecular size humic substances on nitrate uptake and expression of genes involved in nitrate transport in maize (*Zea mays* L.). *J. Exp. Bot.* 55, 803–813.
- Quilty, J.R., Cattle, S.R., 2011. Use and understanding of organic amendments in Australian agriculture: a review. *Soil Res.* 49, 1–26.
- R Development Core Team, 2005. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Ridgeway, G., 2013. *Generalized Boosted Regression Models*. Documentation on the R package 'gbm', version 2.1. <http://code.google.com/p/gradientboostedmodels/>.
- Rodriguez-Lucena, P., Hernandez-Apaolaza, L., Lucena, J.J., 2010. Comparison of iron chelates and complexes supplied as foliar sprays and in nutrient solution to correct iron chlorosis of soybean. *J. Plant Nutr. Soil Sci.* 173, 120–126.
- Russell, L., Stokes, A.R., Macdonald, H., Muscolo, A., Nardi, S., 2006. Stomatal responses to humic substances and auxin are sensitive to inhibitors of phospholipase A(2). *Plant Soil* 283, 175–185.
- Schefe, C.R., Patti, A.F., Clune, T.S., Jackson, R., 2008. Organic amendments increase soil solution phosphate concentrations in an acid soil: a controlled environment study. *Soil Sci.* 173, 267–276.
- Schluckebier, J.G., Martin, C.A., 1997. Effects of above-ground pot-in-pot (PIP) placement and humic acid extract on growth of crape myrtle. *J. Environ. Hortic.* 15, 4.
- Senesi, N., 1994. The fractal approach to the study of humic substances. In: Senesi, N., Miano, T.M. (Eds.), *Humic Substances in the Global Environment and Implications on Human Health*. Proceedings of the 6th International Meeting of the International Humic Substances Society. IHSS, Bari, Italy, pp. 3–41.
- Shepherd, T.G., Saggar, S., Newman, R.H., Ross, C.W., Dando, J.L., 2001. Tillage-induced changes to soil structure and organic carbon fractions in New Zealand soils. *Aust. J. Soil Res.* 39, 465–489.
- Simpson, M.J., Chefetz, B., Hatcher, P.G., 2003. Phenanthrene sorption to structurally modified humic acids. *J. Environ. Qual.* 32, 1750–1758.
- Sladký, Z., 1959. The application of extracted humus substances to overground parts of plants. *Biol. Plant.* 1, 6.
- Smith, S.E., Read, D.J., 2008. *Mycorrhizal Symbioses*, third ed. Academic Press Ltd., Cambridge.
- Sparling, G.P., Wheeler, D., Vesely, E.T., Schipper, L.A., 2006. What is soil organic matter worth? *J. Environ. Qual.* 35, 548–557.
- Stevenson, F.J., 1994. *Humus Chemistry: Genesis, Composition, Reactions*. John Wiley & Sons Inc., New York.
- Sutton, R., Sposito, G., 2005. Molecular structure in soil humic substances: the new view. *Environ. Sci. Technol.* 39, 9009–9015.



- Swift, R.S., Sparks, D., Page, A., Helmke, P., Loeppert, R., Soltanpour, P., Tabatabai, M., Johnston, C., Sumner, M., 1996. Organic matter characterization. In: Sparks, D.L., et al. (Ed.), *Methods of Soil Analysis. Part 3—Chemical Methods*. Soil Science Society of America, Madison, WI, pp. 1011–1069.
- Tan, K.H., Binger, A., 1986. Effect of humic-acid on aluminum toxicity in corn plants. *Soil Sci.* 141, 20–25.
- Tatzber, M., Stemmer, M., Spiegel, H., Katzlberger, C., Zehetner, F., Haberhauer, G., Garcia-Garcia, E., Gerzabek, M.H., 2009a. Spectroscopic behaviour of C-14-labeled humic acids in a long-term field experiment with three cropping systems. *Aust. J. Soil Res.* 47, 459–469.
- Tatzber, M., Stemmer, M., Spiegel, H., Katzlberger, C., Zehetner, F., Haberhauer, G., Roth, K., Garcia-Garcia, E., Gerzabek, M.H., 2009b. Decomposition of carbon-14-labeled organic amendments and humic acids in a long-term field experiment. *Soil Sci. Soc. Am. J.* 73, 744–750.
- Trevisan, S., Pizzeghello, D., Ruperti, B., Francioso, O., Sassi, A., Palme, K., Quaggiotti, S., Nardi, S., 2010. Humic substances induce lateral root formation and expression of the early auxin-responsive IAA19 gene and DR5 synthetic element in *Arabidopsis*. *Plant Biol.* 12, 604–614.
- USDA, 2013. Fertilizer use and price. <http://www.ers.usda.gov/data-products/fertilizer-use-and-price.aspx#26727> (accessed 10.12.13.).
- Valdrighi, M.M., Pera, A., Agnolucci, M., Frassinetti, S., Lunardi, D., Vallini, G., 1996. Effects of compost-derived humic acids on vegetable biomass production and microbial growth within a plant (*Cichorium intybus*)-soil system: a comparative study. *Agric. Ecosyst. Environ.* 58, 133–144.
- Vallini, G., Pera, A., Avio, L., Valdrighi, M., Giovannetti, M., 1993. Influence of humic acids on laurel growth, associated rhizospheric microorganisms, and mycorrhizal fungi. *Biol. Fertil. Soils* 16, 1–4.
- Vaughan, D., Macdonald, I.R., 1976. Some effects of humic acid on cation uptake by parenchyma tissue. *Soil Biol. Biochem.* 8, 415–421.
- Vaughan, D., Malcolm, R.E., 1985. Influence of humic substances on growth and physiological processes. In: Vaughan, D., Malcolm, R.E. (Eds.), *Soil Organic Matter and Biological Activity*. Kluwer Academic Publishers, Dordrecht.
- Viechtbauer, W., 2010. Conducting meta-analyses in R with the metafor package. *J. Stat. Softw.* 36, 1–48.
- Young, C.C., Chen, L.F., 1997. Polyamines in humic acid and their effect on radical growth of lettuce seedlings. *Plant Soil* 195, 143–149.
- Zhang, X.Z., Schmidt, R.E., 2000. Hormone-containing products' impact on antioxidant status of tall fescue and creeping bentgrass subjected to drought. *Crop Sci.* 40, 1344–1349.
- Zhu, Y.G., Cavagnaro, T.R., Smith, S.E., Dickson, S., 2001. Backseat driving? Accessing phosphate beyond the rhizosphere—depletion zone. *Trends Plant Sci.* 6, 194–195.



## 8 References

- ABAD, M., FORNES, F., GARCÍA, D., CEGARRA, J. & ROIG, A. 1991. Effects of humic substances from different sources on growth and nutrient content of cucumber plants. *In: ALLARD, B., BORÉN, H. & GRIMVALL, A. (eds.) Humic Substances in the Aquatic and Terrestrial Environment*. Springer, Berlin-Heidelberg.
- ABDEL-MAWGOUD, A., EL-GREADLY, N., HELMY, Y. & SINGER, S. 2007. Responses of tomato plants to different rates of humic-based fertilizer and NPK fertilization. *Journal of Applied Sciences Research*, 3, 169-174.
- ADANI, F., GENEVINI, P., ZACCHEO, P. & ZOCCHI, G. 1998. The effect of commercial humic acid on tomato plant growth and mineral nutrition. *Journal of Plant Nutrition*, 21, 561 - 575.
- AHMED, O., AMINUDDIN, H. & HUSNI, M. 2006. Effects of urea, humic acid and phosphate interactions in fertilizer microsites on ammonia volatilization and soil ammonium and nitrate contents. *International Journal of Agricultural Research*, 1, 25-31.
- AHMED, O., ROSLIZA, S., MAJID, N. M. & JALLOH, M. B. 2012. Effect of N, P and K humates on dry matter of *Zea mays* and soil pH, exchangeable ammonium and available nitrate. *African Journal of Biotechnology*, 11, 9566-9571.
- AKINREMI, O. O., JANZEN, H. H., LEMKE, R. L. & LARNEY, F. J. 2000. Response of canola, wheat and green beans to Leonardite additions. *Canadian Journal of Soil Science*, 80, 437-443.
- AL-KANANI, T., MACKENZIE, A. & BLENKHORN, H. 1990. Volatilization of ammonia from urea-ammonium nitrate solutions as influenced by organic and inorganic additives. *Fertilizer Research*, 23, 113-119.
- ALBIACH, R., CANET, R., POMARES, F. & INGELMO, F. 2000. Microbial biomass content and enzymatic activities after the application of organic amendments to a horticultural soil. *Bioresource Technology*, 75, 43-48.
- ANDERSON, C. R., CONDRON, L. M., CLOUGH, T. J., FIERS, M., STEWART, A., HILL, R. A. & SHERLOCK, R. R. 2011. Biochar induced soil microbial community change: implications for biogeochemical cycling of carbon, nitrogen and phosphorus. *Pedobiologia*, 54, 309-320.
- ANDREW, C. 1976. Effect of calcium, pH and nitrogen on the growth and chemical composition of some tropical and temperate pasture legumes. I. Nodulation and growth. *Crop and Pasture Science*, 27, 611-623.
- ARANCON, N. Q., EDWARDS, C. A., LEE, S. & BYRNE, R. 2006. Effects of humic acids from vermicomposts on plant growth. *European Journal of Soil Biology*, 42, S65-S69.
- ARSHAD, M. & FRANKENBERGER JR, W. 1991. Microbial production of plant hormones. *Plant and Soil*, 133, 1-8.

- ASENJO, M. C. G., GONZÁLEZ, J. L. & MALDONADO, J. M. 2000. Influence of humic extracts on germination and growth of ryegrass. *Communications in Soil Science and Plant Analysis*, 31, 101 - 114.
- ASGHARI, H. R. & CAVAGNARO, T. R. 2012. Arbuscular mycorrhizas reduce nitrogen loss via leaching. *PLoS one*, 7, e29825.
- ASLI, S. & NEUMANN, P. 2010. Rhizosphere humic acid interacts with root cell walls to reduce hydraulic conductivity and plant development. *Plant and Soil*, 336, 313-322.
- ATIYEH, R. M., LEE, S., EDWARDS, C. A., ARANCON, N. Q. & METZGER, J. D. 2002. The influence of humic acids derived from earthworm-processed organic wastes on plant growth. *Bioresource Technology*, 84, 7-14.
- AUSTRALIAN BUREAU OF AGRICULTURAL AND RESOURCE ECONOMICS AND SCIENCES. 2013. *Australian agricultural and grazing industries survey (AAGIS)* [Online]. Available: <http://daff.gov.au> [Accessed 29 August 2014].
- AUSTRALIAN BUREAU OF STATISTICS. 2013. *Agricultural resource management practices Australia* [Online]. Available: <http://www.abs.gov.au> [Accessed 12 August 2014].
- AYUSO, M., HERNÁNDEZ, T., GARCIA, C. & PASCUAL, J. A. 1996. Stimulation of barley growth and nutrient absorption by humic substances originating from various organic materials. *Bioresource Technology*, 57, 251-257.
- BAGGS, E. M. 2008. A review of stable isotope techniques for N<sub>2</sub>O source partitioning in soils: recent progress, remaining challenges and future considerations. *Rapid Communications in Mass Spectrometry*, 22, 1664-1672.
- BARTRAM, A. K., LYNCH, M. D., STEARNS, J. C., MORENO-HAGELSIEB, G. & NEUFELD, J. D. 2011. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. *Applied and Environmental Microbiology*, 77, 3846-3852.
- BELL, M., GARSIDE, A., MAGAREY, R. & HOGARTH, D. Effect of breaks on sugarcane growth: relations between glasshouse and field studies. Proceedings of the 2000 Conference of the Australian Society of Sugar Cane Technologists held at Bundaberg, Queensland, Australia, 2 May to 5 May 2000., 2000. PK Editorial Services, 68-76.
- BHOGAL, A., HATCH, D. J., SHEPHERD, M. A. & JARVIS, S. C. 1999. Comparison of methodologies for field measurement of net nitrogen mineralisation in arable soils. *Plant and Soil*, 207, 15-28.
- BIDEGAIN, R. A., KAEMMERER, M., GUIRRSE, M., HAFIDI, M., REY, F., MORARD, P. & REVEL, J. C. 2000. Effects of humic substances from composted or chemically decomposed poplar sawdust on mineral nutrition of ryegrass. *The Journal of Agricultural Science*, 134, 259-267.
- BIRCH, A. N. E., GRIFFITHS, B. S., CAUL, S., THOMPSON, J., HECKMANN, L. H., KROGH, P. H. & CORTET, J. 2007. The role of laboratory, glasshouse and field scale experiments in understanding the interactions between genetically modified crops and soil ecosystems: A review of the ECOGEN project. *Pedobiologia*, 51, 251-260.

- BOHN, H. L., MYER, R. A. & OCONNOR, G. A. 2002. *Soil chemistry*, New Jersey, US, John Wiley & Sons.
- BOLAN, N., HEDLEY, M. & WHITE, R. 1991. Processes of soil acidification during nitrogen cycling with emphasis on legume based pastures. *Plant and Soil*, 134, 53-63.
- BOMMARCO, R., KLEIJN, D. & POTTS, S. G. 2013. Ecological intensification: harnessing ecosystem services for food security. *Trends in Ecology & Evolution*, 28, 230-238.
- BREMNER, J. & BLACKMER, A. M. 1978. Nitrous oxide: emission from soils during nitrification of fertilizer nitrogen. *Science*, 199, 295-296.
- BÜNEMANN, E. K., SCHWENKE, G. D. & VAN ZWIETEN, L. 2006. Impact of agricultural inputs on soil organisms—a review. *Soil Research*, 44, 379-406.
- CACCO, G. & DELL'AGNOLA, G. 1984. Plant growth regulator activity of soluble humic complexes. *Canadian Journal of Soil Science*, 64, 225-228.
- CAI, Y., PENG, C., QIU, S., LI, Y. & GAO, Y. 2011. Dichromate digestion–spectrophotometric procedure for determination of soil microbial biomass carbon in association with fumigation–extraction. *Communications in Soil Science and Plant Analysis*, 42, 2824-2834.
- CALVO, P., NELSON, L. & KLOPPER, J. 2014. Agricultural uses of plant biostimulants. *Plant and Soil*, 1-39.
- CAMERON, K., DI, H. & MOIR, J. 2013. Nitrogen losses from the soil/plant system: a review. *Annals of Applied Biology*, 162, 145-173.
- CANELLAS, L. P., BALMORI, D. M., MÉDICI, L. O., AGUIAR, N. O., CAMPOSTRINI, E., ROSA, R. C., FAÇANHA, A. R. & OLIVARES, F. L. 2013. A combination of humic substances and *Herbaspirillum seropedicae* inoculation enhances the growth of maize (*Zea mays* L.). *Plant and soil*, 366, 119-132.
- CANELLAS, L. P., OLIVARES, F. L., OKOROKOVA-FAÇANHA, A. L. & FAÇANHA, A. R. 2002. Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H<sup>+</sup>-ATPase activity in maize roots. *Plant Physiology*, 130, 1951-1957.
- CANELLAS, L. P., PICCOLO, A., DOBBSS, L. B., SPACCINI, R., OLIVARES, F. L., ZANDONADI, D. B. & FAÇANHA, A. R. 2010. Chemical composition and bioactivity properties of size-fractions separated from a vermicompost humic acid. *Chemosphere*, 78, 457-466.
- CAPORASO, J. G., KUCZYNSKI, J., STOMBAUGH, J., BITTINGER, K., BUSHMAN, F. D., COSTELLO, E. K., FIERER, N., PENA, A. G., GOODRICH, J. K. & GORDON, J. I. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7, 335-336.
- CAVALETTI, L., MONCIARDINI, P., BAMONTE, R., SCHUMANN, P., ROHDE, M., SOSIO, M. & DONADIO, S. 2006. New lineage of filamentous, spore-forming, gram-positive bacteria from soil. *Applied and Environmental Microbiology*, 72, 4360-4369.
- CHAN, K. Y. 2010. Assessing P fertiliser use in vegetable production: Agronomic and environmental implications. *Australian Journal of Soil Research*, 48, 674-681.

- CHAU, J. F., BAGTZOGLU, A. C. & WILLIG, M. R. 2011. The effect of soil texture on richness and diversity of bacterial communities. *Environmental Forensics*, 12, 333-341.
- CHEN, Y. & AVIAD, T. 1990. Effects of humic substances on plant growth. In: MACCARTHY, P., CLAPP, C. E., MALCOLM, R. L. & BLOOM, P. R. (eds.) *Humic Substances in Soil and Crop Sciences: Selected Readings*. Madison: Soil Science Society of America.
- CHEN, Y., CLAPP, C., E & MAGAN, H. 2004a. Mechanisms of plant growth stimulation by humic substances: the role of organo-iron complexes. *Soil Science and Plant Nutrition*, 50, 1089-1095.
- CHEN, Y., DE, N., MARIA & AVIAD, T. 2004b. Stimulatory effects of humic substances on plant growth. *Soil Organic Matter in Sustainable Agriculture*. Boca Raton: CRC Press.
- ÇIMRIN, K., KARACA, D. & BOZKURT, M. 2001. The effect of NPK and humic acid applications on growth and nutrition of corn plant (*Zea mays* L.). *Ankara University Journal of Agricultural Science*, 7, 95-100.
- CLEMENTE, R. & BERNAL, M. P. 2006. Fractionation of heavy metals and distribution of organic carbon in two contaminated soils amended with humic acids. *Chemosphere*, 64, 1264-1273.
- CLOUGH, T. J., CONDRON, L. M., KAMMANN, C. & MÜLLER, C. 2013. A review of biochar and soil nitrogen dynamics. *Agronomy*, 3, 275-293.
- COSTAGLIOLA, A., ROPERTO, F., BENEDETTO, D., ANASTASIO, A., MARRONE, R., PERILLO, A., RUSSO, V., PAPPARELLA, S. & PACIELLO, O. 2014. Outbreak of fatal nitrate toxicosis associated with consumption of fennels (*Foeniculum vulgare*) in cattle farmed in Campania region (Southern Italy). *Environmental Science and Pollution Research*, 21, 6252-6257.
- DAKORA, F. & PHILLIPS, D. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil*, 245, 35-47.
- DALAL, R. C., WANG, W., ROBERTSON, G. P. & PARTON, W. J. 2003. Nitrous oxide emission from Australian agricultural lands and mitigation options: a review. *Soil Research*, 41, 165-195.
- DEERY, D., MCLACHLAN, G. & HUTCHINSON, P. 2006. *Soil solution sampler construction and installation guide* [Online]. Available: [www.clw.csiro.au](http://www.clw.csiro.au) [Accessed 24 July 2014].
- DEL RIO, T. G., ABT, B., SPRING, S., LAPIDUS, A., NOLAN, M., TICE, H., COPELAND, A., CHENG, J.-F., CHEN, F. & BRUCE, D. 2010. Complete genome sequence of *Chitinophaga pinensis* type strain (UQM 2034T). *Standards in Genomic Sciences*, 2, 87.
- DENG, Y., CUI, X., HERNÁNDEZ, M. & DUMONT, M. G. 2014. Microbial diversity in hummock and hollow soils of three wetlands on the Qinghai-Tibetan plateau revealed by 16S rRNA pyrosequencing. *PLoS one*, 9, e103115.
- DESANTIS, T. Z., HUGENHOLTZ, P., LARSEN, N., ROJAS, M., BRODIE, E. L., KELLER, K., HUBER, T., DALEVI, D., HU, P. & ANDERSEN, G. L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, 72, 5069-5072.
- DI, H. & CAMERON, K. 2002. Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. *Nutrient Cycling in Agroecosystems*, 64, 237-256.

- DONG, L., CÓRDOVA-KREYLOS, A. L., YANG, J., YUAN, H. & SCOW, K. M. 2009. Humic acids buffer the effects of urea on soil ammonia oxidizers and potential nitrification. *Soil Biology and Biochemistry*, 41, 1612-1621.
- DUPLESSIS, G. L. & MACKENZIE, A. F. 1983. Effects of leonardite applications on phosphorus availability and corn growth. *Canadian Journal of Soil Science*, 63, 749-751.
- DURIE, R. 1991. *The science of Victorian brown coal: structure, properties, and consequences for utilization*, Oxford, Butterworth-Heinemann.
- DUVAL, J. R., DAINELLO, F. J., HABY, V. A. & EARHART, D. R. 1998. Evaluating leonardite as a crop growth enhancer for turnip and mustard greens. *HortTechnology*, 8, 564-567.
- EDGAR, R. C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*, 10, 996-998.
- EDGAR, R. C., HAAS, B. J., CLEMENTE, J. C., QUINCE, C. & KNIGHT, R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, 27, 2194-2200.
- ERB, K. H., MAYER, A., KRAUSMANN, F., LAUK, C., PLUTZAR, C., STEINBERGER, J. & HABERL, H. 2012. *The interrelations of future global bioenergy potentials, food demand and agricultural technology*, Cambridge, Cambridge University Press.
- ERRO, J., URRUTIA, O., SAN FRANCISCO, S. & GARCIA-MINA, J. M. 2007. Development and agronomical validation of new fertilizer compositions of high bioavailability and reduced potential nutrient losses. *Journal of Agricultural and Food Chemistry*, 55, 7831-7839.
- ERTANI, A., FRANCIOSO, O., TUGNOLI, V., RIGHI, V. & NARDI, S. 2011. Effect of commercial lignosulfonate-humate on *Zea mays* L. metabolism. *Journal of Agricultural and Food Chemistry*, 59, 11940-11948.
- EYHERAGUIBEL, B., SILVESTRE, J. & MORARD, P. 2008. Effects of humic substances derived from organic waste enhancement on the growth and mineral nutrition of maize. *Bioresource Technology*, 99, 4206-4212.
- FAGBENRO, J. A. & AGBOOLA, A. A. 1993. Effect of different levels of humic acid on the growth and nutrient uptake of teak seedlings. *Journal of Plant Nutrition*, 16, 1465-1483.
- FAGERIA, N. K. 2010. *The Use of Nutrients in Crop Plants*, Florida, Taylor & Francis.
- FANG, C., MONCRIEFF, J. B., GHOLZ, H. L. & CLARK, K. L. 1998. Soil CO<sub>2</sub> efflux and its spatial variation in a Florida slash pine plantation. *Plant and Soil*, 205, 135-146.
- FEIBERT, E. B., SHOCK, C. C. & SAUNDERS, L. D. 2003. Nonconventional additives leave onion yield and quality unchanged. *HortScience*, 38, 381-386.
- FERNÁNDEZ-ESCOBAR, R., BENLLOCH, M., BARRANCO, D., DUEÑAS, A. & GAÑÁN, J. A. G. 1996. Response of olive trees to foliar application of humic substances extracted from leonardite. *Scientia Horticulturae*, 66, 191-200.
- FIERER, N., BRADFORD, M. A. & JACKSON, R. B. 2007. Toward an ecological classification of soil bacteria. *Ecology*, 88, 1354-1364.

- FIERER, N. & JACKSON, R. B. 2006. The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 626-631.
- FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. 2009. *Global agriculture towards 2050* [Online]. Available: <http://www.fao.org> [Accessed 25 August 2014].
- FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. 2011. *Current world fertilizer trends and outlook to 2015* [Online]. Available: <http://www.fao.org> [Accessed 25 August 2014].
- FORSTER, J. C. 1995. Soil nitrogen. In: ALEF, K. & NANNIPIERO, P. (eds.) *Methods in Applied Soil Microbiology and Biochemistry*. San Diego: Academic Press.
- FORTUN, C., RAPSCH, S. & ASCASO, C. 1985. Action of humic acid preparations on leaf development, mineral elements contents and chloroplast ultrastructure of ryegrass plants. *Photosynthetica*, 19, 294-299.
- FRASER, M. D., SPEIJERS, M. H., THEOBALD, V. J., FYCHAN, R. & JONES, R. 2004. Production performance and meat quality of grazing lambs finished on red clover, lucerne or perennial ryegrass swards. *Grass and Forage Science*, 59, 345-356.
- FROST, C., HOEPPNER, J. & FOWKES, W. 1959. Source and general properties of humic acids from lignitic materials. *Journal of Chemical and Engineering Data*, 4, 173-176.
- FUERST, J. A. & SAGULENKO, E. 2011. Beyond the bacterium: planctomycetes challenge our concepts of microbial structure and function. *Nature Reviews Microbiology*, 9, 403-413.
- FULKERSON, B., BLACKLOCK, M. & NELSON, N. 1997. *Managing Pastures* [Online]. Available: <http://www.dpi.nsw.gov.au/agriculture/livestock/dairy-cattle/feed/publications/managing-pastures> [Accessed 14 July 2014].
- FULKERSON, W. & DONAGHY, D. 2001. Plant-soluble carbohydrate reserves and senescence-key criteria for developing an effective grazing management system for ryegrass-based pastures: a review. *Animal Production Science*, 41, 261-275.
- GIONGO, A., CRABB, D. B., DAVIS-RICHARDSON, A. G., CHAULIAC, D., MOBBERLEY, J. M., GANO, K. A., MUKHERJEE, N., CASELLA, G., ROESCH, L. F. & WALTZ, B. 2010. PANGEA: pipeline for analysis of next generation amplicons. *The ISME journal*, 4, 852-861.
- GIOVANNETTI, M. & MOSSE, B. 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytologist*, 84, 489-500.
- GODFRAY, H. C. J. & GARNETT, T. 2014. Food security and sustainable intensification. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 369, 20120273.
- GOURLEY, C. J. P., ALLAN, D. L. & RUSSELLE, M. P. 1993. Defining phosphorus efficiency in plants. *Plant and Soil*, 155-156, 289-292.
- GOVINDASMY, R. & CHANDRASEKARAN, S. 1992. Effect of humic acids on the growth, yield and nutrient content of sugarcane. *Science of The Total Environment*, 117-118, 575-581.



- GRIFFITH, S. M. & STREETER, D. J. 1994. Nitrate and ammonium nutrition in ryegrass: changes in growth and chemical composition under hydroponic conditions. *Journal of Plant Nutrition*, 17, 71-81.
- GRYNDLER, M., HRŠELOVÁ, H., SUDOVÁ, R., GRYNDLEROVÁ, H., ŘEZÁČOVÁ, V. & MERHAUTOVÁ, V. 2005. Hyphal growth and mycorrhiza formation by the arbuscular mycorrhizal fungus *Glomus claroideum* BEG 23 is stimulated by humic substances. *Mycorrhiza*, 15, 483-488.
- GULIS, G., CZOMPOLYOVA, M. & CERHAN, J. R. 2002. An ecologic study of nitrate in municipal drinking water and cancer incidence in Trnava District, Slovakia. *Environmental Research*, 88, 182-187.
- HAAS, B. J., GEVERS, D., EARL, A. M., FELDGARDEN, M., WARD, D. V., GIANNOUKOS, G., CIULLA, D., TABBAA, D., HIGHLANDER, S. K. & SODERGREN, E. 2011. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Research*, 21, 494-504.
- HARTZ, T. K. 2010. Humic substances generally ineffective in improving vegetable crop nutrient uptake or productivity. *HortScience*, 45, 906-910.
- HATCHER, P. G. 1987. Chemical structural studies of natural lignin by dipolar dephasing solid-state <sup>13</sup>C nuclear magnetic resonance. *Organic Geochemistry*, 11, 31-39.
- HATCHER, P. G., MACIEL, G. E. & DENNIS, L. W. 1981. Aliphatic structure of humic acids; a clue to their origin. *Organic Geochemistry*, 3, 43-48.
- HAYES, M., SWIFT, R., WARDLE, R. & BROWN, J. 1975. Humic materials from an organic soil: A comparison of extractants and of properties of extracts. *Geoderma*, 13, 231-245.
- HAYES, M. H. B., MACCARTHY, P., MALCOLM, R. L. & SWIFT, R. S. 1989. *Humic substances II. In search of structure*, John Wiley & Sons
- HAYES, T. M., HAYES, M. H. B., SKJEMSTAD, J. O. & SWIFT, R. S. 2008. Compositional relationships between organic matter in a grassland soil and its drainage waters. *European Journal of Soil Science*, 59, 603-616.
- HAYNES, R. & WILLIAMS, P. 1993. Nutrient cycling and soil fertility in the grazed pasture ecosystem. *Advances in Agronomy*, 49, 119-199.
- HEDGES, J. I., COWIE, G. L., ERTEL, J. R., JAMES BARBOUR, R. & HATCHER, P. G. 1985. Degradation of carbohydrates and lignins in buried woods. *Geochimica et Cosmochimica Acta*, 49, 701-711.
- HILL, G., MITKOWSKI, N., ALDRICH-WOLFE, L., EMELE, L., JURKONIE, D., FICKE, A., MALDONADO-RAMIREZ, S., LYNCH, S. & NELSON, E. 2000. Methods for assessing the composition and diversity of soil microbial communities. *Applied Soil Ecology*, 15, 25-36.
- HILTBRUNNER, D., SCHULZE, S., HAGEDORN, F., SCHMIDT, M. W. I. & ZIMMERMANN, S. 2012. Cattle trampling alters soil properties and changes soil microbial communities in a Swiss sub-alpine pasture. *Geoderma*, 170, 369-377.
- HOBEN, J. P., GEHL, R. J., MILLAR, N., GRACE, P. R. & ROBERTSON, G. P. 2011. Nonlinear nitrous oxide (N<sub>2</sub>O) response to nitrogen fertilizer in on-farm corn crops of the US Midwest. *Global Change Biology*, 17, 1140-1152.

- HOULBROOKE, D. J. 2011. Land-use intensification in New Zealand: Effects on soil properties and pasture production. *Journal of Agricultural Science*, 149, 337-349.
- INTERNATIONAL HUMIC SUBSTANCES SOCIETY. 2008. *Isolation of IHSS soil fulvic and humic acids* [Online]. Available: <http://www.ihss.gatech.edu/soilhafa.html> [Accessed 19 March 2014].
- ISELL, R. 2002. *The Australian soil classification* Melbourne, CSIRO Publishing
- JACKSON, L. E., BURGER, M. & CAVAGNARO, T. R. 2008. Roots, nitrogen transformations, and ecosystem services. *Annual Review of Plant Biology*, 59, 341-363.
- JALALI, M. & RANJBAR, F. 2009. Effects of sodic water on soil sodicity and nutrient leaching in poultry and sheep manure amended soils. *Geoderma*, 153, 194-204.
- JANSSEN, P. H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and Environmental Microbiology*, 72, 1719-1728.
- JINDO, K., MARTIM, S. A., NAVARRO, E. C., PÉREZ-ALFOCEA, F., HERNANDEZ, T., GARCIA, C., AGUIAR, N. O. & CANELLAS, L. P. 2012. Root growth promotion by humic acids from composted and non-composted urban organic wastes. *Plant and Soil*, 353, 209-220.
- JONES, C. A., JACOBSEN, J. S. & MUGAAS, A. 2007. Effect of low-rate commercial humic acid on phosphorus availability, micronutrient uptake, and spring wheat yield. *Communications in Soil Science and Plant Analysis*, 38, 921-933.
- JUMPPONEN, A. 2007. Soil fungal communities underneath willow canopies on a primary successional glacier forefront: rDNA sequence results can be affected by primer selection and chimeric data. *Microbial Ecology*, 53, 233-246.
- JUPP, A. & NEWMAN, E. 1987. Morphological and anatomical effects of severe drought on the roots of *Lolium perenne* L. *New Phytologist*, 105, 393-402.
- KÄMPFER, P. 2011. Family II. Chitinophagaceae fam. nov. Kämpfer, Lodders and Falsen 2010. *Bergey's Manual of Systematic Bacteriology: Volume 4: The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes*, 4, 351.
- KASIM, S., AHMED, O. H., MAJID, N. M., YUSOP, M. K. & JALLOH, M. B. 2009. Effect of organic based N fertilizer on dry matter (*Zea mays* L.), ammonium and nitrate recovery in an acid soil of Sarawak, Malaysia. *American Journal of Applied Sciences*, 6, 1289.
- KEENEY, D. & OLSON, R. A. 1986. Sources of nitrate to ground water. *Critical Reviews in Environmental Science and Technology*, 16, 257-304.
- KELEMEN, S., AFEWORKI, M., GORBATY, M. & COHEN, A. 2002. Characterization of organically bound oxygen forms in lignites, peats, and pyrolyzed peats by X-ray photoelectron spectroscopy (XPS) and solid-state <sup>13</sup>C NMR methods. *Energy & Fuels*, 16, 1450-1462.
- KHALED, H. & FAWY, H. A. 2011. Effect of different levels of humic acids on the nutrient content, plant growth, and soil properties under conditions of salinity. *Soil and Water Research*, 6, 21-29.

- KIM, K., NEMEC, S. & MUSSON, G. 1997. Effects of composts and soil amendments on soil microflora and *Phytophthora* root and crown rot of bell pepper. *Crop Protection*, 16, 165-172.
- KIRK, J. L., BEAUDETTE, L. A., HART, M., MOUTOGLIS, P., KLIRONOMOS, J. N., LEE, H. & TREVORS, J. T. 2004. Methods of studying soil microbial diversity. *Journal of Microbiological Methods*, 58, 169-188.
- KIRN, A., KASHIF, S. & YASEEN, M. 2010. Using indigenous humic acid from lignite to increase growth and yield of okra (*Abelmoschus esculentus* L.). *Soil and Environment*, 29, 187-191.
- KLINDWORTH, A., PRUESSE, E., SCHWEER, T., PEPLIES, J., QUAST, C., HORN, M. & GLÖCKNER, F. O. 2012. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, gks808.
- KNOWLES, O., ROBINSON, B., CONTANGELO, A. & CLUCAS, L. 2011. Biochar for the mitigation of nitrate leaching from soil amended with biosolids. *Science of the Total Environment*, 409, 3206-3210.
- KÖNIG, E., SCHLESNER, H. & HIRSCH, P. 1984. Cell wall studies on budding bacteria of the *Planctomyces/Pasteuria* group and on a *Prosthecomicrobium* sp. *Archives of Microbiology*, 138, 200-205.
- KYSELKOVA, M. & MOENNE-LOCCOZ, Y. 2012. *Pseudomonas* and other microbes in disease-suppressive soils. In: LICHTFOUSE, E. (ed.) *Organic fertilisation, soil quality and human health*. Springer.
- LAMAR, R., OLK, D. C., MAYHEW, L. & BLOOM, P. R. 2013. Evaluation of a proposed standardized analytical method for the determination of humic and fulvic acids in commercial products. *Functions of Natural Organic Matter in Changing Environment*. Springer.
- LANDEWEERT, R., HOFFLAND, E., FINLAY, R. D., KUYPER, T. W. & VAN BREEMEN, N. 2001. Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *Trends in Ecology & Evolution*, 16, 248-254.
- LATTA, R., COCKS, P. & MATTHEWS, C. 2002. Lucerne pastures to sustain agricultural production in southwestern Australia. *Agricultural Water Management*, 53, 99-109.
- LAUBER, C. L., HAMADY, M., KNIGHT, R. & FIERER, N. 2009. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology*, 75, 5111-5120.
- LAVERMAN, A., ZOOMER, H., VAN VERSEVELD, H. & VERHOEF, H. 2000. Temporal and spatial variation of nitrogen transformations in a coniferous forest soil. *Soil Biology and Biochemistry*, 32, 1661-1670.
- LEE, Y. S. & BARTLETT, R. J. 1976. Stimulation of plant growth by humic substances. *Soil Science Society of America Journal*, 40, 876-879.
- LEGGETT, J. E. & GILBERT, W. A. 1969. Magnesium Uptake by Soybeans. *Plant Physiology*, 44, 1182-1186.
- LI, C.-Z. 2004. *Advances in the science of Victorian brown coal*, Oxford, Elsevier.

- LITTLE, K. R., ROSE, M. T., JACKSON, W. R., CAVAGNARO, T. R. & PATTI, A. F. 2014. Do lignite-derived organic amendments improve early-stage pasture growth and key soil biological and physiochemical properties? *Crop and Pasture Science*, Advance online publication, DOI: 10.1071/CP13433.
- LORIA, R., KERS, J. & JOSHI, M. 2006. Evolution of plant pathogenicity in *Streptomyces*. *Annual Review in Phytopathology*, 44, 469-487.
- LUCAS, R. & DAVIS, J. F. 1961. Relationships between pH values of organic soils and availabilities of 12 plant nutrients. *Soil Science*, 92, 177-182.
- LUCKERT, M. 2014. Market and government failures in the competition for land for biofuel production in Canada. *Biofuels*, 5, 211-218.
- LUO, J., LEDGARD, S. F. & LINDSEY, S. B. 2013. Nitrous oxide and greenhouse gas emissions from grazed pastures as affected by use of nitrification inhibitor and restricted grazing regime. *Science of The Total Environment*, 465, 107-114.
- MACCARTHY, P. 2001. The Principles of Humic Substances. *Soil Science*, 166, 738-751.
- MADIGAN, M., COX, S. S. & STEGEMAN, R. A. 1984. Nitrogen fixation and nitrogenase activities in members of the family *Rhodospirillaceae*. *Journal of Bacteriology*, 157, 73-78.
- MAO, J., OLK, D., CHEN, N., DINNES, D. & CHAPPELL, M. 2013. Chemical Properties of Humic and Fulvic Acid Products and Their Ores of Origin. In: XU, J., WU, J. & HE, Y. (eds.) *Functions of Natural Organic Matter in Changing Environment*. Springer Netherlands.
- MARTIN, J. C., OVERSTREET, R. & HOAGLAND, D. R. 1946. Potassium fixation in soils in replaceable and nonreplaceable forms in relation to chemical reactions in the soil. *Soil Science Society of America Journal*, 10, 94-101.
- MASELLA, A. P., BARTRAM, A. K., TRUSZKOWSKI, J. M., BROWN, D. G. & NEUFELD, J. D. 2012. PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics*, 13, 31.
- MEIJIDE, A., CARDENAS, L., BOL, R., BERGSTERMANN, A., GOULDING, K., WELL, R., VALLEJO, A. & SCHOLEFIELD, D. 2010. Dual isotope and isotopomer measurements for the understanding of N<sub>2</sub>O production and consumption during denitrification in an arable soil. *European Journal of Soil Science*, 61, 364-374.
- MENG, L., DING, W. & CAI, Z. 2005. Long-term application of organic manure and nitrogen fertilizer on N<sub>2</sub>O emissions, soil quality and crop production in a sandy loam soil. *Soil Biology and Biochemistry*, 37, 2037-2045.
- MERINO, A. N., PÉREZ-BATALLÓN, P. & MACÍAS, F. 2004. Responses of soil organic matter and greenhouse gas fluxes to soil management and land use changes in a humid temperate region of southern Europe. *Soil Biology and Biochemistry*, 36, 917-925.
- MIANO, T., MARTIN, J. & SPOSITO, G. 1988. Fluorescence Spectroscopy of Humic Substances. *Soil Science Society of America Journal*, 52, 1016-1019.
- MIRANDA, K. M., ESPEY, M. G. & WINK, D. A. 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide*, 5, 62-71.

- MORA, V., BACAICOA, E., ZAMARREÑO, A. M., AGUIRRE, E., GARNICA, M., FUENTES, M. & GARCÍA-MINA, J. M. 2010. Action of humic acid on promotion of cucumber shoot growth involves nitrate-related changes associated with the root-to-shoot distribution of cytokinins, polyamines and mineral nutrients. *Journal of Plant Physiology*, 167, 633-642.
- MORALES, S. E., COSART, T. F., JOHNSON, J. V. & HOLBEN, W. E. 2009. Extensive phylogenetic analysis of a soil bacterial community illustrates extreme taxon evenness and the effects of amplicon length, degree of coverage, and DNA fractionation on classification and ecological parameters. *Applied and Environmental Microbiology*, 75, 668-675.
- MUKHERJEE, A., LAL, R. & ZIMMERMAN, A. 2014. Effects of biochar and other amendments on the physical properties and greenhouse gas emissions of an artificially degraded soil. *Science of The Total Environment*, 487, 26-36.
- MUNNS, D. 1965. Soil acidity and growth of a legume. II. Reactions of aluminium and phosphate in solution and effects of aluminium, phosphate, calcium, and pH on *Medicago sativa* L. and *Trifolium subterraneum*, in solution culture. *Crop and Pasture Science*, 16, 743-755.
- MUSCOLO, A., BOVALO, F., GIONFRIDDO, F. & NARDI, S. 1999. Earthworm humic matter produces auxin-like effects on *Daucus carota* cell growth and nitrate metabolism. *Soil Biology and Biochemistry*, 31, 1303-1311.
- MUYZER, G. & SMALLA, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*, 73, 127-141.
- MYLONAS, V. & MCCANTS, C. 1980. Effects of humic and fulvic acids on growth of tobacco. *Plant and Soil*, 54, 485-490.
- N.S.W. DEPARTMENT OF PRIMARY INDUSTRIES. 1997. *Establishing pastures* [Online]. Available: <http://www.dpi.nsw.gov.au/> [Accessed 15 August 2014].
- NACKE, H., THÜRMER, A., WOLLHERR, A., WILL, C., HODAC, L., HEROLD, N., SCHÖNING, I., SCHRUMPF, M. & DANIEL, R. 2011. Pyrosequencing-based assessment of bacterial community structure along different management types in German forest and grassland soils. *PloS one*, 6, e17000.
- NARDI, S., CONCHERI, G., DELL'AGNOLA, G. & SCRIMIN, P. 1991. Nitrate uptake and ATPase activity in oat seedlings in the presence of two humic fractions. *Soil Biology and Biochemistry*, 23, 833-836.
- NARDI, S., MUSCOLO, A., VACCARO, S., BAIANO, S., SPACCINI, R. & PICCOLO, A. 2007. Relationship between molecular characteristics of soil humic fractions and glycolytic pathway and krebs cycle in maize seedlings. *Soil Biology and Biochemistry*, 39, 3138-3146.
- NARDI, S., PANUCCIO, M., ABENAVOLI, M. & MUSCOLO, A. 1994. Auxin-like effect of humic substances extracted from faeces of *Allolobophora caliginosa* and *A. rosea*. *Soil Biology and Biochemistry*, 26, 1341-1346.
- NARDI, S., PIZZEGHELLO, D., MUSCOLO, A. & VIANELLO, A. 2002. Physiological effects of humic substances on higher plants. *Soil Biology and Biochemistry*, 34, 1527-1536.

- NATIONAL FARMERS' FEDERATION. 2011. *Farm Facts 2011* [Online]. Available: <http://www.nff.org.au/farm-facts.html> [Accessed 19 March 2014].
- NAUSHAD, H. S. & GUPTA, R. S. 2013. Phylogenomics and molecular signatures for species from the plant pathogen-containing order *Xanthomonadales*. *PLoS one*, 8, e55216.
- NEBBIOSO, A. & PICCOLO, A. 2011. Basis of a humeomics science: chemical fractionation and molecular characterization of humic biosuprastructures. *Biomacromolecules*, 12, 1187-1199.
- NEBBIOSO, A. & PICCOLO, A. 2012. Advances in humeomics: Enhanced structural identification of humic molecules after size fractionation of a soil humic acid. *Analytica Chimica Acta*, 720, 77-90.
- NEBBIOSO, A., PICCOLO, A., LAMSHÖFT, M. & SPITELLER, M. 2014. Molecular characterization of an end-residue of humeomics applied to a soil humic acid. *RSC Advances*, 4, 23658-23665.
- NIELSEN, S., MINCHIN, T., KIMBER, S., VAN ZWIETEN, L., GILBERT, J., MUNROE, P., JOSEPH, S. & THOMAS, T. 2014. Comparative analysis of the microbial communities in agricultural soil amended with enhanced biochars or traditional fertilisers. *Agriculture, Ecosystems & Environment*, 191, 73-82.
- NIKBAKHT, A., KAFI, M., BABALAR, M., XIA, Y. P., LUO, A. & ETEMADI, N.-A. 2008. Effect of humic acid on plant growth, nutrient uptake, and postharvest life of gerbera. *Journal of Plant Nutrition*, 31, 2155-2167.
- OADES, J., VASSALLO, A., WATERS, A. & WILSON, M. 1987. Characterization of organic matter in particle size and density fractions from a red-brown earth by solid state <sup>13</sup>C NMR. *Soil Research*, 25, 71-82.
- OLK, D. C., DINNES, D. L., CALLAWAY, C. & RASKE, M. 2013. On-farm evaluation of a humic product in Iowa (US) maize production. In: XU, J., WU, J. & HE, Y. (eds.) *Functions of Natural Organic Matter in Changing Environment*. Springer Netherlands.
- PAKSOY, M., TÜRKMEN, Ö. & DURSUN, A. 2013. Effects of potassium and humic acid on emergence, growth and nutrient contents of okra (*Abelmoschus esculentus* L.) seedling under saline soil conditions. *African Journal of Biotechnology*, 9.
- PANG, L. S. K., VASSALLO, A. M. & WILSON, M. A. 1990. Chemistry of alkali extraction of brown coals—I. Kinetics, characterisation and implications to coalification. *Organic Geochemistry*, 16, 853-864.
- PARANYCHIANAKIS, N., TSIKNIA, M., GIANNAKIS, G., NIKOLAIDIS, N. & KALOGERAKIS, N. 2013. Nitrogen cycling and relationships between ammonia oxidizers and denitrifiers in a clay-loam soil. *Applied Microbiology and Biotechnology*, 97, 5507-5515.
- PARFITT, R., GILTRAP, D. & WHITTON, J. 1995. Contribution of organic matter and clay minerals to the cation exchange capacity of soils. *Communications in Soil Science & Plant Analysis*, 26, 1343-1355.
- PASCUAL, J., GARCÍA, C. & HERNANDEZ, T. 1999. Lasting microbiological and biochemical effects of the addition of municipal solid waste to an arid soil. *Biology and Fertility of Soils*, 30, 1-6.

- PATEL, R. K. & JAIN, M. 2012. NGS QC Toolkit: a toolkit for quality control of next generation sequencing data. *PLoS one*, 7, e30619.
- PERRY, G. J., ALLARDICE, D. J. & KISS, L. T. 1984. The chemical characteristics of Victorian brown coal. In: SCHOBERT, H. H. (ed.) *The Chemistry of Low-Rank Coals*. USA: American Chemical Society.
- PHILIPPOT, L., HALLIN, S. & SCHLOTTER, M. 2007. Ecology of denitrifying prokaryotes in agricultural soil. *Advances in Agronomy*, 96, 249-305.
- PHILLIPS, J. & HAYMAN, D. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society*, 55, 158-161.
- PICCOLO, A. 2002. The supramolecular structure of humic substances: A novel understanding of humus chemistry and implications in soil science. *Advances in Agronomy*, 75, 57-134.
- PICCOLO, A., CELANO, G. & PIETRAMELLARA, G. 1993. Effects of fractions of coal-derived humic substances on seed germination and growth of seedlings. *Biology and Fertility of Soils*, 16, 11-15.
- PICCOLO, A., NARDI, S. & CONCHERI, G. 1992. Structural characteristics of humic substances as related to nitrate uptake and growth regulation in plant systems. *Soil Biology and Biochemistry*, 24, 373-380.
- PICCOLO, A., PIETRAMELLARA, G. & MBAGWU, J. 1996. Effects of coal derived humic substances on water retention and structural stability of Mediterranean soils. *Soil Use and Management*, 12, 209-213.
- PICCOLO, A., PIETRAMELLARA, G. & MBAGWU, J. S. C. 1997. Use of humic substances as soil conditioners to increase aggregate stability. *Geoderma*, 75, 267-277.
- PIJNENBORG, J. W., LIE, T. & ZEHNDER, A. 1990. Nodulation of lucerne (*Medicago sativa* L.) in an acid soil: pH dynamics in the rhizosphere of seedlings growing in rhizotrons. *Plant and Soil*, 126, 161-168.
- PUGLISI, E., FRAGOULIS, G., RICCIUTI, P., CAPPÀ, F., SPACCINI, R., PICCOLO, A., TREVISAN, M. & CRECCHIO, C. 2009. Effects of a humic acid and its size-fractions on the bacterial community of soil rhizosphere under maize (*Zea mays* L.). *Chemosphere*, 77, 829-837.
- QADIR, M., OSTER, J. D., SCHUBERT, S., NOBLE, A. D. & SAHRAWAT, K. L. 2007. Phytoremediation of sodic and saline-sodic soils. *Advances in Agronomy*, 96, 197-247.
- QUAISER, A., OCHSENREITER, T., LANZ, C., SCHUSTER, S. C., TREUSCH, A. H., ECK, J. & SCHLEPER, C. 2003. *Acidobacteria* form a coherent but highly diverse group within the bacterial domain: evidence from environmental genomics. *Molecular Microbiology*, 50, 563-575.
- QUILTY, J. R. & CATTLE, S. R. 2011. Use and understanding of organic amendments in Australian agriculture: a review. *Soil Research*, 49, 1-26.
- QUINCE, C., LANZEN, A., DAVENPORT, R. J. & TURNBAUGH, P. J. 2011. Removing noise from pyrosequenced amplicons. *BMC Bioinformatics*, 12, 38.

- RATHMANN, R., SZKLO, A. & SCHAEFFER, R. 2010. Land use competition for production of food and liquid biofuels: An analysis of the arguments in the current debate. *Renewable Energy*, 35, 14-22.
- RAUTHAN, B. & SCHNITZER, M. 1981. Effects of a soil fulvic acid on the growth and nutrient content of cucumber (*Cucumis sativus*) plants. *Plant and Soil*, 63, 491-495.
- RAVISHANKARA, A., DANIEL, J. S. & PORTMANN, R. W. 2009. Nitrous oxide (N<sub>2</sub>O): the dominant ozone-depleting substance emitted in the 21st century. *Science*, 326, 123-125.
- ROBERTSON, G. P., GROSS, K. L., HAMILTON, S. K., LANDIS, D. A., SCHMIDT, T. M., SNAPP, S. S. & SWINTON, S. M. 2014. Farming for Ecosystem Services: An Ecological Approach to Production Agriculture. *BioScience*, bio037.
- ROBSON, A. 2012. *Soil acidity and plant growth*, San Diego, Academic Press.
- ROESCH, L. F., FULTHORPE, R. R., RIVA, A., CASELLA, G., HADWIN, A. K., KENT, A. D., DAROUB, S. H., CAMARGO, F. A., FARMERIE, W. G. & TRIPLETT, E. W. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. *The ISME Journal*, 1, 283-290.
- ROS, G. H., TEMMINGHOFF, E. J. M. & HOFFLAND, E. 2011. Nitrogen mineralization: a review and meta-analysis of the predictive value of soil tests. *European Journal of Soil Science*, 62, 162-173.
- ROSE, M. T., PATTI, A. F., LITTLE, K. R., BROWN, A. L., JACKSON, W. R. & CAVAGNARO, T. R. 2014. A meta-analysis and review of plant-growth response to humic substances: practical implications for agriculture. *Advances in Agronomy*, 124, 37-89.
- RÖSKE, K., SACHSE, R., SCHEERER, C. & RÖSKE, I. 2012. Microbial diversity and composition of the sediment in the drinking water reservoir Saidenbach (Saxonia, Germany). *Systematic and Applied Microbiology*, 35, 35-44.
- RUMPEL, C., KNICKER, H., KÖGEL-KNABNER, I., SKJEMSTAD, J. O. & HÜTTL, R. F. 1998. Types and chemical composition of organic matter in reforested lignite-rich mine soils. *Geoderma*, 86, 123-142.
- SAIT, M., DAVIS, K. E. & JANSSEN, P. H. 2006. Effect of pH on isolation and distribution of members of subdivision 1 of the phylum *Acidobacteria* occurring in soil. *Applied and Environmental Microbiology*, 72, 1852-1857.
- SANAPAREDDY, N., HAMP, T. J., GONZALEZ, L. C., HILGER, H. A., FODOR, A. A. & CLINTON, S. M. 2009. Molecular diversity of a North Carolina wastewater treatment plant as revealed by pyrosequencing. *Applied and environmental microbiology*, 75, 1688-1696.
- SANDERSON, M., SKINNER, R., BARKER, D., EDWARDS, G., TRACY, B. & WEDIN, D. 2004. Plant species diversity and management of temperate forage and grazing land ecosystems. *Crop Science*, 44, 1132-1144.
- SAVAGE, K. E. & DAVIDSON, E. A. 2003. A comparison of manual and automated systems for soil CO<sub>2</sub> flux measurements: Trade-offs between spatial and temporal resolution. *Journal of Experimental Botany*, 54, 891-899.



- SCHLOSS, P. D., GEVERS, D. & WESTCOTT, S. L. 2011. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PloS one*, 6, e27310.
- SCHNITZER, M. & SKINNER, S. 1965. Organo-metallic interactions in soils: 4. Carboxyl and hydroxyl groups in organic matter and metal retention. *Soil Science*, 99, 278-284.
- SCHNÜRER, J., CLARHOLM, M. & ROSSWALL, T. 1985. Microbial biomass and activity in an agricultural soil with different organic matter contents. *Soil Biology and Biochemistry*, 17, 611-618.
- SCHOLZ, M. B., LO, C.-C. & CHAIN, P. S. 2012. Next generation sequencing and bioinformatic bottlenecks: the current state of metagenomic data analysis. *Current Opinion in Biotechnology*, 23, 9-15.
- SCHREMPF, H. 2008. Streptomycetaceae: Lifestyle, genome, metabolism and habitats. *eLS*, DOI: 10.1002/9780470015902.a0020393.
- SCHULTEN, H.-R., PLAGE, B. & SCHNITZER, M. 1991. A chemical structure for humic substances. *The Science of Nature*, 78, 311-312.
- SCHULTEN, H.-R. & SCHNITZER, M. 1993. A state of the art structural concept for humic substances. *The Science of Nature*, 80, 29-30.
- SENESI, N., MIANO, T. M., PROVENZANO, M. R. & BRUNETTI, G. 1991. Characterization, differentiation, and classification of humic substances by fluorescence spectroscopy. *Soil Science*, 152, 259-271.
- SHARIF, M., KHATTAK, R. A. & SARIR, M. S. 2002. Effect of different levels of lignitic coal derived humic acid on growth of maize plants. *Communications in Soil Science and Plant Analysis*, 33, 3567 - 3580.
- SKJEMSTAD, J., CLARKE, P., TAYLOR, J., OADES, J. & NEWMAN, R. 1994. The removal of magnetic materials from surface soils - a solid state <sup>13</sup>C CP/MAS NMR study. *Soil Research*, 32, 1215-1229.
- SMITH, C., PEOPLES, M., KEERTHISINGHE, G., JAMES, T., GARDEN, D. & TUOMI, S. 1994. Effect of surface applications of lime, gypsum and phosphogypsum on the alleviating of surface and subsurface acidity in a soil under pasture. *Soil Research*, 32, 995-1008.
- SMITH, K. A. & LI, S. 1993. Estimation of potentially mineralisable nitrogen in soil by KCl extraction. *Plant and Soil*, 157, 167-174.
- SMITH, M., CONTE, P., BERNIS, A., THOMSON, J. & CAVAGNARO, T. 2012. Spatial patterns of, and environmental controls on, soil properties at a riparian-paddock interface. *Soil Biology and Biochemistry*, 49, 38-45.
- SMITH, M. S., FRYE, W. W. & VARCO, J. J. 1987. Legume winter cover crops. *Advances in Soil Science*. New York: Springer.
- SMITH, S., SCHEPERS, J. & PORTER, L. 1990. Assessing and managing agricultural nitrogen losses to the environment. *Advances in Soil Science*. New York: Springer.
- SMITH, S. E. & READ, D. J. 2010. *Mycorrhizal symbiosis*, London, Academic.

- SPACCINI, R. & PICCOLO, A. 2007. Molecular characterization of compost at increasing stages of maturity. 2. Thermochemolysis-GC-MS and <sup>13</sup>C-CPMAS-NMR spectroscopy. *Journal of Agricultural and Food Chemistry*, 55, 2303-2311.
- STALEY, T., WRIGHT, R. & HERN, J. 1989. Comparative growth responses of perennial forage legumes in hill-land ultisols and their relationship to soil acidity-related factors. *Journal of Plant Nutrition*, 12, 245-262.
- STEFFAN, R. J., GOKSOYR, J., BEJ, A. K. & ATLAS, R. M. 1988. Recovery of DNA from soils and sediments. *Applied and Environmental Microbiology*, 54, 2908-2915.
- STEINER, C., DAS, K., MELEAR, N. & LAKLY, D. 2010. Reducing nitrogen loss during poultry litter composting using biochar. *Journal of Environmental Quality*, 39, 1236-1242.
- STEVENSON, F. J. 1994. *Humus chemistry: genesis, composition, reactions*, New York, John Wiley & Sons.
- SUDDICK, E., STEENWERTH, K., GARLAND, G., SMART, D. & SIX, J. 2011. Discerning agricultural management effects on nitrous oxide emissions from conventional and alternative cropping systems: a California case study. In: GUO, L., GUNASEKARA, A. & MCCONNELL, L. (eds.) *Understanding Greenhouse Gas Emissions from Agricultural Management*. Washington DC, USA: American Chemical Society.
- SWIFT, R. S. 1996. Methods of soil analysis. Part 3-Chemical methods. In: SPARKS, D. L. (ed.). Soil Science Society of America
- SWIFT, R. S. 1999. Macromolecular properties of soil humic substances: fact, fiction, and opinion. *Soil Science*, 164, 790-802.
- TAHIR, M. M., KHURSHID, M., KHAN, M. Z., ABBASI, M. K. & KAZMI, M. H. 2011. Lignite-derived humic acid effect on growth of wheat plants in different soils. *Pedosphere*, 21, 124-131.
- TAN, K., HIMMELSBACH, D. & LOBARTINI, J. 1992. The significance of solid-state <sup>13</sup>C NMR spectroscopy of whole soil in the characterization of humic matter. *Communications in Soil Science & Plant Analysis*, 23, 1513-1532.
- TAN, K. H. & NOPAMORNBODI, V. 1979. Effect of different levels of humic acids on nutrient content and growth of corn (*Zea mays* L.). *Plant and Soil*, 51, 283-287.
- THIELEMANN, T., SCHMIDT, S. & PETER GERLING, J. 2007. Lignite and hard coal: Energy suppliers for world needs until the year 2100—An outlook. *International Journal of Coal Geology*, 72, 1-14.
- TILMAN, D., BALZER, C., HILL, J. & BEFORT, B. L. 2011. Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences*, 108, 20260-20264.
- TIPPING, E. 2002. *Cation binding by humic substances*, Cambridge, Cambridge University Press.
- TISDALL, J. M. & OADES, J. M. 1982. Organic matter and water-stable aggregates in soils. *Journal of Soil Science*, 33, 141-163.
- TREVISAN, S., FRANCIOSO, O., QUAGGIOTTI, S. & NARDI, S. 2010. Humic substances biological activity at the plant-soil interface. *Plant Signaling & Behavior*, 5, 635-643.

- TUOMISTO, H., HODGE, I., RIORDAN, P. & MACDONALD, D. 2012. Does organic farming reduce environmental impacts? A meta-analysis of European research. *Journal of Environmental Management*, 112, 309-320.
- VALDRIGHI, M. M., PERA, A., AGNOLUCCI, M., FRASSINETTI, S., LUNARDI, D. & VALLINI, G. 1996. Effects of compost-derived humic acids on vegetable biomass production and microbial growth within a plant (*Cichorium intybus*)-soil system: a comparative study. *Agriculture, Ecosystems & Environment*, 58, 133-144.
- VALLINI, G., PERA, A., AVIO, L., VALDRIGHI, M. & GIOVANNETTI, M. 1993. Influence of humic acids on laurel growth, associated rhizospheric microorganisms, and mycorrhizal fungi. *Biology and Fertility of Soils*, 16, 1-4.
- VAN ZWIETEN, L., KIMBER, S., MORRIS, S., CHAN, K., DOWNIE, A., RUST, J., JOSEPH, S. & COWIE, A. 2010a. Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. *Plant and Soil*, 327, 235-246.
- VAN ZWIETEN, L., KIMBER, S., MORRIS, S., DOWNIE, A., BERGER, E., RUST, J. & SCHEER, C. 2010b. Influence of biochars on flux of N<sub>2</sub>O and CO<sub>2</sub> from Ferrosol. *Soil Research*, 48, 555-568.
- VAN ZWIETEN, L., KIMBER, S. W. L., MORRIS, S. G., SINGH, B. P., GRACE, P. R., SCHEER, C., RUST, J., DOWNIE, A. E. & COWIE, A. L. 2012. Pyrolysing poultry litter reduces N<sub>2</sub>O and CO<sub>2</sub> fluxes. *Science of The Total Environment*, 465, 279-287.
- VAN ZWIETEN, L., SINGH, B., JOSEPH, S., KIMBER, S., COWIE, A. & CHAN, K. Y. 2009. Biochar and emissions of non-CO<sub>2</sub> greenhouse gases from soil. In: LEHMANN, J. & JOSEPH, S. (eds.) *Biochar for Environmental Management: Science and Technology*. London: Earthscan.
- VANCE, E. D., BROOKES, P. C. & JENKINSON, D. S. 1987. An extraction method for measuring soil microbial biomass C. *Soil Biology and Biochemistry*, 19, 703-707.
- VARANINI, Z. & PINTON, R. 1995. Humic substances and plant nutrition. *Progress in Botany*. New York: Springer.
- VERLINDEN, G., COUSSENS, T., DE VliegHER, A., BAERT, G. & HAESAERT, G. 2010. Effect of humic substances on nutrient uptake by herbage and on production and nutritive value of herbage from sown grass pastures. *Grass and Forage Science*, 65, 133-144.
- VERLINDEN, G., PYCKE, B., MERTENS, J., DEBERSAQUES, F., VERHEYEN, K., BAERT, G., BRIES, J. & HAESAERT, G. 2009. Application of humic substances results in consistent increases in crop yield and nutrient uptake. *Journal of Plant Nutrition*, 32, 1407-1426.
- VISSER, S. A. 1985a. Effect of humic acids on numbers and activities of micro-organisms within physiological groups. *Organic Geochemistry*, 8, 81-85.
- VISSER, S. A. 1985b. Physiological action of humic substances on microbial cells. *Soil Biology and Biochemistry*, 17, 457-462.
- WAGNER, M. R., LUNDBERG, D. S., COLEMAN-DERR, D., TRINGE, S. G., DANGL, J. L. & MITCHELL-OLDS, T. 2014. Natural soil microbes alter flowering phenology and the intensity of selection on flowering time in a wild *Arabidopsis* relative. *Ecology Letters*, 17, 717-726.

- WANG, C., LU, H., DONG, D., DENG, H., STRONG, P., WANG, H. & WU, W. 2013. Insight into the effects of biochar on manure composting: Evidence supporting the relationship between N<sub>2</sub>O emission and denitrifying community. *Environmental Science & Technology*, 47, 7341-7349.
- WANG, J., JENKINS, C., WEBB, R. I. & FUERST, J. A. 2002. Isolation of Gemmata-like and Isosphaera-like planctomycete bacteria from soil and freshwater. *Applied and Environmental Microbiology*, 68, 417-422.
- WANG, Y., SHENG, H.-F., HE, Y., WU, J.-Y., JIANG, Y.-X., TAM, N. F.-Y. & ZHOU, H.-W. 2012. Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. *Applied and Environmental Microbiology*, 78, 8264-8271.
- WARD, N. L., CHALLACOMBE, J. F., JANSSEN, P. H., HENRISSAT, B., COUTINHO, P. M., WU, M., XIE, G., HAFT, D. H., SAIT, M. & BADGER, J. 2009. Three genomes from the phylum *Acidobacteria* provide insight into the lifestyles of these microorganisms in soils. *Applied and Environmental Microbiology*, 75, 2046-2056.
- WATTS-WILLIAMS, S. J. & CAVAGNARO, T. R. 2012. Arbuscular mycorrhizas modify tomato responses to soil zinc and phosphorus addition. *Biology and Fertility of Soils*, 48, 285-294.
- WEIHERMÜLLER, L., SIEMENS, J., DEURER, M., KNOBLAUCH, S., RUPP, H., GÖTTLEIN, A. & PÜTZ, T. 2007. In situ soil water extraction: A review. *Journal of Environmental Quality*, 36, 1735-1748.
- WENG, H. H., TSAI, S. S., WU, T. N., SUNG, F. C. & YANG, C. Y. 2011. Nitrates in drinking water and the risk of death from childhood brain tumors in Taiwan. *Journal of Toxicology and Environmental Health, Part A*, 74, 769-778.
- WHEELER, D. & DODD, M. 1995. Effect of aluminium on yield and plant chemical concentrations of some temperate legumes. *Plant and Soil*, 173, 133-145.
- WHITELEY, G. M. & PETTIT, C. 1994. Effect of lignite humic acid treatment on the rate of decomposition of wheat straw. *Biology and Fertility of Soils*, 17, 18-20.
- WIJLER, J. & DELWICHE, C. 1954. Investigations on the denitrifying process in soil. *Plant and Soil*, 5, 155-169.
- WILSON, M. A., VINCENT VERHEYEN, T., VASSALLO, A. M., HILL, R. S. & PERRY, G. J. 1987. Selective loss of carbohydrates from plant remains during coalification. *Organic Geochemistry*, 11, 265-271.
- WONG, M. T. F., NORTCLIFF, S. & SWIFT, R. S. 1998. Method for determining the acid ameliorating capacity of plant residue compost, urban waste compost, farmyard manure, and peat applied to tropical soils. *Communications in Soil Science and Plant Analysis*, 29, 2927 - 2937.
- WORLDOMETERS. n.d. *Current world population* [Online]. Available: <http://www.worldometers.info/> [Accessed 25 August 2014].
- YAZAWA, Y., WONG, M. T. F., GILKES, R. J. & YAMAGUCHI, T. 2000. Effect of additions of brown coal and peat on soil solution composition and root growth in acid soil from

- wheatbelt of western Australia. *Communications in Soil Science and Plant Analysis*, 31, 743 - 758.
- YONEBAYASHI, K. & HATTORI, T. 1989. Chemical and biological studies on environmental humic acids II. <sup>1</sup>H-NMR and IR spectra of humic acids. *Soil Science and Plant Nutrition*, 35, 383-392.
- YU, K. & ZHANG, T. 2012. Metagenomic and metatranscriptomic analysis of microbial community structure and gene expression of activated sludge. *PLoS one*, 7, e38183.
- ZAMAN, M. & BLENNERHASSETT, J. 2010. Effects of the different rates of urease and nitrification inhibitors on gaseous emissions of ammonia and nitrous oxide, nitrate leaching and pasture production from urine patches in an intensive grazed pasture system. *Agriculture, Ecosystems & Environment*, 136, 236-246.
- ZELLER, S. L., KALININA, O., BRUNNER, S., KELLER, B. & SCHMID, B. 2010. Transgenex environment interactions in genetically modified wheat. *PLoS One*, 5, e11405.
- ZHOU, J., BRUNS, M. & TIEDJE, J. 1996. DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology*, 62, 316-322.