Does the application of vermicompost solid and liquid extracts influence the growth, N-nutrition and soil microbial diversity of the legume, *Lupinus angustifolius*?

By

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DECLARATION

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ABSTRACT

Vermicomposts (VCs) are the solid excreta of earthworms, known to contain plant available nutrients, large amounts of microbial life and diversity, and plant growth regulating hormones. VCs may play an integral role in the nitrogen nutrition of *Lupinus angustifolius* and function to reduce the reliance of legume crops on chemical fertilizers. Furthermore, the effects of the combination of VC solids and VC teas on legume growth and N nutrition, is unknown. The aim of the present study was to determine the role of varying concentrations of chicken manure VC, with and without the additions of varying VC tea concentrations, on the substrate bacterial functional diversity, plant biomass and N nutrition of the legume, *L. angustifolius*.

In the first experiment the plants were grown in pots under glasshouse conditions and VC was substituted into the quartz sand growth media at rates of 5%, 10% and 100%. Furthermore, rhizobia inoculated and non-inoculated groups were established within the VC treatments. The plants were harvested after 30 days and analysed for tissue nutrient concentrations and biomass production. The VC-containing substrates were assessed for wide-spectrum soil analyses, nematode diversity and microbial diversity via Biolog EcoPlates. In the second experiment the plants were grown under similar conditions for 50 days and amended with 5% VC in the growth media as well as 50ml of aerated VC teas. The teas were brewed for 24 hours in concentrations of 4%, 10% and 20% (v/v), with molasses and kelp extract added as sugar sources.

The combined treatments of 5% VC and rhizobia inoculation yielded the greatest biomass response. Furthermore the addition of VC allowed for bacterial nitrogen fixation within non-rhizobia treatments. However, changes in VC concentration had no effect on bacterial guild structure but were found to effect nematode funtional diversity. The additions of VC teas to soil media containing 5% VC had no effect on biomass production but were found to influence bacterial nitrogen fixation. Lower concentration teas increased BNF while the 20% tea reduced this parameter significantly over 50 days. The 20% tea also contained significantly greater bacterial functional diversity than the 5% and 10% teas.

The findings of study indicate that the combined treatment of VC solids and teas do not increase the plant biomass of *L. angustifolius*, but that the additions of 20% teas result in greater microbial diversity in the soil. This in turn may lead to increases in soil fertility. Furthermore,

additions of high concentration vermicompost teas (20%) shift the dependence of the plant from atmospheric N sources to soil N sources.

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Environmental damage due to conventional agricultural practices

Over the last two decades the issue of environmental damage due to agricultural practices has received an increasing amount of public attention, specifically the damage caused by agrochemicals and their un-sustainability as a resource utilized into the future (Sharma *et al*., 2005). Furthermore, conventional crop protection practices utilizing heavy doses of pesticides, fungicides and herbicides have largely killed of native soil life, leaving the soil barren (Sharma *et al*., 2005) thus reducing the potential for effective nutrient cycling within the soil (Bardgett, 2005) and as a result further increasing the reliance on fertilizer inputs to achieve the desired yields. These increased inputs of inorganic fertilizers have led to negative localized effects including a change in the composition of microbial community structures within agricultural soils as well as a decrease in microbial diversity (Reilly *et al*., 2013). This decreased diversity may further compound the previously mentioned reduction in nutrient cycling. Regional effects of excessive fertilizer application include the eutrophication of water bodies. One of the causal agents of this phenomenon have been identified as the leaching of inorganic fertilizers from agricultural soils, predominantly phosphates (PO_4) and nitrates (NO_3), as these compounds are water soluble. Between 40 to 60% of N that is added to the soil as fertilizer is utilized by the intended plants (Paustian *et al*., 1992; Parton and Rasmussen 1994), the remainder is left in the soil or lost, either through off-gassing or leaching. Thus upwards of 40% of the applied NO_3 and

PO_4 fertilizers are lost into water systems or remain in the soil and thus not utilized as intended, driving up the cost of crop production and causing environmental damage.

The global population is expected to increase by half by the year 2050 and in response to this growth the global grain demand is expected to double (Cassman, 1999; Alexandratos, 1999). If previous agricultural practices are used as a guide we would need to increase our inorganic fertilizer input (predominantly N, P and K) threefold in order to achieve the required grain yields (Tilman *et al*., 2002). Increased input of this magnitude could have severe effects on the environment and thus the future sustainability of inorganic fertilizers, used in isolation from other amendments, is questionable.

The production of ammonia (NH_4) is an industrial process that is highly energy intensive, typically powered by the burning of fossil fuels. In addition, the phosphate cycle is completed over a geological time-scale and due to current agricultural practices the current rate of PO_4 consumption is higher than that of production (not to be confused with extraction) . Since the compound is mined, there are also severe environmental implications to be considered, a clear example of this is the near complete environmental destruction of the tiny Pacific island nation of Nauru due to phosphate mining activities (Weeramantry, 1992).

As a result of strip mining of PO_4 on the island of Nauru for nearly one hundred years the majority of indigenous flora and fauna which inhabited the island prior to the commencement of mining operations are locally extinct, including economically important species (Manner *et al*., 1984). Furthermore, due to mining related deforestation the rainfall patterns of the island have changed resulting in a drier, hotter island with a reduced water table (Manner *et al*., 1984). Short-term economic growth drove this environmental destruction. This is evident as Nauru boasted the

highest per-capita income of any sovereign state in the world during the 1960's-1970's. The per capita GDP was estimated at approximately US $31,000 (McQuade, 1975) largely as a result of its mining operations. More recently the remaining PO_4 reserves on the island were essentially deemed uneconomically viable to mine. As a result of this and many bad investment choices made by an ineffective government, the economy has all but collapsed (Connell, 2006). According to the Nauru Bureau of Statistics it achieved a GDP per capita of US $3614 in 2010-2011 (2013). Placing it in the bottom two-thirds of global per capita GDP currently (Central Intelligence Agency, 2013). The story of this tiny island nation should serve as a warning of the risks of environmental degradation at the cost of mineral extraction and mining operations.

Nauru is currently considered a failed state (Connell, 2006), with minimal prospects at making a recovery.

Typically economic factors have been the drivers for environmentally friendly change within agriculture. These economic incentives may come from either consumer pressure (Allen and Kovach, 2000) or, as a result of government regulation, the inclusion of environmental remediation costs into the production costs of the farmer. An increasing amount of contemporary research is showing that the state of the environment and particularly soil health (Tilman, 1998), ultimately influences overall agricultural productivity. The problem of environmental degradation is clearly linked to economics and thus decisive action is required to preserve soil health in order to ensure the food security of a growing global population.

Organic amendments and the effects on the environment, soils and crops

In light of the previously mentioned issues there has been a significant drive to find alternative fertilizer sources which can amend or replace the usage of chemical fertilizers. Various sources

of organic amendments exist, the most important of which are highlighted in a review by Quilty and Cattle (2011). Thermophyllic composts and vermicomposts have received much research attention due to the fact that they utilize plant and animal waste products in their manufacture and stabilize and homogenize heterogeneous organic materials, thereby increasing their suitability for agricultural practices (Follet *et al*., 1981; Quilty and Cattle, 2011; Atiyeh *et al*., 2000).

Catanzaro *et al*. (1998) showed that slow release fertilizers reduce nutrient leaching and vermicomposts (VCs) have been shown to leach less nutrients compared to synthetic fertilizers (Jouquet *et al*., 2011). The reduction in nutrient leaching in VCs is due to the ongoing microbial action within the substrate which facilitates nutrient cycling, ultimately resulting in a slow release of nutrients over time.

Furthermore the low salt content of VCs have also been shown to reduce salinity induced plant stress (Lazcano *et al*., 2008), a common affliction in plants fertilized excessively or for a long period of time with thermophyllic composts, manures and chemical fertilizers. Furthermore a study by Flores *et al*. (2006) also found that vermicomposts reduced the uptake of lead (Pb) into agricultural crops, thus limiting the risk to human health upon consumption.

Composts and vermicomposts have been shown to increase the numbers of *Trichoderma* (a soil fungus of which the majority of species are avirulent plant symbionts) species, enteric bacteria, and thermophyllic microorganisms in the soil while reducing the number of plant pathogens such as *Phytophthora* and *Pythium* species when compared to soils receiving inorganic chemical fertilizers (Bulluck III *et al*., 2002). Furthermore these organic amendments have been shown by

the same authors to improve the physical characteristics of the soil by decreasing bulk density and increasing cation exchange capacity, organic matter content and total carbon content as well as increasing the concentrations of Ca, K, Mg and Mn when compared to chemically fertilized soils.

In terms of crop yields VCs have been shown to produce similar (Manivannan *et al*., 2009; Srivastava *et al*., 2012) or better yields (Manivannan *et al*., 2009; Arancon *et al*., 2003; 2005) when used instead of synthetic chemical fertilizers. Furthermore yield increases were noted when VCs and chemical fertilizers were used in conjunction (Bachman and Metzger, 2008; Manivannan *et al*., 2009; Singh *et al*., 2011; Srivastava *et al*., 2012; Arancon *et al*., 2004), thereby reducing the both the amounts of chemical fertilizers and vermicomposts required.

Currently there exists a mindset amongst many farmers that organic amendments are not substantiated by scientific evidence (Quilty and Cattle, 2011) and thus may be ineffectual. Admittedly VCs require further research into a wider variety of crops and their effects in different soil types. However, it can be safely stated that VCs are a viable alternative or supplement to chemical fertilizers across many crop types. Currently they remain slightly uncompetitive due to the large amounts required relative to chemical fertilizers, but from an economic point of view costs are similar. Thus it is of the utmost importance that not only continuous research be conducted into vermicomposts but that this information is effectively transmitted to the public in general. Such action may stimulate the changing of traditional mindsets and drive public pressure on farmers and lawmakers to increase the utilization of vermicomposts in agriculture.

Vermicompost production and physical characteristics

Vermicomposts are the solid excreta of earthworms, produced through the mesophyllic metabolism of organic matter under aerobic conditions (Edwards, 1998). Casts, or castings, are the final product of the vermicomposting process. They are a homogenous, finely divided, peat-like substance which exhibit good water retention characteristics, high porosity, drainage and aeration (Edwards and Burrows, 1988; Edwards 1998). The vermicomposting process is best carried out in an environment that maintains moisture levels between 40-50%, a temperature between 20-30ºC, a pH of 7 and is kept dark for the duration of the process (Kale, 1995). Epigeic and anecic earthworm species such as *Eisenia fetida*, *Lumbricus rubelus*, *Eudrilus eugeniae*, *Perionyx excavatus* and *Eisenia andrei* (Edwards, 1995; Haimi, 1990) are used commercially for vermicompost production in various regions of the world, with *E. fetida* being the most commonly utilized (Haimi 1990).

Chemical constituency

VCs influence the fertility of soils in multiple ways, including the increase of available mineral nutrients, the establishment of a diverse and abundant microbial community which drives increased nutrient cycling, the production of plant growth regulating hormones (phytohormones) as secondary metabolites of microbial action, the production of humic and fulvic acids, the sequestration of heavy metals and by improving the physical structure of the soil.

The chemical constituency of vermicomposts varies greatly according to the source material utilized in its production as can be seen in Table 1.1. However a full complement of the macro and micronutrients required for plant growth are present in vermicomposts regardless of source material, albeit in varying amounts. Vermicomposts have been shown to contain most nutrients

in plant available forms such as nitrates, ammonia, phosphates, exchangeable cations of calcium and iron as well as soluble potassium (Edwards, 1998).

Mineral nutrition

VCs have been shown to contain higher amounts of plant available N (in the forms of NH_4, NO_3 and NO_2) than the source material, regardless of material type (Pramanik *et al*., 2007; Suthar, 2009). This is due to the increased rates of mineralization occurring as a result of the combined effect of the large microbial abundance and diversity present in the gut of the earthworm as well as the digestive processes of the annelid (Fischer *et al*., 1997; Dominguez, 2004). However N, and other mineral nutrient content varies according to the nutrient content of the source material (Pramanik *et al*., 2007).

Exchangeable potassium, calcium, magnesium and sodium have been shown to be significantly increased in VCs compared to that of certain soil types, the transit of the soil through the gut of the earthworm is most likely responsible for this (Basker *et al*.,1993). The same authors also found that earthworm species and soil type has a large role to play in the availability of the above mentioned nutrients present in VCs.

C to N ratio

The role of inorganic nitrogen and organic carbon in the cellular functioning of living organisms is critical to their survival and growth. Furthermore the ratio in which these two are present in the cell is also of the utmost importance. For optimum nutrition earthworms typically require a ratio of 25:1 (C:N) in the substrate that they feed on (Butt, 1993). The same author also noted that different species of earthworm respond differently to different forms of N, and thus the C:N ratio

for optimum nutrition and digestion may vary according to earthworm species. Thus in order to utilize the vermicomposting process efficiently the C:N content of the substrate as well as the requirements of the earthworm species utilized should be known. Ndegwa and Thompson (2000) investigated the effect of substrate (biosolids amended with paper mulch) C:N ratio, using the composting earthworm *Eisenia fetida*, on the quality of the final vermicompost product. They found that total N and P to be maximum, soluble N and P to be reduced and volatile substances to be at a minimum at an initial substrate C:N ratio of 25:1; indicating the potential of the final product to be a viable agricultural fertilizer with a reduced environmental pollution capacity. Furthermore the authors concluded from this study that the earthworm,species, as well as the available C and N content of the source material, but not the absolute content, is important factors to take into account when trying to produce the most agriculturally relevant vermicompost.

pH

The pH of different vermicomposts was shown to vary according to source material, as seen in Table 1.2. However it has been suggested by Hogg *et al*. (2002) that this variance in vermicompost pH is not detrimental to the growth of most plants, provided it is within the range of 6.5-8.0. It has been suggested that vermicomposting alters the pH of the substrate compared to that of the source material towards neutral/acidic conditions through a number of actions (Mitchell, 1997; Gunadi and Edwards, 2003; Ndegwa *et al*., 2000; Atiyeh *et al*., 2000; Gupta and Garg 2008). The mineralization of nitrogen and phosphorous into nitrates/nitrites and orthophosphates as well as the bioconversion of organic substances into organic acid intermediate species causes a reduction in the substrate pH (Ndegwa *et al*., 2000; Dominguez, 2004). Furthermore the production of humic and fulvic acids in the vermicomposting process

may lead to an increase in pH of the system through the production of ammonium ions. This results in a shift towards overall pH neutrality of the vermicomposting system (Pramanik *et al*., 2007).

Humic and Fulvic Acids

The humic acid (HA) content of organic wastes and vermicomposts is well documented (Albanell *et al*. 1988; Petrussi *et al*. 1988; Hervas *et al*. 1989; Senesi *et al*. 1992; Garcia *et al*. 1995; Masciandaro *et al*. 1997; Elvira *et al*. 1998), with a body of evidence indicating their ability to increase plant growth independent of mineral nutrition (Atiyeh *et al*. 2002; Arancon *et al*., 2003) and function to increase plant growth more than commercially produced humic acids (Arancon *et al*., 2006). These substances increase plant growth through a hormone-like effect, similar to that of exogenous auxin (Quaggiotti *et al*. 2004; Dobbss *et al*. 2010), via the promotion of root growth and the enhancement of plasma membrane H^+-ATPase activity (Nardi *et al*., 2002; Canellas *et al*., 2002). The increased plant growth due to additions of HAs derived from VCs typically are exhibited at low concentrations (<1000mg.kg^{-1}) of HAs and increase plant growth through both direct actions, as described above, or indirect actions (Arancon *et al*., 2003). These indirect actions include increasing the metabolic rate of soil microorganisms, altering the dynamics of soil nutrient uptake and changing the physical properties of the surrounding soil. Furthermore Canellas *et al*., (2002) found that HAs derived from VCs contain exchangeable auxin groups. These growth promoters are typically transient in the soil and thus when bonded to the macrostructure of the HAs they become much more persistent in the soil and act in concert with HAs to promote plant growth.

Microbial constituency

The biooxidation and stabilization of organic wastes through vermicomposting changes the microbial profile of the source material throughout the process, increasing microbial diversity and abundance as well as microbial functional diversity within the substrate (Vivas *et al*., 2009; Aira *et al*., 2007). The biochemical degradation of the substrate during the vermicomposting process is largely due to the action of the microbes found within the gut of the earthworm (Dominguez, 2004), with physical changes such as substrate grinding being attributed to the earthworm as well as substrate modification through the action of digestive enzymes secreted inside the gut (Edwards and Fletcher, 1988). The worms are known to feed on fungi but largely gain their nutrition from the symbiotic associations with the microflora contained within their gut and not from the ingestion of organic residues (Doube and Brown, 1998). Mucous excretions along the gut wall contain nitrogen-rich amino acids which serve as a food source for the internal flora.

Passage of microbes through the gut of the earthworm results in quantitative and qualitative changes in the microbial community (Pedersen and Hendriksen 1993), with certain groups of microorganisms capable of surviving passage through the gut of the earthworm while others are not. Bacteria and actinomycete populations have been found to increase during the passage through the gut of three separate earthworm species (*Lumbricus terrestris*, *Allolobophora caliginosa* and *Allolobophora terrestris*) while fungi and yeast populations remain consistent with that of the surrounding soil (Parle, 1963), interestingly though the same author found that fungal and yeast populations increased rapidly over time in the excreted cast while bacterial and actinomycete populations remained constant (Parle, 1963a). The author hypothesized that bacterial and actinomycete population increases seen in the gut of the earthworms could be as a

result of the grinding action of the gizzard, resulting in a finely divided substrate with an increased surface area to volume ratio, thus increasing the sites vulnerable to microbial attack and metabolism. The proliferation of certain microbial species and the depression of others is indicative of selective feeding by the earthworm. It has been experimentally shown that earthworms feed preferentially; with fungi and protozoans being of the greatest importance, algae of moderate importance and bacteria of minor importance as a source of nutrients (Edwards and Fletcher, 1988). Positive circumstantial evidence of the dependence of earthworms on microbes for their nutrition is given by the similarity of microbial community structures within the earthworm gut to that of the native soil or organic matter that the worm ingests. If earthworms were solely dependent on the metabolic action of microbes on ingested materials for their nutrition one would expect to find a difference in the microbial community present in their gut compared to that of the ingested materials (Edwards and Fletcher, 1988). Recent molecular and culture-based approaches have further strengthened the hypothesis that microbial populations are largely similar in the surrounding soil and earthworm casts, as the majority of the microbial community passes through the gut of the earthworm unchanged, with large increases in relative abundance only observed in certain species (Furlong *et al.*, 2002). The previously mentioned groups which do not survive passage through the gut of the earthworm have received attention as some of these organisms are noted as plant and/or human pathogens.

Some VCs are prepared from manures and thus there exists a safety concern that human pathogens may survive the vermicomposting process and contaminate the crops to which they are applied. Human pathogens such as faecal coliforms, *Escherichia coli* as well as species from the *Salmonella* genus, enteric virusses and helminth ova, are common in human and animal manures, where they exist in large numbers. Dominguez (2004) found that after 60 days of

vermicomposting faecal coliform numbers in biosolids dropped from 39 000 MPN (Most Probable Number)/g to 0 MPN/g. The author also found that over the same time period the number of *Salmonella* species dropped from less than 3MPN/g to less than 1MPN/g. Eastman *et al*. (2001) found a fourfold reduction in faecal coliforms, 1.75 times reduction in *Salmonella* species, 2.5 times reduction in enteric virus and a 3.2 times reduction in helminth ova due to the vermicomposting of municipal biosolids over after the duration 144 hours.

The reductions of plant pathogens to agricultural crops and soils are also of particular importance. The vermicomposting process and additions of VCs to soils, have been shown to reduce numbers of certain pathogens. Arancon *et al*. (2004) investigated the effects of VC additions to soil on pest populations and associated damage to pepper, tomato and cabbage plants. The authors found that the additions of VC decreased losses of dry weight to pepper plants (*Capscicum annuum* L.) due to infestations of aphids (*Myzus persicae* Sulz.) and mealy bugs (*Pseudococcus* spp.) as well as decreasing the populations of these pests significantly, when substituted into the soil media at 20% and 40%. Furthermore, the authors also found that mealy bug populations on tomato plants (*Lycopersicon esculentum* Mill.) were decreased significantly in response to VC additions to the soil leading to a significant reduction is shoot dry weight losses. Cabbage plants (*Brassica oleracea* L.) also suffered significantly less leaf area loss due to cabbage white caterpillar (*Pieris brassicae* L.) infestations in response to the additions of VC (Arancon *et al*., 2004). Toyota and Kimura (1994) found that propagule numbers of the soil-borne plant pathogen, *Fusarium oxysporum*, were decreased in the casts of the *Pheretima* species of earthworm. Field trials comparing the tropic diversity of nematodes in VC treated and chemical fertilizer treated soils found that the addition of VCs significantly

reduced the number of plant pathogenic nematodes present in the soil compared to the chemical fertilizer treatment (Arancon *et al*., 2002).

Growth effects

A study was conducted in which the growth effects of vermicompost and chemical fertilizers (NPKs) were compared on beans (*Phaseolus vulgaris*) in two different soils (Manivannan *et al*., 2009). It was found that vermicompost significantly improved the physical structure of the soils, increased macro and micro nutrient, as well as soil organic carbon content, In addition, it increased microbial activity, crop protein content, growth and yields. A combination treatment of 50% vermicompost (2.5 tons per hectare) and 50% (recommended NPK dosage) synthetic chemical fertilizer yielded the greatest soil macro and micro nutrient content as well as crop growth and yield, indicating that reducing synthetic fertilizer input and augmenting it with VC is a viable alternative. Similarly, it was found that a combination treatment of VC, organic mulch and inorganic fertilizer led to increased growth and crop yields of French bean, as well as a reduction in water usage and fertilizer application (Singh *et al*., 2011).

During a two season long experiment using tomatoes (*Lycopersicon esculentum*), bell peppers (*Capsicum anuum grossum*) and strawberries (*Fragaria* spp.), comparing VCs made from recycled paper waste, market food waste and cattle manure with inorganic fertilizers, it was found that in all of the VC treated plots the marketable fruit yields of the tomatoes were greater than those in the plots which received inorganic fertilizers (Arancon *et al*., 2003). In the plots in which peppers were grown the VC treatments exhibited significantly greater shoot weights, leaf areas and marketable fruit yields than the inorganic fertilizer treatments. Similar findings were presented for strawberries.

Sainz *et al*. (1997) found that substitutions of 10% and 50% VC derived from composted urban wastes significantly increased the dry matter yields of both red clover and cucumber plants, relative to both compost and control treatments. Furthermore, the additions of VC reduced arbuscular mycorrhizal fungi (AM) colonization rates of the roots of the afore mentioned crops. AM fungi are known to increase the nutrient content of colonized host plants, through the increased surface area of nutrient uptake (Ibijbijen *et al*., 1996), but carry an increased carbon cost to the host (Mortimer *et al*., 2005). AM fungi are known to benefit plant growth and are of particular importance in no-till agricultural systems, thus the use of vermicomposts should be utilized with AM colonization rates kept in mind, since both offer benefits to the farmer. Sainz *et al*. (1997) found that a 10% substitution of VC to soil increased the amount of AM root colonization in red clover (*Trifolium pratense* L.) while 50% and 100% substitutions successively, reduced colonization. With cucumber plants (*Cucumis sativus* L.) any additions of VC reduced AM colonization. As indicated by the previously mentioned study additional research into the interaction between plant species, VC and AM are required to maximize the agronomic potential of utilizing both VC and AM in concert to maximize soil health and crop production in an environmentally friendly fashion.

Vermicompost tea

Vermicompost teas (VCTs) are produced via aqueous extraction of vermicompost. Common practices include addition of microbial growth enhancers, such as molasses and kelp extracts, and to aerate the suspension for a period of time, typically more than 12 hours. This process is known as “brewing” and it is undertaken to increase the abundance and diversity of the microbial population already present in the VC. The aeration of the tea during the brewing process

facilitates the growth of aerobic bacteria, while excluding the growth of anaerobic bacteria, which include many plant and human pathogenic species (Ingham, 2003). The addition of sugars such as molasses and kelp extract serve as a food source for the microbial populations and in tandem with the abundance of oxygen facilitate microbial growth (Kannangara *et al*., 2006). It is known that due to the production processes involved some of the microbes present in the teas are beneficial to plants (Fritz *et al*., 2012) and can be utilized to outcompete pathogens and pests in agricultural soils and on plant surfaces, when applied as a foliar application or soil drench (Ingham, 2003; Edwards *et al*., 2007). Furthermore many microbes found to occur in VCs are known to produce phytohormones as secondary metabolites; these substances further stimulate plant growth (Tomati *et al*., 1988). In essence VCTs are liquid fertilizers which are easier to apply to crops than VC solids and reduce the amount of solids required in order to achieve a positive growth response, simplifying the job of the farmer as well as saving him money.

Chemical constituency

VCTs typically contain all of the macro and micronutrients found in the source VC. Pant *et al*. (2012) found that humic acid (HA), total N, NO_3, NH_4, K, Ca and Mg concentrations increased in VCTs relative to the source VC used in production. In contrast, P and micronutrient concentrations were found to decrease in the VCT compared to the original VC. In a further study by Pant *et al*. (2012) it was shown that the chemical constituency varied significantly between the maturity and type of VC sources utilized in the production of VCTs. Thus as is logically expected, the source material from which the VC was produced influences the chemical constituency of the VCT. Furthermore an increase in VCT nutrient concentration is to be expected by increasing the ratio of VC to water.

The tea brewing process, whether aerobic or anaerobic, typically results in a slight increase in pH with teas tending towards neutrality (Pant *et al*., 2009; Arancon *et al*., 2012; Pant *et al*., 2012; Pant *et al*., 2012). The addition of microbial growth enhancing agents, such as kelp extract and humic acids, may be partly liable for the increase in pH (Pant *et al*., 2009).

Phytohormones such as auxins, cytokinins and gibberellins have also been found to occur in VCTs, however the prevalence of certain groups seems to be linked to the source material of the parent VC. In a study conducted by Arancon *et al*. (2012) it was found that VCT derived from chicken manure VC did not contain the auxin N-(indole-3-yl-acetyl)-leucine, while VCT derived from food waste VC contained more than 180 $ng.L^{-1}$ of this phytohormone. Similarly, the chicken manure based VCT did not contain any cytokinins, but three different gibberellins (GA4, GA24 and GA34), while the food waste VCT contained one cytokinin (isopentyladenosine) and one gibberellin species (GA 24). Pant *et al*. (2012) found that VCT derived from aged chicken manure VC (cured for 3 months) contained a greater diversity of gibberellins (GA4, GA24 and GA34) than VCT derived from fresh chicken manure VC, which contained only GA4 and GA34. However no significant differences in the concentrations of the two common gibberellins were found. Food waste derived VCT was also found to contain the two gibberellins GA4 and GA34 albeit in significantly greater concentrations than both the chicken manure VCTs. The authors found no traces of auxins or cytokinins in any of the tested VCTs. A wide range of factors, including the species of earthworm used in the composting process, may likely influence the production and persistence of phytohormones in VCs, many of which remain unexplored.

Microbial constituency

Compost and vermicompost teas typically contain the same suite of microbes as the parent compost if extracted under aerobic conditions (Ingham, 2003). Due to the chemical differences

in source material, it is expected that microbial population dynamics should differ between various VCs and composts. Kannangara *et al*. (2006) showed that significantly different amounts of bacteria and fungi are present in different sources of compost and VC. However, quantitative evidence of how these numbers change in the tea brewing process still remains understudied. There has been discussion in the literature concerning the effect of aeration of bacterial numbers. The addition of different carbon sources such as kelp extract, molasses, wheat flour etc. to the tea brewing process were shown to be a significant factor driving increases in bacterial and fungal abundance (Fritz *et al*. 2012), not the process of aeration (Pant *et al*., 2011). However, Pant *et al*. (2009) found that neither the addition of sugars nor aeration increased microbial abundance. The difference in conclusions drawn between these studies may be due to the different organic materials used in the vermicomposting process, since Fritz *et al*. (2012) utilized a pre-composted mixture of green waste and cattle manure while Pant *et al*. (2009) analysed the effects on chicken manure VC.

Limited research has been conducted on the diversity of microbial community assemblages in VCTs. The interaction between VC source material, VC concentration in teas and the effects of aeration and sugar additions results in a complex suite of factors which are likely to influence microbial abundance and diversity. Analyzing microbial abundance is fairly straight forward and is achieved through a number of methods, including epiflouresence microscopy, substrate induced respiration, culture based methods or flow cytometry. Conversely, the analysis of microbial diversity is both expensive and labour intensive. Denaturing gel gradient electrophoresis (DGGE) makes use of molecular probes to identify microbial species, however this process is not very efficient when analyzing a community assemblage potentially comprised of hundreds of species. Recently the Biolog Ecoplate system has been utilized to study bacterial

diversity within composts and agricultural soils (Reilly *et al*., 2013; Jouquet *et al*., 2011; Frac *et al*., 2012), this is a more cost effective and pragmatic system which makes use of functional grouping approach, based on sole carbon source utilization. The limitation of the Biolog system is that individual species cannot be identified; a community level physiological profile (CLPP) is established instead. Furthermore, fungal and prokaryote assemblages are also excluded (Preston-Mafham *et al*., 2002).

Utilizing the Biolog Ecoplate system, the passage of organic material through the gut of the earthworm has been shown to alter both the abundance and diversity of the bacterial community assemblage (Aira *et al*., 2006). The authors found that while the vermicomposting process reduced microbial abundance and activity it increased metabolic diversity. This increased metabolic diversity results in a wider variety of bacterial diversity, which may play a role in soil fertility (Bardgett 2005a).

CLPP analyses of different types of VC are still relatively scarce in the literature and even more so when used to evaluate VCTs. It is largely unknown how CLPPs in VCs and VCTs will differ as a result of different VC sources, aeration, sugar additions etc. This is a field which is likely to grow since the system is both inexpensive and simple to use.

Reduction of pathogens in Vermicompost Teas

A key concern raised is the proliferation of plant and animal pathogens in VCTs as a result of the addition of sugars and the aeration process. VCs, and the vermicomposting process, have been shown to reduce the levels of soil borne pathogens. However, if even small populations exist within the VC favorable conditions created through the addition of sugars or aeration may lead to the proliferation of pathogens. Kannangara *et al*. (2006) found that a non-pathogenic *E. coli*

strain (K12- MG1655) proliferated in response to increasing concentrations of kelp extract and molasses in VCTs. However this was only noted when teas were inoculated with the bacteria, since the source VC contained no detectable amounts of *E. coli*. Furthermore, in inoculated teas, *E. coli* populations were found to be significantly lower in those which were aerated. The same trend was noted in VCTs, when compared to teas derived from thermophyllic composts. Interestingly the addition of carrot juice to the teas was found the further reduce *E. coli* populations, without inhibiting the growth of other bacterial groups. Thus, even though sugar amended VCTs contain a labile nutrient pool conducive to *E. coli* growth, the proliferation of this bacterium is reduced even when inoculated. However, the human pathogenic *E. coli* strain (O157:H7) is frequently harbored in animal derived wastes (Hutchison *et al*., 2004) and known to cause symptoms in humans from the ingestion of as few as 10 cells (Chart, 2000). Growing salad vegetables such as lettuce, which are frequently consumed raw, in soil contaminated with *E. coli* O157:H7, may lead to the growth of the pathogen on leaf surfaces (Abdul-Raouf *et al*., 1993) or for it to become internalized in the plant tissue (Solomon *et al*. 2002). A situation such as this caused a massive public health scare in 2012 when cucumbers grown in Germany were contaminated with a pathogenic strain of *E. coli*. Thus, even though the vermicomposting process has been shown to reduce pathogen levels to that of a United States Environmental Protection Agency (USEPA) recognized class A biosolid (Eastman *et al*., 2001), and the correct brewing of VCTs further reduces this load, it is of the utmost importance that a rigorous testing schedule for pathogens is maintained.

Plant growth effects

VCTs have been shown to increase plant mass (Pant *et al*., 2012; Arancon *et al*., 2007), yields (Pant *et al*., 2011; Pant *et al*., 2012; Pant *et al*., 2009; Arancon *et al*., 2007), seed germination

rate (Arancon *et al*., 2012) as well as soil microbial diversity and activity (Fritz *et al*., 2012; Pant *et al*., 2009). The increase in growth has been attributed by Pant *et al*. (2009) to an increase in available mineral nutrients, particularly N, provided by VCTs. However, the presence of humic acids and phytohormones in VCTs are also likely to elicit a growth response, independent of mineral nutrient availability. Thus, a complex set of interacting factors are responsible for increases in plant growth. The field of research into VCTs remains rather small at this stage but is growing rapidly, as numerous authors are investigating VCTs as an augmentative treatment to chemical fertilizers.

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Figures and Tables - Chapter 1

Table 1.1. Nutrient concentration of vermicomposts derived from various source materials. * indicates data from (Arancon *et al*., 2005), **(Arancon *et al*., 2004), † (Atiyeh *et al*., 2001), ×(Gupta and Garg, 2009), \ indicates no data for selected parameter

VC type	N (%)	P (%)	K (%)	B (mg/kg)	Ca (mg/kg)	Fe (mg/kg)	Mg (mg/kg)	Mn (mg/kg)	Na (mg/kg)	S (mg/kg)	Zn (mg/kg)	pH
Food	1.3	2.7	9.2	23	18614	23264	4364	610	842	2587	279	7.40**
Cow	1.9	4.7	1.4	58	23245	3454	5802	160	3360	5524	516	6.40
Paper	1	1.4	6.2	31	9214	17811	7661	447	613	1929	127	7.70×
Green	0.86	0.13	0.18	9.56	14500	10535.80	1200	59.23	283.07	\	49.52	6.30
Chicken	1.37	1.02	0.15	0.00	69900	7313.58	4900	421.50	471.81	\	497.85	6.50
Swine manure†	2.36	4.50	0.40	\	8600	800	500	\	\	\	\	5.68

Chapter 2: General Introduction

South Africa has established itself over the last 300 years as a proud agricultural nation, satisfying the majority of our domestic agricultural requirements and supplying more than half of the maize requirements of southern Africa (Scotcher 2010). However the current population of South Africa is approximately 50 million and is growing by 2% per annum (Statistics South Africa, 2015), projected to reach 82 million by the year 2035. This population growth will place massive stress on the agricultural system. The area of land available for agriculture is effectively saturated (Open data for Africa, 2009) and thus increases in food production will have to come from agricultural intensification and innovations, rather than land use expansion. Additionally, farmers are faced with the problems of decreases in soil fertility due to past practices, climate change and economic difficulties due to economy of scale (Goldblatt 2010). In light of these issues, and order to maintain profitability and productivity, farmers are becoming more aware of the need to make their operations more sustainable, particularly in terms of soil health and increases in efficiency.

The use of VCs in domestic agriculture is extremely limited (Ungerer, 2013), with the majority of soil C replenishment being achieved through the additions of thermophyllic composts. Vermicomposting, as a technology, is generally accepted to produce a product superior to that of thermophyllic composting (Munroe, 2007) however some authors have found that no generalization can be made (Tognetti *et al*., 2005; Atiyeh *et al*., 2000). In spite of this there exist a number of factors which have been limiting the use of VCs in domestic agriculture. These include the relatively high cost of application (VC solids are applied at rates between 3-10 tons per hectare (Singh *et al*., 2011; Arancon *et al*., 2004; Arancon *et al*., 2005), translating to a cost of between R3600-R12000 per hectare)and cultural biases against unknown organic products. Despite this there exists a large body of literature indicating that VCs promote growth across a wide variety of crops (Arancon *et al.*, 2004; Arancon *et al.*, 2006; Arancon *et al.*, 2008; Arancon *et al.*, 2003; Arancon *et al.*, 2005; Arancon *et al.*, 2003; Arancon *et al.*, 2003; Atiyeh *et al.*, 2000; Atiyeh *et al.*, 2002; Bachman and Metzger 2008; Atiyeh *et al.*, 2000) and as such would increase the net production of the farmer. One could argue that the farmer would see increases in soil

health and crop yield to such a degree that it is in fact cost effective to add VCs even at high concentrations, but in South Africa farmers have not yet been exposed to VC technologies long enough to have faith in their use. It is thus vital that domestic research is conducted and the results of which are accurately conveyed to the agricultural community.

VC teas, while not as expensive as VC solids in terms of application are also approached with apprehension by South African farmers. These biases exist mainly because there has been a lack of education by government and research institutions to the farmers concerning the efficacy and benefits of VC products. In South Africa, private entities have typically led the charge in terms of VC production and research while government institutions have fallen behind. However, this is changing and government institutions are starting to fund VC research, a prime example being this study. If VCs applications are too be adopted as a commonly used practice in South African agriculture, public institutions will have to support private enterprises in conveying research results to end users, thereby adding credibility to their findings. The South African government has recently created the National Organic Waste Composting Strategy (Department of Environmental Affairs, 2013) aimed at reducing the amount of organic waste entering landfills and converting it to a value added product to be used in agriculture. This document details the norms and standards to be used in the composting process and the acceptable parameters of a number of factors in the final product. This document does not however detail the vermicomposting process or provide any useful information regarding its efficacy and use in agriculture. As such, the distribution of the bulk of information concerning VCs and their use has typically fallen within the academic community or the very limited VC production operations of the private sector. Consequently information regarding the use of VCs has not been adequately dispersed to the farming community.

Lupin production in the Western Cape has a long history; the earliest record of their use dates back to 1897, when Mr JP de Waal noted in the 11th volume of The Journal of Agriculture of the Cape of Good Hope that they responded best to seed inoculation and dense sowing. Their use became widespread after 1949 when a soft-shelled variety, suitable for animal feeds and crop rotations, was successfully bred in the Malmesbury area. Since then they have been used widely across the Western Cape as both an animal feed and a green manure. The narrow leaved lupin (*Lupinus angustifolius*) grows well in sandy to sandy-loam soils and contains approximately 30-

32% crude protein and 13% fibre in the dried seeds, making it an excellent sheep feed. Cultivation of *L. angustifolius* peaked between 1962 when approximately 198 000 hectares of farmland was allocated to this crop, however it subsequently decreased to less than 20 000 hectares by 1983 due to the increasing price of wheat and the fluctuations in the wool price. Currently the area under cultivation of *L. angustifolius* fluctuates between 15 000 to 20 000 hectares (Agenbag, 2008).

Due to rising costs farmers are faced with increased pressure to diversify their operations, with many in the Western Cape maintaining sheep herds as well as wheat, barley, oats and maize (in irrigated areas). Legumes such as lucerne and lupins are very important as cover and rotation crops since they replace soil N through BNF and can be used as animal feeds. Lupins have been extensively bred in the Western Cape with regards to their potential as a sheep feed but no work has been conducted concerning their interaction with VCs and VC teas. It is known that VCs improve the physical and chemical structure of sandy loam and clay loam soils as well as increasing the yield, protein and sugar content of *Phaseolus vulgaris* seeds (Manivannan *et al*., 2009). Additions of VC have also been shown to increase germination time, N uptake (Rodríguez-Quiroz *et al*., 2011) and reduce the required fertilizer input of *P. vulgaris* (Singh *et al*., 2011).

It may be that similar results could be found in *L. angustifolius*, since it is also a legume. Of particular interest would be the influence of VCs on BNF rates, since the crop is used as a green manure in a rotation. Furthermore the addition of VC to the soil will increase soil C content and microbial life, aiding in increasing the rate of nutrient cycling and productivity of the crop.

VC teas are favoured among many organic growers since they are easier to transport and apply than solid VCs (Arancon *et al*., 2007) even though they are contain lower concentrations of mineral nutrients. However, one of the main reasons for adding VCs to the soil is to introduce organic C. Thus VC solids and teas should be applied in concert, to reduce the amount of solids that need to be applied and thus saving costs to the farmer. Furthermore, it is currently unknown how the combination treatment of VC solids and teas will affect the N nutrition of legumes, specifically *L. angustifolius.*

The aim of this thesis will be to investigate the most efficient use of VC in terms of biomass production of *L. angustifolius.* Our hypothesis is that VC teas, in conjunction with VC solids, would provide sufficient N to alter the legume dependence on BNF. To address this hypothesis, two separate studies will be conducted, the first to determine the optimum use of VC solids and the second to determine if VC teas can be used in conjunction with VC solids to increase affect N nutrition and biomass production.

Experiment 1

Objective To determine if increasing concentrations of VC in solid form, have any effect on plant biomass production, BNF and soil bacterial diversity.

Experiment 2

Objective: To determine if increasing combinations of VC in tea and solid forms, have any effect on plant biomass production, inorganic N forms, BNF and soil bacterial diversity.

The end goal of this project would be to investigate what the most efficient use of VC solids and teas are when used in combination, in terms of biomass production of *L. angustifolius* and the influences of these VC additions on soil microbial diversity and BNF of the plant.

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Chapter 3: Optimising vermicompost concentrations for the N nutrition and production of the legume Lupinus angustifolius.

COMPOST SCIENCE & UTILIZATION Article in Press 2015

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Abstract

Vermicomposts (VCs) are the solid excreta of earthworms, known to contain plant available nutrients, large amounts of microbial life and diversity, and plant growth regulating hormones. (VCs) may play an integral role in the nitrogen nutrition of *Lupinus angustifolius* and function to reduce the reliance of legume crops on chemical fertilizers. The aim of the present study was to determine the role of varying concentrations of chicken manure VC on the biomass production and N nutrition of the legume, *L. angustifolius*. We investigated the effect of increasing concentrations of VC's, in conjunction with commercial rhizobial inoculum additions, on the biomass yield of *L. angustifolius*. Plants were grown in various mixtures of VC, rhizobial inoculum and quartz sand under glasshouse conditions. After 30 days the plants were harvested and analysed for tissue nutrient concentrations. The VC-containing substrates were assessed for wide-spectrum soil analyses and microbial diversity via Biolog EcoPlates. The combined treatments of 5% VC and rhizobia inoculation yielded the greatest biomass response. Furthermore the addition of VC allowed for bacterial nitrogen fixation within non-rhizobia treatments. Nematode numbers and diversity grew with increases in VC concentrations, likely driven by similar increases in abundance of their microbial prey. However, changes in VC concentration had no effect on bacterial guild structure. In conclusion VC concentrations should be an important consideration for substrate nutrient availability, microbial abundance, and bacterial nitrogen fixation.

Introduction

Industrialization of agriculture has led to substantial increases in food production over the last 80 years, a movement collectively referred to as the green revolution. This, coupled with ever

increasing reach of transport networks, has allowed for an increase in the global human population. However, it has been argued that this growth has been unsustainable, as modern agriculture depends heavily on non-renewable fossil fuels to sustain high levels of production (Altieri, 2012). In response to this an area of research that has received much attention is the chemical fertilizer industry. Three most limiting macronutrients required for plant growth, Nitrogen (N), Phosphorous (P) and Potassium (K) are produced industrially (N) or mined in very large amounts (P and K), to sustain our current levels of food production. Depletion of P is of particular concern since the P-cycle takes place over a geological time frame. Although there are no signs of short to medium-term depletion of the resource, worst case scenarios predict that way may deplete between 60-70% of our global P-supply by the end of the century. However, under the best case scenario depletion should not occur for the next 100-200 years (Van Vuuren *et al*., 2010), yet the fact remains that P-depletion is a reality that future generations will have to face should better management of our P-resources not be implemented. Furthermore, in recent years it has become apparent that the excessive use of agrochemicals (fertilizers and pesticides) has led to significant environmental damage (McLaughlin and Mineau, 1995) and negatively affected human health (Stallones and Beseler, 2002; Cole *et al*., 2000). Thus there are multiple factors driving the need for an alternative form of fertilizer substance which can inherently reduce input costs, curb dependence on non-renewables and offset environmental degradation. Vermicompost (VC) has been proposed as a viable supplement to reduce chemical fertilizer input and in some cases even an alternative, and for the last twenty years a significant amount of research has been conducted on this substance (Adhikary, 2012; Arancon *et al*., 2006; Arancon *et al*., 2003; Edwards *et al*., 2006; Manivannan *et al*., 2009).

Vermicomposts are the product of the mesophyllic biodegradation and stabilization of organic materials through the interactive actions of microbes and earthworms. The end product is a finely divided, homogenous substance which is rich in organic compounds, microbial life, mineral nutrients, phytohormones and humic acids (Edwards and Burrows 1988; Edwards, 1998). Vermicomposts can be produced from many different waste sources including plant material, farmyard manures, paper and food wastes as well as municipal solid wastes and thus can vary greatly in complexity of composition and hence the growth response that they elicit in plants (Arancon *et al*., 2004; Arancon *et al*., 2005; Arancon *et al*., 2008). In recent years the

vermicompost industry has grown substantially as it is a low-cost, low-tech industry and is likely to grow further as organic agriculture principals are becoming more commonplace in conventional agriculture with a growing number of South African farmers utilizing the resource, primarily as a soil conditioner.

The factors that drive plant growth as a result of the addition of VC's are numerous and complex, but can be broadly classified into four categories. These are, mineral nutrient content (Arancon *et al*., 2008), microbial abundance and diversity (Anastasi amd Varese, 2005), plant phytohormone production as a result of microbial action (Tomati *et al*., 1988; Edwards, 1995) and humic/fulvic acid content (Atiyeh *et al*., 2002; Aguiar *et al*., 2013; Arancon *et al*., 2003; Arancon *et al*., 2006).

Traditionally mineral nutrition has been the component of plant fertilizers that have received the largest portion of research, as it is easily quantifiable and many methods exist to trace the utilization of nutrients within the plant. VCs contain relatively large amounts of N immediately available for plant uptake (Parmelee and Crossley, 1988; Ruz-Jerez *et al*., 1992) and thus are recognized as a viable supplement to a chemical fertilizer application regime (Srivastava *et al*., 2012). Plant available N contained in VCs is predominantly in the forms of nitrate (NO_3) and ammonium (NH_4). Typically fresh castings contain higher amounts of NH_4 than NO_3. This ratio equalizes over time due to nitrification and cast desiccation, the latter process resulting in the encapsulation of the organic matter contained within the casts, preventing further N conversion (Decaens *et al*, 1999). Furthermore VCs contain greater amounts of available C, Mg, Ca, P and K compared to their source materials (Daniel and Anderson 1992; Lavelle *et al.*, 1992; Basker *et al.*, 1993). However, pound for pound, VCs do contain far less plant available mineral nutrition than synthetic chemical fertilizers, but are comparable to those found in other secondary sources such as manures (Troeh and Thompson 2005). An aspect of the mineral nutritional component that is often overlooked is the large pool of nutrients that are contained within organic complexes and sequestered within microbial tissues (Bardgett, 2005). This pool of nutrients is available for slow release via mineralization by microbial nutrient cycling through the action of soil animals such as mites and nematodes.

Various methods exist to enumerate the total microbial load contained within soils and these techniques have been modified to analyze microbial abundance within both thermophyllic

composts and VCs. These methods include standard dilution-plating techniques (Lorch *et al*., 1995), DGGE analyses (Vivas *et al*., 2009), microbial biomass C (Vance *et al*., 1987) and CO_2 evolution analysis (Anderson, 1982). Thus choosing a unit to quantify absolute microbial abundance in VCs is problematic. However, using a combination of techniques, VCs have been shown by Tognetti *et al*. (2005) to contain significantly lower amounts of microbial abundance than thermophilic composts, while Lazcano *et al*. (2008) found no difference between the two types of compost. In contrast, Vivas *et al*. (2009), as well as Sen and Chandra (2009), found the microbial abundance of VCs to be more than thermophilic composts. Not surprisingly, it was contended that microbial abundance and diversity varies with VC maturity and source material (Tognetti *et al*., 2005) and that further research into this field was required. Arguably more useful than bacterial abundance data is that concerning bacterial diversity. Aira *et al*. (2005; 2007) found that the bacterial community level physiological profiles (CLPP) found in pig slurry pre and post vermicomposting were significantly different and that the vermicomposting process increased the rate of carbon mineralization as a result of the increases in microbial diversity. The Biolog EcoPlate system measures the consumption of 32 different carbon sources to obtain carbon utilization profiles of the microbial communities involved. This system has been used to determine the effects of VC applications and physical management practices on bacterial diversity in the soil (Gomez *et al*., 2006). The Biolog EcoPlate system has been widely used in many ecological applications but is not without its faults, however if suitable precautions are taken the system is effective in comparing CLPPs rather than characterizing them (Preston-Mafham *et al*., 2002).

Besides mineral nutrition VCs further stimulate plant growth through the microbially mediated release of phytohormones (Tomati *et al*., 1988; Frankenberger and Arshad 1995). In a study conducted by Barea *et al*. (1976) on 50 different microbial isolates occurring in the rhizosphere of various plants it was found that 90% could produce kinetin-like substances, 86% auxins and 58% gibberellins which were reported to influence plant growth directly. More recently similar results have been found by Ahmad *et al*. (2008). It has been shown that VCs contain a large diversity of bacterial and fungal biomass which are known to produce these secondary metabolites and are thus capable of influencing plant growth across a variety of crops (Arancon, Edwards, *et al*. 2004; A. P. Pant *et al*. 2012; Arancon *et al*. 2012).

A second class of growth inducing organic compounds contained within VCs are humic acids (HAs). It has been shown that these compounds significantly influence plant growth and development independent of mineral nutrition (Atiyeh *et al*. 2002). The action of HAs have been shown to potentiate the effects of phytohormones on plant growth (Phuong and Tichy, 1976) or have a similar effect in their absence (Nardi *et al*. 1988). Furthermore HAs have been shown to increase legume biomass and nodule mass (Tan and Tantiwiramanond 1989) and thus may indirectly play a role in N availability in the soil through increased biological nitrogen fixation (BNF). BNF is the conversion of atmospheric N_2 gas to plant-available NO_3 via symbiotic associations between nodule forming bacteria and a host plant. This contributes greatly to the N nutrition of the host. The amount of BNF that occurs is indicative of the reliance of the host plant on soil-derived mineral N and thus as BNF increases so the dependence on mineral N decreases.

Nematodes are the most abundant multicellular organisms on earth. They are capable of surviving in any environment that provides a source of organic C and under some of the harshest conditions on earth, including the dry valleys of Antarctica where five endemic species have been found (Virginia and Wall, 1999). The *in-situ* analysis of nematode community structure has also been used as a bio-indicator assay in both terrestrial and aquatic environments (Malhotra *et al*.,, 2002; Neher, 1997), since they are primary or intermediary consumers and respond rapidly to environmental disturbance or enrichment (Bongers and Ferris, 1999). Nematodes are primarily classified into functional groups according to their mouthpart morphology and feeding behavior (Siddiqi 1986). They feed on a wide variety of organisms and are typically classified as bacteriovores, fungivores, herbivores, omnivores, carnivores or parasites, depending on their ecological function. It is well known that VCs are rich in microbial diversity and abundance, and as such it is expected that a large diversity of nematodes should also be present in the substrate. Typically researchers quantify microbial diversity through a number of methods, as discussed earlier, but none have yet investigated the possibility of using nematode diversity indices as a proxy for microbial diversity. The presence of a large variety of nematode functional groups within a VC could be used as an indicator of the ecosystem complexity contained within the product. Furthermore, sampling the soil prior to and after the addition of VC could indicate the changes occurring within the soil food web, giving the researcher an idea of the impact that the addition of the VC has on the soil biota. The strength of this system is that a skilled technician

with a microscope can analyze the samples and generate results faster than any culture based methods investigating microbial diversity. This will provide the end user with practical data that can be translated into rapid management decisions on the farm.

The legume *L. angustifolius* has been well studied by numerous research groups globally and can thus be used as a model legume. This is of great importance for agriculture as legumes such as peas, beans and peanuts are grown in significant amounts globally. Therefore understanding the growth responses of *L. angustifolius* to VC will offer insights into its usage and research potential with other legume crops. Inoculation with symbiotic N-fixing rhizobacteria has been shown to increase the biomass output of numerous legume species including that of *L. angustifolius* (Lange and Parker 1960; Stepkowski *et al*. 2005). As such the inoculation of lupin seeds with various species from the rhizobia genus is standard agricultural practice.

Thus far there has been a large body of work performed on the physiological mechanisms of N uptake on *L. angustifolius*, however no studies have been published concerning the effect of vermicompost on biomass production of *L. angutifolius*. VC is known to contain large amounts of organic N and this study aims to contribute to our understanding of its uptake and utilization. Furthermore, the effects of the interaction between rhizobia bacteria and VC on the biomass production of *L. angustifolius* are also unknown. The goal of this study is to determine how chicken manure VC, and the microbial diversity contained within it, affects soil N availability and the consequences that this has on legume N nutrition. In addition we aim to optimize the application ratio of chicken manure VC to quartz sand, under glasshouse conditions, to achieve maximal biomass production of *L. angustifolius*.

Methods

Cultivation and harvest

Chicken manure, aged for approximately one year, in windrows exposed to the elements, was used as a substrate for the VC in this study. The vermicomposting process, using the compost earthworm *Eisenia fetida*, took place in windrows approximately 1 meter high by 1 meter wide by 50 m long on the commercial vermicomposting farm, Wormworks (Simondium, Western

Cape). The process took 3 months to complete and homogenized samples were taken throughout the windrows to use in the study.

The experiment was carried out in a temperature controlled (18-25°C) glass house at Stellenbosch University during late autumn/early winter. The VC was mixed with autoclaved quartz sand in each of the following ratios (vermicompost: sand): 5%, 10% and 100% (v/v). The mixture was placed in medium sized seedling trays (150 ml wells), and wetted down to field capacity prior to planting.

Two parallel experiments were run, in one the plants were grown in varying ratios of VC and sand only (NR), while in the second (R) a rhizobia innoculant was added to the sand and VC mixtures..Within the NR experiment a sample size of 10 was used while in the R experiment the sample size was 6 . Seed tray wells in the R experiment were filled with the vermicompost/sand mixture and the seeds were covered in rhizobia innoculant (Registration No. L 1729, Stimuplant CC.) prior to planting.

After planting the seedlings also received approximately 50 ml of filtered water containing more inoculum to ensure nodulation of the legume roots occurred. Control treatments were grown in 100% quartz sand; R controls received *rhizobium* innoculant and the NR controls received no additives besides water.

2 Seeds of *L. angustifolius* were planted in each well and each seed was planted approximately 2 cm deep and covered with the soil mixture.

Two weeks after planting the seedlings were thinned to one seedling per plug and twelve days after this the seedlings were harvested, separated into roots and shoots, stored in brown paper bags and placed in the oven at 50 ºC for one week to dry out completely. Small nodules were noted on some of the plants at the time of harvest, indicating the initiation of the BNF symbiosis. As such N isotope analyses were deemed necessary.

Nutrient analyses

The dried plant material was ground into a fine powder using a mixer mill (Retsch MM400) and

submitted for nutrient analysis. The sand/vermicompost mixture for each treatment was also ground and submitted for soil analysis. The submitted samples were analysed for their respective C, N and P concentrations by a commercial laboratory, using inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyser with suitable standards (BemLab, De Beers Road, Somerset West, SA).

The 15N analyses were carried out at the Archeometry Department, University of Cape Town. The isotopic ratio of 15N was calculated as ı=1000‰ ($R_{sample}/R_{standard}$), where R is the molar ratio of the heavier to the lighter isotope of the samples and standards is as defined by Farquhar *et al*. (1989). Between 2.100 and 2.200 mg of each milled sample were weighed into 8 mm × 5 mm tin capsules (Elemental Micro-analysis Ltd., Devon, UK) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons instruments SpA, Milan, Italy). The 15N values for the nitrogen gas released were determined on a Finnigan Matt 252 Mass Spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift: two in-house standards (Merck Gel and Nasturtium) and the IAEA (International Atomic Energy Agency) standard $(NH_4)_2SO_4$.

Percentage nitrogen derived from atmosphere (%NDFA) is a measure of the reliance of the plant on BNF in relation to the uptake of soil nitrogen. It was calculated by using the following equation (Mortimer *et al*., 2009):

$$\%NDFA = 100\left(\frac{\delta 15\text{N reference plant} - \delta 15\text{N legume}}{\delta 15\text{N reference plant} - \text{B}}\right)$$

Where the reference plant was *Virgilia divaricata* grown under the same glasshouse conditions, in the absence of a BNF symbiont, relying on all of its N-nutrition from soil sources. The B value is the δ15N natural abundance of the N derived exclusively from BNF of the above-ground tissue

of nodulated *Lupinus angustifolius*, grown in a N-free culture. The B value of *L. angustifolius* was determined as -1.6 (Magadlela *et al*., 2014).

Vermicompost ecology

Microbial counts

Total numbers of bacteria and fungi contained within the pure (100%) vermicomposts were determined using the BD FACSAria™ flow cytometer (Franklin Lakes, NJ). Dry vermicompost material was sifted through a 1 mm mesh sieve to remove the large particles and homogenize the material. Five ml of the sifted material was measured into a falcon tube and 10 ml of distilled water was added to the vermicompost. The suspension was then vortexed for 60 seconds to ensure separation of the microbes from the soil particles. The Syto 9 dye BacLight Bacterial Live/Dead viability and counting kit (Life Technologies™, Johannesburg) was used to enumerate bacterial cell numbers. Two ~1ml samples of the VC suspension were centrifuged in a microcentrifuge at 10,000□ □g for 1-3 minutes to pellet the cells. The supernatants were then removed and resuspended separately in 1 ml of 0.85% (w/v) NaCl (PPS) solution (for the live-cell suspension) and in 1 ml of 70% isopropyl alcohol (for the dead-cell suspension). The samples were then incubated at room temperature for 30 minutes, mixed every 15 minutes. Both samples were then pelletized via centrifuge at 10,000□ □g for 1- 3 minutes. The samples were then washed in 1 ml of PPS and pelletized again via centrifuge at 10,000□ □g for 1- 3 minutes. Both samples were then resuspended in 1 ml of PPS.

Staining of the bacterial samples then proceeded as follows: A 977 µl aliquot of PPS was measured into a flow cytometry analysis tube. Then 1.5µl of 3.34 mM SYTO 9 nucleic acid stain and 1.5 µl of 30 mM propidium iodide were then added to the same tube. 10µl of the previously prepared bacterial suspension was then added to the staining solution in the tube as well and incubated in the dark at 22 ºC for 15 minutes. The microsphere standard (component C of the kit) was then resuspended by vortexing, followed by sonication in a water bath for 5 minutes. 10µl of this suspension was then added to the bacterial staining solution in the flow cytometry tube, making up a total volume of 1000µl. This procedure was conducted for both the live and dead stained samples.

Four single-color controls — two live-cell and two dead-cell bacterial suspensions — were required for setting up the flow cytometer, with the microsphere standard added to a minimum of one of the tubes. The control samples were prepared in the same manner as the bacterial staining samples detailed above except one live-cell and one dead-cell suspension were stained with the SYTO 9 stain only and the remaining two control samples were stained with propidium iodide stain only.

The stained bacterial samples were then assayed using the flow cytometer at a wavelength of 488 nm according to the procedure laid out in Molecular probes (2004).

Fungal cell (filamentous and yeast) enumeration was conducted using the same methodology as the bacterial cell counts. However, Calcofluor White staining was used to differentiate between yeasts and fungi (20µl Calcofluor and 20µl potassium hydroxide). *Trimella globispora* was used as a control for the filamentous fungi and *Saccharomyces cerevisiae* was used as the yeast control, *Escherichia coli* was used as the bacterial control.

We used the FITC fluorescent parameter to detect the cells stained by the Syto 9 dye (i.e. all live cells) and we used the DAPI parameter to detect the cells stained with Calcofluor White (i.e. Yeast and fungi). The SYTO 9 dye is excited by the 488nm laser and has an excitation wavelength of 485nm and emission wavelength of 498nm. It is detected in the FITC channel with a 502 LP (long pass) filter and 530/30 BP (band pass) filter. Calcofluor White is excited by the 405nm laser and has an excitation wavelength of 355nm and emission wavelength of 433nm. It is detected on the DAPI filter with a 502 LP filter and 450/40 BP filter.

Biolog EcoPlate C-guild structure and Community level physiological profile analyses

Samples from all VC treatments were subjected to community level physiological profiling (CLPP) (Garland and Mills 1991). VC suspensions were prepared by transferring 5 ml of VC to 25 ml deionized water contained in 50 ml Pyrex tubes. Each suspension was shaken at 200 rpm for 30 min, further diluted to 10^{-3} $g.ml^{-1}$, after which 100 µl aliquots of this dilution were used to inoculate the wells of Biolog EcoPlates (Biolog, Haywood, CA, USA). The inoculated plates, each designed to test the utilization of 31 different substrates, were incubated in the dark at 21°C (± 0.5°C) for 96 h. Utilization of the carbon source in each well, indicated by a reduction of the tetrazolium dye, was recorded every 12 h at 590 nm using a MicroPlate Reader (Biotek,

Winooski, VT). To correct for possible background color effect, the absorbance reading of the control well was subtracted from the actual reading. The appropriate observation period was chosen as the time at which most of the substrate was used or the appearance of fungal growth in the different wells. Thus observations ceased after 96 hours and absorbance values at this time point were used to calculate the indices. The absorbance value of each well at 96 h was divided by the average well colour development (AWCD), which was calculated as the mean of all absorbance values for all wells containing a carbon substrate (Garland and Mills, 1991).

Microbial functional diversity was calculated as the Shannon diversity (H_0) index determined by substrate utilization according to (Zak *et al*., 1994):

$$Ho = -\sum Pi - \ln(pi)$$

Where *Pi* is calculated by dividing the corrected values by the total colour change (AWCD) recorded for all 31 substrates.

The Shannon evenness (E) index of catabolic diversity was calculated according to the equation (Derry *et al*., 1998):

$$E = H^{'}/lnS$$

Where S (substrate richness) is the number of substrates used by the microbial communities contained within the vermicompost treatments. Substrate richness was determined by the summation of all wells that had an absorbance of 0.25 or higher than that of the control well. Soil microbial functional diversities for the various vermicompost treatments were also examined by dividing the 31 substrates into five categories according to their chemical nature, i.e.

carbohydrates, carboxylic acids, amines and amides, amino acids and polymers (Preston-Mafham *et al*. 2002).

Nematode ecology

Nematodes were extracted from 250 ml of each sample by the Cobb's decanting and sieving method, through consecutive 90, two 53 and 45 µm sieves followed by a modified Baermann funnel extraction for 48 hours (Cobb, 1918; Southey, 1986). The extracted nematodes were then allowed to settle for 60 minutes and the supernatant was siphoned off. Samples were concentrated to 20 ml after which nematodes were enumerated. Subsamples of 1 ml were mounted on slides for identification up to family level at high magnification. Nematodes were then allocated to feeding groups according to (Yeates *et al*. 1993).

Statistical Analyses

One-way analysis of variance (ANOVA) tests were used to evaluate significant differences between all parameters tested. Ratio data was Ln converted prior to ANOVA analysis. Means were separated with the Fishers LSD test using the Statistica 12 software package. Statistical significance was determined at the 95% level ($P<0.05$).

Results

Nutrient Analyses

The pH of the vermicompost and soil mixtures did not differ significantly with changes in vermicompost concentration; however a minor, but non-significant, decrease in pH was noted in the 100% sample (Fig. 1.A).

Substrate N, P and K content

No significant difference in total N content of the substrate was found between the treatments containing lower VC concentrations (5 and 10%). However, the total N content of the 100% VC substrate, was found to be over ten times greater than that of the substrates containing lower VC concentrations (Fig.1.B). Furthermore, across all three VC concentrations it was found that N partitioning was overwhelmingly locked into organic forms of N (Tab.1). However the proportion of N maintained in inorganic forms (NH_4 of NO_3), did differ between treatments (Fig. 5). In the substrates containing 5% and 10% VC, significantly greater amounts of inorganic N

were contained in NH_4 than in NO_3. Conversely in the 100% VC substrate significantly greater amounts of NO_3 was present compared to NH_4.

A similar trend was found for both P and K content as was present for N content. No significant differences were found between the 5 and 10% treatments with the 100% treatment containing significantly greater amounts of the macronutrients (Fig.1.C-D). Overall K content was an order of magnitude lower than P and N content in the respective treatments.

Biomass analyses

Both the addition of VC and rhizobia bacteria significantly increased total plant mass over that of the control treatment. However significant biomass increases in R treatments were only achieved when used in conjunction with vermicompost (Fig. 2), as can be seen by there being no significant difference between the two sand control groups. The addition of VC only yielded a significantly greater biomass response when added as 100% VC.

The addition of VC at a concentration of 5% had no significant effect on biomass production ($P>0.05$), in the NR treatment group (Fig. 2). Conversely however, the R treatment group exhibited significantly greater biomass production than both the NR treatment as well as the control group ($P<0.05$).

Within the 10% VC treatment group the plants exposed to the NR treatment did not differ significantly from the NR control group; however the R treatment produced significantly greater amounts of biomass than both the NR treatment as well as both control treatments (Fig. 2).

Within the 100% VC group (Fig. 2) the NR treatment produced significantly greater amounts of biomass than both the R treatment and the two controls ($P<0.05$). No significant differences were found between the R and control treatments.

Root: Shoot ratio's

The division of resources by a plant between above and below ground tissues is indicative of nutrient availability and thus a change in nutrition may lead to a shift in this ratio (Poorter and Nagel, 2000). R treatments invested significantly more in root growth than both its control and NR treatments at the low VC concentrations of 5 and 10% (Fig. 3). This difference between the groups was not evident at 100% VC as significantly less root growth occurred at this

concentration for both R and NR treatments than in all other treatments, including the controls. As the concentration of VC in the soil media increased from 10 to 100% a decrease of approximately 10 % occurred in the root: shoot ratio of the NR treatment and 25% for the R treatment. Thus at the high VC concentrations significantly greater amounts of shoot growth was observed and at the lower concentrations of 5 and 10% significantly greater root growth occurred, across both NR and R treatments.

N nutrition

Nitrogen derived from atmosphere (NDFA)

No significant differences in NDFA were found for R or NR treatments at the low concentrations of 5 and 10% VC (Fig. 4), however significantly less NDFA was observed at both R and NR treatments of 100% VC.

Organic and inorganic N

Of the N that was available to the plants for uptake from the soil/VC mixture it was found that a very large proportion (>99%) was sequestered in organic complexes (Tab. 1), thus less than a single percentage of the total N was available for immediate uptake by the plants. Of this pool of inorganic N it was found that available N was in one of two forms, Nitrate (NO_3) or ammonium (NH_4). At the low concentrations of 5 and 10% VC it was observed that significantly greater amounts of available N were in the NO_3 pool than in the form of NH_4. The opposite was found in the 100% treatment (Fig. 5). Furthermore, similar total amounts of inorganic N were found in the 5 and 10% treatments, while a significantly larger amount of total available N was present in the 100% treatment (Data not shown).

Vermicompost ecology

Microbial counts

Bacterial and fungal counts were found to differ significantly, with greater amounts of bacteria than fungi, however only 100% VC treatments were analyzed (Fig 6).

Carbon guild utilization and Community level physiological profile analyses

No significant differences were found between the different treatments within any of the C-containing guilds in the Biolog EcoPlate kit. (Fig 7. A-E).

Average well colour development (AWCD) for all the treatments followed a similar trend (fig.8. A), with colour development only starting after 24 hours of incubation and increasing over time. Only at one point was a significant difference in AWCD found between the treatments, this was after 60 hours of incubation. At this point the 100% treatments exhibited significantly greater AWCD than the 5% treatment.

Substrate richness (fig.8. B). was found to increase significantly over time for all treatments. Treatments were found to differ significantly at three time points, these being after 48, 60 and 84 hours of incubation. After 48 hours the 10% treatment was found to exhibit greater substrate richness than the 5% treatment, the 100% treatment was not significantly different from either. After 60 hours both the 10% and 100% treatments were significantly richer than the 5% treatment and after 84 hours the 10% treatment was significantly richer than both. However, after 96 hours of incubation no difference was found between any of the treatments.

Shannon's evenness (Fig.8. C) was maximal for all treatments at 36 hours and this was the only point where significant differences could be found. 5% and 10% VC exhibited a significantly greater degree of Shannon's evenness than the 100% treatment, indicating that a significantly greater degree of microbial activity took place across the utilized substrates in the 5 and 10% treatments.

Nematode ecology

The relative abundance of the nematode functional groups present varied with the concentration of VC in the soil (Fig. 9). Nematode abundance decreased with a decrease in organic matter. No nematodes were present in treatments containing less than 10% VC. Rhabditidae (bacterial feeding nematodes) were present in all treatments. Other nematode families identified include the plant parasitic families Criconematidae and Tylenchidae, the bacterial feeding families Panagrolaimidae, Monhysteridae and Diplogasteridae, a fungivorous family (Paraphelenchidae) and an omnivorous family Dorylaimidae.

Discussion

These results contribute to the understanding of the combined effect of chicken manure VC and rhizobia inoculation on the growth and N-nutrition of the legume *L. angustifolius*, particularly when VC is applied at low concentrations.

Biomass allocation was found to differ significantly among treatments due to variation in VC application rates as well as the addition of symbiotic N-fixing bacteria. BNF occurred across all treatments, indicating that the VC used in this study contained either free-living or symbiotic diazotrophic bacteria. However, biomass production within concentration groups differed significantly due to the addition of Rhizobia innoculant, with R treatments producing greater amounts of biomass. However, no differences in NDFA were found between the 5% and 10% treatments. Thus it is conceivable that the C-costs to the host plant may have varied between diazotroph partners present in the NR and R treatments, explaining the reduction in biomass in the NR treatments (5% and 10%). It is understood that diazotrophic symbionts carry a C-cost to the host plant (Harris *et al*., 1985) and furthermore it is known that a variation in symbiont C-costs to the host also exists, depending on which symbiont has formed the association (SkØt *et al*., 1986). *L. angustifolius* is capable of forming symbioses with a wide variety of *Bradyrhizobium* species (Stepkowski *et al*., 2005), some of which may have been present in the VC. Thus the reduction in biomass in the NR treatments may be due to a symbiosis forming with a bacterial partner that has a less efficient rate of C exchange than those present in the commercial innoculant. Currently there exists very little literature concerning the diversity of N-fixing bacteria present within VC, thus any speculation as to which species of these microbes are present remains, at best, only an educated guess. Further future research into this field, using DGGE and COMPOCHIP™ microarray technologies, is required to determine diazotrophic diversity in VCs.

The reduction in NDFA for both R and NR 100% VC treatments is likely due to the large amount of readily available soil N present in the soil media. In hydroponic experiments, NO_3 concentrations of over 5mM have been shown to reduce nodule size (Saito *et al*., 2014) while in field experiments it has been shown that additions of NO_3 fertilizer reduce BNF (Hungria *et al*. 2006). It is cheaper for the plant in terms of C to not form a N-fixing symbiotic association since its N requirements can be met by the uptake of soil N alone. Support to this is given by the significant reduction in root: shoot ratio of both 100% treatments compared to the controls. A reduction of this ratio is indicative of increased nutrition, as it implies increased shoot growth (Poorter and Nagel, 2000). The plant will allocate a greater proportion of its available resources to shoot growth when nutrients are in excess and a large root system is not required, in

accordance with the functional equilibrium model (Brouwer, 1962; 1963). The significantly greater root: shoot growth ratio exhibited by the R over the NR treatments in the 5% and 10% VC groups cannot be due to differences in soil mineral nutrient availability, since these treatments received equal amounts of VC. Bearing in mind that the NR treated plants also exploited similar amounts of NFDA as the R treatments, one cannot assume that increased BNF led to increased root mass, as is typically the case (Huang and Erickson, 2007). Ergo the reductions in root:shoot ratios in the NR treatments are as a result of an increase in shoot biomass relative to that of the roots. As predicted by the functional equilibrium model of (Brouwer, 1962; 1963), a plant will increase its shoot growth relative to that of its root when the shoot suffers a reduction in above ground resources, such as CO_2 and light. In this case however the plant experiences a C-sink and thus it is conceivable that it increases shoot growth allocation to counter this. Further investigation into the C-costs of VC derived diazotrophic symbionts to *L. angustifolius* is required.

The substrate nutrient content of N, P and K in the 100% treatments were exponentially greater than those found in the 5% and 10% treatments. Soil microflora and microfauna are responsible for the cycling of nutrients and increases in abundance and diversity of both bacteria and fungi have been shown to increase nutrient availability to plants (Bardgett, 2005). Thus this finding may indicate that microbial mineralization within the VC could account for the increases in nutrient availability, as found by Aira *et al*. (2007).

The large degree of variation in NH_4 and NO_3 content between the 100% and the two, low VC concentration treatments is interesting as there is a change in ratio between these two nutrients not just an increase in abundance as one would expect. This indicates that the availability of C may be influencing nitrification rates. Soils receiving large amounts of organic material have larger labile pools of C, greater soil N supplying potential and greater microbial activity compared to soils receiving less organic matter (Kramer *et al*., 2002; Gunapala and Scow, 1998). Soils receiving greater amounts of VC have been shown to contain larger amounts of microbial N than those receiving less VC (Arancon *et al*., 2004) and as such, have a greater N supplying potential. However, the input of C may increase the immobilization of NO_3^-, reducing its availability for immediate plant uptake (Recous *et al*., 1990; Stark and Hart, 1997). This microbial immobilization may be rapidly cycled due to the greater amounts of nematode

diversity in the 100% treatments than in 5% and 10% groups, resulting in greater amounts of predation upon bacterial and fungal populations. The predatory actions of nematodes contribute to soil nutrient cycling (Bardgett, 2005b) and function to increase the availability of nutrients such as NO_3^- and NH_4^+ to plants (Ingham *et al*., 1985). This would explain the increase in the relative abundance of both NO_3^- and NH_4^+ in the 100% VC group. The increase in NO_3^- relative to NH_4^+ may be driven indirectly by a decrease in microbial abundance due to predation and thus a subsequent decrease in mineralization. This is supported by the findings of (Hart *et al*., 1994) which indicate that in coniferous soils NO_3^- production increases as microbial immobilization of N decreases. In a similar fashion the reduced ratio of NO_3^- to NH_4^+, exhibited in the low VC concentration groups, may be as a result of the preferential microbial immobilization of NO_3^- and a lack of nematodes. Typically microbes in agricultural soils assimilate less NH_4^+ than NO_3^- (Burger and Jackson, 2003), the net result being greater amount of NH_4^+ than NO_3^-. A lack of understanding of the dynamics of N transformation in VCs currently exists and warrants further research, especially when taking the entire soil food web into account.

The relatively large abundance of both bacteria and fungi within the VC is indicative of the high rates of potential mineralization and other microbially mediated actions. Comparisons of functional diversity within the five C-containing guilds of the Biolog EcoPlates yielded no significant differences and thus the different VC concentrations appear to have no effect on microbial community structure. As such, changes in plant biomass as a result of different VC treatments are likely due to microbial abundance rather than microbial functional diversity. AWCD increases significantly over time for all treatments, in all likelihood due to a lag phase in growth and after 96 hours of incubation shows no sign of a stationary phase emerging. This is indicative of the utilization of a wide range of C-containing substrates and thus a diverse array of microbial flora for all VC treatments. The difference found between the 5% and 100% treatment after 60 hours is most likely due to the difference in microbial abundance between these two treatments. As found in the guild analyses though, this seems to have no effect on functional diversity. Credence to this is given by the lack of differences between treatments in substrate richness after 96 hours, once again differences at earlier time points are likely due to microbial abundance differences. The differences in Shannon's evenness after 36 hours between the high VC treatment and two low treatments is to be expected, as similar levels of evenness group

together when plotted, with lower levels of variation attaining a higher evenness value (Hill, 1973).

As with soil microbial ecology, nematode ecology provides insight into the complexity of the trophic cascade within VC's. The low carbon content of the 5% VC treatment would have reduced the microbial content, and thus the prey populations of predatory, bacteriovore and fungivore nematodes. This explains why no detectable amounts of nematodes were present in this treatment. Within the 10% treatment the presence of 4 different functional groups of nematodes indicate a diversity of microbial guilds. However the population is very small compared to that in the 100% treatment, in which bacteriovores, herbivores and fungivores are represented in the largest amounts. The large amounts of bacteria and fungi present within 100% VC are thus likely the cause of the large nematode population in this treatment. Using nematode maturity index (MI) as an indicator of soil enrichment is a method employed widely in soil and water health analyses (Bongers and Ferris, 1999). A similar technique could be used in the VC industry to provide a reliable method of determining the nematode functional diversity contained within the product. Furthermore temporal analyses using this method could aid in determining the effects of VC on soil life over time. Additional studies dedicated to elucidating the effects of VC source material and concentration in the soil on nematode functional diversity will help to understand how this system can be used in agriculture as a rapid analysis tool.

In conclusion, we found that the substitution of vermicompost into quartz sand growth media significantly increases biomass accumulation in *L. angustifolius*. The most efficient method of biomass production under the conditions used in this study was found to be inoculation with commercially available rhizobia and the addition of chicken manure vermicompost mixed in a ratio of 5% VC to 95% sand. Interesting patterns of inorganic N utilization were found particularly in terms of NO_3^- availability in the presence of high concentrations of carbon. VC's contain a large abundance and diversity of microbial life and thus nitrification and N immobilization are to be expected. Furthermore, it was found that VC concentration in the substrate media had no effect on microbial guild structure but did influence nematode functional diversity and abundance. The implications of this study are that VC and rhizobia substitutions into the potting media should be an important consideration for substrate nutrient availability, microbial abundance, and BNF when cultivating *L. angustifolius*.

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Figures and Tables – Chapter 3

Table 1. Organic vs inorganic Nitrogen partitioning within chicken manure vermicompost at the concentrations of 5%, 10% and 100% vermicompost to sand. N=3.

	Total N	
VC Treatment	**Organic (%)**	**Inorganic (%)**
5%	**99,74**	**0,26**
10%	**99,77**	**0,23**
100%	**99,87**	**0,13**

Table 2.
31 Carbon sources contained within the Biolog™ Ecoplate kit and the carbon containing guild to which they belong.

Control	Carbohydrates	Polymers	Carboxylic & acetic acids	Amino acids	Amines/amides
Water	Pyruvic acid Methyl Ester	Tween 40	D-Glucoseaminic Acid	L-Argenine	Phenylethyl-amine
	D-Celloboise	Tween 80	D-Galactonic Acid γ-Lactone	A-Asparagine	Putrescine
	α-D-Lactose	α-Cyclodextrin	D-Galacturonic Acid	L-Phenylalanine	
	β-Methyle-D-Glucoside	Glycogen	2-Hydroxy Benzoic Acid	L-Serine	
	D-Xylose		4-Hydroxy Benzoic Acid	L-Threonine	
	i-Erythritol		γ-Hydroxybutyric Acid	Glycyl-L-Glutamic Acid	
	D-Mannitol		Itaconic Acid		
	N-Acetyl-D-Glucoseamine		α-Ketobutiric Acid		
	Glucose-1-Phosphate		D-Malic Acid		
	D,L-α-Glycerol Phosphate				

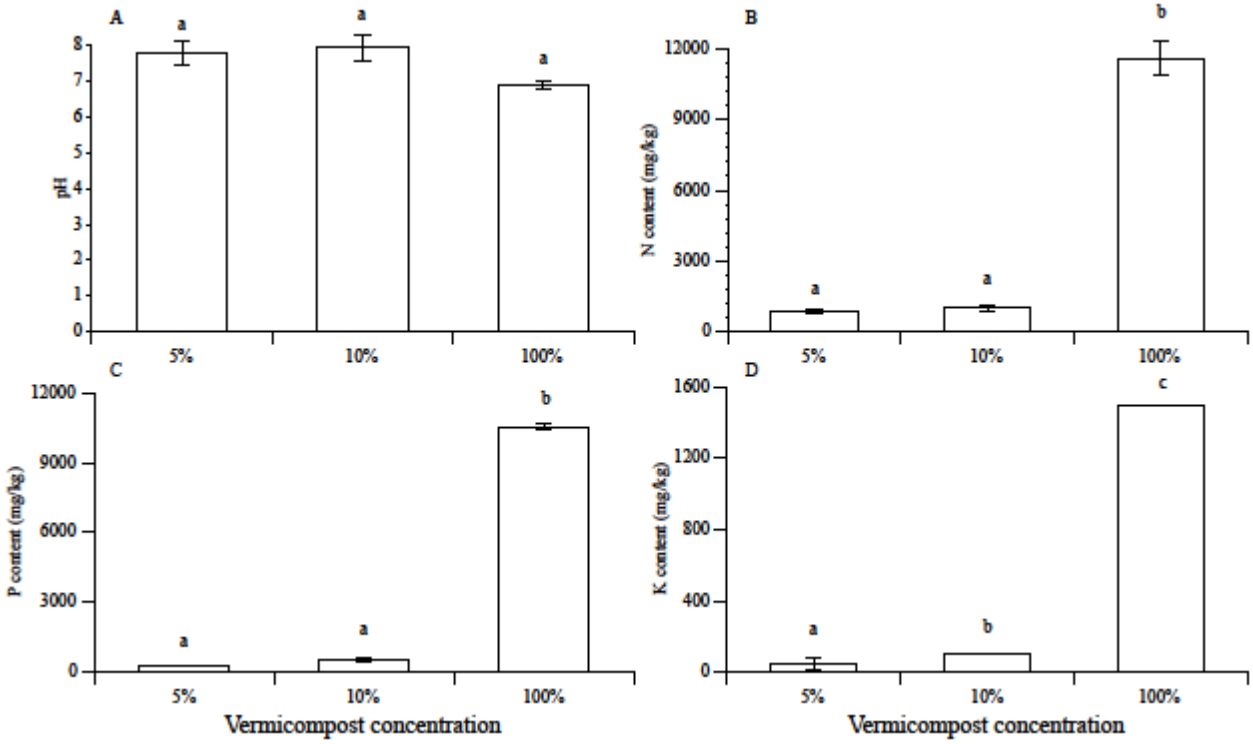

Figure 1.
Soil nutrient content was analyzed for macro and microelements at VC concentrations of 5%, 10% and 100%. A. pH. B. Nitrogen concentration. C. Phosphate concentration. D. Potassium concentration. Different letters indicate significant differences within each graph (P<0.05), n=3. Standard errors indicated by the error bars.

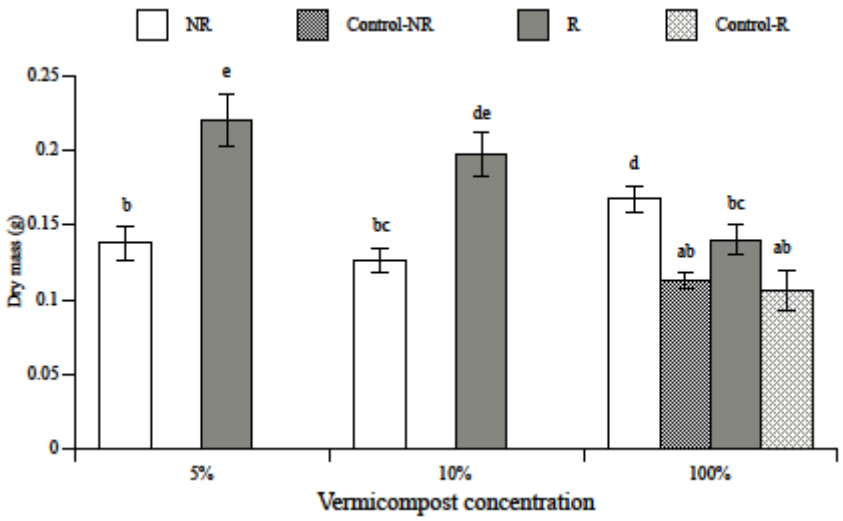

Figure 2.
Total plant dry mass as a function of increasing VC to sand concentrations for chicken manure vermicompost, both with and without *Rhizobium* inoculation.
Sterile and inoculated quartz sand used as controls. Different letters indicate significant differences. Standard error indicated by error bars (P<0.05), n=9.

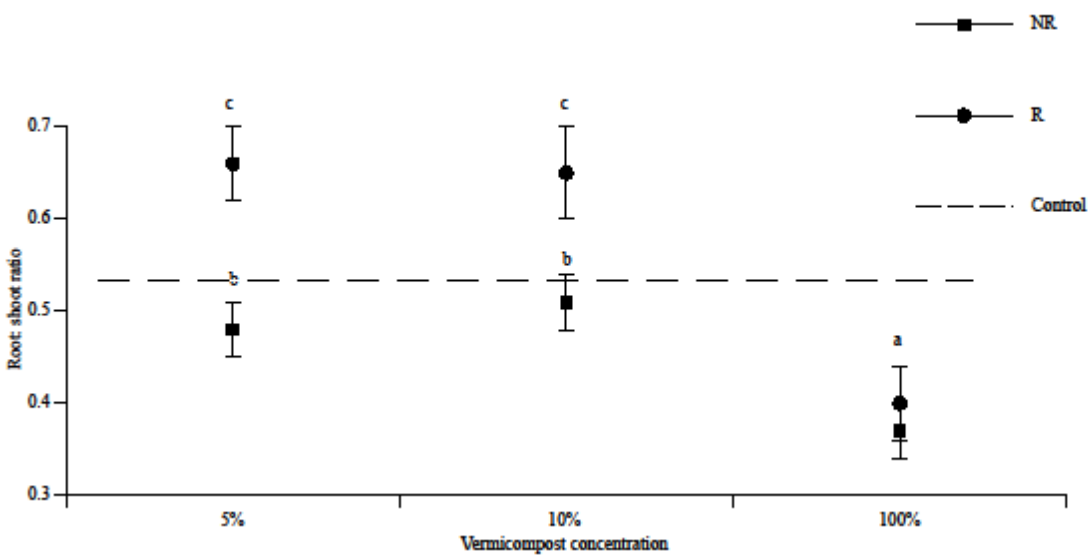

Figure 3.
Mean root: shoot ratio, with std errors, of *L. angustifolius* grown in chicken manure vermicompost across a variety of concentrations (5, 10 and 100% vermicompost. Control groups were grown in 100% quartz sand without additional nutrition, either with *Rhizobium* inoculation (R) or without (NR) Significant differences are indicated by different letters Control groups were not significantly different from each other. NR n=10, R n=6.

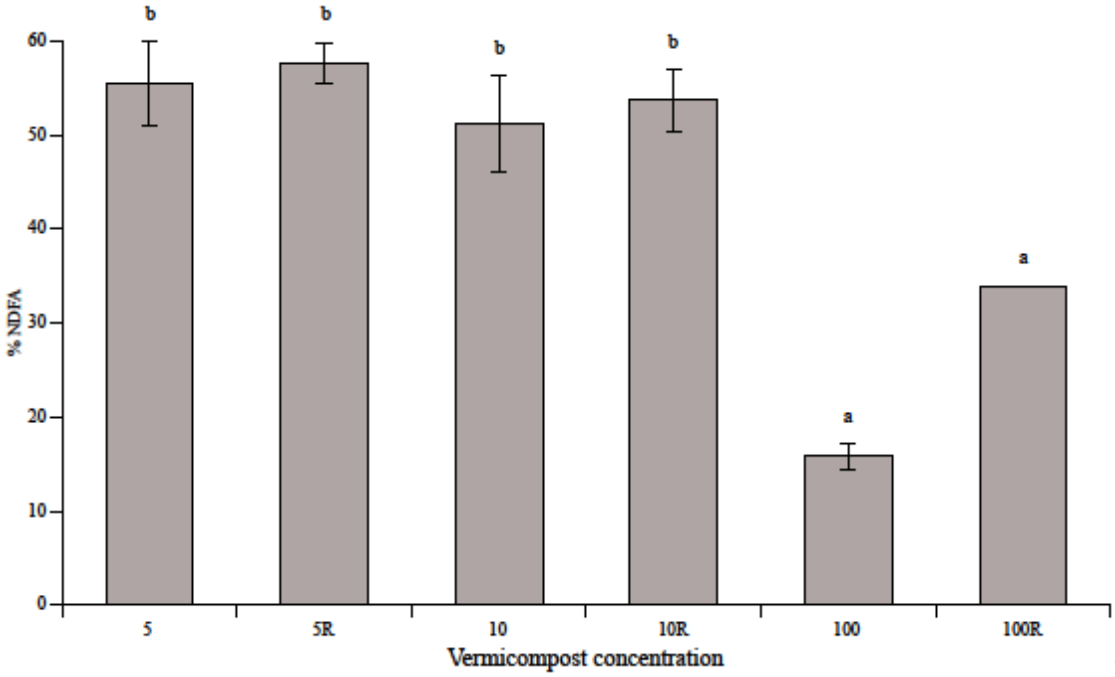

Figure 4. Nitrogen derived from atmosphere (NDFA) gives an indication of the amount of biological nitrogen fixation (BNF) that occurs within both *Rhizobium* (R) and non-*Rhizobium* treatment plants via symbiotic means. Vermicompost type and concentration also has a clear effect on the amount of BNF that occurs. Significant differences are indicated by different letters. P<0.05, n=3.

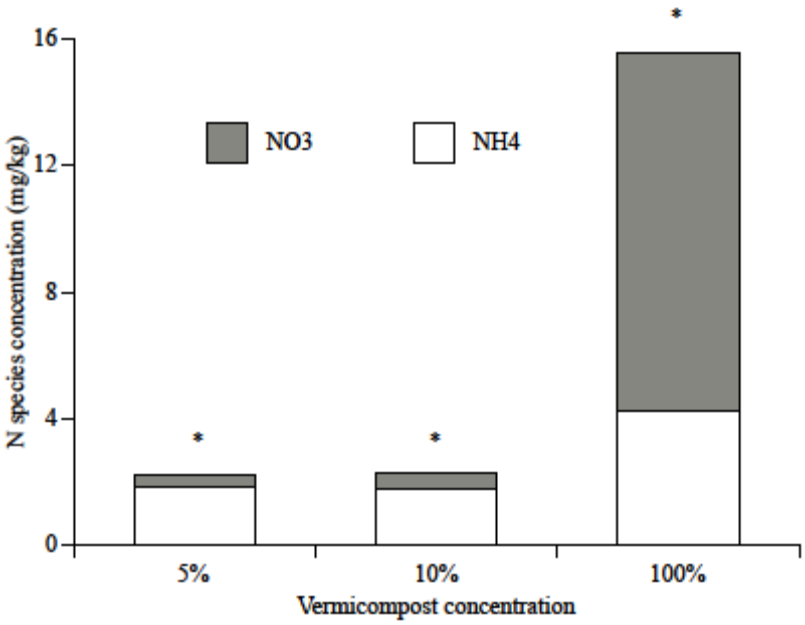

Figure 5.
Inorganic N (Ammonium and Nitrate) partitioning within chicken manure vermicompost at the increasing concentrations of 5%, 10% and 100%. Asterisk (*) indicates significant differences between NO_2 and NH_3 within each treatment. P<0.05, n=3.

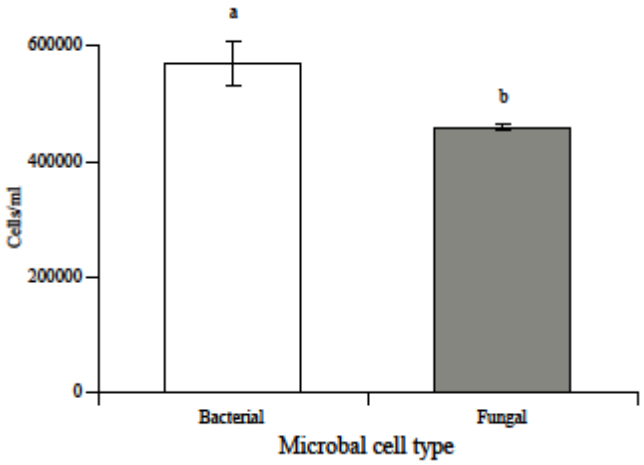

Figure 6. Bacterial (B) and fungal (F) cell counts within 100% chicken vermicompost analyzed via flow cytometry. Different letters indicate significant differences between groups. N=3, $p<0.05$.

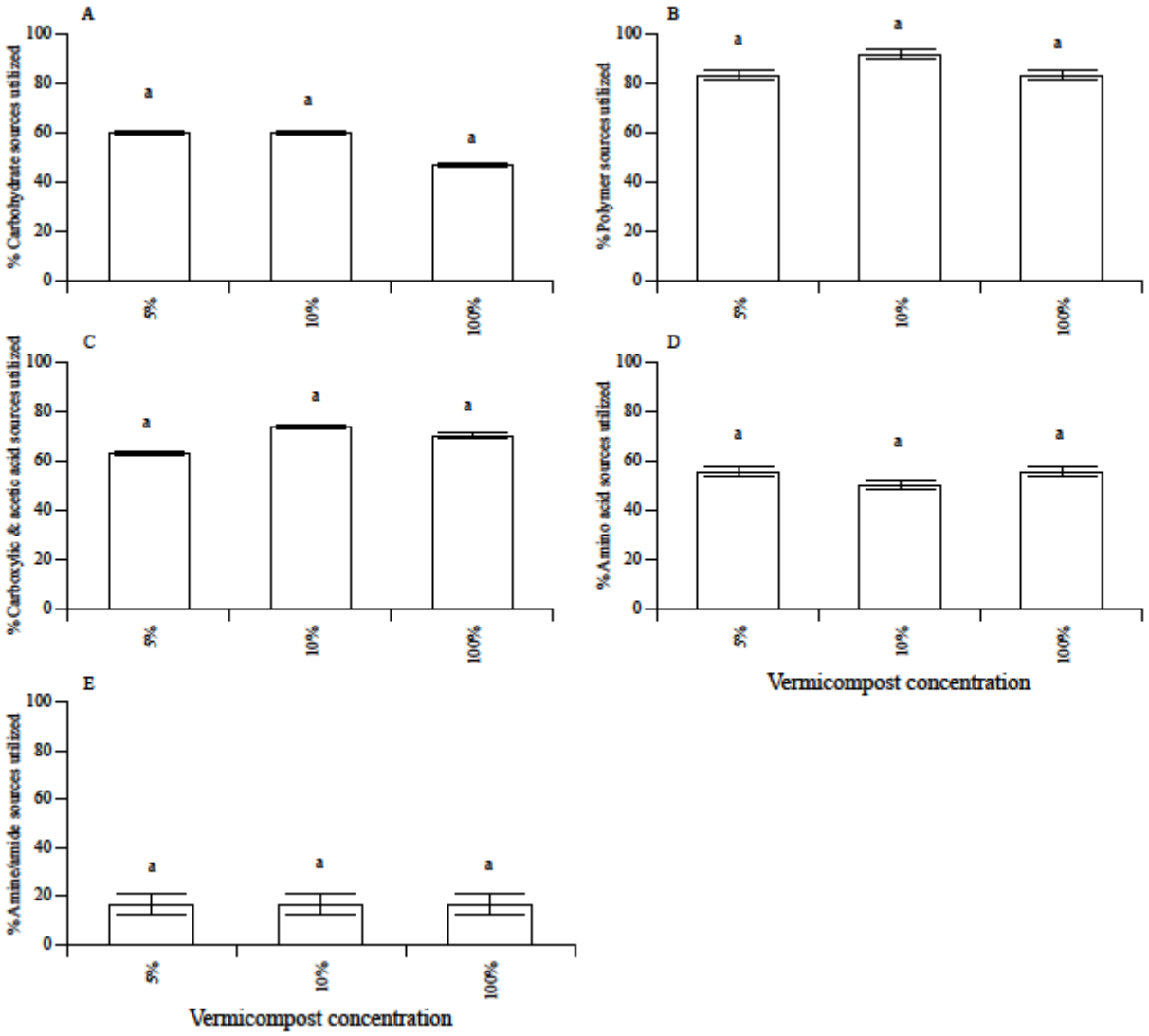

Figure 7.
Carbon containing guild utilization across 5%, 10% and 100% chicken manure VC treatments. A. Carbohydrate guild. B. Polymer guild. C. Carboxylic and acetic acids guild. D. Amino acid guild. E. Amines and amides guild. Different letters indicate significant differences. P<0.05, n=3.

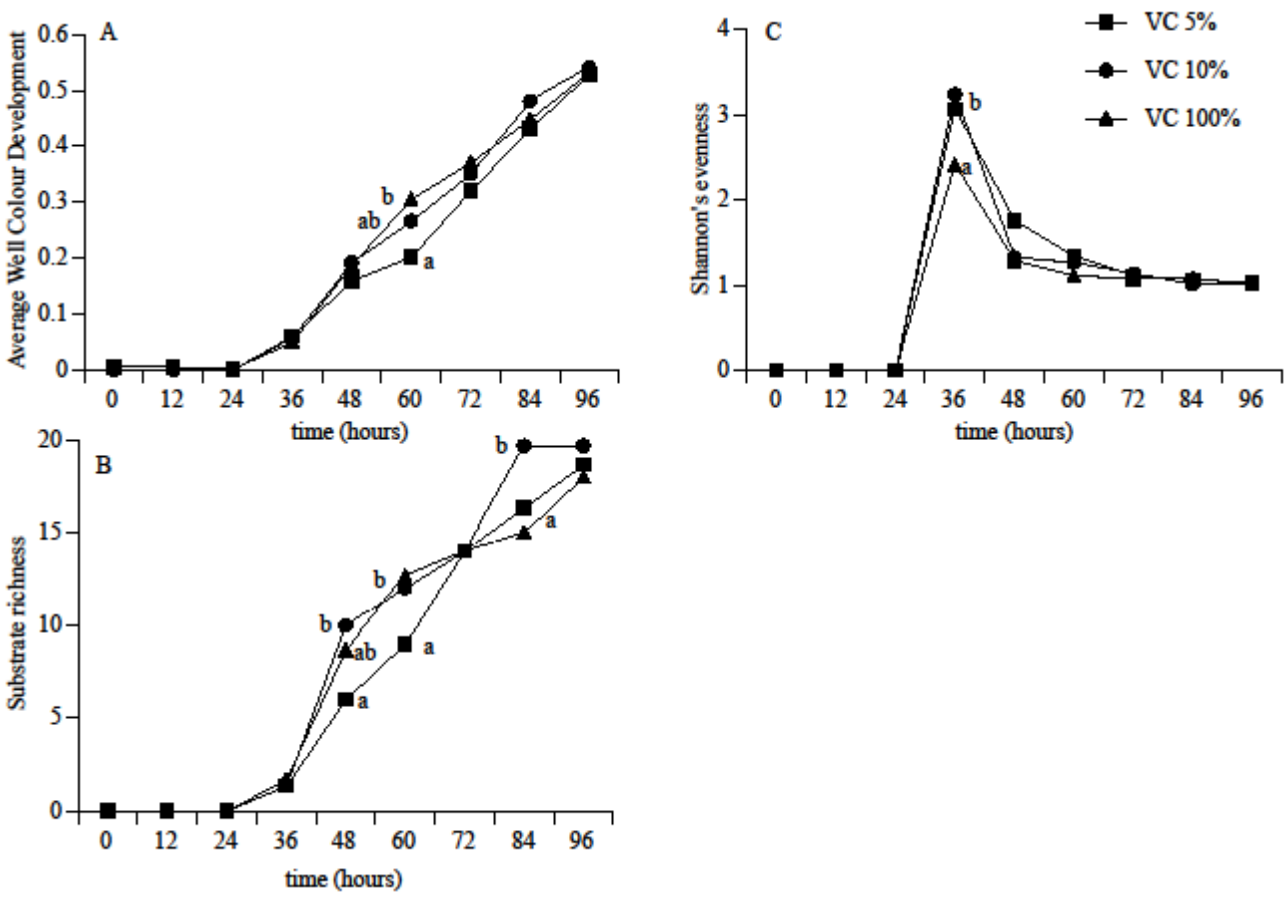

Figure 8.
Microbial community physiological profiles established by using Biolog Eco-plates for chicken manure vermicompost at three concentrations (5%, 10% and 100%) over the course of 96 hours. A. Average well colour development (AWCD), B. Substrate richness, C. Shannon's evenness index. N=3. Temperature 21°C ±0.5.

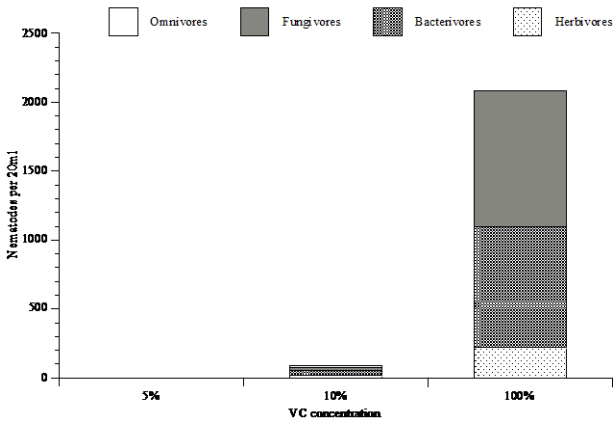

Figure 9.
Soil nematode count across functional groups within chicken manure vermicompost across concentrations of 5%, 10% and 100%.

Chapter 4: Vermicompost concentrations in teas influence microbial functional diversities and N nutrition of the legume Lupinus angustifolius.

Abstract

The effect of the combination treatment of vermicompost (VC) solids and teas on legume growth is unknown. This study investigated the effects of this combination on the biomass production and N nutrition of the legume, *L. angustifolius*. In addition, the effects of VC concentration on microbial functional diversity in the teas as well the effects of tea additions on soil nutrient content were investigated. VC teas, produced from aged chicken manure VC, were prepared in concentrations of 4%, 10% and 20% (v/v). Sugars were added and the teas were aerated for 24 hours. These were fed to the plants at a rate of 50ml per plant per week for 50 days. Additions of teas did not significantly influence biomass production, regardless of concentration. 20% teas were found to significantly decrease atmospheric nitrogen dependence, while the 4% and 10% treatments significantly increased this parameter. 20% teas contained significantly greater amounts of functional bacterial diversity than the 4% and 10% treatments, resulting in the metabolism of a significantly greater diversity of carbon containing compounds. No differences in microbial abundances or nutrient concentrations were found. This study indicates that the combined treatment of VC solids and teas do not increase the plant biomass of *L. angustifolius*, but that the additions of 20% teas result in greater microbial diversity in the soil. This in turn may lead to increases in soil fertility. Furthermore, additions of high concentration vermicompost teas (>20%) shift the dependence of the plant from atmospheric N sources to soil N sources.

Keywords: Vermicompost teas, Biolog *Eco-plates*, Flow cytometry, NDFA, Microbial Functional Diversity, Organic agriculture

Introduction

Vermicomposts (VCs) are the product of the mesophilic biodegradation of organic residues through the joint action of earthworm ingestion and microbial actions within the gut of the earthworm. The end product is a stable, finely divided, peat-like substance which has good drainage, aeration and water holding capacity as well as high amounts of microbial activity, making them very good soil conditioners (Edwards and Burrows, 1988; Edwards, 1998).

VCs have been shown to increase yields in a number of agricultural crops including beans (Manivannan *et al*., 2009), peppers and strawberries (Arancon *et al*., 2003), onions (Srivastava *et al*. 2012) and tomatoes (Atiyeh *et al*., 2000). Increases in the number of flowers have also been noted in plants of horticultural value (Arancon *et al*., 2008). In addition, VCs have also been shown to increase plant biomass, soil microbial activity and soil microbial biomass (Chaoui *et al*., 1999; Arancon *et al*., 2003; Manivannan *et al*., 2009).

Soil biota play integral roles in ecosystems through the formation of soils (Rillig and Mummey, 2006), Nitrogen (Clarholm, 1985) and Carbon cycling as well as the nutrient uptake of plants (Smith and Read, 1997; Sprent, 2001). Thus they are vitally important for the success of agriculture. VCs have been utilized successfully as an augmentative (Srivastava *et al*. 2012) or in some cases, replacement, for chemical fertilizers (Arancon *et al*., 2003). The latter mentioned authors have argued that the availability of macronutrients in VC cannot be solely responsible for the observed increases in plant growth. They found that secondary metabolites present in the VC excreted by the large and diverse microbial population as well as humic acids may be driving increases in plant biomass production.

VC teas (VCTs) are aqueous extracts of VCs, typically aerated over the course of 24 hours with sugar additives to stimulate the growth of aerobic bacteria, yeasts and protozoa. They have been shown to increase plant growth, crop yields, plant nutritive quality as well as increase soil biological activity and reduce crop damage due to pathogens and pests (Edwards *et al*., 2007; Edwards *et al*., 2006; Fritz *et al*., 2012; Pant *et al.*, 2009; Pant *et al*., 2011).

The Biolog Eco-plate system has been utilized by a number of authors investigating the functional groups of bacteria present in VC (Aira *et al,. 2007*; Doan *et al.*, 2013). It has been found that the vermicomposting process significantly alters the community level physiological profile (CLPP) of both the source material and the soil to which it is applied. Furthermore this system has been utilized to indicate that VC amended soils have a significantly greater abundance and diversity of bacterial species present than those treated with synthetic chemical fertilizers (Frac *et al.*, 2012).

Currently there exists a fairly large amount of literature on the beneficial effects of VC in agriculture (Adhikary 2012) with a smaller body of literature investigating VCTs. However, few

studies have been conducted on the combination treatment of the two resources, with the intention of maximizing the efficiency of such a combination in terms of plant growth and resource utilization. Furthermore there is a lack of understanding of how such a combination will influence functional microbial groups as well as the availability of N sources and plant growth efficiencies. In this study we aim to investigate how the legume, *Lupinus angustifolius*, utilizes N sources provided by a combination treatment of VC solids and tea over time as well as the effects of this on biomass production. In addition we aim to investigate the effect of VCT concentrations on the functional bacterial groups present in the soil by means of the Biolog Eco-plates system.

Materials and Methods

Growth medium preparation, tea preparation, planting and harvest

Lupinus angustifolius seeds were germinated in quartz sand for 12 days in the glasshouse of the Natural Science department at Stellenbosch University, South Africa. Two days after planting a slurry of rhizobia bacteria (Stimuplant) was added poured over the ungerminated seedlings in the seed trays. Seedlings were watered daily with reverse osmosis filtered water. After this germination period they were transplanted into small plastic pots (1L), these pots contained a ratio of 95% sterile quartz sand to 5% chicken manure VC (Wormworks, Simondium), each pot was also treated with a slurry of rhizobia (Stimuplant) and distilled water, to induce nodulation. The pots were then separated into the following treatments: 4%, 10% and 20% tea. A control treatment was also planted which was designated to receive no tea but an equal amount of filtered water. Furthermore each tea treatment was divided into 5 successive harvests, separated by 10 days each. A sample size of 3 plants was maintained for each tea treatment within each harvest. Upon harvest the plants were separated into roots, stems, leaves and nodules and placed in the oven until their mass had stabilized; dry weights were recorded. Mass fractions of stems, roots and leaves were calculated and were compared to the control treatment as well as an initial treatment (n=6) which was harvested when transplanted from the seed trays.

Teas of three different concentrations were made: 4%, 10% and 20% (v/v). The teas were made by placing the required amount of VC in a fine mesh nylon bag (<100µm) which was then placed inside a perforated (hole size=8mm diameter) PVC tube, with an aeration line fixed inside the

bottom of it. A cotton cheese cloth was fixed around the outside of the tube to catch any large particulate material that may have escaped through the first filter. 10L of filtered water, 25ml of kelp extract (Kelpac Co. Glen Cairn) and 50ml of raw black-strap molasses (Agricol) were mixed together poured into a 20L bucket, the tube containing the filters and VC was added to this. A second aeration line was placed inside the bucket and total aeration was maintained at a rate higher than $20L.min^{-1}$ to ensure that aerobic conditions were maintained for the 24 hour brewing period. The temperature of the tea was kept between 13-16°C by passing the aeration line through a heat exchange before entering the tea brewer. After brewing for 24 hours, samples of each tea was taken to determine microbial abundance and perform CLPP analyses. Each plant then received 50ml of its respective tea every 7 days immediately after the completion of the brewing process.

Chemical and biological analyses

The plant tissues were ground in liquid nitrogen and sent for N15/N14 isotope analysis at the University of Cape Town (Department of Archeometry). The isotopic ratio of d15N was calculated as d 1⁄4 1000% ($R_{sample}/R_{standard}$), where R is the molar ratio of the heavier to the lighter isotope of the sample and standards as defined by Farquhar *et al*. (1989). The oven-dried plant components were milled in a Wiley mill using a 0.5-mm mesh (Arthur H Thomas). Between 2.100and 2.200 mg of each sample was weighed into 8 mm □ 5 mm tin capsules (Elemental Micro- analysis Ltd., Devon, UK) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyzer (Fisons Instruments SpA, Milan, Italy). The d15N values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyzer by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift: two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard—$(NH_4)_2SO_4$.

A sample of the soil from each replicate and harvest was taken and frozen at -20°C and upon completion of the experiment sent for chemical analysis at BEM labs (Somerset West, Western Cape), analyses included pH, cation exchange capacity, total N, P and K as well as NH_4 and NO_3 concentration.

Tea samples were analyzed for microbial abundance directly after the completion of the brewing process at the Central Analytical Facility (Stellenbosch University) and quantified via flow cytometry using the BD FACSAria™ flow cytometer (Franklin Lakes, NJ). Control treatments were made by adding 5ml of chicken manure VC to 10ml of distilled water and vortexing for 1 minute. All samples were sieved through 50µl filter paper before analysis. The Syto 9 dye BacLight Bacterial Live/Dead viability and counting kit (Life Technologies™, Johannesburg) was used to enumerate bacterial cell numbers. Two ~1ml samples of the VC suspension were centrifuged in a microcentrifuge at 10,000□ □g for 1-3 minutes to pellet the cells. The supernatants were then removed and resuspended separately in 1 ml of 0.85% (w/v) NaCl (PPS) solution (for the live-cell suspension) and in 1 ml of 70% isopropyl alcohol (for the dead-cell suspension). The samples were then incubated at room temperature for 30 minutes, mixed every 15 minutes. Both samples were then pelletized via centrifuge at 10,000□ □g for 1- 3 minutes. The samples were then washed in 1 ml of PPS and pelletized again via centrifuge at 10,000□ □g for 1- 3 minutes. Both samples were then resuspended in 1 ml of PPS.

Staining of the bacterial samples then proceeded as follows: A 977 µl aliquot of PPS was measured into a flow cytometry analysis tube. Then 1.5µl of 3.34 mM SYTO 9 nucleic acid stain and 1.5 µl of 30 mM propidium iodide were then added to the same tube. 10µl of the previously prepared bacterial suspension was then added to the staining solution in the tube as well and incubated in the dark at 22 ºC for 15 minutes. The microsphere standard (component C of the kit) was then resuspended by vortexing, followed by sonication in a water bath for 5 minutes. 10µl of this suspension was then added to the bacterial staining solution in the flow cytometry tube, making up a total volume of 1000µl. This procedure was conducted for both the live and dead stained samples.

Four single-color controls — two live-cell and two dead-cell bacterial suspensions — were required for setting up the flow cytometer, with the microsphere standard added to a minimum of one of the tubes. The control samples were prepared in the same manner as the bacterial staining samples detailed above except one live-cell and one dead-cell suspension were stained with the SYTO 9 stain only and the remaining two control samples were stained with propidium iodide stain only.

The stained bacterial samples were then assayed using the flow cytometer at a wavelength of 488 nm according to the procedure laid out in Molecular probes (2004).

Fungal cell (filamentous and yeast) enumeration was conducted using the same methodology as the bacterial cell counts. However, Calcofluor White staining was used to differentiate between yeasts and fungi (20µl calcofluor and 20µl potassium hydroxide). *Trimella globispora* was used as a control for the filamentous fungi and *Saccharomyces cerevisiae* was used as the yeast control, *Escherichia coli* was used as the bacterial control.

We used the FITC fluorescent parameter to detect the cells stained by the Syto 9 dye (i.e. all live cells) and we used the DAPI parameter to detect the cells stained with Calcofluor White(i.e. Yeast and fungi). The SYTO 9 dye is excited by the 488nm laser and has an excitation wavelength of 485nm and emission wavelength of 498nm. It is detected in the FITC channel with a 502 LP (long pass) filter and 530/30 BP (band pass) filter. Calcofluor White is excited by the 405nm laser and has an excitation wavelength of 355nm and emission wavelength of 433nm. It is detected on the DAPI filter with a 502 LP filter and 450/40 BP filter.

Three additional tea extractions of 4%, 10% and 20% were made in the same method utilized for the treatments; these were used to investigate the substrate utilization pattern of the tea samples, using Biolog Eco-plates (Biolog Inc., Hayward, CA, USA) and the same methodology of (Reilly *et al*. 2013). In addition to the teas, a control treatment of pure VC mixed with quarter strength Ringers solution (1:10 w/v) was made. Once aerated for 24 hours, the teas were also diluted to a ratio of 1:10 (v/v) with quarter strength Ringers solution. The solutions were then shaken for 15 minutes at 150 r.p.m. and then left to settle for 15 minutes. A 10 ml aliquot of each sample was taken and of that 100µl was pippetted into a microplate and read in the spectrophotometer (BioTek) at a wavelength of 590nm. Samples were further diluted to a common absorbance value of 0.08. Once the necessary dilutions were performed 100µl of each treatment was pippeted into each well in the Biolog Eco-plate, a separate plate was used for each treatment, allowing for triplicate replication. Absorbance was measured at 590nm every 24 hours for 7 days.

Tea samples taken for chemical analysis were taken directly after completion of the brewing process and placed frozen at -20°C to arrest microbial activity. These samples were analysed by BEM Labs (Somerset West), analyses included N, P and K as well as NH_4 concentrations.

Statistical analyses

Two-way analysis of variance (ANOVA) of biomass parameters, soil nutrient content, plant nutrient content and CLPP data were performed on main treatment effects and their interaction with time. The one-way ANOVA test was used to test for significance between treatments in microbial abundance in the different teas. Means were separated with the Fishers LSD test using the Statistica 12 software package. Statistical significance was determined at the 95% level ($P<0.05$).

Results

Chemical properties of VCTs and soil media

The chemical analyses of the different teas indicated that there was no difference in the concentrations of N, P, K or NH_4 between treatments (Tab.1.1). In the soil media significant differences were found in K concentrations, with that of the 10% and 20% treatments being significantly greater than the control. No differences were found in any of the other parameters tested (Tab.1.2).

Biomass

No significant differences were found in any plant parts due to tea treatments prior to 40 days after transplanting (Addendum A, Fig 1). Differences were most pronounced after 50 days (Fig. 1.1). At this point it was found that additions of tea had no effect significant on root growth. Additions of 4% tea significantly reduced stem growth when compared to both other treatments and the control. No significant differences were found in terms of leaf growth due to tea additions. Nodule growth was significantly affected by the addition of teas; both the 4% and 20% treatments produced significantly more nodule mass than the 10% and control treatments. Total plant mass was not significantly affected by tea additions.

The plants were divided into 3 groups for growth kinetic evaluations: Root (RMF), leaf (LMF) and stem (SMF) mass fractions (Fig 1.2). After 50 days of growth it was found that there was no

significant difference between RMF due to tea additions. However a significantly larger proportion of growth was found in the LMF of the 4% treatment compared to the initial values. Conversely the same treatment exhibited significantly smaller SMF then any of the other treatments.

Plant nitrogen assimilation and utilization

The amount of nitrogen derived from atmosphere (NDFA) within the plant was significantly different in all treatments, including the control (Fig 2.1) over the course of 50 days. The 4% treatment utilized the greatest proportion of NDFA, followed by the 10% and then the 20% treatments, with the control utilizing the least. Both the control (40 days) and the 20% treatment (50 days) reached 0% NDFA.

The SNAR's of the different treatment groups were found to not differ significantly 50 days after transplanting (Fig 2.2.A). Differences were however found in the earlier stages of the experiment (data not shown). The 20% treatment was found to have a significantly greater SNUR than both the control as well as the 4% treatment (Fig 2.2.B). The SNUR of the 10% treatment, while not differing from the control, was also found to be significantly greater than that of the 4% treatment.

Microbial abundance

No significant differences were found between treatments for neither bacterial, nor yeast cell counts. A large degree of variance was found within both bacterial and yeast cell counts. Mean bacterial numbers were found to be in excess of 10 times greater than that of yeasts (Tab 2.1).

Biolog CLPP analyses

Average well colour development (AWCD) analyses indicated that the largest degree of substrate metabolism occurred in the control and 20% tea treatments. These two treatments displayed significantly greater amounts of AWCD than the 10% treatment, which in turn was significantly greater than the 4% treatment (Fig 3.1).

The carbohydrate guild in the Biolog Eco-plate system is comprised of 8 compounds, of which significant differences in utilization between tea treatments were found in 5 different compounds, one compound was not utilized at all (Tab.2.1). CLPP analyses of carbohydrate

utilization (Fig 3.2) indicated that the control and 20% tea treatments utilized significantly greater amounts of Pyruvic acid Methyl Ester (A) and D-Mannitol (D) than the 4% and 10% treatments. The consumption of i-Erythritol (B) was significantly greater within the 20% treatment than in all other treatments. For this compound no difference was found between the control and 10% treatment and between the 4% and 10% treatments. The control treatment utilized significantly greater amounts of β-Methyl-D-Glucoside (C) than any other treatments while no differences were found between the other treatments for this compound. The control treatment utilized significantly greater amounts of N-Acetyl-D-Glucosamine (E) than all other treatments, additionally the 20% treatment also utilized significantly greater amounts of the same compound than the 4% and 10% treatments. For all of the compounds within the carbohydrate guild that were utilized by the various tea treatments only i-Erythritol (B) was utilized in greater amounts by the 20% treatment, for all others the control treatment was most effective.

Within the polymer guild significant differences in utilization between tea treatments were found in 2 of the 4 compounds (Tab 2.1). CLPP analyses (Fig 3.3) of Tween 80 (A) indicated that the control and 20% treatment utilized significantly larger amounts of the compound over time than the 4% and 10% treatments. Tween 40 (B) was utilized to a significantly greater degree by the control treatment than by any of the other treatments; however the 10% treatment also utilized significantly greater amounts of the polymer than the 4% treatment.

CLPP analysis of the carboxylic and acetic acids guild indicated that of the 9 compounds with the guild, two were not utilized by any of the treatments and significant differences in utilization profiles between treatments were only found in one compound: 4-Hydroxy Benzoic Acid (Tab 2.1). The control treatment (Fig 3.4) utilized significantly greater amounts of this compound over time than all the other treatments. The 20% tea treatment, while utilizing significantly less than the control, still utilized significantly more of the substrate than the 4% treatment.

Within the amino acid guild it was found that of the total of 6 compounds, all were utilized, yet only 2 were utilized to a significantly different degree between tea treatments (Tab 2.1). The control treatment (Fig 3.5) utilized significantly greater amounts of A-Asparagine (A) than any of the other treatments. The 20% treatment also utilized significantly greater amounts of the

substrate than both the 4% and 10% treatments. The same trend was found for the second substrate within the guild, L-Serine (B).

The final guild investigated by means of the Biolog Eco-plate system was that of the amine/amides. Both compounds contained within the guild were utilized, however only the utilization of Putrescine varied significantly between tea treatments (Tab 2.1). CLPP analyses indicated (Fig 3.6) that the substrate was utilized to a significantly greater degree over time by the control, 20% and 10% treatments than the 4% treatment.

Discussion

The positive growth effects of VC on plants, even in relatively low concentrations in the soil, is well documented (Arancon *et al.*, 2003a; Arancon *et al*., 2003b; Arancon *et al.*, 2004; Atiyeh *et al*., 2000a; Atiyeh *et al*., 2000b; Gutiérrez-Miceli *et al*., 2008; Manivannan *et al*., 2009). Similarly the additions of aqueous extracts of VCTs have also yielded increases in plant biomass and/or yields (Edwards *et al*., 2006; Arancon *et al*., 2007; Pant *et al*., 2009; Arancon *et al*., 2012; Pant *et al*., 2012a; Pant *et al*., 2012b). However the combination treatment of VC and VCTs remains relatively unexplored.

In this study we found no differences in the concentrations of N, P, K or NH_4 found in the teas, regardless of the concentration of VC used in their production. Arancon *et al*. (2012) investigated the chemical properties of chicken manure based, 20% VCT, and found the total N concentration to be almost three times higher than that recorded in this study. However they recorded lower NH_4 and P concentrations and similar concentrations of K. The chemical constituency of VC has been shown to change over time (Atiyeh *et al*., 2000) and depending on the maturity of the product, a wide variety of macronutrient concentrations have been found to occur, particularly in terms of NH_4 and NO_3 concentrations. Thus differences in nutrient concentrations in teas are to be expected if VCs of different source material and maturity are utilized. Supporting this, Welke (2008) found that an increasing ratio of compost to water increased NO_3, NO_2, Ca, K, Mg and S content in the teas. Thus the lack of differences in macronutrient concentrations between treatments found in this study is intriguing; however the relatively large degree of variation between replicates may explain this finding.

The lack of significant differences in macronutrient concentrations in the soil media over time indicates that the concentration of VCT does not influence soil nutrient concentration, except in the case of Potassium. Small volumes (50ml) of tea were added on a weekly basis for 50 days, and although not significant, it was noted that nutrient levels in the soil increased after these additions (Data not shown). The significantly higher K concentration in the tea treatments over the control may be as a result of the additions of kelp extract and molasses to the teas during the brewing process, as kelp extract is known to contain high concentrations of K (Pant *et al*., 2009). Furthermore, soil K content during the first ten days and across all treatments, (data not shown) was at its greatest, decreasing from this point onwards. Soluble K may have been flushed out of the soil media by successive tea additions, explaining the decrease in content over time. The relatively large ratio of NH_4 to NO_3 in the soil media, across all treatments, is indicative of low levels of nitrification. Typically as VC matures NO_3 content exceeds that of NH_4, due to the formation of conditions within the substrate that favour nitrification (Atiyeh *et al*., 2000). Our findings are in contrast to what is generally found in the literature and may be as a result of high rates of nitrate immobilization or low rates of nitrification in the VC.

The additions of teas did not significantly alter biomass production except in the cases of stem and nodule growth. The 4% tea treatment, albeit decreasing stem growth, increased the leaf mass fraction with the net result being no change in overall plant biomass when compared to the other treatments. The significantly greater nodule biomass of the 4% treatment is likely to have influenced the amount of bacterial nitrogen fixation, and as a result increased the amount of NDFA in this treatment, compared to the control. However the 20% treatment also had significantly greater nodule biomass than the control after 50 days yet after the same time period utilized no NDFA. Interestingly this treatment, as well as the other tea treatments, relied heavily upon NDFA for the first 30 days, while the control treatment did not. Although not significant, the mean NO_3 content of the control soils was higher than that of all of the tea treatments. This may have decreased reliance on bacterial nitrogen fixation (BNF) in this treatment. Further investigation into the possible mechanisms influencing increased BNF due to tea additions is required. The overall findings of the additions of teas and the effects on biomass production are in agreement with those of (Fritz *et al*., 2012) in that they have no effect on total plant biomass. This study incorporated a mixture of 5% VC in the growth medium and since no increases in

available N and P were found due to the additions of tea it is understandable, from a mineral nutrition perspective, why no differences in plant growth were found.

Within the VC tea treatments, the reliance on nitrogen derived from the atmosphere (NDFA) was inversely related (broadly) to the VC tea concentration. In particular, the 4 and 10% treatments had more reliance on nitrogen derived from the atmosphere (NDFA) over the 50 day time period than soil/mineral N, which was favoured by the 20% and control treatments. Initially relatively large amounts of nitrogen was derived from the atmosphere for all treatments, however this was to be expected since all plants were inoculated with symbiotic N-fixing bacteria. The decrease in dependence over time, particularly in the 20% and control treatments, indicate that soil N was made available differentially between treatments over time. The specific nitrogen utilization rate (SNUR) was shown to increase with VC tea concentrations, indicating an increase in N use efficiency. The increase in SNUR is driven by either decreasing C-costs or an increase in N uptake, however no significant differences were found in the specific nitrogen acquisition rates (SNAR) between treatments. This indicates that a decrease in C-cost is most likely responsible for the increased SNUR in the 20% treatment

Average well colour development (AWCD) of the Biolog Eco-plates indicated that the control and 20% teas utilized a significantly greater diversity of carbon sources than the 4 and 10% treatments. Across all guilds (carbohydrates, polymers, carboxylic and acetic acids, amino acids and amines/amides) the control and 20% tea treatments utilized significantly greater amounts of individual substrates. This indicates that within these treatments a greater bacterial diversity, capable of metabolizing a wider variety of compounds, occurs. The significantly greater degree of metabolism of substrates within the amino acid and amine/amide guild by the 20% and control treatments is indicative of the utilization of the large pool of organic N by the microbes contained within these teas. Amino acids have been shown to constitute over 85% of total soil N (Martens and Loeffelmann, 2003).Thus relative to the 4% and 10% treatments, the metabolism of these compounds in the 20% and control treatments indicate the potential for increased soil N cycling if a healthy soil-food web exists, or N immobilization if soil bacteriovores are absent (Bardgett, 2005).

The greater SNUR exhibited by the 20% tea treatment may thus be as a result of the preferential uptake of cycled N made available in the soil through the increased metabolism of N-containing organic compounds such as amino acids, amines and amides. It is conceivable that the microbial metabolism of these amino acids result in the initial immobilization of N, followed by a slow, long-term release of N as the microbes complete their life cycle or are predated upon. This would explain why NDFA reliance in the 20% and control treatments was initially high but fell quickly after 30 days. The bulk of the literature investigating the functional groups of bacteria contained within VC has been focused on VC solids or the effects of the addition of these products on soil microbial diversity (Aira *et al*., 2007; Aira *et al*., 2005; Doan *et al*., 2013); the effects of the additions of teas has remained unexplored. Thus further investigation into this field is required.

The lack of differences in microbial abundance values between tea treatments is at odds with the idea that an increase in VC concentration will lead to an increase in microbial number. Bacterial cell numbers were found to be at least an order of magnitude greater than yeast cell numbers. Although not significant it appears that aeration with sugar additives decreases active fungi numbers, supporting the findings of (Pant *et al*., 2009). These authors found that during the extraction process bacterial numbers were significantly reduced while in this study it was found that extraction had no effect on bacterial cell counts. However the large degree of variation found within treatments suggests that a larger sample size is required to draw accurate conclusions on this issue. Furthermore limited research has been conducted into this area, with the bulk of microbial abundance comparisons being drawn between VC types and the effect of aeration and not the effect of concentration. The use of flow cytometry is also a novel approach to cell enumeration in VCTs; most studies in the past have utilized epifluorescence microscopy.

In conclusion the findings in this study indicate that the additions of chicken manure based VCT to a substrate containing 5% chicken manure VC had no effect on plant biomass after 50 days of growth, regardless of tea concentration. Nodule biomass was significantly increased in the 20% and 4% treatments; however the mechanisms for this remain to be elucidated. No differences in mineral nutrient contents were found between the different tea concentrations, except in the case of K, which is most likely due to the additions of kelp extract and molasses. NDFA was found to be significantly greater in the low VC tea concentration treatments over 50 days, when compared to the control and the 20% treatment. Despite there being no differences in bacterial cell numbers

in the various teas it was found that the 20% tea contained a significantly greater functional diversity of bacteria than the 4% and 10% treatments and was capable of accessing a wider variety of C-containing compounds. The control and 20% tea treatments were capable of utilizing amino acids, and thus may have derived a portion of the N requirements from such sources. This plausibly explains the reduction in NDFA in these treatments. Furthermore this finding indicates that bacterial functional diversity in VCT is concentration dependent. However, pure VC that was not aerated, but simply suspended in water, contained an even greater variety of functional diversity than the 20% tea treatment. The findings of this study question the efficacy of the brewing process of VCTs in order to increase the agronomic potential of VC, particularly when used in conjunction with VC solids in the soil media. However the additions of high concentrations of VC tea have been found to alter the source from which the legume *L. angustifolius* acquires its N as well as increasing the functional diversity of bacteria in the soil.

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Figures and Tables – Chapter4

Table 1.1. Chemical properties of vermicompost tea. Values are given as means (n=3). Different letters in the same column indicate significant differences between parameters (P<0.05).

Treatment	N ($mg.L^{-1}$)	P ($mg.L^{-1}$)	K ($mg.L^{-1}$)	NH_4 ($mg.L^{-1}$)
4%	44.00^a	25.79^a	61.90^a	2.09^a
10%	48.00^a	20.61^a	46.60^a	1.44^a
20%	49.33^a	25.70^a	47.95^a	2.06^a

Table 1.2. Chemical properties of growth media, treated with weekly doses of vermicompost tea. Samples were taken every 10 days for 50 days. All substrates contained 5% vermicompost (v/v) and were inoculated with Rhizobium nodule-forming bacteria. Values given as means (n=5). Different letters in the same column indicate significant differences between parameters (P<0.05).

Mean value over 50 days

Treatment	N (mg/kg)	P (mg/kg)	K (mg/kg)	NH_4 (mg/kg)	NO_3 (mg/kg)	CEC (pH 7) cmol(+)/kg	Available N (mg/kg)
4%	1640^a	81.584^a	0.09^{ab}	5.424^a	0.712^a	1.016^a	6.136^a
10%	1560^a	476.044^a	0.11^b	5.97^a	1.12^a	1.24^a	7.09^a
20%	1620^a	320.59^a	0.096^b	6.04^a	1.444^a	1.204^a	7.484^a
Control	1580^a	313.338^a	0.036^a	6.092^a	1.524^a	1.152^a	7.616^a

Table 2.1. Cellular abundance of bacteria and yeasts from three different concentrations of vermicompost teas (n=3). Significant differences indicated by different letters (P<0.05).

Treatment	Cell count ($cells.ml^{-1}$)					
	Bacteria		Std error	Yeast		Std error
4%	$3.2X10^{-6}$	a	$7.5 x10^{-5}$	$2.4 x10^{-5}$	a	$7.4 x10^{-4}$
10%	$8.7X10^{-6}$	a	$4.5X10^{-6}$	$1.2X10^{-6}$	a	$7.9 x10^{-5}$
20%	$1.6X10^{-6}$	a	$1.0X10^{-6}$	$1.8 x10^{-5}$	a	$1.2 x10^{-5}$
Control	$2.1X10^{-6}$	a	$8.1x10^{-5}$	$4.6 x10^{-5}$	a	$4.8 x10^{-4}$

Table 2.1. Carbon containing compounds within the Biolog Ecoplate and their utilization by the different tea treatments. Certain compounds were not utilized at all by any of the tea treatments while some were utilized significantly (P<0.05) more in certain treatment groups.

C-containing compound	Not utilized	Significant difference in utilization between tea treatments (P<0.05)
Carbohydrates		
Pyruvic acid Methyl Ester		x
D-Celloboise		
α-D-Lactose		
β-Methyl-D-Glucoside		x
D-Xylose		
i-Erythritol		x
D-Mannitol		x
N-Acetyl-D-Glucoseamine		x
Glucose-1-Phosphate	x	
Polymers		
Tween 40		x
Tween 80		x
α-Cyclodextrin		
Glycogen		
Carboxylic & acetic acids		
D-Glucoseaminic Acid		
D-Galactonic Acid γ-Lactone		
D-Galacturonic Acid		
2-Hydroxy Benzoic Acid	x	
4-Hydroxy Benzoic Acid		x
γ-Hydroxybutyric Acid	x	
Itaconic Acid		
α-Ketobutiric Acid		

D-Malic Acid	
Amino acids	
L-Argenine	
A-Asparagine	x
L-Phenylalanine	
L-Serine	x
L-Threonine	
Glycyl-L-Glutamic Acid	
Amines/amides	
Phenylethyl-amine	
Putrescine	x

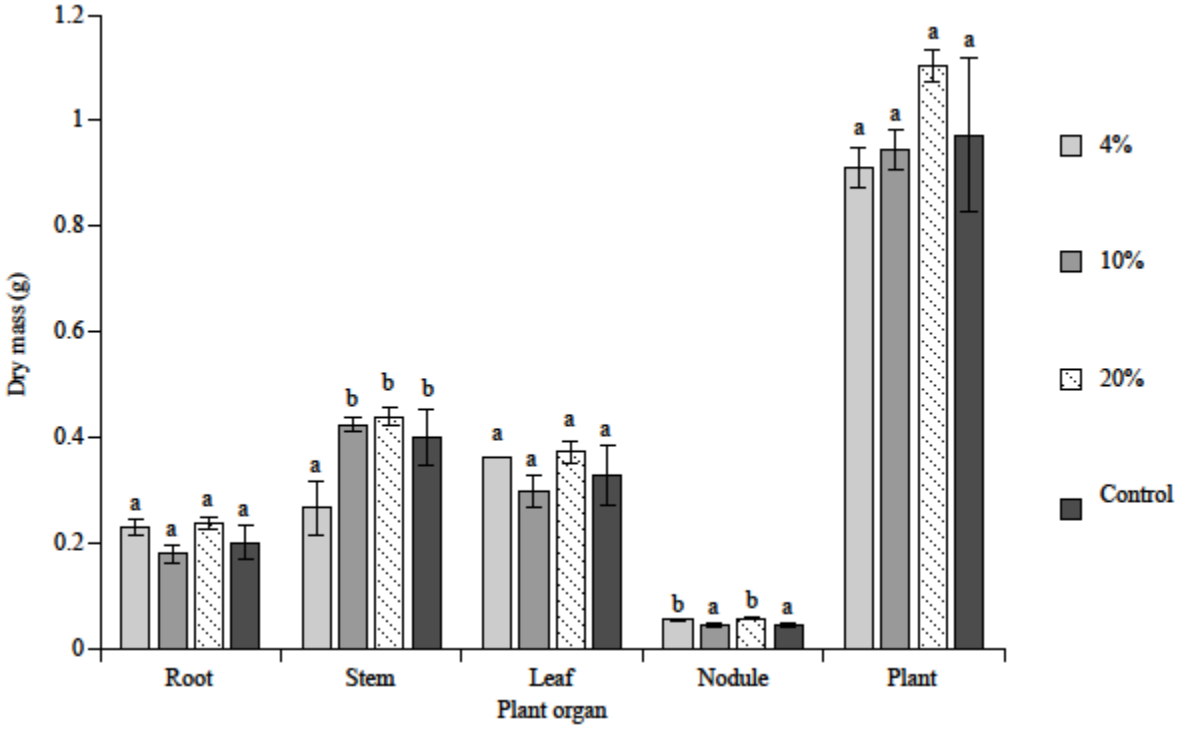

Figure 1.1. The effect of three different vermicompost tea treatments on biomass. Dry mass of plant roots, stems, leaves, nodules and total plant mass 50 days after transplanting (n=3). Different letters indicate significant differences (P<0.05) within plant organ type. Error bars indicate standard error.

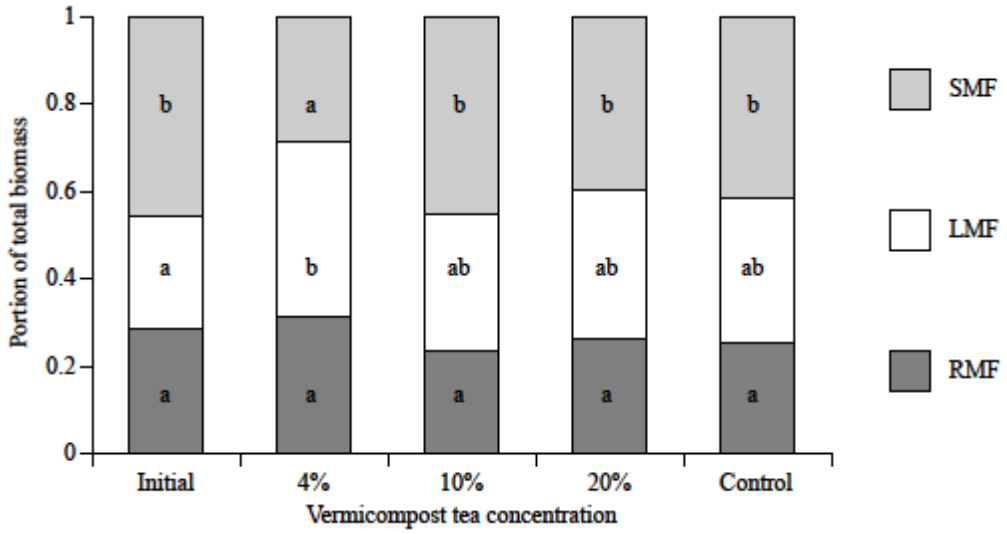

Figure 1.2. Mass fractions of different plant tissue types 50 days after planting. SMF- Stem Mass Fraction. LMF-Leaf Mass fraction. RMF-Root Mass Fraction. Treatment: Initial values were obtained upon transplanting, all other values 50 days after this (n=3). Significant differences indicated by different letters within mass fraction groups (P<0.05).

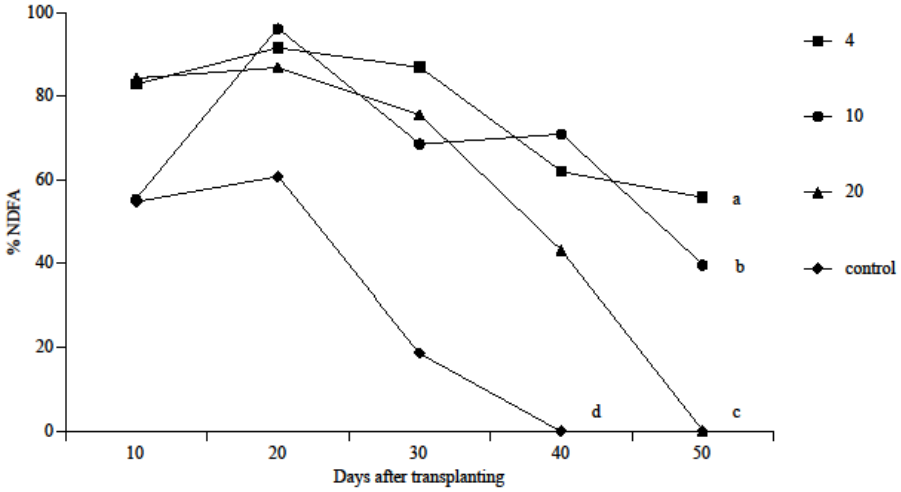

Figure 2.1. % Nitrogen derived from atmosphere (%NDFA) in the total plant. Samples were taken every 10 days after planting. Three different tea treatments were investigated as well as a control, which received only water. Significant differences in %NDFA content indicated by different letters (P<0.05).

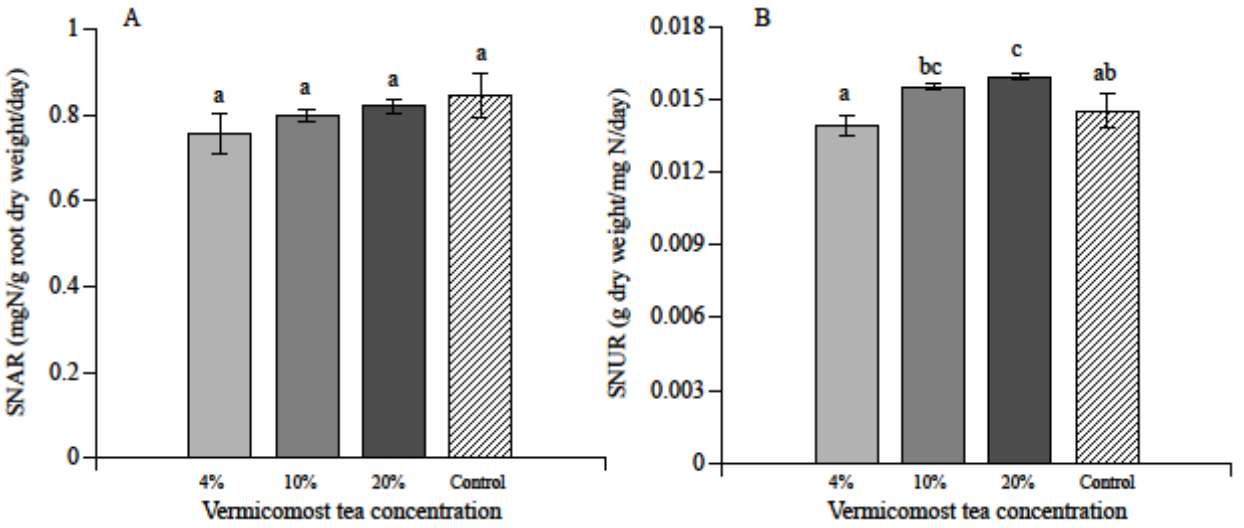

Figure 2.2. Specific nitrogen acquisition rate A. (SNAR) and Specific nitrogen utilization rate B. (SNUR) of *L. angustifolius* in response to the addition of different tea treatments (n=3) 50 days after transplanting. Significant differences indicated by different letters ($p<0.05$).

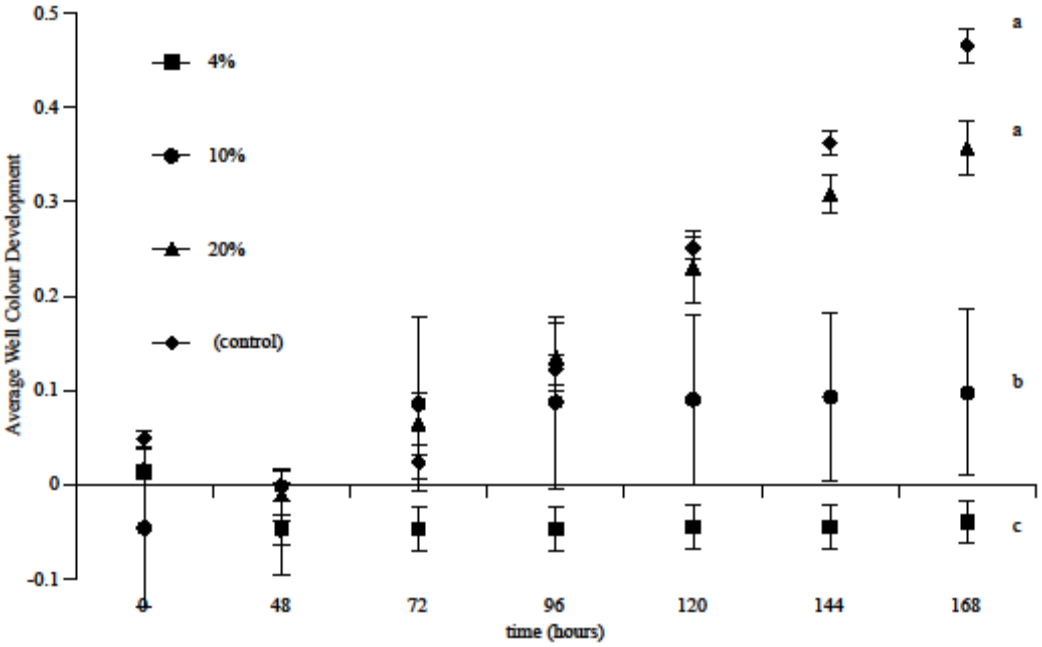

Figure 3.1. Average well colour development (AWCD) of three vermicompost teas (4%, 10% and 20% v/v) and a 100% vermicompost control over the course of 168 hours. Different letters indicate significant differences (P<0.05) between treatments over the entire time period. Error bars indicate standard error.

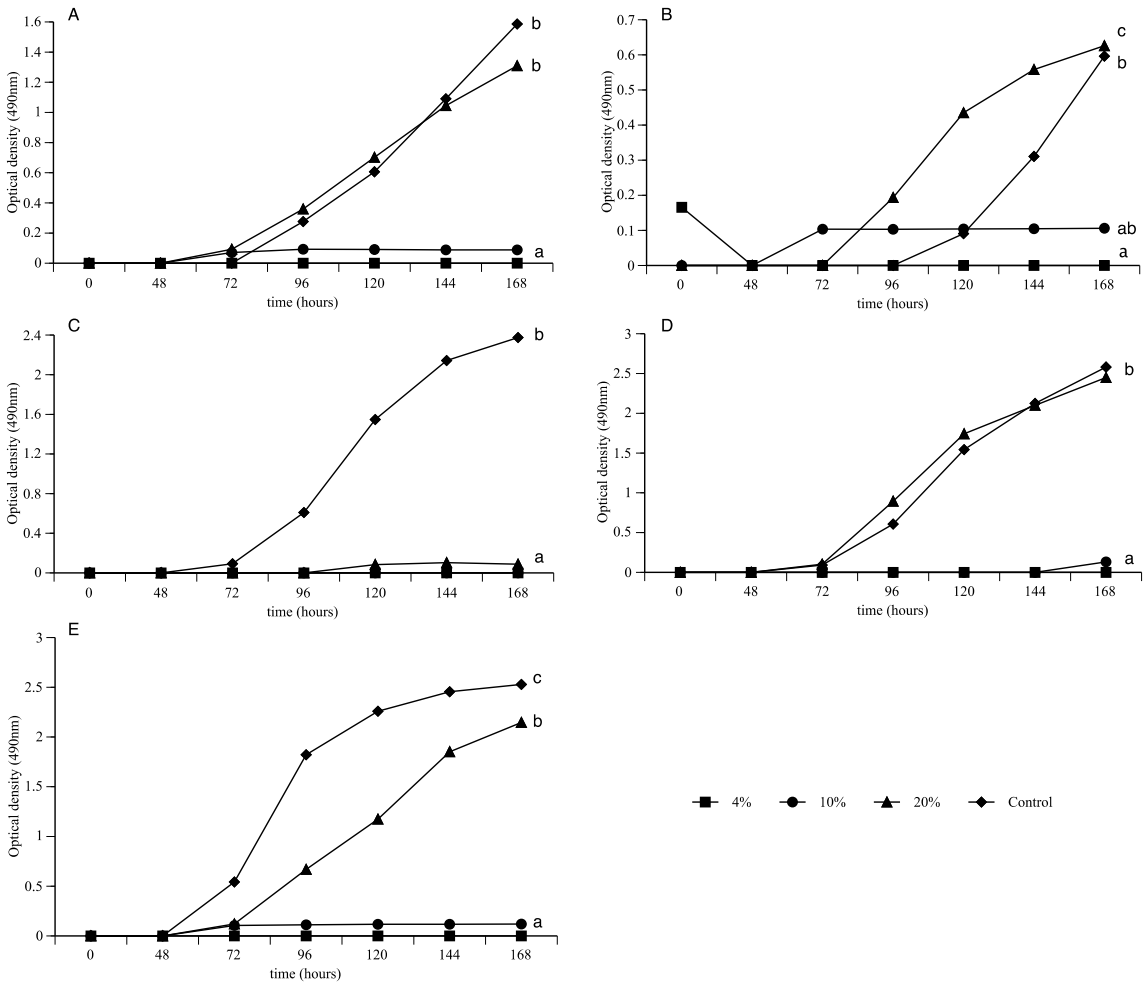

Figure 3.2. Carbon source utilization profile for five different carbohydrates within the Biolog Ecoplate system, across three tea treatments (n=3). A. Pyruvic acid Methyl Ester. B. i-Erythritol. C. β-Methyl-D-Glucoside. D. D Mannitol. E. N-Acetyl-D-Glucosamine. Significant differences (P<0.05) indicated by different letters.

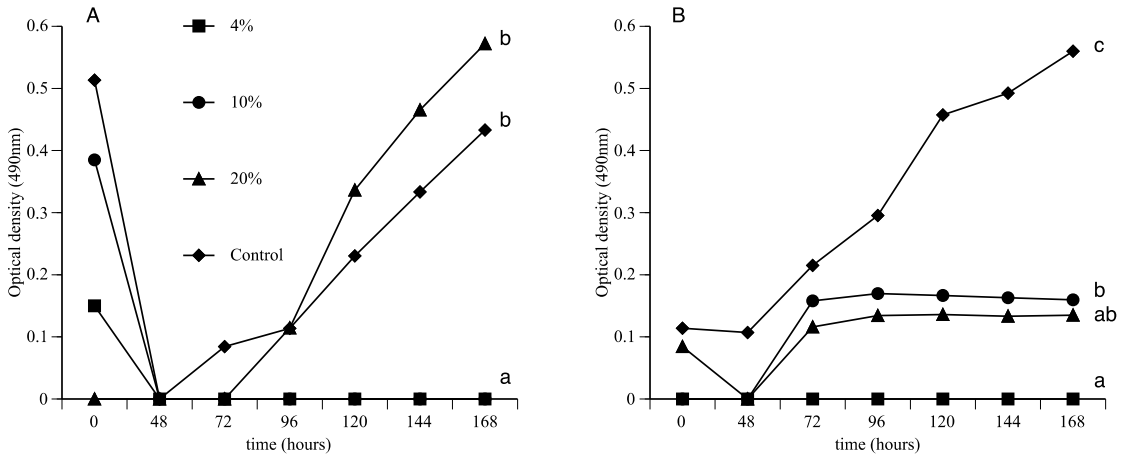

Figure 3.3. Carbon source utilization profile for two polymers within the Biolog Ecoplate system, across various vermicompost tea treatments. A. Tween 80. B. Tween 40. Significant differences (P<0.05) indicated by different letters.

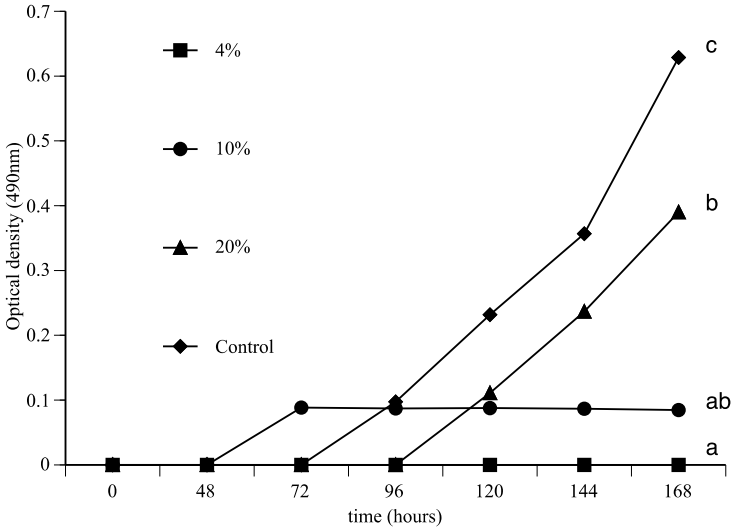

Figure 3.4. Carbon source utilization profile for the carboxylic and acetic acid guild, within the Biolog Ecoplate system. Within the guild, only the utilization of one compound, 4-Hydroxy Benzoic Acid, varied significantly between the four different tea treatments. Significant differences (P<0.05) indicated by different letters.

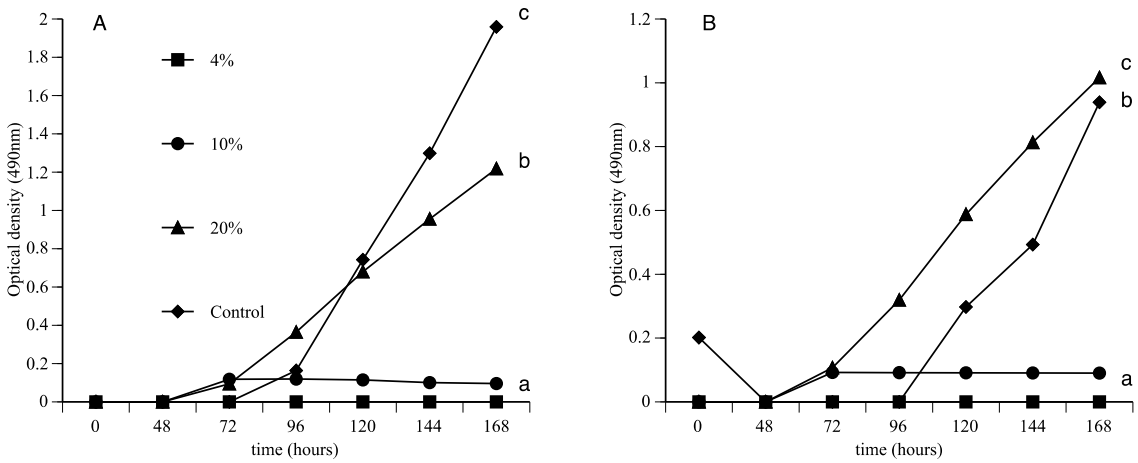

Figure 3.5. Carbon source utilization profile for the amino acid guild, using the Biolog Ecoplate system. Within the guild, only the utilization of two compounds, A. A-Asparagine and B. L-Serine, varied significantly between the 4 different tea treatments. Significant differences (P<0.05) indicated by different letters.

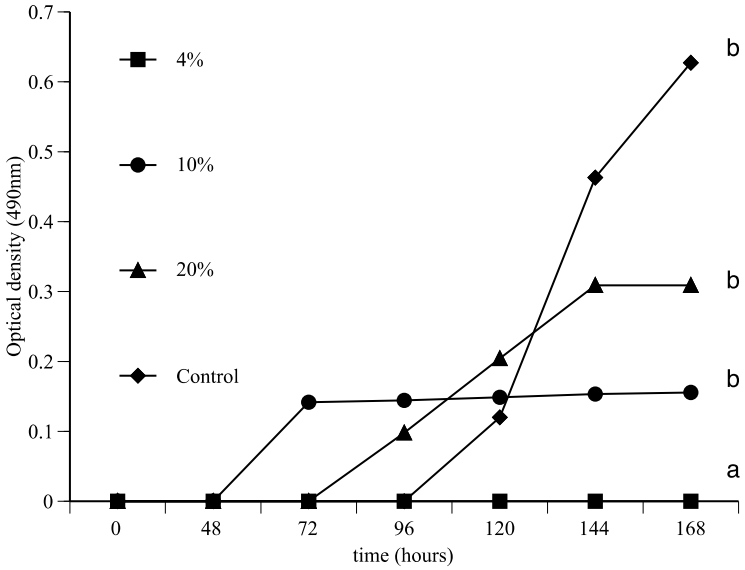

Figure 3.6. Carbon source utilization profile for the amines/amides guild, using the Biolog Ecoplate system. Within the guild, only the utilization of one compound, Putrescine, varied significantly between the 4 different tea treatments. Significant differences (P<0.05) indicated by different letters.

Addendum A

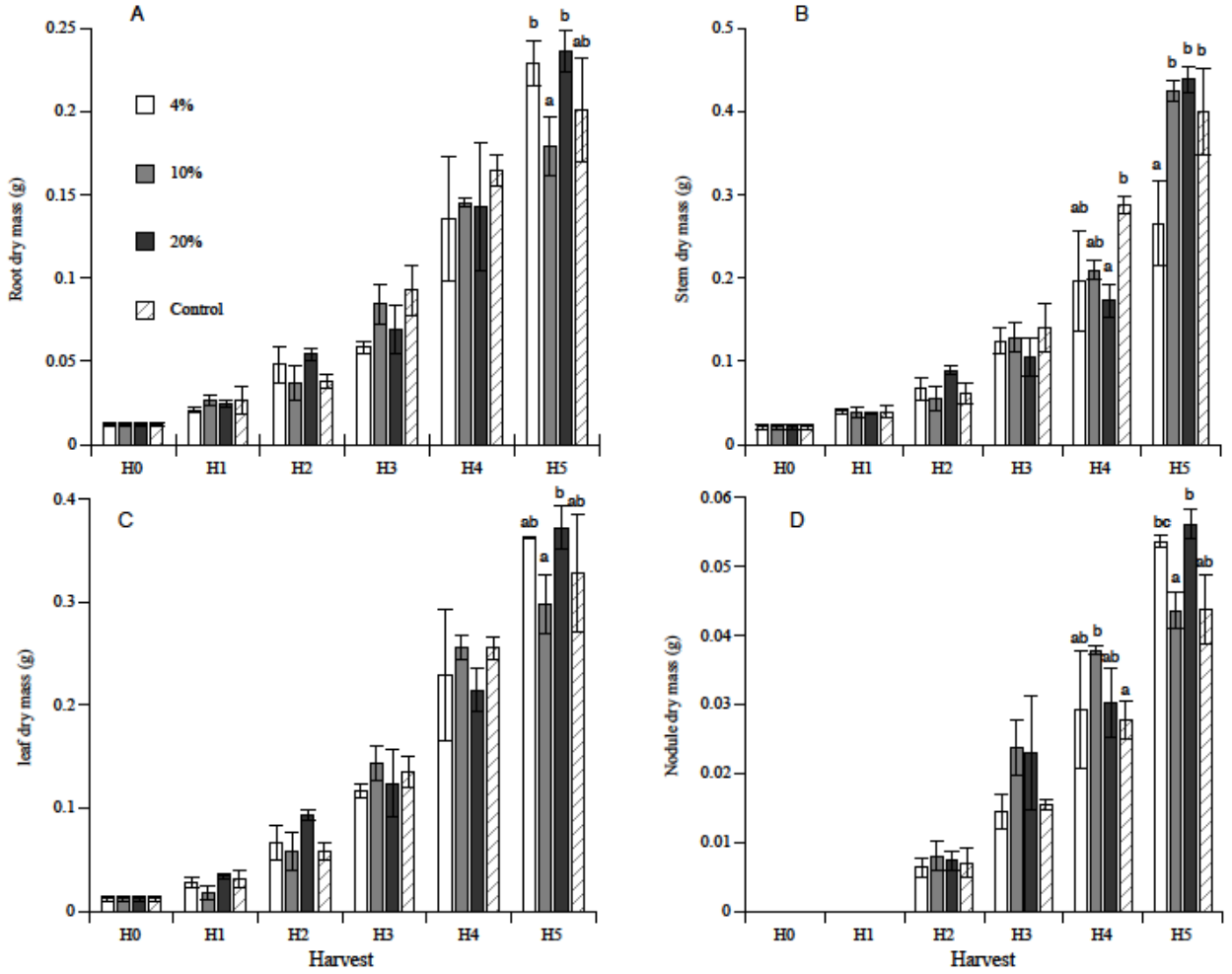

Figure 1. Biomass allocation of different plant organs over 50 days, 5 successive harvests and 4 different treatments. A. Roots, B. Stems, C. Leaves, D. Nodules. Treatments were comprised of 3 increasing tea concentrations and a control, which received no tea (NT). Harvests were separated by 10 days. Significant differences are indicated by different letters ($P<0.05$), treatments that do not have letters indicate no significant differences within that particular harvest. Error bars indicate standard error.

Chapter 5: General Discussion

The fundamental reason why sustainable agriculture technologies exist is to reduce the damage that we are causing to our soils and broader environment through our current agricultural practices. In order to offset this damage and to return fertility to degraded soils, we need to make use of a variety of technologies. Vermicompost is one of these technologies.

The fact that VCs are produced from organic wastes that would otherwise not be utilized productively, l*et al*one as an organic fertilizer, makes it inherently more sustainable than chemical fertilizers. However the question still remains, are VCs capable of providing crops with enough nutrition to maintain the yields that we currently enjoy through the application of chemical fertilizers? Unfortunately the answer is not simple because the action of VCs on plant growth is complex. VCs function through numerous ways to illicit growth responses in plants; these include the mineral nutritional component, which is incidentally the only way that chemical fertilizers cause a growth response. On this front, VCs cannot match chemical fertilizers. The concentration of nutrients is simply not as high as those found in chemical fertilizers and as such, far greater amounts of the substance is required. For example, the chemical fertilizers ammonium nitrate (Nitrogen), triple superphosphate (Phosphate) and muriate of potash (Potassium) typically contain 33%, 46% and 60% of their constituent macronutrients respectively. Chicken manure VC contains about 1.3%N, 1%P and 0.15%K. Thus in order to equal the amount of N contained in 1kg of chemical fertilizer one would require about 25kg of VC. To match the P content you would require 46kg and for the K content, 400kg. However, we do know that VCs promote plant growth through other methods as well. One of these methods is through the action of humic acids, which are well known for their growth effects on plants, when used at low concentrations (Nardi *et al*., 2002). Another component known to drive plant growth is the vast microbial diversity (van der Heijden *et ali.*, 2008) contained within VCs (Pathma and Sakthivel, 2012), which produce a wide variety of hormone-like substances which also influence plant growth (Nardi *et al*., 1988; Tomati *et al*., 1988). Combined, these actions increase the efficacy of VC greatly above what it would be if growth promotion was driven solely by the mineral nutrient

component (Arancon *et al*., 2008). Besides driving plant growth VC also improves soil health (Ansari and Sukhraj, 2010), however, if VCs, or any other organic amendment for that matter, are to be adopted and commonly used within agriculture they must not only prove to be efficacious but also cost effective. This is the fundamental problem that all organic amendments are faced with in our market driven economy. Unfortunately, the historical emphasis of agriculture was placed on yields, not on the maintenance of soil health. This attitude has, in part, led us to the situation we find ourselves in today, where agricultural land degradation is a real threat to our food security (Timm-Hoffman and Todd, 2000). As a result of declining soil health and increasing consumer pressure many South African farmers are now starting to take note of organic amendments such as VCs (Ungerer, 2013) as viable supplements to their chemical fertilizer application regime.

Experiments 1 & 2

The Narrow leaved lupin (*L. angustifolius*) is a N-fixer that is used primarily as a rotation crop in the Western Cape. This study shows that when a combination of chicken manure VC and *Rhizobium* bacteria are used in the cultivation process, biomass is increased. The mechanisms driving the increase in biomass are not entirely clear since opposing trends were found between the R and NR treatments. At the low concentration of 5% VC, maximum biomass accumulation in the R treatment was found. This indicates that the response is due to an interaction between the rhizobial inoculant and the VC. The response is most likely a complex one, being driven by more than one factor and should be investigated in future research since it will aid in the understanding of legume nutrition in sustainable agricultural practices. VCs and their influence on plant biomass production and crop yields have been investigated as well as their potential to augment (Manivannan *et al*., 2009), or even replace (Arancon *et al*., 2003), chemical fertilizer use in the field. Thus the next step in terms of the effects of VC on lupin growth is to investigate biomass production in the field. Additionally, the interaction of VCs with chemical fertilizer input should be investigated, since it is possible that a percentage of the fertilizer requirements could be substituted with VC, improving soil health and reducing chemical fertilizer input.

The use of the Biolog EcoPlate system in determining differences in bacterial metabolic diversity is a well known technique (Preston-Mafham *et al*., 2002), used in environmental remediation (Derry *et al*., 1998) and agricultural analyses to investigate changes in bacterial community structures (Frac *et al*., 2012; Gomez *et* al., 2006). This technique serves as a useful tool to characterise the quality of VC, since it indicates the level of bacterial diversity within the product, serving as an indication of potential nutrient cycling. Furthermore, this is of particular use in field trials, when assessing how agricultural soil bacterial diversity changes due to the addition of VC. Current soil analyses do not incorporate the Biolog EcoPlate system into their reports on soil fertility; this oversight thus excludes bacterial diversity. More holistic analyses such as the Solvita test kit incorporate biological parameters such as microbial respiration rate as a measure of soil biological activity; not offering any insights into the complexity of the soil food web which is driven by the bacterial diversity in the soil.

Testing for nematode diversity indicated that nematode diversity increases with an increase in VC concentration. Nematodes have already been identified as effective indicator species used for identifying environmental change (Bongers and Ferris, 1999) but their use as an indicator species for microbial diversity in VC is novel. Nematodes are identified through morphology and as such are far easier to categorize taxonomically than bacteria and fungi, the reason being that they can be identified under a microscope. No culturing is required. Thus analyzing nematode functional diversity may be a rapid and reliable means of determining potential nutrient cycling and soil health.

The finding that VCTs had no effect on biomass production was in contrast with a number of studies (Arancon *et al*., 2012; Arancon *et al*., 2007; Pant *et al*., 2009) but supported by the findings of (Fritz *et al*. (2012). However it is very difficult to compare results between studies since often the source material, sugar additions and physical design of the tea brewing apparatus are different. What was clear from the findings in this chapter though is that there were no differences in nutrient concentrations in the various teas, regardless of VC concentration. Furthermore, the additions of these teas had no effect on the nutrient content of the growth media, relative to the control. Thus the mineral nutrition component of VCTs could not have influenced the biomass production of the plant. Differences in mass allocation and stem biomass

production of the 4% treatment were noted; however these had no effect on the overall plant mass.

Despite finding that VCTs did not increase biomass accumulation it was found that 20% VCT and the control tea decreased the dependency of the plant on NDFA, while the low concentration teas (4% and 10%) still relied on atmospheric N for between 45 and 60% of their N requirements. If low concentration VCTs stimulate increased reliance on NDFA further research into this area is very important. The mechanism driving nodule N production is suggested to be due to the transport of NO_3 from the young leaves to the symbionts, signalling when adequate N is present and inhibiting N_2 fixation (Streeter and Wong, 1988). This action could be responsible for the continued BNF occurring in the low concentration VCTs but it does not explain why BNF decreased in the 20% treatment, since no differences in NO_3 content were found in the soil media. The significantly greater bacterial diversity found in the 20% treatment is likely the cause of reduced BNF; however the mechanisms remain to be elucidated in future studies. N enzyme assays could be used to investigate the rates of organic N mineralization in the future as this may support the hypothesis that increased microbial diversity relatives to increases in nutrient cycling.

The significantly greater bacterial functional diversity found in the 20% tea has practical implications for field trials. No tea treatment increased biomass but application of 20% VCT may lead to increases in soil bacterial functional diversity. As stated earlier, increased diversity leads to a more diverse soil food web (Bardgett, 2005) which in-turn influences nutrient cycling and soil fertility. Thus a study investigating the complete life cycle of the plant my find differences between VCT treatments.

To summerize, the vermicomposts show great potential to be used as an agent to alter the N nutrition of *L. angustifolius*. Relatively small concentrations of VC in the soil media stimulate increases in biomass production and increased reliance on BNF. Furthermore the additions of VCTs, particularly 20% concentration, increase bacterial functional diversity. However, while the additions of these teas have no effect on biomass production they also alter the N nutrition of the lupin; low concentration teas stimulate BNF while high concentration teas (20%) reduce it.

Conclusion

In spite of the new findings in this area of VC's, there have been some limitation in the study. Due to financial constraints a number of parameters were not investigated. DGGE analysis of the microbial community would have given interesting insights into the individual species or genera present in the VC and VCTs. Of particular interest would have been determining which bacterial species were responsible for nodulating in the NR treatments of data chapter 1. Phytohormone analysis of both the VC and VCTs would have provided interesting information about the hormonal effect of VCs and VCTs, as found by (Pant *et al*., 2012). Furthermore the use of Biolog Fungal identification (FF) microplate would have indicated fungal functional diversity, giving a more holistic view of microbial functional diversity. As stated earlier, assays for enzymes such as nitrogenase, urease and phosphatases would have helped to understand how nutrient cycling was taking place within the substrates. It would have also been more informative to run both trials to the completion of the life cycle of the plant, so that yields might have been analysed.

This study has opened quite a few future research avenues. Primarily, the findings of this study need to be confirmed by field trials. One foreseeable difficulty may lie in how to convert the percentage VC values used in the pot trials to kg/ha values used in the field. However, if the findings of this study are found to hold in field trials then the implications for farmers growing *L. angustifolius* are rather positive. Farmers could improve nodulation through the combined use of a relatively small quantity of VC and inoculation with the symbiont that is already commercially available. Furthermore the loss in bacterial functional diversity that the farmer would sustain through the addition of only 5% VC could be mitigated by the addition of 20% VCT. Through this combined treatment of VC solids and tea high levels of BNF, biomass accumulation and bacterial diversity could be achieved, all the while potentially reducing the reliance on chemical fertilizers.

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