Chemical and Microbial Characteristics of Vermicompost Leachate and their Effect on Plant Growth

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STATEMENT OF ORIGINALITY

The content of this thesis is the product of my own work. All assistance received and sources have been acknowledged. This thesis has not been submitted for any other degree or purpose.

Kathy Donohoe

Publications

Donohoe, K., Wilson, N.L., Deaker, R. (2017) Effect of different fruit and vegetable substrates on vermicomposting earthworms *Eisenia fetida*. Completed manuscript to be submitted

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Contents

Abstract

There is increasing interest in reducing chemical fertiliser use in favour of organic amendments. Vermicompost is an amendment resulting from the breakdown of organic waste by earthworms. The benefits of vermicompost to plant growth have been well established. However, less evidence is available on the benefits of vermicompost leachate, the liquid by-product. In addition, little is known about the effect of different organic substrates fed to worm-beds on the properties of vermicompost leachate. In this study, various organic worm-bed substrates were tested for their effect on earthworms and properties of leachate including the chemical and microbial composition and the capacity to promote plant growth.

The effect of eighteen individual organic worm-bed substrates on earthworm population and mass was measured over eight weeks. Earthworm growth and survival varied significantly with substrates with some being lethal and others providing good support. T-RFLP analysis of leachate produced from six substrates at four and eight weeks indicated differences in bacterial and fungal diversity.

A subset of three substrates, banana, green bean and rockmelon, providing a range of effects was selected for further testing. Banana provided poor support to earthworms, rockmelon was intermediate, and green bean provided the best support for earthworm growth and survival. The three substrates were used, alone and in combination. Earthworm population, mass, life cycle stage and cocoon production were measured over sixteen weeks and microbial community structure using ARISA was analysed in leachate collected at ten weeks. Bacterial diversity varied with worm-bed substrate. Leachates were stored for one year under refrigeration and at room temperature. Chemical and microbial properties of leachates were studied for changes following storage. Leachates produced from worm-bed substrate combinations of banana and rockmelon or green bean and rockmelon were both microbiologically and chemically stable for one year following refrigeration.

Banana, green bean and rockmelon leachate were applied to soybean, serradella and wheat. Leachate was sterilised in experiments with soybean, by either autoclaving or filtration, however microbial populations were reduced and full sterilisation was not achieved using these approaches. After eight weeks of growth, unsterile green bean leachate increased nodule numbers. Effects on shoot dry weight varied with sterilisation technique and presence of introduced commercial rhizobial strains for soybean. Leachate had no effect on root dry weight or shoot nutrients.

Banana, green bean and rockmelon leachate were applied to serradella and wheat and compared with chemical fertiliser. Leachate had no effect on nodule numbers at five weeks but green bean leachate increased nodule numbers of serradella at ten weeks. Shoot and root dry weight of both serradella and wheat was highest in plants treated with nutrient solution, however, there was no difference in shoot and root dry weights of serradella after ten weeks indicating plant growth was able to catch up probably through nitrogen fixation.

The ability of green bean leachate to increase nodulation was studied further by applying to serradella grown in sand. The experiment aimed to determine whether the effect was microbiological or chemical. Green bean leachate was compared with a combination of green bean, rockmelon and banana leachate, a synthetic nutrient solution prepared using inorganic nutrient analysis of green bean leachate from previous experiments and a standard nutrient solution used for legume growth (CRS). All treatments were then further split and either inoculated with microbial cells pelleted from the green bean leachate or left uninoculated. CRS nutrient solution produced the greatest plant height and shoot dry weight at all harvest times. Root dry weight and nodules per plant were highest in CRS after ten weeks. These results can be attributed to more complete supply of nutrients from CRS compared to other treatments. When CRS was removed from the analysis, treatment of plants with green bean leachate resulted in the greatest plant height, shoot dry weight, root dry weight at both harvest times and nodules per plant after ten weeks. Inoculation of plants with pelleted cells from green bean leachate increased nodule fresh weight in plants treated with CRS.

It is clear from these results that leachate has the potential to improve legume nodulation in serradella and soybean. The exact mechanism needs further investigation but the results indicate that the effect may be the result of microorganisms in the green bean leachate rather than the chemical composition. However, leachate has a complex organic chemical composition which was not measured in this study. The plant experiments were carried out in the glasshouse and yield parameters were not measured. Measurement of these effects in the field across several environments and legume genotypes would be an important future direction as well as combining green beans with other commonly vermicomposted substrates such as manures or plant tissues from other legume species. Improved nodulation by legumes has the potential to increase biologically fixed nitrogen in farming systems. This along with increased recycling of nutrients through vermicomposting will reduce dependence on chemical inputs in agriculture and support a more sustainable approach to crop production.

Chapter 1 - Literature review

1.1 Organic amendments to promote sustainable agriculture

Fertile soils are crucial for sustainable food crop production. Few agricultural soils are naturally fertile, requiring regular addition of plant-essential nutrients such as nitrogen (N), phosphorus (P) and potassium (K) (Hilton & Dawson, 2011; Rathod et al, 2013). These nutrients are available in variable quantities through natural recycling but are routinely supplied to crops by large-scale chemical fertiliser use. The intensification of crop production, with removal of nutrients in harvested commodities, and long-term chemical fertiliser use degrades soil fertility, reduces soil organic matter, microbial activity and water holding capacity (Chinsamy et al., 2013; Verma et al., 2014). In addition, chemical fertilisers are costly, prices are increasing, and availability is limited (Chinsamy et al., 2013; Verma et al., 2014).

The consequences of agricultural intensification are far-reaching, from increased erosion, pollution of groundwater and the surrounding environment, to more global concerns of impact on atmospheric constituents and climate. Lack of diversity in cropping can reduce biodiversity and increase the abundance of insect pests and microbial pathogens, leading to greater crop losses and requirement for increased use of agrochemicals (Matson et al., 1997).

Organic amendments may be an economically viable, ecologically sustainable method of supplying nutrients (Oliva-Llaven et al., 2010). Soil fertility could be improved with organic waste application such as by-products of farming or municipal activities (Diacono & Montemurro, 2010). Organic amendments include crop residues, manure, composted and uncomposted agricultural, industrial, and municipal wastes (Matson et al., 1997; Quilty & Cattle, 2011). Addition of these substances has been shown to increase microbial activity in soil (Dick, 1992), soil organic carbon, aggregate stability, water holding capacity, as well as the long-term availability of nutrients. They can increase plant yield (Diacono & Montemurro, 2010) and reduce the impact of pests and disease (Quilty & Cattle, 2011).

The conventional composting method involves arranging organic materials into windrows and manually turning three times over a period of six months. Turning is necessary to maintain aeration but releases odours when the windrow is disturbed (De Bruyn et al., 2009). There are many organic amendments available and selecting one can be a challenge. There is a lack of direct comparisons

between amendments, a lack of guidelines for their use and their mechanisms of action are poorly understood. An application positively affecting one plant or soil type will very likely not have the same impact elsewhere. This review will focus on vermicompost as an organic amendment.

1.2 Vermicompost can promote plant growth

Vermicompost is the stable product of organic waste processing by earthworms (Doan et al., 2013). Certain earthworm species ingest and fragment organic solids through a grinding gizzard. Vermicomposting involves the joint action of earthworms and microorganisms (Ayyobi, 2014; Gholipoor, 2014). Vermicompost leachate is an organic liquid produced from digested material and earthworm casts which can be used as a liquid fertiliser (Chinsamy et al., 2013; León-Anzueto et al., 2011). Figure 1.1 shows research interest in these products has increased substantially over the last few decades. Citation trends show the increasing impact of this research.

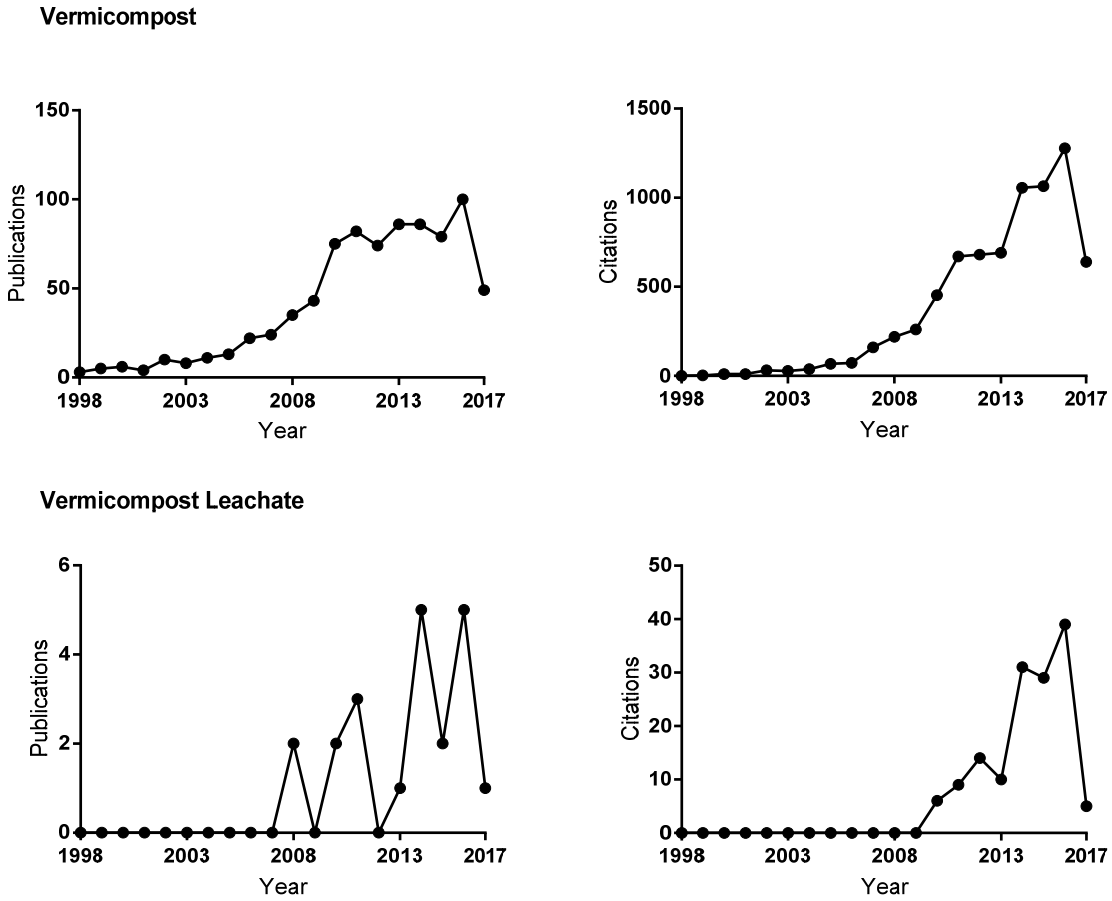

Figure 1.1. Publications and citations involving vermicompost, vermicompost leachate and plant growth.

Many studies indicate that vermicompost has a largely positive effect on plant growth. The majority of work on vermicompost involves a mix of vermicompost and chemical fertiliser in various ratios (Arancon et al., 2004; Arancon et al., 2005; Kale et al., 1992; Kumari & Ushakumari, 2002; Lazcano et al., 2012; Parthasarathi et al., 2008; Rathod et al., 2013; Verma et al., 2014). For example, a field trail was conducted in Bangladesh by Alam (2014) using the Chhiata series of Grey Terrace Soil. Recommended chemical fertiliser was compared with reduced amounts of chemicals supplemented with vermicompost or conventional compost from cow manure and food waste. The organic amendment volumes were 0%, 25%, 50%, 75% and 100%. Alam (2014) found a mixture of 75% chemical fertiliser and 25% vermicompost produced the largest tomato plant and fruit yield.

Alam (2014) and others (Dinani et al., 2014; Rao et al., 2010; Rathod et al., 2013) have recommended reducing chemical fertiliser use by supplementing with vermicompost. While such recommendations reduce chemical fertiliser use, application rates are still high to achieve positive effects in these studies. High volumes of chemical fertiliser producing the best yields are common in such studies. In economic terms, high chemical fertiliser produced the best return on investment (Alam, 2014). A reluctance to dramatically reduce or even replace chemical fertiliser is therefore understandable. However, these are short-term, single harvest studies. It is possible that economic benefits of organic amendments would increase in subsequent harvests as improved soil fertility continues to raise yields. Longer-term studies are required to confirm this but it is important to note that while the cost of fertiliser is expected to rise, vermicompost should remain an economical alternative.

While it is generally believed that vermicompost only provides long-term benefits, there are many studies that show otherwise. Vermicompost increases the yield of grains such as wheat and maize (Rathod et al., 2013) and legumes (Gholipoor et al., 2014; Sinha et al., 2010a), growth, germination and flowering of ornamentals (Asciutto et al., 2006; Atiyeh et al., 2000b; Bachman & Metzger, 2008; Chamani et al., 2008; Gajalakshmi & Abbasi, 2002; Sangwan et al., 2010; Vanmathi & Selvakumari, 2012) and the growth and yield of fruits (Arancon et al., 2004; Atiyeh et al., 2000a; Bachman & Metzger, 2008; Joshi & Vig, 2010; Singh et al., 2008; Tejada et al., 2008) and vegetables (Arancon et al., 2005; Hammermeister et al., 2006; Pant et al., 2009; Rao et al., 2010). Vermicompost combats drought stress in chickpeas (Gholipoor et al., 2014), increases red pigment in chilli peppers used for grinding (Małgorzata & Georgios, 2009), retards skin browning in bananas (Padmavathiamma, Li, & Kumari, 2008) and increases photosynthetic pigments and rate (Ayyobi et al., 2013; Małgorzata &

Georgios, 2009; Nadi et al., 2011; Quaik et al., 2012b). These benefits are observed very soon after organic amendment addition and are generally increased when compared to chemical fertiliser.

Chemical fertiliser contains immediately available nutrients (Dinani et al., 2014) which can compare unfavourably with the slower release nutrients of vermicompost (Diacono & Montemurro, 2010). An unfortunate drawback of this is that vermicompost has been shown to reduce yield in comparison with chemical fertiliser immediately after application, with yield increases seen only in subsequent harvests (Ramesh et al., 2009). Long-term studies are therefore important in assessing organic amendments as changes will be gradual. Long-term studies of conventional compost have shown repeat applications dramatically improved soil fertility (Diacono & Montemurro, 2010). Very few long-term studies exist using vermicompost and none applied vermicompost without some form of supplement, either chemical or organic. A two year trial of vermicompost and manure showed increased wheat yield immediately following organic amendment. Crucially, maize yields in year two showed further increases over chemical fertiliser use due to the residual effect of organics (Rathod et al., 2013). Such an immediate residual effect of vermicompost application is not always observed (Behera, 2009). Most studies on the effect of vermicompost over multiple growing seasons involve re-application of vermicompost, masking any residual effect.

1.3 Vermicompost can increase soil fertility and change microbiology

Soil analyses likely reflect the complicated nature of the plant-amendment-soil interactions, with each amendment affecting different aspects of soil fertility (Rathod et al., 2013). The difficulty in using such data to assign benefits to vermicompost is compounded by the blended nature of most of the treatments. It is generally agreed that vermicompost increases soil organic carbon (Parthasarathi et al., 2008; Ramesh et al., 2009). Many studies have found vermicompost to increase available P in soil (Arancon et al., 2006; Islas-Espinoza et al., 2013; Lazcano et al., 2012; Parthasarathi et al., 2008; Ramesh et al., 2009; Rathod et al., 2013) as well as K (Kumari & Ushakumari, 2002; Najafi-Ghiri, 2014; Parthasarathi et al., 2008; Ramesh et al., 2009). Soil mineral N is the main limiting plant nutrient (Tilman et al., 1996) and the impact of vermicompost on soil N is less clear. Some studies showed no change in available soil N (Arancon et al., 2004, 2006, 2005; Singh et al., 2008) while others found an increase in N (Ansari, 2008; Chamani et al., 2008; Hammermeister et al., 2006; Parthasarathi et al., 2008; Ramesh et al., 2009). The impact of vermicompost on soil N cannot be definitively stated. The slow release of nutrients from vermicompost (Islas-Espinoza et al., 2013) may

render direct comparisons between these studies invalid as they all involve different soil types and different time frames.

An often overlooked factor in studies combining vermicompost with chemical fertiliser is the importance of the microbial community. Synthetic N sources, including ammoniacal fertilisers produced by the Haber-Bosch process, do not support microbes (Jack et al., 2011) and a key difference between vermicompost and chemical fertiliser or other amendments is the association of microbes and earthworms. Earthworms eliminate some microorganisms and alter the organic worm-bed substrate to create a new environment for others (Doan et al., 2013; Gholipoor et al., 2014; Yang et al., 2014). As a result, the final microbial community differs greatly from that of the worm-bed substrate (Huang et al., 2013; Yasir et al., 2009).

The organic worm-bed substrate used in the vermicomposting process influences the microbial population of the end product, although the mechanisms and the degree of influence are not understood. Several studies have shown that different substrates change population size and level of activity (Arancon et al., 2006, 2004, 2005; Vivas et al., 2009). Identification of microbial strains in these trials was not undertaken and the exact nature of the population differences remains unknown. Microorganisms from the substrate can survive the vermicomposting process (Pramanik et al., 2009) although the degree to which this occurs has not been elucidated. However, it may be possible to produce a vermicompost that contains beneficial microbes through careful selection of worm-bed substrate.

The microbial population of vermicompost can be further changed by microbial inoculation of the worm-bed substrate. These changes vary with both inoculant and substrate used (Pramanik et al., 2009). Inoculation experiments like those conducted by Pramanik et al. (2009) are limited to very few substrates with no identification of the microbial strains involved. While it is clear that vermicompost microbial populations can be changed by inoculation the nature of these changes remain unclear. Pramanik et al. (2009) did not determine whether these changes were beneficial to plant growth. Also unknown is whether the inoculant organism survived the vermicomposting process to persist in the final product. There is some evidence that inoculant microbes survive in conventional compost when inoculated after the thermophilic stage (Postma et al., 2003) but this cannot be extrapolated to the vermicomposting process as the two processes are very different.

The question of why one would want to change the microbial population of vermicompost is important to consider. Amending soil with vermicompost adds microbes to the soil, increasing the diversity of the soil population (Doan et al., 2013; Lazcano et al., 2012). Bacteria have been isolated from vermicompost with the potential to tolerate high temperatures and salinity, produce plant growth promoting hormones, solubilise P, utilise a variety of C sources, degrade fungal pathogens (Pathma & Sakthivel, 2013) and even increase plant yield (Gopalakrishnan et al., 2014). If such organisms can be transferred to the soil or plant it could provide manifold benefits to plant growth and soil fertility.

Vermicompost-derived changes to the microbial population are more far-reaching than the initial amendment. It has been established that microbial communities in the rhizosphere are modified after amendment of plants with vermicompost. While survival of the vermicompost microbial population in the rhizosphere was of relatively short duration, the indigenous rhizosphere population was changed in response to the introduced organisms (Jack et al., 2011). This work involved population diversity studies, so no specific organisms were identified.

Vermicompost application has been demonstrated to increase microbial activity in soil regardless of soil type, plant species or organic worm-bed substrate (Arancon et al., 2006; Atiyeh et al., 2000b; Ghosh et al., 2008; Lazcano et al., 2012; Rathod et al., 2013). However, Arancon et al. (2006) found that soil type affected amendment-derived changes in the rhizosphere population. The authors concluded that there were likely to be many factors that influenced the rhizosphere populations in this study, from resident soil microbes at the two field sites responding to introduced microbes in different ways, to differing physical and chemical conditions in the soil. Organic carbon provided by organic amendments plays a key role in establishing the predominant microbes in the rhizosphere (Lazcano et al., 2012). The organic substrate used in vermicomposting may have a direct bearing on the quantity and quality of organic carbon in the composted product and therefore have an indirect effect on the rhizosphere population. Better understanding of the changes that occur could help elucidate the role of these changes in plant growth and soil fertility. It may be possible to engineer desired rhizosphere communities if these relationships were better understood.

Conclusions about the impact of vermicompost on microbial populations are further complicated by the many different methodologies for studying the effect of vermicompost on plant growth and microbial communities. Amendment-derived microbes were found to colonise germinating seeds and remain associated with roots (Jack et al., 2011). Reported methods include transplanting young

plants or sowing seeds into amended soil but the two methods have not been directly compared and may produce very different results.

The rhizosphere population undergoes multiple changes after amendment with vermicompost before stabilising into a new community. This new rhizosphere population could be due to the early influence of introduced organisms on the rhizosphere community (Jack et al., 2011). According to Jack et al. (2011) amendment microorganisms may change the trajectory of rhizoshere community succession. A further complication is introduced when plants are transplanted into pre-amended soil. In much of the literature the time between vermicompost application and transplanting or seed sowing isn't specified. There may be a time lag, particularly in field trials where these steps would be time-consuming. Field trials conducted by Kale et al. (1992) involved transplanting seedlings into soil amended three weeks prior. This type of experimental system may result in a different microbial population than earlier transplantation. Soil microbes react rapidly to changes brought about by organic amendments (Lazcano et al., 2012). The time frame for microbial population stabilisation is unknown and likely differs with each system, but a three week post-amendment population may be very different to a more recent population. Interestingly, Kale et al. (1992) found a decrease in *Actinomycetes* after amendment, a population which other researchers have found to increase (Huang et al., 2013; Yasir et al., 2009).

1.4 The effect of vermicompost on plant growth varies with organic worm-bed substrate and plant species

The effects of vermicompost amendments are variable and complex. The interactions between soil, plant and amendment are poorly understood. A field trail was conducted in India by Roy et al. (2010) using colluvial and alluvial mixed soil generated from Gondwana rock. Vermicompost from goatweed and paddy straw had a significantly different impact on germination of maize, okra and common bean. The mechanisms behind this change are unknown. A field trial was conducted in the United States by Arancon et al. (2004) using two vermicomposts applied to Doles silt loam. Vermicompost from food waste had different effects on growth and yield of strawberries than vermicompost from paper. The vermicomposts differed in nutrient content but it is unknown whether this is reflected in uptake by plants. In a field trial in India, Ramesh et al. (2009) trialed combinations of vermicompost and animal manures compared with chemical fertiliser. The organic amendments reduced durum wheat and mustard grain yield but had no effect on isabgol and chickpea grain yield. This variation was attributed at least in part to different plant nutrient requirements (Ramesh et al., 2009).

It has also been demonstrated that vermicompost from different worm-bed substrates has variable effects on the same plant species and soil type. Arancon et al. (2005) used a field trail to study vermicompost from three substrates on capsicum. Over three parameters measured, worm-bed substrate effect wasn't uniform and no substrate produced superior vermicompost. The results of Arancon et al. (2005) indicate that using one vermicompost likely involves compromising some aspect of plant growth. Such a trial does not take into account soil fertility, doubtless involving more compromises upon substrate selection. The measurement of additional plant growth parameters may produce a clearer picture of the impact of worm-bed substrate choice, it may well have the opposite effect.

1.5 Liquid leachate from vermicompost can have plant growth benefits

Most studies on vermicompost as an organic amendment use the composted product only. Few studies use vermicompost leachate. The number of publications and citations for both amendments are shown in Figure 1.2. Vermicompost has a much higher publication rate and research impact. Vermicompost leachate can be obtained in convenient volumes by flushing water through the system. Its existence in a liquid form facilitates a variety of application methods. The reluctance to use vermicompost leachate may be because data is limited and the results more variable than vermicompost.

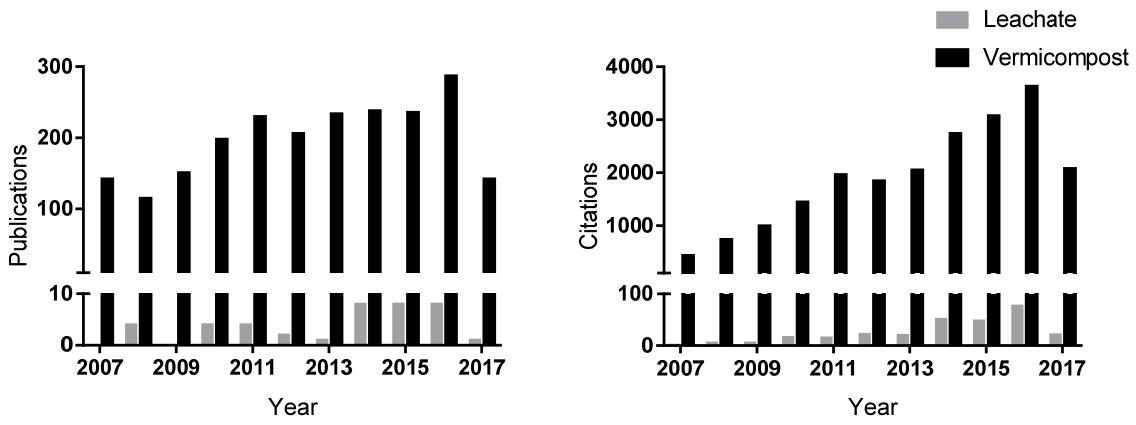

Figure 1.2. Publications and citations involving vermicompost and vermicompost leachate.

Large vermicompost units suitable for commercial production are in use. Aira et al. (2011) used a 4 x 1.5 x 1 m metal container. Frederickson (2002) used a 6.6 x 1.5 x 1 m breeze block bed. Common commercial vermicompost units are manufactured in China by Vermicrobe (Figure 1.3). The bins at the Sonoma valley worm farm are shown in Figure 1.4. This farm in San Francisco grows organic produce using vermicompost, and sells solid and liquid vermicompost products. There are many vermicompost products commercially available in Australia. For example, Circular Food in Somerton, Vic sell vermicompost liquid in 500 mL to 1000 L packs. Wormtec in Oxenford, QLD sell 2.5 L to 1000 L packs. Intune Earth in Hickeys Creek, NSW sell 2 L to 200 L packs. In addition, many organisations use vermicompost to reduce the amount they spend on waste disposal (Figure 1.5). These large vermicompost units can be flushed with water to obtain sufficient leachate for broad acre application, as evidenced by field trials such as those reported by Ayyobi et al. (2013).

Figure 1.3. Standard modular earthworm pits from Vermicrobe.

Figure 1.4. Sonoma valley worm farm.

Figure 1.5. Hungry bins at Mount Eden Corrections Facility, New Zealand.

Some studies test leachate in combination with chemical fertiliser. For example, a pot trial conducted by Garcia-Gomez, Dendooven, & Gutiérrez-Miceli (2008) combined different volumes of composted cow manure and paddy straw leachate with NPK. Chemical fertiliser was responsible for most of the variation in maize growth. This study did not apply leachate alone, but many studies do.

Several studies found vermicompost leachate improved some aspects of plant growth, although to a lesser degree than chemical fertiliser. For example, cow manure and maize straw leachate increased maize shoot N, P and K. The increase from chemical fertiliser was greater (Garcia-Gomez et al., 2008). However, leachate treatments were superior to no amendment (Aremu et al., 2012; Aremu et al., 2014; Ayyobi et al., 2014). No soil nutrient or microbial population analyses following leachate addition have been published. More information is needed on vermicompost leachate before it can be more widely adopted.

Published studies on application of vermicompost leachate to plants are summarised in Table 1.1. Vermicompost leachate has positive effects on yield and quality of grasses (Garcia-Gomez et al., 2008; León-Anzueto et al., 2011), vegetables (Ansari, 2008; Gutiérrez-Miceli et al., 2011), fruits (Arthur et al., 2012; Oliva-Llaven et al., 2010; Singh et al., 2010), grains (Garcia-Gomez et al., 2008) and herbs (Quaik et al., 2012b). Leachate breaks seed dormancy in jute, a process normally requiring boiling water (Ayanlaja et al., 2001), stimulates salt stress tolerance mechanisms in tomato (Chinsamy et al., 2013) and improves germination of aged Eucalyptus seeds (Kandari et al., 2011). Leachate reverses some growth retardation and chlorophyll loss caused by nutrient deficiencies in bulbous plants (Aremu et al., 2014).

In many cases the positive effects of leachate were dosage-specific but whether a high or low dose is optimal is impossible to predict without testing on plants. Increased leachate dosage increased lemongrass essential oil yield (León-Anzueto et al., 2011) whereas reducing dosage increased spinach and onion yield (Ansari, 2008) and leachate increased red kidney bean roots regardless of dosage (Singh, 2014). More often dosage effects are less clear cut and a compromise is required. For example, a 1:5 dilution of commercial leachate produced the greatest Eucalyptus plant height. The 1:10 dilution produced the greatest shoot dry weight and the 1:20 dilution produced the greatest leaf number (Kandari et al., 2011).

In many cases vermicompost leachate improves some but not all plant parameters measured. Cow manure leachate combined with chemical fertiliser was applied to peat moss in a trial by Gutiérrez-Miceli et al. (2008). Treatments increased sorghum height and dry weight but had no effect on stem diameter. Cow manure leachate was sprayed on foliage of tomato grown in peat moss in a trial by Oliva-Llaven et al. (2010). Treatments increased stem diameter and fruit soluble solids but had no effect on plant height and dry weight. The published studies found on the effects of vermicompost leachate on plants are all short-term studies limited to a single harvest. Possibly more uniform vermicompost leachate benefits would be found in subsequent harvests.

Table 1.1 Positive effects of vermicompost leachate from a range of worm-bed substrates and earthworms tested on plants.

Organic substrate	**Earthworm species**	**Plant species**	**Positive impact on plants**	**Increased plant N, P, K**	**Reference**
Cattle manure and paddy straw	*Perionyx excavatus*, *Lampito mauritii* Kinberg	Spinach (*Spinacia oleracea*), Onion (*Allium cepa*), Potato (*Solanum tuberosum*)	Increased vegetable yield	Not measured	Ansari (2008)
Commercial. Not reported	Unknown	Banana (*Musa* Williams)	Increased offshoot number and shoot length	Not measured	Aremu et al. (2012)
Commercial. Garden waste	*Eisenia fetida*	Bulbous plants (*Eucomis autumnalis*, *Tulbaghia ludwigiana*, *Tulbaghia violacea*)	Reversed detrimental growth in nutrient deprived plants, including leaf reduction, reduced bulb size and chlorophyll reduction	Not measured	Aremu et al. (2014)
Commercial. Not reported	Unknown	Tomato (*Lycopersicon esculentu*m Mill. 'Heinz-1370')	Increased shoot length and fresh and dry weight	Not measured	Arthur et al. (2012)
Not reported	*Eudrilus eugeniae*	Jute (*Chorchorus olitoriu*s L.)	Broke seed dormancy	Not measured	Ayanlaja et al. (2001)
Cattle manure	*E. fetida*	Peppermint (*Mentha piperita* L., Lamiaceae)	Increased plant height, leaf area and chlorophyll	Not measured	Ayyobi et al. (2013)
Cattle manure	*E. fetida*	French Dwarf Bean (*Phaseolus vulgaris* L.)	Increased pod number, plant dry weight and internode length	Not measured	Ayyobi et al. (2014)

Organic substrate	Earthworm species	Plant species	Positive impact on plants	Increased plant N, P, K	Reference
Cattle manure	*E. fetida*	*Stevia rebaudiana* (Bertoni)	Enhanced photosynthetic efficiency	Not measured	Bidabadi et al. (2016)
Cattle manure	*E. fetida*	Pomegranate (*Punica granatum* cv. Rabab and Malas Daneh Ghermez)	Increased leaf area, shoot and root weight. Reduced Na^+ accumulation in salt stressed plants	Not measured	Bidabadi, Dehghanipoodeh, & Wright (2017)
Commercial. Garden waste	*E. fetida*	Tomato (*L. esculentum* Mill. 'Heinz-1370')	Increased leaf number, leaf area and stem thickness in salt-stressed plants	Not measured	Chinsamy et al. (2013)
Cattle manure, composted	*E. fetida*	Sorghum (*Sorghum bicolor* (L.) Moench)	Increased stem height	No	Gutiérrez-Miceli et al. (2008)
Sheep manure	Unknown	Radish (*Raphanus sativus* L.).	Increased plant height	Not measured	Gutiérrez-Miceli et al. (2011)
Commercial. Not reported	*E. fetida*	Eucalyptus (*Eucalyptus dunnii*, *Eucalyptus nitens, Eucalyptus smithii*)	Improved germination of aged seeds. Increased seedling height and fresh weight	Not measured	Kandari et al. (2011)
Not reported	Unknown	Potato (*S. tuberosum*)	Increased tuber yield	Not measured	Kasal et al. (2015)
Cattle manure, composted	*E. fetida*	Lemongrass (*Cymbopogon citratus* (DC) Stapf.)	Increased essential oil yield and shoot dry weight	Not measured	León-Anzueto et al. (2011)

Organic substrate	**Earthworm species**	**Plant species**	**Positive impact on plants**	**Increased plant N, P, K**	**Reference**
Not reported	Unknown	Tomato (*Solanum lycopersicum* L.)	Affected fruit phenolic content and antioxidant capacity	Not measured	López-Martínez et al. (2016)
Commercial. Garden waste	*E. fetida*	African foxglove (*Ceratotheca triloba* (Bernh.) Hook.f. (Pedaliaceae))	Increased protein and photosynthetic pigment of N, P, K deficient plants	Not measured	Masondo et al. (2016)
Cattle manure, composted	*E. fetida*	Tomato (*L. esculentum* Mill)	Increased stem diameter. Affected fruit pH and soluble solids	Not measured	Oliva-Llaven et al. (2010)
Commercial. Chicken manure	Unknown	Pak choi (*Brassica rapa* cv. Bonsai, Chinensis group)	Increased shoot fresh weight, leaf number	Yes	Pant et al. (2009)
Not reported	Unknown	Rockmelon (*Cucumis melo*. L)	Increased fruit antioxidant capacity and phenolic content	Not measured	Preciado-Rangel et al. (2015)
Cattle manure, partially composted	*E. eugeniae*	Indian Borage (*Plectranthus ambionicus*)	Increased root and shoot size and chlorophyll level	Not measured	Quaik et al. (2012b)
Cattle manure, pre-digested, and grass waste	*E. fetida, Eisenia andrei, E. eugeniae*	Tea (UPASI-9)	Increased leaf number, plant height, root length, root weight	Not measured	Radhakrishnan & Mahendran (2009)

Organic substrate	Earthworm species	Plant species	Positive impact on plants	Increased plant N, P, K	Reference
Cattle manure	*E. fetida*	Strawberry (*Fragaria ananassa* Duch.)	Increased leaf number and dry weight. Reduced fruit malformation and grey mould	Yes	Singh et al. (2010)
Vegetable waste	*E. fetida*	Strawberry (*F. ananassa* Duch.)	Increased leaf number and dry weight. Reduced fruit malformation and grey mould	Yes	Singh et al. (2010)
Cattle manure and vegetable waste	*E. fetida*	Strawberry (*F. ananassa* Duch.)	Increased leaf number and dry weight. Reduced fruit malformation and grey mould	Yes	Singh et al. (2010)
Commercial. Not reported	Unknown	Red kidney bean (*P. vulgaris* L.)	Increased seedling axis growth and root number	Not measured	Singh et al. (2014)
Cattle manure	*E. fetida*	Tomato (*L. esculentum cv.* Momotaro)	Increased plant height and chlorophyll content	No	Tejada et al. (2008)
Green waste (vegetable, herbal and grass)	*E. fetida*	Tomato (*L. esculentum cv.* Momotaro)	Increased plant height and chlorophyll content	Yes, N only	Tejada et al. (2008)

1.6 Toxicity and safety of vermicompost leachate

As well as the potential benefits, potential toxicity to plants needs to be considered. There is an often cited theory that undiluted vermicompost leachate can be detrimental to germination and growth (Garcia-Gomez et al., 2008; Gutiérrez-Miceli et al., 2008; Kandari et al., 2011). Many researchers apply only dilute product. Aremu et al. (2012) applied 1:5, 1:10 and 1:20 dilutions of commercial leachate to banana grown in a mixture of sand, soil and vermiculite. Aremu et al. (2014)

applied a 1:10 dilution of commercial leachate to three species of bulbous plants grown in quartz sand. Radhakrishnan & Mahendran (2009) applied a 1:10 dilution of leachate from cow manure and grass clippings to tea. Reasons for choosing a particular dilution are not given in these studies.

Other researchers have used undiluted leachate. Arthur et al. (2012) applied undiluted commercial leachate to perlite used to grow tomato. Ayyobi et al. (2013) applied undiluted cow manure leachate to loam used to grow peppermint in a field trial in Iran. Singh et al. (2010) sprayed strawberry foliage with undiluted leachate from cow manure, green waste and a mixture of cow manure and green waste in a field trial in India. No evidence of phytotoxicity was reported in these studies.

Undiluted leachate has been shown to inhibit germination and growth of cress (Garcia-Gomez et al., 2008) and radish (Gutiérrez-Miceli et al., 2011; Pittaway, 2014), and germination of sorghum (Pittaway, 2014) and impatiens (Asciutto et al., 2006). Potential toxicity is attributed to high salt or pH and dilutions recommended (Asciutto et al., 2006; Frederickson, 2002; Pittaway, 2014). However, inhibition varies with plant species. For example, green waste vermicompost reduced watercress germination but had no effect on lettuce (Morales-Corts et al., 2014). Cow manure leachate inhibited radish germination but had no effect on cress (Gutiérrez-Miceli et al., 2011).

There is a potential trade-off between germination and yield where concentration is concerned. While concentrated leachate may inhibit germination, it can also produce the largest plants (Asciutto et al., 2006; Gutiérrez-Miceli et al., 2008). The possible inhibitory effect of high salt vermicompost leachate is further complicated by differing responses from plants depending on their nutritional requirements and salt tolerance. Leachate found safe for one species can inhibit more saline sensitive species and vice versa (Gutiérrez-Miceli et al., 2011; Pittaway, 2014). Of the few germination inhibition studies conducted, even fewer quantified the soluble salt content of the leachate. Those that did are shown in Table 1.2. There is no difference in soluble salt content of the leachates giving different degrees of inhibition. This indicates that salt content is not the sole determining factor and that plant species and worm-bed substrate likely play a role in inhibition. It is recommended that germination percentage be determined on a case by case basis prior to vermicompost leachate use.

Many authors have stated that vermicomposting alleviates concerns over the safety of using recycled organic wastes, particularly on edible plants. Studies have found that earthworms remove heavy metals and pathogens from the substrate (Gutiérrez-Miceli et al., 2007; Singh et al., 2011; Srivastava et al., 2005). Others have found that leachate is free of human and plant pathogens and

concluded that the passage of liquid through the organic material acts as a filter to aid pathogen removal (Frederickson, 2002; Gutiérrez-Miceli et al., 2011; Huang et al., 2013).

Vermicompost leachate from cow manure has been found to be free of faecal coliforms, *Salmonella* sp. and *Shigella* sp. (Garcia-Gomez et al., 2008; Gutiérrez-Miceli et al., 2008, 2011). Other pathogens were not tested in these studies. Aira et al. (2011) similarly showed reduced faecal coliforms and faecal enterococci, but found the vermicomposting process had no effect on *Clostridium* sp., total coliforms or Enterobacteria. The vermicomposting process in the study by Aira et al. (2011) was incomplete and it is possible the unaffected pathogens may be reduced upon further earthworm action. A range of pathogens may need to be considered before cow manure leachate can be declared pathogen free and safe for plant application.

Table 1.2. Electrical conductivity and pH of vermicompost leachate tested for germination inhibition.

Organic Worm-bed substrate	**EC (dS m^{-1})**	**pH**	**Plant species**	**Germination inhibition**	**Reference**
Potato slurry	2.6	7.7	Cress (*Lepidum sativum*)	No	Frederickson (2002)
Cattle manure, composted, and maize straw	2.6	7.8	Cress (*L. sativum*)	Yes	Garcia-Gomez et al. (2008)
Sheep manure	2.6	7.8	Cress (*L. sativum*)	No	Gutiérrez-Miceli et al. (2008)
Cattle manure, composted	2.6	7.8	Cress (*L. sativum*)	No	Gutiérrez-Miceli et al. (2011)
Cattle manure, composted	2.6	7.8	Radish (*R. sativus* L)	Yes	Gutiérrez-Miceli et al. (2011)
Cattle manure, composted	2.6	7.8	Cress (*L. sativum*)	No	Gutiérrez-Miceli et al. (2017)

1.7 Does vermicompost leachate improve plant nutrition?

It is uncertain whether vermicompost leachate impacts plant-tissue nutrient concentration. Cow manure and paddy straw leachate had no effect on shoot N, P, K of maize (Garcia-Gomez et al., 2008). Sheep manure leachate had no effect on shoot N, P, K of sorghum (Gutiérrez-Miceli et al., 2008). Chicken manure leachate increased pak choi shoot N, P, K (Pant et al., 2009). Green waste

and cow manure leachate increased strawberry fruit P, K and shoot N (Singh et al., 2010). The degree of N increase varied with plant species (Hammermeister et al., 2006). Additional effects on shoot nutrients are detailed in Table 1.1. Most studies focused on N,P and K, however Pant et al. (2009) also reported an increase in pak choi shoot Ca, Mg, S, Mn, Cu, B and Zn.

There are very few reports where nutrient levels of vermicompost leachates were characterised. Leachates from different worm-bed substrates contain different N, P, K levels (Garcia-Gomez et al., 2008; Gutiérrez-Miceli et al., 2008; Tejada et al., 2008). It is unknown whether the nutrient content of substrate is a direct reflection of available nutrient in vermicompost leachate. Vermicompost contains higher N, P, K than the substrate (Suthar, 2007). The nutrient levels of very few vermicompost leachates have been characterised. Many leachates contain similar nutrient contents. For example, potato waste leachate contains the same N and P concentration as cow manure leachate. Vegetable waste leachate contains the same N and K as cow manure and vegetable waste leachate (Table 1.3).

Table 1.3. Characterised vermicompost leachate with reported pH, macronutrient content and worm-bed substrate.

Organic Substrate	**pH**	**N (g/L)**	**P (g/L)**	**K (g/L)**	**Reference**
Potato slurry	7.7	0.2	0.2	0.9	Frederickson, (2002)
Cattle manure, composted	7.8	0.2	0.2	0.8	Gutiérrez-Miceli et al. (2008)
Sheep manure	7.8	0.9	0.2		Gutiérrez-Miceli et al. (2011)
Cattle manure, composted	7.8	0.2	0.2	0.8	Gutiérrez-Miceli et al. (2017)
Cattle manure, composted	7.5	15	0.9		León-Anzueto et al. (2011)
Cattle manure, composted	7.8	0.2	0.2	0.8	Oliva-Llaven et al. (2010)
Cattle manure	6.7	0.8	0.6	0.6	Singh et al. (2010)
Vegetable waste	7.5	0.7	0.4	0.5	Singh et al. (2010)
Cattle manure and vegetable waste	7.1	0.7	0.5	0.5	Singh et al. (2010)

Leachate application method may influence effectiveness. Leachate can be applied to soil pre- or post-sowing, to seeds, or directly to plants. Foliar application is used to rapidly supply nutrients to plants, aiming to maximise plant nutrients and avoid nutrients entering the soil (Zaller, 2006). In much of the literature, the application method is not described in detail. Unless the method is specifically stated as foliar application, for the purposes of this review, soil application is assumed. Foliar application increased strawberry leaf size, fruit yield and quality, reduced fruit malformation and increased leaf and fruit nutrients (Singh et al., 2010). Foliar application increased tomato leaf chlorophyll (Tejada et al., 2008). Foliar application is not always beneficial, as Zaller (2006) found foliar application to have minimal impact on tomato growth, fruit yield or nutrients. There is insufficient available data to confirm the effectiveness of foliar application.

It is unlikely that foliar application will benefit soil fertility or nutrient supply to succeeding generations of plants. Foliar application may remove the beneficial effects of microbes from the soil. Deciding on an application method may involve a trade-off between nutrient supply to soil and nutrient supply to plants. Although foliar application has also been observed to play a role in pathogen suppression and disease reduction (Singh et al., 2010; Zaller, 2006), the mechanism by which disease incidence was reduced has not been elucidated.

1.8 Does vermicompost leachate improve plant disease resistance?

Several studies have demonstrated the ability of vermicompost to inhibit plant fungal pathogen growth *in vitro* (Gopalakrishnan et al., 2010; Pathma & Sakthivel, 2013; Sathianarayanan & Khan, 2008; Yasir et al., 2009). The inhibitory effects were not uniform, varying with worm-bed substrate and pathogen (Gopalakrishnan et al., 2010). This anti-fungal action is likely to be microbial in nature as autoclaving vermicompost reduces or removes the inhibition potential (Asciutto et al., 2006; Yasir et al., 2009).

In a study of one hundred and ninety three bacterial isolates from vermicompost, Pathma & Sakthivel (2013) found fifty percent showed antifungal activity against plant pathogens, including *Fusarium*, *Colletotrichum*, *Sarocladium* and *Macrophomia* sp. While many isolates showed wide ranging inhibitory action, others had a much narrower action, and no isolate could inhibit all fifteen pathogens tested. A suite of microbes is necessary for anti-fungal activity. In this study, anti-fungal activity was observed in controlled conditions with a single bacterial isolate tested against a single pathogen. The interaction of these two organisms may be very different outside the laboratory.

There are few *in planta* tests of the disease suppressive potential of vermicompost. The limited published data indicates that vermicompost mixed with potting mix reduces damping-off and root rot of impatiens by inhibiting pathogen growth (Asciutto et al., 2006). Adding vermicompost to soil also reduces the numbers of and damage caused by insects and mites (Arancon et al., 2007). These experiments were performed in controlled conditions with plants in protective cages to prevent access by other potential pests. These effects are also dose specific, with only high doses achieving positive results (Asciutto et al., 2006; Edwards et al., 2010). No such tests have been carried out using vermicompost leachate applied to soil. So, while foliar application of vermicompost leachate may reduce disease but not contribute to soil fertility, it is possible that vermicompost leachate application to soil may achieve both.

1.9 Additional considerations when choosing an organic worm-bed substrate

There are infinite combinations of substrate that may change the nature of the resulting vermicompost leachate. Designing a substrate must also consider the impact on the health and productivity of the earthworm population. A study by Suthar (2007) of six different worm-bed substrates found that varying substrate affects earthworm growth but has no effect on mortality or reproduction rates. The study found that an increased earthworm growth rate wasn't necessarily reflected by a high substrate or vermicompost nutrient content, although high substrate N correlated with high earthworm growth rate (Suthar, 2007). Certain worm-bed substrates, therefore, may limit earthworm growth. When modifying the substrate, the impact of substrate choice on earthworm growth needs to be considered as small earthworm size may limit the volume of substrate they consume. This study by Suthar (2007) was limited by substrate choice, which was lacking in diversity. The substrates used were largely crop residue and manure mixes with little variation in nutrient content. More extreme differences in substrate may have more of an impact on earthworm populations. Worm-bed substrates with a greater range in N concentration would be of particular interest to test the correlation between worm growth rate and N found by Suthar (2007).

Another limitation of the trial by Suthar (2007) is the utilisation of the earthworm species *Perionyx sansibaricus*, not commonly found in vermicomposting trials. It is known that different species have different rates of substrate consumption (Sinha et al., 2012) and they may well differ in their response to substrate nutrients. The correlation between substrate N and growth, for instance, may not hold true for other species. Most studies on vermicomposting and vermicompost leachate use *Eisenia fetida* (Table 1.1) a leaf-litter-dwelling, or epigeic, species, chosen for its high rate of organic

matter consumption and reproduction (Frederickson, 2002; Sinha et al, 2010b). According to Frederickson (2002) worm-bed substrate can affect reproduction rate, although the earthworm species used was not specified. Mixtures of sewage waste, cow manure and straw with different moisture contents were examined for effect on *E. fetida* by Contreras-Ramos, Escamilla-Silva, & Dendooven (2005). Moisture content significantly affected earthworm mass. Any changes to the substrate would need to be monitored for its effects on the chosen species growth and reproduction.

The pH of the worm-bed substrate may also need to be taken into consideration, although this has yet to be definitively established. It is generally thought that maintaining a favourable pH in a vermicomposting system is necessary (Singh et al., 2011) but the required pH has not been defined. Earthworms are absent or rare in soil with a pH less than four (Sinha et al., 2012) although earthworms in vermicompost may be more tolerant of acidity. Sathianarayanan & Khan (2008) reported vermicomposting using *Eudrilus eugeniae* neutralised acidic substrates. Unfortunately, the pH of these substrates was not reported, so the degree of acidity this species of earthworms can process is unclear. *E. fetida* are capable of vermicomposting a substrate with a pH of 4.2 to a near neutral product (Nadi et al., 2011). This may indicate that the worm-bed substrate pH is less important than commonly thought, although whether this vermicompost from acidic worm-bed substrate is beneficial to plants to the same degree as other less acidic substrates was not tested. Neither of these studies determined whether consuming acidic substrates had adverse effects on the earthworm populations. Further testing is required to determine whether the pH of the worm-bed substrate is a crucial factor in substrate selection.

1.10 Conclusion

There are no standard guidelines for vermicompost leachate use due to their variable nature (Gutiérrez-Miceli et al., 2011). Changing the worm-bed substrate changes many characteristics of the resulting vermicompost and vermicompost leachate. While several studies have tested more than one substrate, no research has attempted to design an ideal substrate or to identify which components of the substrate determine characteristics of the vermicompost. In the literature, reasons for a choice of worm-bed substrate are seldom given, likely as it is considered more a means to an end rather than an important variable. This is particularly true of vermicompost leachate experiments (Table 1.1) where a lack of substrate variability is obvious and limiting. While vermicompost leachate from a narrow range of substrates has proven positive effects on plant growth more work is needed to determine the impact of vermicompost leachate on soil and plant nutrients and soil microbiology.

Aims

The aims of this study were as follows.

1. Test a range of organic worm-bed substrates to identify substrates with beneficial effects on earthworms.
2. Study the effect of worm-bed substrate on leachate microbial communities and nutrient composition.
3. Study the effect of leachate storage on microbial and chemical composition.
4. Determine whether leachates from different worm-bed substrates improved plant growth when used as soil amendments.
5. Determine whether leachate effect on plants varied with plant species and addition of commercial legume inoculants.
6. Determine whether leachate effect on plants is due to chemical or microbial composition of leachate.

Chapter 2 - Effect of worm-bed substrate on earthworm survival using small scale worm farms

2.1 Introduction

There is increasing interest in reducing chemical fertiliser use by using organic fertilisers instead (Quaik et al., 2012a). A large range of organic fertilisers, some certified, are available in Australia, but there is little evidence of their efficacy (Quilty & Cattle, 2011). Earthworm processed organic waste, or vermicompost, is a potential source of nutrients available to plants as well as a means of disposing of organic waste (Atiyeh et al., 2000a).

Epigeic, or leaf litter, earthworms are the only earthworms suitable for vermicompost (Domínguez, 2004). *E. fetida*, commonly known as tiger worms, are temperate climate earthworms (Edwards, 1988), *Eisenia andreii*, or red worms, are sub-tropical (Windust, 1997), and *Perionyx excavatus,* or blue worms, are tropical (Edwards, 1988). These earthworms require aerobic conditions and a worm-bed substrate that is not water saturated (Domínguez, 2004). *E. fetida* can tolerate a wide moisture range (Domínguez, 2004) and a low pH (Windust, 1997). They also have a wide temperature tolerance, are easy to handle, and generally become the dominant species in mixed earthworm populations (Edwards, 1988). *E. fetida* produce more hatchlings per cocoon than *E. andreii* (Sheppard, 1988) and tolerate a higher population density (Kale et al., 1992). *P. excavatus* can survive drier conditions than *Eisenia* sp. and are more suitable to warmer climates (Windust, 1997).

E. fetida, *E. Andrei* and *P. excavatus* can be fed most organic wastes, although some need pre-treatment and not all support earthworms equally. Early vermicompost studies used sewage sludge as a worm-bed substrate. Unprocessed sludge was toxic to *E. fetida*, the variable moisture content presented difficulties with drainage and maintaining aerobic conditions, and aged sludge provided poor nutrition (Edwards & Arancon, 2004). Many vermicompost studies use *E. fetida* fed on animal manure substrates (Rahimi & Karimi, 2016; Ravindran & Mnkeni, 2017; Sharma & Garg, 2017; Singh et al., 2016). Cow manure is a popular substrate because of ease of use, but it can contain unfavourable components including inorganic salts and chemicals used for cleaning and cattle drenching (Edwards & Arancon, 2004; Windust, 1997). The amount of substrate consumed is related to earthworm mass, with earthworms consuming in excess of their body weight each day under ideal conditions (Munroe, 2007). Maximum life expectancy of *E. fetida* and *E. Andrei* is five years

under ideal conditions (Kale, 2004). In order to maintain vermicompost systems for longer than this period, reproduction is necessary. Earthworm mass and earthworm numbers can be used to indicate substrate suitability.

Earthworm life cycle consists of three phases, cocoon, juvenile and adult (Jefferies & Audsley, 1988). A glandular swelling, the clitellum, develops over several segments of adult earthworms and these are necessary for reproduction (Sims & Gerard, 1985). The optimum vermicompost temperature varies with earthworm species (Aston, 1988) but optimum temperature for *E. fetida* and *P. excavatus* cocoon production is 25°C (Neuhauser, Loehr, & Malecki, 1988). Organic waste pass through the earthworm gut where they are decomposed by the muscular grinding of the gizzard (Padmavathiamma et al., 2008). Wastes are ground with the aid of small stones in the gizzard (Sinha et al., 2010b).

Vermicompost products are commercially available, but it is impossible to know how they are made or their composition. Analysis of a commercial vermicompost by Buchanan, Russell & Block (1988) revealed a large amount of gravel and sand. Vermicompost systems are easy to establish although upkeep can be challenging and worm-bed substrate will affect the growth and reproduction of earthworms. The materials used in commercial vermicompost operations include sewage sludge, animal manure, paper pulp, garden and vegetable waste (Frederickson, 2002) although the relative benefits of the various substrates is unknown. The aim of this work was to test a range of organic worm-bed substrates as earthworm foods. A variety of worm-bed substrates were used to test the effect on earthworm health by measuring changes in earthworm size and survival. The ultimate aim of these experiments was to identify defined worm-bed substrates that support earthworm growth and survival so the leachates can be analysed for microbial diversity, chemistry and effects on plants in later experiments. The null hypothesis for these experiments was that earthworm size and survival will not vary with different organic substrates.

2.2 Methods

2.2.1 Vermicompost

Small scale worm farms were established in 1 L capacity open top cylindrical PVC pipe (Figure 2.1). 3 mm drainage holes were drilled in the base. The pipes were covered with pinpricked aluminium foil to provide darkness and allow air flow. Foil was kept in place with rubber bands to prevent earthworms exiting from the top. Worm farms were maintained indoors at 25°C.

Bedding consisting of damp shredded newspaper (24 g dry weight, 2 g per live earthworm) was added to each unit. Bedding was weighed dry then soaked in water, drained and added to the units. Vermicomposting earthworms were obtained from Worm Affair (Midland, Australia). Earthworms were rinsed in distilled water to remove the material they were transported in. They consisted of a mixture of *E. fetida*, *E. andreii* and *P. excavatus*. Twelve juvenile earthworms of mixed species weighing between 0.08-0.16 g each were added to each unit. The species were not recorded but total earthworm mass per unit was recorded. The newspaper bedding and earthworms were covered with two layers of damp newspaper to retain moisture and darkness. Earthworms were allowed to acclimate to their new environment for 1-3 days.

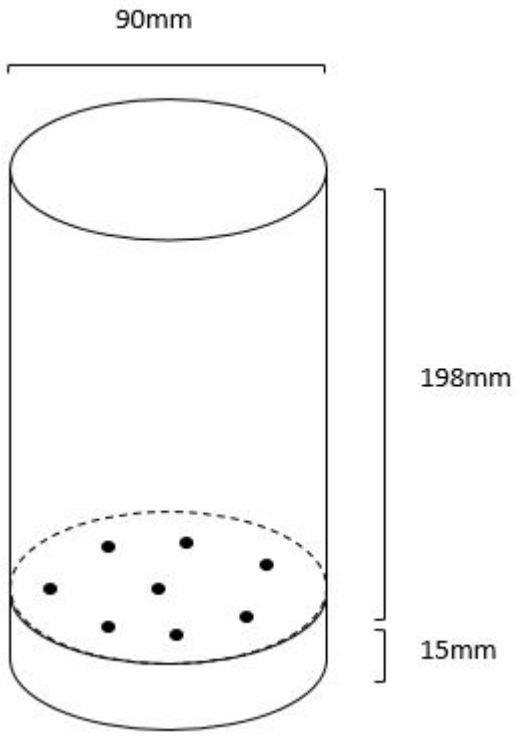

Figure 2.1. Vessels used for small scale worm farms.

Substrates were pureed to ensure uniform particle size (substrates used are listed in Table 2.1) and 18 g (1.5 g per live earthworm) was added on top of the bedding. Propagating sand (1.2 g, Brunnings, St Marys, Australia) was sprinkled over the substrate to aid earthworms in grinding. An additional 12 g of bedding was added to achieve a bedding: substrate ratio of 2:1. Bedding volume was chosen to half fill the units, to supply sufficient material for earthworms to live in and allow enough space for air flow. Substrate volume was chosen to supply sufficient food and minimise the disruption to the vermicompost system by more frequent additions of new substrate.

Due to the high moisture content in celery and rockmelon, pureed substrate drained straight through the units. These substrates were therefore filtered through a single layer of muslin prior to use. Due to the potential toxicity of a low C:N ratio (Christie, 2011) kelp meal and lucerne hay were each combined with paper pulp in a ratio of 1:2 (w/w) prior to use. Shredded newspaper was soaked in hot water then pureed to obtain newspaper pulp. Three replicate worm farms per substrate were established. Remaining substrate was stored at -20°C for future use. A 1:1 (w/v) mixture of substrate and water was used to determine substrate pH (Table 2.1) using a pH Cube Benchtop pH-mV-Temperature Meter (TPS, Brendale, Australia).

Table 2.1. Vermicompost substrates fed to *Eisenia fetida*, *Eisenia andreii* and *Perionyx excavatus*.

Substrate	Treatment	Selection Criteria	pH	Source/Brand
Banana (Cavendish)	Whole (fruit and peel), pureed	High Carbohydrate High C:N High Potassium	4.70	Independent Grocers of Australia (IGA) Alexandria, Australia
Green bean	Frozen, whole, pureed	High Protein High Calcium	6.00	Birds Eye Mentone, Australia
Rockmelon	Whole (fruit, peel and seeds), pureed, filtered through muslin	Medium Carbohydrate Medium C:N	6.80	IGA Alexandria, Australia
Celery	Whole (stalk and leaves), pureed, filtered through muslin	High Sodium	5.75	Redfern Fruit Market Redfern, Australia
Sweet corn	Whole (kernel, cob and leaves), pureed	High Phosphorus	6.32	Redfern Fruit Market
Spinach	Whole, pureed	High Protein Low C:N	6.06	Redfern Fruit Market
Garden pea	Frozen, shelled, pureed	High Protein	7.04	Birds Eye Mentone, Australia
Kelp meal (*Durvillaea potatorum*)	Hydrated 1:3 (w/v)	Organic amendment	6.89	King Island Kelp Industries King Island, Australia
Blueberry	Frozen, pureed	Low pH High C:N	3.35	Creative Gourmet Knoxfield, Australia
Potato	Whole, pureed	High Carbohydrate Low C:N	5.54	Redfern Fruit Market
Sweet potato	Whole, pureed	High Carbohydrate Medium C:N	5.80	Redfern Fruit Market
Cow manure composted & pasteurised	NA	Frequently used substrate	8.75	Australian Native Landscapes (ANL) Terrey Hills, Australia
Lucerne hay	Hydrated 1:3 (w/v), pureed	Frequently used bedding in domestic worm farms Contains N	5.29	Try Organics Dungarubba, Australia
Control	Newspaper pulp	Frequently used bedding in domestic worm farms Low N	6.80	NA

Nutrient data from United States Department of Agriculture (2017).

Worm farms were observed daily up to 30 days to determine whether earthworms were alive. Earthworm number and total mass were measured weekly for eight weeks to determine relative survival. To measure earthworms, unit contents were transferred into a tray and earthworms were removed by hand. Earthworms were counted then combined to obtain total mass. Fresh substrate (1 g per surviving earthworm) and bedding (3 g per surviving earthworm) were added weekly over weeks 1-3 and 5-7. When populations reached 40 earthworms, food and bedding were reduced to 0.5 g and 1.5 g per surviving earthworm, respectively, to avoid overfilling the units. If the top newspaper layers dried out, enough water was added to rewet them. Otherwise, no water was added to the units.

2.2.2 Modifying substrates to increase survival

Several substrates were lethal to earthworms so further tests were carried out using modified substrates to improve earthworm response. Substrates were modified as follows and new treatments are listed in Table 2.2.

1. Lethal substrates sweet corn and garden pea were combined with non-lethal substrates celery and rockmelon.
2. A mixture of foods was used to test a wide range diet. This contained tomato 12%, lettuce 3%, green bean 4%, mushroom 6%, red grape 8%, banana 10%, apple 9%, red capsicum 19%, carrot 11%, avocado 18%. Percentages were by weight of puree.
3. Acidic substrates (apple and the above food mixture) were neutralised before use.

Table 2.2. Modified vermicompost substrates fed to *Eisenia fetida*, *Eisenia andreii* and *Perionyx excavatus*.

Substrate	pH (initial)	pH (final)	Source
Rockmelon: Garden pea 2:1 (w/w)	NA	NA	Per table 2.1
Celery: Sweet corn 2:1 (w/w)	NA	NA	Per table 2.1
Apple, pink lady	3.08	7.00	Redfern Fruit Market
Mixture	4.35	7.00	Redfern Fruit Market

Unless otherwise stated, worm farms were established and maintained using the protocol previously described. Sodium bicarbonate (4% w/v) (McKenzie's Foods, Melbourne, Australia) in water was added to acidic substrates to neutralise pH. Substrate (12 g, 1 g per earthworm) and bedding (36 g,

3 g per earthworm) were added initially. Live earthworms were counted weekly, and additional 1 g per earthworm substrate and 3 g per earthworm bedding were added.

2.2.3 Comparison of earthworm survival between small scale and large scale worm farms

Larger vermicompost units were needed to obtain enough leachate for plant application. The aim of this work was to compare earthworm biomass using the same substrates in different size worm farms.

Small scale worm farms were established in 1 L PVC pipes (as described in Section 2.2.1). Large scale worm farms were established in 5 L plastic buckets (Figure 2.2) (AYVA packaging, Wetherill Park, Australia). The buckets had plastic lids with pinholes. Drainage holes (3 mm) were drilled in the base. Worm farms were maintained indoors at 25°C.

Bedding was added to the worm farms, 48 g moist newspaper was added to small scale worm farms and 240 g to large scale farms. A mixture of *E. fetida*, *E. andreii* and *P. excavatus* were used as previously described. Six mature earthworms, mean weight 0.56 g each, were added to each small scale worm farm and 17 g (approximately 30 earthworms) of mature earthworms were added to each large scale worm farm. The combined mass of earthworms added to each unit was recorded but not earthworm species. The contents were covered with two layers of damp newspaper. Earthworms were allowed to acclimate to their new environment overnight before adding substrate.

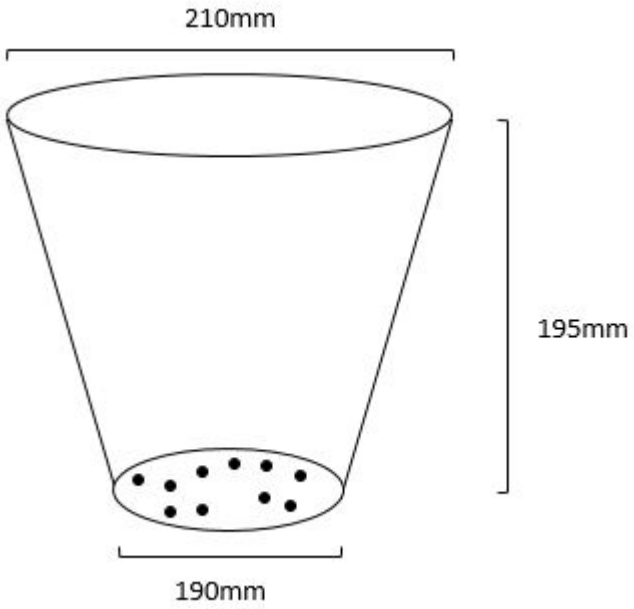

Figure 2.2. Vessels used for large scale worm farms.

Substrate was added to the small units at a rate of 1 g per earthworm and sand at a rate of 0.1 g per earthworm. Five times this was added to the large units. An additional 150 g bedding was added to the large scale units to bring the contents to half of the total volume. The substrates used were celery, green bean and control prepared as described in section 2.2.1. Triplicate worm farms were established for each substrate treatment. Fresh substrate and bedding were added weekly over weeks 1-3 and 5-7. Total earthworm biomass was recorded weekly for five weeks with a final weighing at 60 days. Due to time constraints, small celery and control worm farms were weighed only at 4 weeks and 60 days.

2.2.4 Substrate testing - banana, green bean and rockmelon

Three fresh produce substrates, banana, green bean and rockmelon were selected for further analysis on the basis of their differences in supporting earthworm growth and survival. Banana was observed to be a poor source of nutrition, rockmelon was intermediate and green bean supported high numbers of earthworms. The aim of this work was to combine these substrates to determine if substrate combinations affected earthworm response.

In this experiment earthworms were separated by species and only *E. fetida* were added to worm farms. *E. fetida* stocks were maintained in a mix of fruit and vegetable waste, paper, and composted, pasteurised cow manure until needed. Bedding (36 g per unit) was added to 24 small scale worm farms. Twelve juvenile earthworms weighing between 0.03-0.18 g each were added per unit. Combined mass of earthworms was recorded. Earthworms were left overnight prior to addition of 18 g of substrate listed in Table 2.3.

Table 2.3. Composition of vermicompost treatment substrates.

Treatment	Substrate
P (Control)	Newspaper pulp, pureed
B	Banana, whole, pureed
G	Green beans, whole, pureed
R	Rockmelon, whole, pureed
B/G	1 part banana: 1 part green bean (w:w)
B/R	1 part banana: 1 part rockmelon (w:w)
G/R	1 part green bean: 1 part rockmelon (w:w)
B/G/R	1 part banana: 1 part green bean: 1 part rockmelon (w:w:w)

Rockmelon was filtered through muslin to remove excess liquid before being weighed. Bedding (24 g) and substrate (12 g) were replenished weekly for 3 weeks and then fortnightly. Total earthworm biomass, clitellum development and cocoon production were recorded weekly for 16 weeks.

2.2.5 Data analysis

Earthworm measurements in small scale units were analysed using Linear Mixed Modelling (LMM). Comparisons of small and large scale units were analysed using Analysis Of Variance (ANOVA). All analysed were conducted using Genstat 17th Edition (VSN International Ltd, Hempstead, UK).

2.3 Results

2.3.1 Effect of worm-bed substrates on growth and survival of earthworms

Earthworm survival as a percentage of the starting population is shown in Table 2.4. Six worm-bed substrates led to an increase in earthworm population after 30 days. The earthworm population fed rockmelon increased 0.7-fold, lucerne hay 0.5-fold, cow manure 0.3-fold, paper 0.3-fold, kelp meal 0.2-fold, sweet corn and celery 0.1-fold. Six worm-bed substrates led to a decrease in earthworm population after 30 days. Earthworms fed potato were reduced by 97%, celery by 26%, green bean by 17%, banana by 17%, fruit and vegetable mix by 11%, sweet potato by 3%.

The worm-bed substrates that produced living earthworms for 30 days all did so for another 30 days. Eight worm-bed substrates showed improved survival during the second month. The earthworm population fed sweet corn and celery increased 13.8-fold, green bean 7.5-fold, rockmelon 3.9-fold, lucerne hay 2.9-fold, cow manure 2.9-fold, banana 2.3-fold, sweet potato 0.8-fold, kelp meal 0.4-fold. Three worm-bed substrates continued to reduce earthworm population during the second month. Earthworms fed fruit and vegetable mix were reduced by 89%, paper by 8% and celery by 3%.

Five worm-bed substrates were lethal to earthworms, killing all added earthworms in the first 1 – 3 weeks of the experiment (Table 2.5). Earthworms fed spinach survived for only four days after the food was introduced to the system. Survival when blueberry was used was 10 days, and sweet corn was 26. Earthworms fed garden peas survived for an average of 10 days and a maximum of 13 days. Earthworms fed potato survived for 7 days in two replicates, with the third replicate showing survival after 30 days.

Table 2.4. Earthworm survival fed different worm-bed substrates.

	% Survival	
Worm-bed substrate	**30 days**	**60 days**
Apple	20 ± 3	25 ± 14
Banana	132 ± 43	122 ± 27
Blueberry	0 ± 0	
Celery	74 ± 14	97 ± 3
Cow manure	213 ± 33	220 ± 15
Fruit and vegetable mix	88 ± 8	11 ± 11
Garden pea	0 ± 0	
Green bean	65 ± 11	418 ± 233
Kelp	158 ± 44	113 ± 22
Lucerne	179 ± 36	329 ± 15
Potato	3 ± 3	
Paper	123 ± 19	100 ± 5.4
Rockmelon	199 ± 51	172 ± 54
Rockmelon and garden pea	0 ± 0	
Spinach	0 ± 0	
Sweet corn	0 ± 0	
Sweet corn and celery	106 ± 7	1453 ± 284
Sweet potato	97 ± 3	178 ± 43

Values shown are the mean ± SEM of three replicates. % survival 30 days was calculated based on a starting population of twelve. % survival 60 days was calculated based on the 30 day population.

Table 2.5. Time to death of all earthworms in triplicate worm farms supplied with lethal worm-bed substrates.

Substrate	**Time (days)**
Spinach	4 ± 0
Blueberry	10 ± 0
Garden pea	10 ± 2
Sweet corn	26 ± 0
Potato	15 ± 8

Values shown are mean ± SEM.

2.3.2 Effect of modifying worm-bed substrate on earthworm growth and survival

Combining garden pea with rockmelon to ameliorate the lethal effects of garden pea was unsuccessful, although survival improved over garden pea alone. The earthworms survived for a mean of 16 days and a maximum of 21 days. Combining sweet corn with celery to ameliorate the lethal effects of sweet corn was successful. The earthworms survived the 30 day trial period and increased in number. Neutralising acidic worm-bed substrates allowed earthworms to survive 30 days but decrease in number.

After 60 days earthworms fed sweet corn and celery had a significantly larger population than all other worm-bed substrates ($p < 0.001$). Green bean had a significantly larger population than apple, banana, celery, kelp meal, fruit and vegetable mixture and control (Figure 2.3). Lucerne hay had a significantly larger population than the fruit and vegetable mixture. No other differences were observed.

After 60 days earthworms fed celery had a higher total biomass than all other worm-bed substrates despite the small population. Celery produced few large earthworms. Earthworms fed sweet corn and celery had a higher total biomass than all worm-bed substrates except celery and green bean alone. Sweet corn and celery produced many small earthworms. Earthworms fed green bean had a higher total biomass than all worm-bed substrates except celery, rockmelon and sweet corn and celery. All worm-bed substrates except apple and the fruit and vegetable mixture had a greater total biomass than the control ($p < 0.001$) (Figure 2.4).

As all vermicompost units began with populations of different weights, the fold change in weight over 60 days is shown in Figure 2.3. Green bean and rockmelon showed higher comparative weight gain than the control. The fruit and vegetable mixture showed greater weight loss than celery, sweet corn and celery, rockmelon, sweet potato.

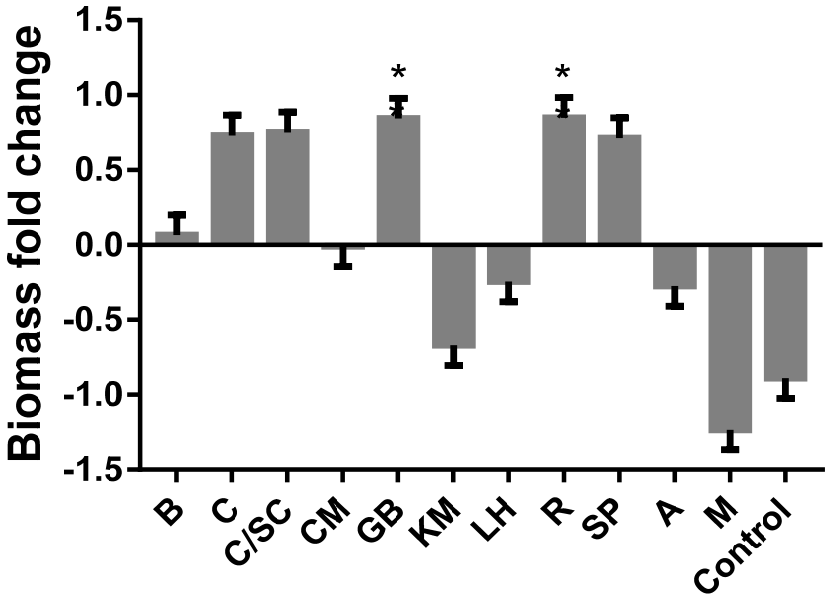

Figure 2.3. Change in total earthworm biomass after 60 days of being fed different worm-bed substrates. Foods were added to a newspaper base with an identical sized starting population. B-banana, C-celery, SC – sweet corn, CM-cow manure, GB-green bean, KM-Kelp meal, LH-lucerne hay, R-rockmelon, SP –sweet potato, A – apple, M – fruit and vegetable mixture, Control - newspaper. Plots of predicted means and SEM calculated using LMM analysis. * indicates value significantly greater than the control ($p < 0.05$).

The progress of lethal worm-bed substrates can be visualised for garden pea (Figure 2.4d), garden pea and rockmelon (Figure 2.4g) and potato (Figure 2.4h). Earthworm reproduction began at different times for different worm-bed substrates (Figure 2.4). Earthworms fed rockmelon began reproducing after 3 weeks. Earthworms fed sweet corn and celery, banana, lucerne began reproducing after 4 weeks. Earthworms fed cow manure, kelp meal began reproducing after 5 weeks, green bean after 6 weeks, sweet potato after 7 weeks. The juvenile earthworms fed paper did not begin reproducing during the 8 weeks shown. These worm farms were discontinued after 17 weeks, and no reproduction was observed.

Control and banana resulted in a decrease in population size. The banana population showed a drop of 30% during weeks 3-4 and a drop of 9% during weeks 6-7 (Figure 2.4g). Kelp meal underwent a slight population decrease during weeks 6-7 and 7-8 of 2% and 11%, respectively. For seven worm-bed substrates, the largest earthworm population was observed at 8 weeks. These were sweet corn and celery, green bean, sweet potato, banana, cow manure, lucerne, rockmelon. The kelp meal population peak was observed at 6 weeks before declining.

Total biomass of earthworms fed sweet potato increased weekly (Figure 2.4). Total biomass of earthworms fed green bean or sweet corn and celery underwent a drop or a plateau in biomass at 4 and 7 weeks (Figure 2.4a,c,f). This decrease corresponded with a small decrease in population size at the 4 week point. However, the population increased during weeks 6-7 for both worm-bed substrates. The highest biomass was recorded after 8 weeks. The control biomass decreased except during weeks 3-4 and 6-7 where there were small increases of 18% and 3%, respectively. The final biomass was 2-fold less than the initial biomass.

Some worm-bed substrates showed a drop in biomass in the first week (Figure 2.4g-k). Banana biomass decreased by 27% in the first week and decreased more slowly in subsequent weeks. During weeks 4-5 and 7-8 there was a small increase of 12% and 11%, respectively. The final biomass was 1.5-fold less than the initial biomass. Cow manure biomass decreased by 28% in the first week. The biomass fluctuated, with increases in weeks 2, 3, 5, 6, 8 and decreases in weeks 4 and 7. The final biomass was level with the initial biomass. Kelp meal biomass decreased by 28% in week 1 and continued to decrease at a smaller rate. There were small increases in weeks 4 and 5. The final biomass was 1.7-fold less than the initial biomass. Lucerne hay biomass decreased 35% in week 1. Weeks 2 and 4 saw smaller decreases. The rest of the time, lucerne hay biomass increased. The final biomass was 1.2-fold less than the start. Rockmelon biomass decreased by 30% in week 1. The only other decrease was in week 7. The rest of the time saw increases, with a large increase of 52% in week 8. The final biomass was 1.3-fold greater than the start. Paper decreased by 40% in week 1. There were very small increases in weeks 3 and 5, the rest of the time saw decreases. The final biomass was 3.4-fold less than the initial biomass.

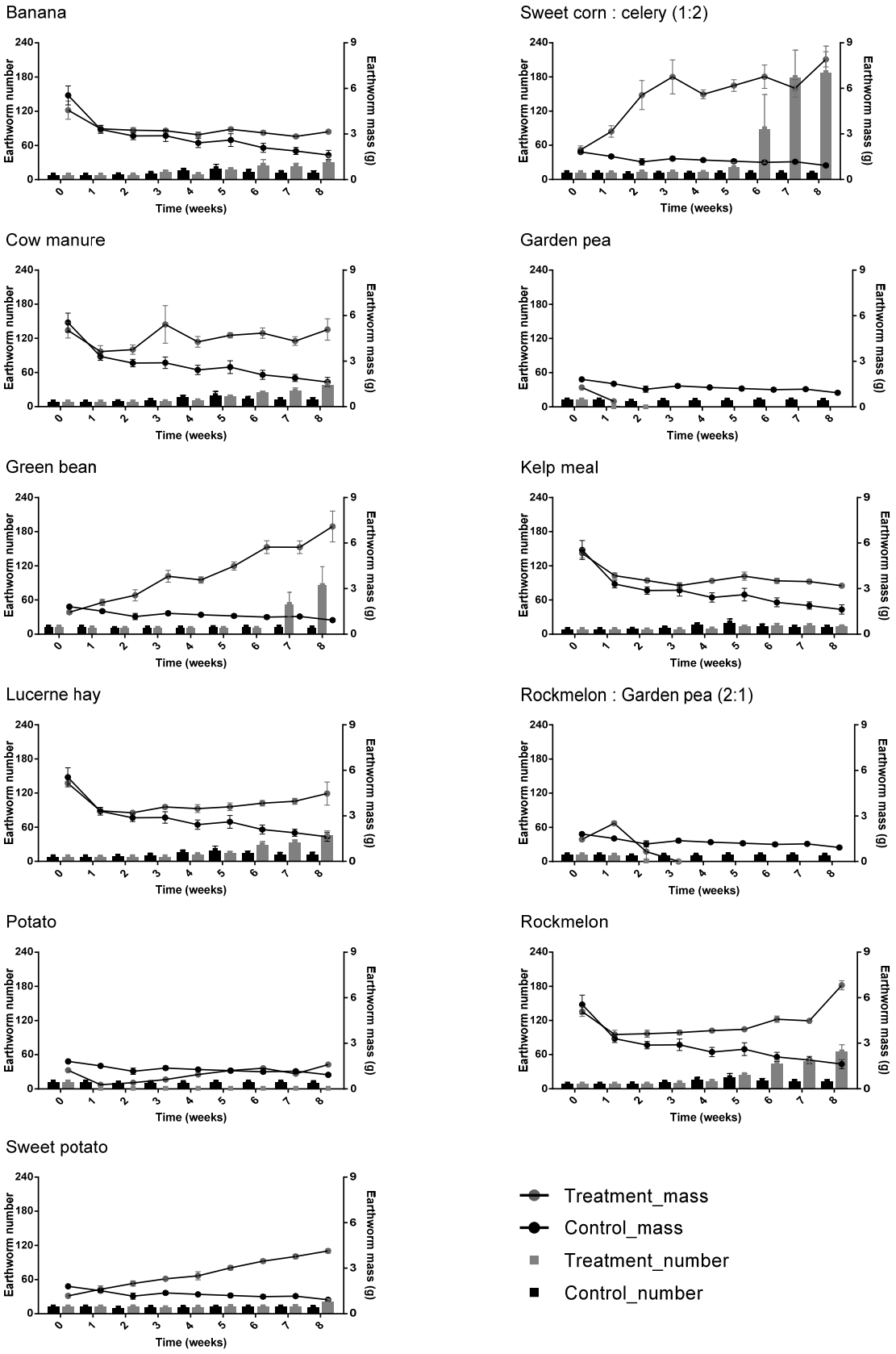

Figure 2.4. Changes to earthworm population and biomass when fed different worm-bed substrates. Values plotted are the mean and SEM of three replicates.

2.3.3 Effect of worm farm scale on growth and survival of earthworms

Larger worm farms were required so that larger quantities of leachate could be acquired for plant growth experiments. As expected, total earthworm mass was higher in larger worm farms. No significant variation was observed between the large and small control units when comparing the biomass change between week 0 and week 8.6 (Figure 2.5). Earthworm biomass decreased weekly, except week 3 in the small unit and week 5 in the large where there were increases of 18 and 15%, respectively. The final biomass of the small units was 15% and 49% of the initial biomass. The final biomass of the large units was 35% of the initial biomass.

Variation between week 0 and week 8 biomass was significantly different between large and small units fed celery ($p < 0.001$). Earthworm biomass increased 2.8-fold in the small units and 0.2-fold in the large units (Figure 2.5a). Biomass in large units decreased 22% from weeks 5 to 8. Biomass in small units increased 23% from weeks 5 to 8.

Variation between week 0 and week 8 biomass was significantly different between large and small units fed green bean ($p < 0.001$). Earthworm biomass of small units increased at each time point, except weeks 4-5 where there was a 7% decrease. The final biomass was 4.0-fold greater than the initial biomass. Earthworm biomass of large units increased at every time point with the final biomass 1.4-fold greater than the initial biomass.

Worm farm size had no significant effect on earthworm growth rate for control, green bean or celery ($p = 0.575$). Celery produced greater 8 week earthworm mass than green bean and control in both large and small units. Green bean produced greater 8 week mass than control in both large and small units. Worm farm size therefore had no effect on the comparative performances of the substrates.

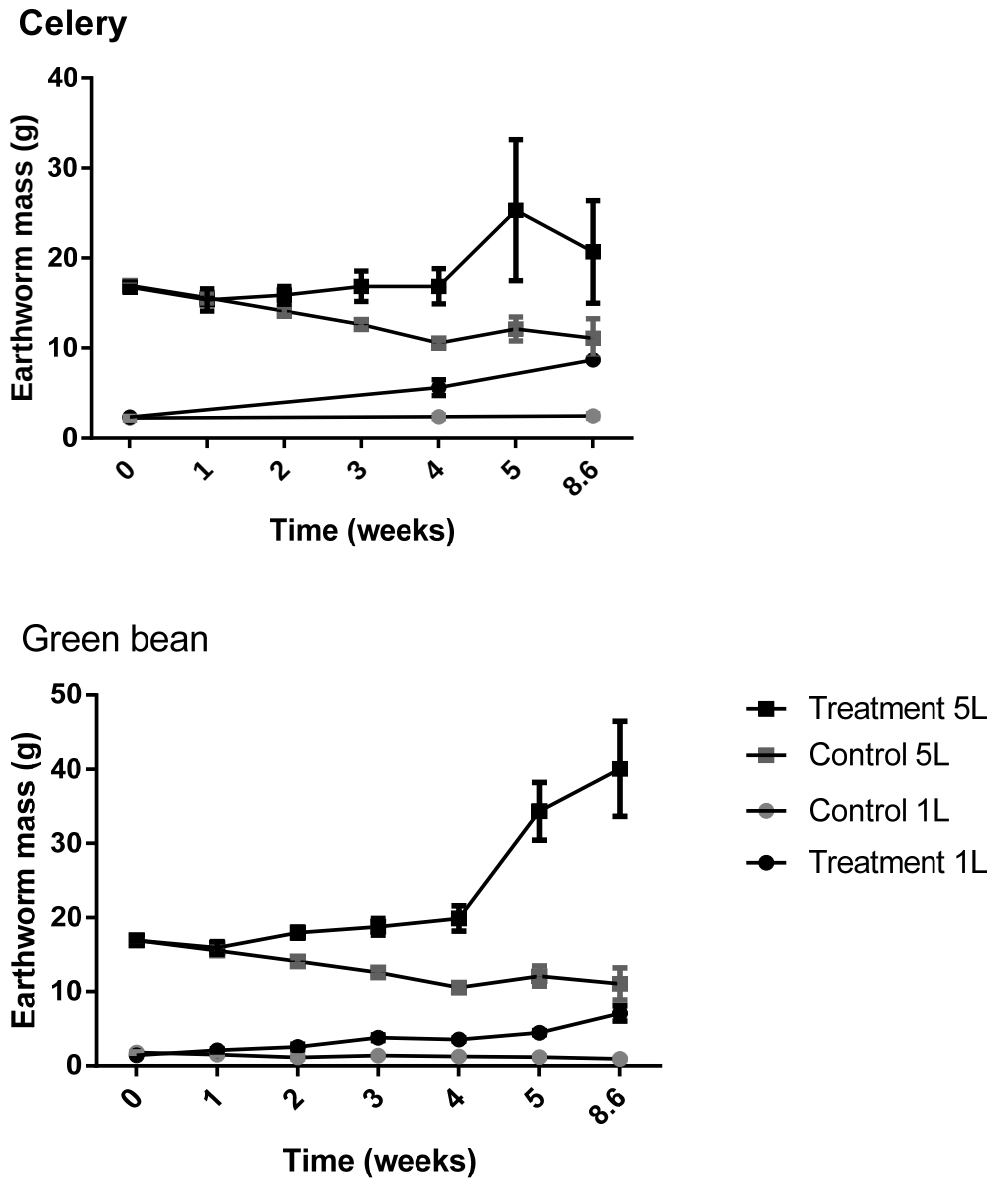

Figure 2.5. Changes to biomass of earthworms fed different worm-bed substrates in different sized worm farms. Worm farm capacity was 1 L or 5 L and earthworms were fed celery or green beans compared to a paper pulp control. Values plotted are means and SEM of three replicates.

2.3.4 Growth, survival and reproductive capacity of earthworms using selected worm-bed substrates in small scale worm farms

As expected, different worm-bed substrates had different effects on earthworms. Figure 2.7 shows the population changes, growth and cocoon production of *E. fetida* in the eight feeding treatments. In the control (P) biomass increased to a mean weight of 0.12 g per earthworm after 16 weeks. The mean starting weight was 0.10 g per earthworm (Table 2.6). In the banana treatment, biomass increased from 0.08 g to a mean weight of 0.13 g per earthworm. Biomass of earthworms fed rockmelon increased from 0.03 g to 0.21 g per earthworm. The other treatments produced a decrease in earthworm mass. Biomass decreased from 0.06 g to a mean of 0.05 g per earthworm in the green bean treatment. In treatment B/R, earthworm biomass decreased from 0.13 g to a mean of 0.04 g per earthworm. In treatment B/G, earthworm biomass decreased from 0.08 g to a mean of 0.01 g per earthworm. In treatment G/R, earthworm biomass decreased from 0.05 g to a mean of 0.02 g per earthworm. In treatment B/G/R, earthworm biomass decreased from 0.10 g to a mean of 0.02 g per earthworm.

In the control (P) total earthworm biomass declined after 16 weeks (Figure 2.7). Total biomass at 16 weeks was significantly lower than all other treatments. Other treatments produced a total biomass increase. In the banana treatment, 16 week total biomass was significantly higher than the control treatment but lower than all other treatments. In the green bean treatment, 16 week total biomass was significantly greater than banana and control treatments as well as mixtures B/G and B/R. The rockmelon treatment had the highest increase in total biomass, significantly greater than all treatments except green bean ($p < 0.001$). In treatment B/R, the 16 week total biomass was greater than B but less than G and G/R. In treatment B/G, 16 week total biomass was greater than P and B but less than G and G/R. In treatment G/R, 16 week total biomass was greater than B, R, B/G, B/R, B/G/R, P. In treatment B/G/R, 16 week total biomass was greater than P and B but less than G/R.

Population decline over time was observed in the control only, with the final population declined by 0.69-fold (Table 2.6). Total population at 16 weeks was significantly lower than other treatments (Figure 2.7). In the banana treatment, mean population increase after 16 weeks was 0.78-fold. The population at 16 weeks was significantly lower than the green bean treatment and mixtures B/G, G/R and B/G/R. In the green bean treatment, population size increased 10.47-fold and the final population was significantly greater than control, banana and rockmelon treatments as well as mixture B/R. In the rockmelon treatment, population size increased 4.25-fold over 16 weeks. The final population was greater than the control treatment but less than green bean treatments and mixtures G/R and B/G/R. In treatment B/R, the total population showed an increase 2.33-fold. The

final population was significantly lower than G, B/G, G/R and B/G/R. In treatment B/G, total population showed an increase 6.67-fold. The final population was significantly greater than P and B. Treatment B/G showed the highest growth rate, significantly higher than all other treatments ($p < 0.001$). In treatment G/R, total population showed an increase 8.94-fold. The final population was significantly greater than B, R, B/R, P. In treatment B/G/R, total population showed an increase 8.94-fold. The final population was significantly greater than P, B, R and B/R.

No cocoon or hatchling production was observed in the control. Reproductive indicators were observed in all other treatments. The treatments had no effect on time to cocoon formation but had an effect on the onset of reproduction as observed by hatchling appearance (Table 2.6). The banana treatment had the earliest population increase. Cocoons were observed after 7 weeks although a population increase indicating reproduction was observed after 1 week. Hatchlings appeared in the banana treatment significantly faster than green bean, rockmelon treatments and mixture B/G. In the green bean treatment the initial population increased after 5 weeks and cocoon production was observed after 7 weeks. Population increase prior to cocoon production was unexpected. Observations were conducted once per week. A likely explanation is that cocoons formed and hatched in the interim between observations, indicating time from cocoon production to hatching could be less than 7 days.

In the rockmelon treatment, cocoons were observed after 7 weeks and population increase began after 10 weeks. Time until hatchling appearance was significantly longer than the banana treatment and mixture B/G/R. In treatment B/R, cocoons were observed after 5 weeks. Population increase began after 6 weeks. In treatment B/G, cocoons were observed after 4 weeks. Population increase began after 7 weeks. In treatment G/R, cocoons were observed after 6 weeks. Population increase began after 5 weeks. In treatment B/G/R, cocoons were observed after 4 weeks. Population increase began after 4 weeks. Earthworms fed worm-bed substrate B/G produced more cocoons on average than those fed banana, rockmelon or mixtures G/R and B/R (Table 2.6). No other variation in cocoon per earthworm was observed.

Initial maturation of earthworms as judged by the appearance of clitella was observed after 1 week for every treatment except rockmelon, which was observed after 2 weeks (Figure 2.7). The time taken for the starting population to mature varied as detailed in Table 2.7. At 5 weeks, treatment B/G was the first to mature completely, significantly faster than banana and green bean treatments and mixtures G/R and B/R. At 9 weeks, the banana treatment was the last to mature completely, taking significantly more time than green bean and mixtures B/G and B/G/R. Treatments G/R, B/R

and B/G/R took 6 weeks to reach maturity, while green bean and rockmelon took 7 weeks. The control population failed to mature completely.

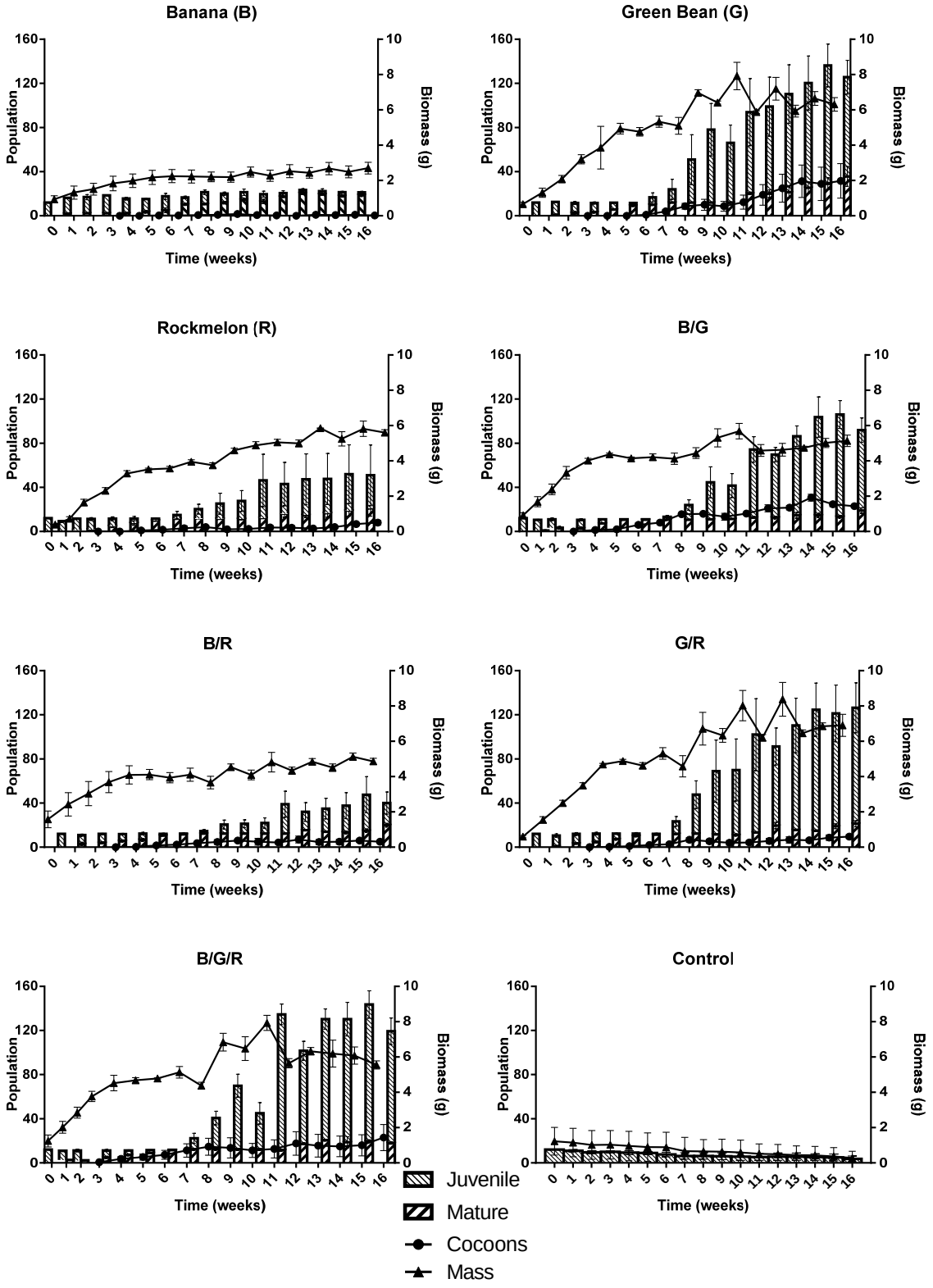

Figure 2.7. Changes to *E. fetida* population size, maturity, total earthworm biomass and cocoon production over time using different treatment worm-bed substrates and mixtures. Values plotted are mean and SEM of three replicates. LMM analysis showed significant treatment variation in population size and mass at 16 weeks ($p < 0.001$) but not cocoon number.

Table 2.6. Development of juvenile *E. fetida* fed different treatment worm-bed substrates.

		Banana (B)	**Green bean (G)**	**Rock-melon (R)**	**G/R**	**B/G**	**B/R**	**B/G/R**	**Control (P)**
Mean earthworm mass (g)	0 weeks	0.08	0.06	0.03	0.05	0.08	0.13	0.10	0.1
	16 weeks	0.13	0.05	0.21	0.02	0.01	0.04	0.02	0.12
Population fold change after 16 weeks		0.78	10.47	4.25	8.94	6.67	2.33	8.94	-0.69
Weeks to maturation of starting population (n = 12)		9 ± 1a	7 ± 0b	7 ± 0abc	6 ± 0.6ab	5 ± 0.3c	6 ± 0.3abd	6 ± 0bc	NA
Weeks to observation of cocoons		7 ± 1.5a	7 ± 0.3a	7 ± 0.7a	6 ± 0.9a	4 ± 0.3a	5 ± 0.3a	4 ± 0.5a	NA
Weeks to observation of hatchlings		1 ± 0c	5 ± 1.9ab	10 ± 2.5a	5 ± 1.7abc	7 ± 0ab	6 ± 1.9abc	4 ± 1.2bc	NA
Mean cocoons per earthworm		0.1 ± 0c	0.6 ± 0.1abc	0.2 ± 0c	0.3 ± 0.1bc	1.1± 0.2a	0.3 ± 0bc	0.9 ± 0.1ab	NA

One-way ANOVA completed, with different letters indicating significant differences ($p < 0.05$).

2.4 Discussion

2.4.1 Earthworm responses to a broad worm-bed substrate range

Earthworm growth and survival varied with worm-bed substrates provided. Some worm-bed substrates supported earthworm growth well, while others only supported earthworms for a limited time. Blueberry, the most acidic worm-bed substrate, supported earthworms for less than two weeks. This may be because the pH was too low for *E. fetida*, *E. andreii* and *P. excavatus*. To prevent this from reoccurring, subsequent acidic substrates (apple and the mixture) were neutralised before use.

Neutralised apple was also a poor worm-bed substrate, indicating that pH alone does not determine substrate efficacy. Apple pulp, or the waste from juicing, is a substrate commonly used by worm farmers (Frederickson, 2002; Hanc & Chadimova, 2014). In this study, apple reduced earthworm biomass after one month. A similar drop was observed by Hanc & Chadimova (2014) after one month followed by a subsequent increase. Hanc & Chadimova (2014) attribute the drop to stress from a new environment and the increase to earthworms adapting to that environment. In their study, substrate was prepared for six weeks, and involved pre-composting apple pulp with straw and cow manure. The purpose was to increase apple pH (Hanc & Chadimova, 2014). However, pre-composted substrate reduced earthworm reproduction by half (Frederickson, 2002). Apple, despite its abundance as an organic waste (Hanc & Chadimova, 2014) is a less than ideal substrate for vermicomposting.

Earthworm responses to mixtures of fruits and vegetables were complex. Mixtures provided better support than some worm-bed substrates during the first month, but earthworm survival declined in the second month. Testing these substrates was further complicated by the variation in pH. Neutralisation of low pH worm-bed substrates (mixture and apple) with sodium bicarbonate may have produced confounded results because of excess sodium. However, the same amount of sodium added to apple did not reduce survival, although survival with apple was consistently low. Contreras-Ramos, Escamilla-Silva, & Dendooven (2005) found no effect of high sodium substrate on *E. fetida*. Celery was the highest sodium substrate used in this study and survival of earthworms was less than one hundred percent after two months, although biomass increased. It is likely that multiple factors caused the mixtures tested here to be less than ideal worm-bed substrates.

Potato and sweet potato were tested in this study to represent substrate with high carbohydrate content. Only sweet potato supported earthworms well. Raw whole sweet potato contains higher

carbohydrate concentration than potato (United States Department of Agriculture, 2017). The nature of the carbohydrates differ, with raw potato containing over two times more starch than raw sweet potato (Scott-Dixon & Pierre, 2017). This starch may be responsible for the poor performance of potato, although several earthworm species, including *E. fetida*, are able to digest starch (Lattaud et al., 1998; Ueda et al., 2008). Adding carbohydrate to cow manure improved *E. fetida* biomass at low concentrations, but had a detrimental effect at high concentrations (Morgan, 1988).

Raw potato was largely lethal in this study. Cooked potato, while not lethal to *E. fetida*, remained mostly uneaten and had to be removed from vermicompost units (Frederickson, 2002; Neuhauser et al., 1980). Frederickson (2002) reported large-scale movement of *E. fetida* out of open vermicompost units after being fed potato. This was attributed to heavy rain causing damp and anaerobic conditions. However, the substrate may have contributed to earthworms leaving the units. In this study, potato contained living earthworms for two weeks on average. In the study by Frederickson (2002), cooked potato waste led to earthworm population decline, with increases beginning after eight months. This increase was attributed to hatchlings forming after eight months. The worm-bed substrate used by Frederickson (2002) was a by-product of potato chip manufacture and was chosen partly because each batch was expected to have consistent composition. Substrate was characterised prior to use, however variation between batches was not tested. In addition, Windust (1997) stated that potato waste is less than ideal for vermicompost production due to the possible spread of potato disease or pests such as nematodes.

Cow manure was included in initial experiments in this study because of its frequent use in vermicompost (Table 1.1). Cow manure supported earthworms well, but was not the best performing worm-bed substrate (Figure 2.4). Earthworm population and biomass increased, and were highest at eight weeks. Similar increases in *E. fetida* (Reinecke & Viijoen, 1990) and *E. andreii* (Elvira et al., 1998) biomass were shown over two months using cow manure. Cow manure is a widely used substrate in the literature (Ansari, 2008; Ayyobi et al., 2014, 2013; Singh et al., 2010; Tejada et al., 2008) and is used in some commercial vermicompost operations (Arancon et al., 2005; Vanmathi & Selvakumari, 2012). Many researchers used composted manure (Garcia-Gomez et al., 2008; Gutiérrez-Miceli et al., 2008; León-Anzueto et al., 2011; Oliva-Llaven et al., 2010; Quaik et al., 2012a) although the presence of pathogens such as *Clostridium*, total coliforms and Enterobacteria in the final product is a possibility (Aira et al., 2011). Faecal contamination in sewage sludge substrate has been shown to persist in the vermicompost (Grantina-Ievina et al., 2013) and the same may be true of cow manure.

Newspaper worm-bed substrate provided adequate earthworm support. Newspaper was non-lethal (Table 2.5) but didn't lead to weight gain or population increase (Figure 2.4). Gupta & Garg (2009) tested various ratios of cow manure and paper waste. Earthworm mass and growth rates were highest using cow manure only, biomass decreasing significantly with the addition of paper. In contrast, Elvira et al. (1998) observed an initial increase in earthworm mass using paper waste substrate although both mass and population later declined. Paper waste is used in many commercial operations (Aira et al., 2011; Arancon, Edwards, & Bierman, 2006; Arancon et al., 2005, 2004, 2006; Sinha et al., 2002) and is considered a substrate well-suited for vermicomposting (Frederickson, 2002). Paper waste consists of cellulose fibres, synthetic polymers, starch, clay and inorganic additives (Yasir et al., 2009) and may be different from the pureed newspaper worm-bed substrate used in this study. Newspaper may have enough nutrients to allow earthworms to survive, but not enough to promote reproduction. While paper had no negative effects on survival, more nutritious substrates are preferable.

In this study sweet corn was selected for its high P concentration (United States Department of Agriculture, 2017) in order to produce a high P leachate which would be beneficial to plants. Unfortunately it did not support growth and survival of *E. fetida*, *E. andreii* and *P. excavatus*. It is not known whether high P was a likely contributor to earthworm decline. A study by Neuhauser et al., (1980) found corn cobs mixed with soil allowed *E. fetida* to survive and promoted survival and weight gain of *Lumbricus terrestris,* although this study only ran for two weeks.

Many of the worm-bed substrates used in this study have not been tested previously in vermicompost. Kelp meal is used as a commercially available organic amendment due to the presence of plant growth hormones and N (Jack et al., 2011; Quilty & Cattle, 2011). *D. potatorum* formed the basis of the first commercial seaweed amendment produced in Australia (Arioli, Mattner, & Winberg, 2015). Seaweeds show plant growth stimulating action and their use in crop production is well established (Khan et al., 2009). Seaweeds can increase crop yield and improve soil structure and microbiology (Arioli et al., 2015). They contain macro and micro-nutrients although many chemical components and their mode of action remain unknown (Khan et al., 2009). Kelp has been used as a compost extract additive. Kelp, humic acid and rock dust have been shown to increase culturable microbes and suppression of *Pythium ultimum* (Scheuerell & Mahaffee, 2004). This study shows that *D. potatorum* can be used as a substrate in vermicomposting, although it is not an optimum substrate, and whether this will confer the reported benefits of seaweed extracts onto leachate is untested.

Three high protein worm-bed substrates were selected in this study, green bean, garden pea and spinach. Green bean provided good support, with increases in population and mass. Garden pea and spinach did not support earthworms. These results may be due to green bean containing the least protein of the three (United States Department of Agriculture, 2017). Combining non-supportive substrates with those providing good support produced variable results. When garden pea was combined with rockmelon earthworm growth and survival was poor. In contrast, sweet corn, which was poor on its own, out performed other worm-bed substrates when combined with celery.

Lucerne hay provided good earthworm support (Table 2.5.), indicating that in addition to bedding, this substrate provides nutrition. Compared with newspaper, lucerne hay may be a preferable bedding material for vermicompost as it supplies more nutrients between feedings. However, intact lucerne hay may contribute differently to earthworm survival than the puree used in this study.

Changing the scale of worm farms effects the earthworm response for some measurements. In this study, upscaling vermicompost units reduced weight gain for earthworms fed celery and green bean. Similar findings were reported by Suthar (2010), with larger scale units reducing mass and cocoon production of *E. fetida* fed sewage sludge and sugarcane waste. Elvira (1998) observed similar reduced growth and reproduction for upscaled *E. andreii* units fed cow manure, paper waste and dairy sludge. Elvira (1998) attributed these differences to lack of temperature control in upscaled units, however many other factors may contribute, including earthworm density, moisture and aeration.

2.4.2 Earthworm response to banana, green bean and rockmelon

Different worm-bed substrates can result in large changes in the size of earthworm populations. Focusing on banana, green bean and rockmelon showed that substrate also influences reproduction onset. To more accurately compare worm-bed substrate effect on reproduction, only *E. fetida* earthworms were used in these studies. It was not expected that this would have an effect on vermicomposting efficiency as *E. fetida* dominated the mixture. Neuhauser, Loehr, & Malecki (1988) showed that a mixed population of earthworms had no advantage over single species in breaking down sewage sludge.

The three substrates were applied individually and in mixtures and no variable effect on the number of cocoons was observed after 16 weeks, whereas the paper control produced no cocoons. Gupta & Garg (2009) also observed no cocoons from *E. fetida* fed paper waste. Elvira et al. (1998) did observe

low level cocoon production from *E. fetida* fed paper waste after 56 days. While substrate doesn't affect cocoon production, different volumes of the same substrate effect *E. fetida* cocoon production (Hand et al., 1988). In this study, hatchlings were observed prior to cocoons in banana and green bean treatments. This indicates that cocoon production to hatching could be less than seven days. Cocoon data is therefore unreliable as many cocoons likely formed and hatched between weekly observations. Reproduction onset as judged by hatchling appearance took between 1 to 10 weeks and varied with worm-bed substrate. Much later reproduction onset (24 weeks) of *E. fetida* fed potato waste was reported by Frederickson (2002).

Earthworm biomass increased with time for all worm-bed substrates except the control. This increase was not always constant and did not always correlate with population number. Frederickson (2002) observed a similar pattern of peaks and troughs in earthworm total biomass using potato waste as a substrate. The mean mass per earthworm decreased for all worm-bed substrates except paper, a pattern also observed by Chaudhuri et al. (2003) when *E. fetida* were fed leaf litter. The weight loss observed in this study was likely due to limitations in available space and food.

The eight worm-bed substrates in this trial caused significant differences in total earthworm biomass and total earthworm population size after 16 weeks. Taken separately, the suitability of each worm-bed substrate in descending order are as follows.

For biomass

G/R > R > G > B/G/R, B/R, B/G > B > P

For population

G, G/R, B/G/R > B/G, R > B, B/R > P

A mixture of green bean and rockmelon may have contained optimum protein and carbohydrate content for earthworm consumption, as well as ideal moisture level and pH. Biomass and population size are important for ensuring optimum substrate consumption (Kale, 2004; Munroe, 2007) but it is unknown whether either measurement is more important. Worm-bed substrate blends showed no advantage over single substrates. Worm-bed substrate G/R achieved maximum biomass and population over 16 weeks. The features of the substrate that led to this result are difficult to determine. High substrate N can elevate reproduction (Windust, 1997) and too much or too little substrate moisture can inhibit reproduction (Neuhauser et al., 1988) and impede earthworm growth (Edwards & Arancon, 2004). Worm-bed substrates too high in inorganic salts or ammonia, including many animal wastes, can be lethal to earthworms (Edwards & Arancon, 2007). Different earthworm species experience differences in weight gain when fed the same substrate (Chaudhuri et al., 2003). Hatching also varies with temperature (Jefferies & Audsley, 1988).

2.5 Conclusion

A limited diet made of a single worm-bed substrate will generally support earthworms. The degree of support varies with substrate and should be tested on a small scale prior to use. A diet of worm-bed substrate blends will also generally support earthworms. The degree of support varies, with a blend not necessarily out-performing single substrates. A 2:1 mixture of celery and sweet corn provides optimum support for a blend of *E. fetida*, *E. andreii* and *P. excavatus*. A 1:1 mixture of green bean and rockmelon provides optimum support for *E. fetida*. These observations are likely to vary with different vermicompost conditions such as earthworm species and temperature. These observations are unlikely to vary with worm farm scale.

Chapter 3 - Effect of worm-bed substrate on leachate microbes using small scale worm farms

3.1 Introduction

Earthworms consume living and decomposing plant material, animal waste, and microorganisms. Some microbes are digested, while others may survive and multiply during passage through the earthworm gut (Sims & Gerard, 1985) passing out with the castings into the vermicompost (Windust, 1997). Earthworms supply fresh organic matter in their mucus, and increase decomposition by facilitating contact between microbes and substrates in their gut (Bernard et al., 2012). The community of culturable earthworm gut microbes differs from the surrounding environment and from earthworm excrement microbes (Byzov et al., 2009).

Several studies have shown that vermicompost addition enhances soil microbe abundance. Adding cow manure vermicompost to saline alkaline soil collected from Tanghai County in China increased soil microbial biomass over chemical fertiliser addition (Wu et al., 2013). Adding buffalo manure vermicompost to alkaline sandy soil collected from the red river delta in Vietnam increased culturable bacteria numbers compared with chemical fertiliser (Doan et al., 2013). Adding sugarcane waste vermicompost to clay loam soil, sandy loam soil and red loam soil increased culturable bacteria numbers in all three soil types compared with no amendment (Parthasarathi et al., 2008). Of particular interest in the study by Parthasarathi et al. (2008) was the finding that addition of chemical fertiliser significantly reduced culturable bacteria numbers in all three soil types. This reduction was observed in comparison with both no amendment control and vermicompost amendment.

The effect of transplanting amended soil was tested in a study by Jack et al. (2011). A mixture of peat moss and vermiculite was amended with four different organic amendments; cow manure vermicompost, conventional cow manure compost, sesame meal or alfalfa meal. Jack et al. (2011) grew tomatoes in amendment filled seedling trays then transplanted plants into unamended Howard gravelly loam soil in a field trial in New York, USA. Transcription Fragment Length Polymorphism (T-RFLP) analysis showed that transplantation from amended soil changed the population diversity of the rhizosphere microbes in the field. The changes observed from conventional compost, sesame and alfalfa meal were of short duration. The rhizosphere microbes reverted to the original population diversity after one month. However, the changes brought about

by vermicompost amendment remained for the duration of the trial, which was over 37 days (Jack et al., 2011) although the exact duration was not given.

Several studies have shown that vermicompost substrate affects microbial communities. Single strand conformation polymorphism was used to compared bacterial population diversity of vermicompost from pig manure, cow manure and chicken manure. The three substrates contained different bacterial populations (Fracchia et al., 2006). Denaturing gradient gel electrophoresis (DGGE) and the microarray method COMPOCHIP were used to compared bacterial population diversity of vermicompost from tomato fruit, winery waste and a mixture of olive-mill waste and sewage sludge. The three substrates contained different bacterial populations (Fernández-Gómez et al., 2012).

Microbes isolated from vermicompost can accelerate the breakdown of organic waste and increase the nutrient content of compost (Mahanta et al., 2014), inhibit growth of fungal pathogens (Marín et al., 2013) and increase plant growth, grain yield and rhizosphere soil nutrients (Gopalakrishnan et al., 2014). However, vermicompost microbial communities have generally not been well characterised, and most studies use culture based techniques (Aira et al., 2011; Grantina-Ievina et al., 2013; Mahanta et al., 2014) which represent only a small portion of microbial diversity (Neher et al., 2013). Sequence identification of dominant culturable microbes identified potential growth promoting microbes as well as potential plant pathogens in vermicompost (Grantina-Ievina et al., 2013).

Some studies have investigated the microbes in liquid extract of vermicompost. This liquid was extracted by immersing commercial vermicompost, which was free of earthworms, in water for several days (Fritz et al., 2012; Marín et al., 2013). Given the differences in microbial community between earthworm guts and castings, this earthworm-free liquid extract is likely to have a different microbial composition to leachate which is collected by passing water through worm farms. No studies to date have been carried out on microbes in leachate. Such studies are necessary as leachate is frequently applied to soil and plants.

The aim of this work was to investigate leachate microbial populations. To determine whether changing worm-bed substrate and vermicomposting time affects the community structure of leachate microbes. The effect of storage temperature after collection of leachates on microbial diversity was also investigated. T-RFLP analysis was used to compare microbial diversity in leachate prepared from different worm-bed substrates and over time. Illumina Next-Generation Sequencing (NGS) was used to identify microbes in some leachates. The null hypothesis for these experiments was that leachate microbial diversity will not vary with different organic worm-bed substrates.

3.2 Methods

3.2.1 Use of T-RFLP to analyse six worm-bed substrates at two time points

3.2.1.1 Vermicompost

Small scale worm farms were established and maintained as described in section 2.2.1. Three independent replicates per worm-bed substrate were established. To remove a potential source of microbial contaminants, no sand was added to any worm farms described in this chapter. Worm-bed substrates used are listed in table 3.1.

Table 3.1. Vermicompost substrates fed to *Eisenia fetida*, *Eisenia andreii* and *Perionyx excavatus*.

Substrate	Treatment	Source
Banana	Whole, pureed	IGA Alexandria, Australia
Green bean	Frozen, whole, pureed	Birds Eye Mentone, Australia
Rockmelon	Whole, pureed, filtered	IGA
Celery	Whole, pureed, filtered	Redfern Fruit Market Redfern, Australia
Sweet potato	Whole, pureed	Redfern Fruit Market
Control	Newspaper pulp	NA

3.2.1.2 Leachate harvest

45 mL of leachate from each worm farm was collected by flushing worm farms with 50 mL of distilled water. Leachates were stored in plastic screw-capped tubes at -80°C prior to analysis. Leachates were sampled 30 days and 60 days after addition of substrate. Sweet corn and celery units were sampled at 30 days only. No substrate was added in the week prior to sampling to ensure that leachate was collected from composted material only.

3.2.1.3 DNA extraction and T-RFLP

Leachates were thawed and cells from 4 mL harvested at 20 000 g for 3 min in an Eppendorf™ 5424 Microcentrifuge (Eppendorf, Hamburg, Germany). Cells were resuspended in 250 µL supernatant. Cells were lysed with the PowerLyzer™ 24 Homogenizer (MoBio, Carlsbad, USA) at 2000 rpm for 5 min. DNA was extracted using the PowerLyzer™ PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, USA) following the manufacturers' instructions. DNA was eluted in 100 µL Tris and stored at -20°C. DNA was visualised by agarose gel electrophoresis using a 1% gel in 1 X TAE buffer. The gel contained 0.01 µg/mL ethidium bromide (Sigma-Aldrich, Castle Hill, Australia) and DNA was visualised by exposure to UV light. DNA was quantified using the NanoDrop™ 2000C Spectrophotometer (Thermo Scientific, Waltham, USA). For amplification of the bacterial 16S ribosomal RNA (rRNA) gene, primers 27F-YM and 1492R were used. For amplification of fungal internal transcribed spacer (ITS), primers ITS1 and ITS4 were used. Primer sequences used in this study are listed in Table 3.2. The forward primers were labelled at the 5' end with fluorescent dyes, 27F-YM with NED™ and ITS1 with 6-carboxyfluorescein (FAM). Primers were synthesised by Integrated DNA Technologies (IDT, Baulkham Hills, Australia). Each PCR contained 1 µL of DNA, 1 X MyTaq™ Red Buffer, 0.4 µM of each primer and 0.5 U MyTaq™ Red DNA polymerase (Bioline, Eveleigh, Australia). Reaction conditions consisted of initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 57°C for 1 min and 72°C for 5 min. PCRs were run in a C1000 Touch™ Thermal Cycler (Bio-Rad, Gladesville, Australia). Amplicons were visualised by electrophoresis, as previously described, using a 1.5% agarose gel. To purify amplicons, 22 µL PCR reaction was mixed with 2.2 µL 3 M sodium acetate (pH 5.2) and 44 µL absolute ethanol (Merck, Frenchs Forest, Australia). Following incubation on ice for 30 min, DNA was pelleted by centrifugation at 20 000 g for 20 min in an Eppendorf™ 5424 Microcentrifuge. DNA was washed in 500 µL 70% (v/v) ethanol in water, followed by repeat centrifugation for 10 min. Dried DNA was resuspended in 22 µL sterile water. Purified amplicons were quantified using the NanoDrop™ 2000C Spectrophotometer (Thermo Scientific, Waltham, USA). Purified amplicon (600 ng) was digested with 10 U *HhaI* (Promega, Alexandria, Australia) in a 15 µL reaction volume at 37°C for 3.5 h. Purified digested amplicons were submitted to the Australian Genome Research Facility (AGRF, Westmead, Australia) for Capillary Separation using the size standard LIZ500.

Table 3.2. Primer sequences.

Name	Sequence	Reference
27F-YM	5' – AGAGTTTGATYMTGGCTCAG - 3'	Frank et al. (2008)
1492R	5' – GGTTACCTTGTTACGACT T - 3'	
ITS1	5' – TCCGTAGGTGAACCTGCGG - 3'	White et al. (1990)
ITS4	5' – TCCTCCGCTTATTGATATGC - 3'	
Pro341F	5'-AATGATACGGCGACCACCGAGATCTACA**ATCGTACG** CGTCGAGCTGACTCCCGCATCTGGTAGA-3'	Modified from Takahashi et al. (2014)
Pro805R	5'-CAAGCAGAAGACCGCATACGAGATAACT**AACTCTCG** GCCTGACTGCCACGCGATAGGCCTCCGA-3'	

3.2.1.4 Data Analysis

Raw data was retrieved and the size of the T-RFs determined using Peak Scanner ™ v2.0 from Applied Biosystems® (Foster City, USA). T-RFLP analysis Expedited (T-REX) (Culman et al., 2009) was used to find true peaks and eliminate background noise (Abdo et al., 2006) then align peaks (Smith et al., 2005). The diversity of the T-RFLP profiles was analysed by the Bray-Curtis dissimilarity index (Bray & Curtis, 1957) for presence-absence data using Past v3.02a (Hammer, Harper, & Ryan, 2001). Non-metric Multidimensional Scaling (nmMDS) ordination plots, on the Bray-Curtis similarity matrix, were used to visualise dissimilarities between communities. Differences between communities were measured using one-way analysis of similarity (ANOSIM) on the Bray-Curtis distance matrices including 10 000 permutations with significant differences considered as $p < 0.01$ (Clarke, 1993).

3.2.2 Use of T-RFLP to study short-term storage effect on leachate microbes

Leachates from four substrates were stored under different conditions for a short time. T-RFLP was used to compare microbial populations after storage. The aim of this work was to determine whether leachate storage conditions affect microbial population diversity.

Small scale worm farms were established and maintained as described in section 2.2.1. Substrates, green bean and sweet potato (Table 3.1) were compared with the control. Three independent replicates per worm-bed substrate were established. Leachate harvest from each worm farm was carried out as in section 3.2.1 at 30 days only. Leachates were divided into three 15 mL aliquots and

stored in plastic screw capped tubes. Leachates were stored indoors at ambient temperature, 4°C and -80°C for six weeks. DNA extraction and T-RFLP analysis were carried out as in section 3.2.1.

3.2.3 Use of T-RFLP to compare leachate microbes from different worm farms fed the same substrate

The experiments described in this chapter involved triplicate worm farms fed the same worm-bed substrate established at the same point in time. It was not known whether leachate microbiology would be the same using an entirely different set of worm farms fed the same substrate. It was not known whether worm farms established in May would produce similar leachate microbiology to worm farms established in August. The aim of this work was to determine whether leachate microbes from the same substrate were stable across different worm farms.

Microbial diversity was measured using T-RFLP from two sets of worm farms supplied with green bean substrate. Green bean was selected to use as a substrate in this experiment from previous results that indicated good earthworm survival. Small scale worm farms were established and maintained as described in section 2.2.1. Independent replicate worm farms were established in triplicate in May 2014. Three months later, a new independent set of replicate worm farms were established in triplicate. Leachate harvest was carried out as described in section 3.2.1 at 30 days. Leachates were stored in plastic screw capped tubes at -80°C prior to use. DNA extraction and T-RFLP analysis were carried out as described in section 3.2.1.

3.2.4 Use of T-RFLP to compare leachate microbes from different size worm farms

Larger vermicompost units were needed to obtain enough leachate for plant application. Two different sized worm farms were fed the same substrate and the leachate microbes analysed. The aim of this work was to determine whether changing the size of the units effects the diversity of the leachate microbial population.

Small and large scale worm farms were established and maintained as described in section 2.2.3. Substrates used were celery, green bean and control (Table 3.1). Three independent replicates per worm-bed substrate were established. 50 mL of leachate was collected by flushing worm farms with 60 mL of distilled water. Leachate was harvested after 60 days. No fresh substrate was added in the week prior to harvest. Leachates were stored in plastic screw-capped tubes at -80°C. DNA extraction and T-RFLP analysis was carried out described in section 3.2.1.

3.2.5 Use of T-RFLP to compare leachate microbes over time

Four substrates were used to analyse the differences between initial leachate microbes and the populations after composting. The aim of this work was to determine whether the leachate microbial population changes over time.

Small and large scale worm farms were established and maintained as described in section 2.2.3. Substrates celery and green bean were compared with the control (Table 3.1). Three independent replicates per worm-bed substrate were established. Biological replicates of leachate were harvested as shown in Figure 3.1. Leachate (50 mL) was collected by flushing worm farms with 60 mL of distilled water. Leachate was harvested after 60 days. No fresh substrate was added in the week prior to harvest. Leachates were stored in plastic screw-capped tubes at -80°C. DNA extraction and T-RFLP analysis was carried out as described in section 3.2.1.

3.2.6 Use of Illumina NGS to identify leachate bacteria

The information provided by both published studies and the current study of microbial diversity are limited. The aim of this work was to identify major bacterial genera from a subset of leachates.

3.2.6.1 Vermicompost

Small scale worm farms were established and maintained as described in section 2.2.1. Substrates banana, green bean, rockmelon were compared to a paper pulp control (Table 3.1). Three independent replicates per worm-bed substrate were established. Biological replicates of leachate were harvested as shown in Figure 3.1. Leachates were harvested by flushing units with deionised water. At time 0, immediately after first substrate addition, 50 mL water was used to collect 45 mL leachate. At 30 days and 60 days, 50 mL water was used to collect 45 mL leachate. Leachates were stored in plastic screw capped tubes at -80°C. Two biological replicate leachates were sampled from each worm farm (0 days and 60 days) DNA extraction and T-RFLP analysis was carried out as described in section 3.2.1.

3.2.6.2 Illumina NGS

DNA extraction was carried out as described in section 3.2.1. DNA was quantified using the Quant-iT™ PicoGreen® dsDNA Assay kit (Life Technologies, Carlsbad, USA). The V3-V4 region of 16S rDNA was amplified using modified universal prokaryotic primers Pro341F and Pro805R. In addition to the V3-V4 specific priming regions, these primers were complementary to standard Illumina forward and reverse primers. The reverse primer also contained a 6-bp indexing sequence to allow for multiplexing. Primer sequences are detailed in Table 3.2 with the Illumina adapter underlined and

the index sequence in bold. PCR reactions contained 10 ng DNA template, 1 X MyTaq™ Reaction Buffer, 0.2 µM of each universal primer, 0.5 U MyTaq™ DNA polymerase (Bioline, Eveleigh, Australia). Reaction conditions consisted of an initial 95°C for 5 min followed by 20 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1.5 min and a final extension of 72°C for 5 min. PCRs were run in a C1000 Touch™ Thermal Cycler (Bio-Rad, Gladesville, Australia). The product of this reaction was diluted 50 X and 1 µL used as template for Illumina primers. Reaction conditions were the same as the previous PCR, with an annealing temperature of 65°C. PCR products were visualised by electrophoresis as in section 3.2.1, using a 1.5% agarose gel. Purified amplicon (5 ng) was submitted to Micromon (Clayton, Australia) for sequencing using the Illumina MiSeq.

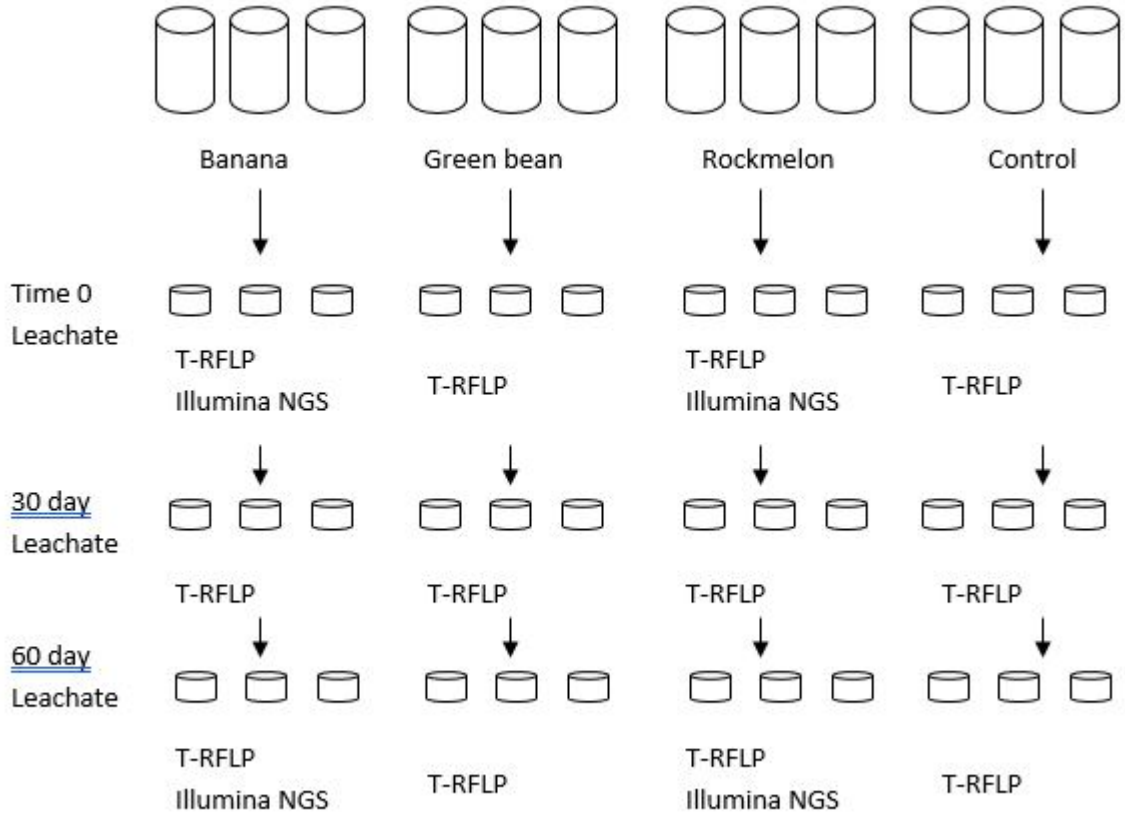

Figure 3.1. Experimental design and analysis of worm farm leachates from different substrates.

3.2.6.3 Data Analysis

The sequencing data was processed using Quantitative Insights into Microbial Ecology (QIIME) (Caporaso et al., 2010) version 1.9.1. Sequences were clustered in operational taxonomic units (OTUs) at 97% similarity using the open reference picking protocol. To normalise data for comparisons among different samples and to avoid the effect of variable sample size on the diversity analyses, a randomly subsampled data set of 4799 reads per sample was created, based on the lowest number of reads per sample.

3.3 Results

3.3.1 Leachate microbial diversity from different worm-bed substrates

Worm-bed substrate had no effect on species richness as indicated by the number of terminal restriction fragments (T-RFs) but did change the community structure as measured by T-RFLP. All worm-bed substrates showed distinct bacterial and fungal profiles and differentiation increased over time.

The number of T-RFs of leachate microbes from various worm-bed substrates are listed in Table 3.3. The number of T-RFs, which indicates species number and relative abundance, was not affected by substrate for banana, green bean, celery, rockmelon, sweet corn/celery or the control. There was no significant effect on bacterial or fungal T-RF numbers at 30 days and 60 days vermicomposting time.

Worm-bed substrate had an effect on microbial community structure of leachate as indicated by ANOSIM results (Table 3.4). Leachates from banana, green bean, celery, rockmelon, sweet corn/celery and control had significantly separate bacterial communities after 30 days. The low R value indicates the populations were separate but overlapping. All six worm-bed substrates had significantly different fungal communities at 30 days with some overlap. Similarly, bacterial and fungal communities for all worm-bed substrates were significantly different after 60 days. The increased R values indicate greater separation than the 30 day communities for bacteria and fungi.

Table 3.3. T-RFs from vermicompost leachate fed different worm-bed substrates.

	30 days		60 days	
Substrate	**Bacterial T-RFs**	**Fungal T-RFs**	**Bacterial T-RFs**	**Fungal T-RFs**
Banana	110 ± 21	14 ± 6	118 ± 27	34 ± 9
Green bean	93 ± 2	24 ± 2	80 ± 5	27 ± 3
Celery	126 ± 13	20 ± 2	139 ± 34	27 ± 3
Rockmelon	129 ± 10	19 ± 3	112 ± 12	22 ± 6
Sweet corn & celery	79 ± 10	18 ± 3	NA	NA
Control	113 ± 18	16 ± 4	120 ± 14	26 ± 1

Leachate harvested at 30 and 60 days and T-RFLP profiles of 16S rRNA bacterial and ITS fungal regions completed. Data shown are mean ± SEM of three replicates. One-way ANOVA showed no significant variation between substrates at each harvest ($p < 0.05$). Sweet corn & celery was not sampled at 60 days.

Table 3.4. Analysis of similarity of leachate microbial communities from banana, green bean, celery, rockmelon, sweet corn/celery and paper control.

	30 days		60 days	
	p	R	p	R
Bacteria	0.0001	0.4687	0.0001	0.6756
Fungi	0.0001	0.549	0.0001	0.7296

3.3.2 Leachate microbial diversity after short-term storage

Leachates from worm farms fed green bean, sweet potato and control were stored under different conditions for six weeks. Storage temperature had no effect on bacterial community structure of green bean leachate or control leachate. Storage of sweet potato leachate at -80°C, 4°C and room temperature for six weeks resulted in significantly different bacterial communities. The low R value indicates these populations are separate but overlapping. Storage temperature had no effect on fungal communities in green bean leachate, sweet potato leachate or control leachate (Table 3.5).

Table 3.5. Analysis of similarity of leachate microbial communities under different storage conditions.

	Bacteria		Fungi	
	p	R	p	R
Green bean	0.0587	0.2593	0.0754	0.1564
Sweet potato	0.0177	0.394	0.9126	0.2428
Control	0.9201	-0.1728	0.7828	-0.1934

Species richness following six week storage at different temperatures is shown in Table 3.6. Storage temperature had no effect on bacterial species richness of sweet potato, control or green bean leachate. Storage temperature had no effect on fungal species richness of sweet potato or control leachate. Storage at 4°C significantly reduced fungal species richness in green bean leachate. No differences in fungal species richness were observed between green bean leachate stored at ambient temperature and -80°C.

Table 3.6. T-RFs from vermicompost leachate from different worm-bed substrates.

Substrate	Storage	Bacterial T-RFs	Fungal T-RFs
Green bean	Ambient	83 ± 6	26 ± 3a
	4°C	88 ± 13	9 ± 5b
	-80°C	93 ± 2	26 ± 3a
Sweet potato	Ambient	80 ± 5	23 ± 4a
	4°C	79 ± 11	26 ± 3a
	-80°C	78 ± 10	23 ± 2a
Control	Ambient	89 ± 5	5 ± 4a
	4°C	92 ± 17	6 ± 5a
	-80°C	98 ± 9	14 ± 6a

Leachates were harvested at 30 days then stored under different conditions for six weeks. T-RFLP profiles of 16S rRNA bacterial and ITS fungal regions were completed. Data shown are mean ± SEM of three replicates. One-way ANOVA completed with different letters indicating differences within substrate ($p < 0.05$).

3.3.3 Leachate microbial diversity from different worm farms fed the same worm-bed substrate

The microbial community structures in leachate from worm farms established at different times using green bean are unchanged. The results indicate that establishing the worm farms at different times using a different earthworm population but the same worm-bed substrate did not alter leachate microbial communities. Worm farms established in May 2014 showed the same bacterial ($p = 0.0984$, $R = 0.8889$) and fungal ($p = 0.1037$, $R=0.6667$) populations as worm farms established in August 2014. This similarity is indicated by the high p values which show no statistically significant differences. Bacterial and fungal species richness did not vary between these worm farms (Table 3.7).

Table 3.7. T-RFs from vermicompost leachate from replicate worm farms.

	Bacterial T-RFs	**Fungal T-RFs**
May 2014	113 ± 10	26 ± 9
August 2014	93 ± 2	24 ± 2

Number of T-RFs determined from T-RFLP profiles of 16S rRNA bacterial and ITS fungal regions from DNA extracted from leachate. Data shown are mean ± SEM of three replicates. One-way ANOVA showed no variation between replicates ($p < 0.05$)

3.3.4 Effect of worm farm size on leachate microbes

Different size vermicomposting units were used to analyse effect of scale on leachate microbes. Changing worm farm scale had no effect on bacterial or fungal population diversity of green bean leachate, celery leachate or control leachate. This similarity is indicated by the high p values which show no statistically significant differences (Table 3.8).

Table 3.8. Analysis of similarity of leachate microbial communities from different size worm farms.

	Bacteria		**Fungi**	
	p	R	p	R
Green bean	0.1028	0.9259	0.1035	0.9259
Celery	0.0962	0.7778	0.1018	0.6296
Control	0.094	0.7037	0.104	0.7407

Worm farm scale had no effect on bacterial species richness in green bean, celery or control leachate. Worm farm scale had no effect on fungal species richness in celery leachate. Scaling up worm farms significantly increased fungal species richness in green bean leachate and reduced fungal species richness in control leachate (Table 3.9).

Table 3.9. T-RFs from vermicompost leachate from different size worm farms.

Worm-bed substrate	Unit size	Bacterial T-RFs	Fungal T-RFs
Green bean	1 L	101 ± 6	6 ± 5a
	5 L	94 ± 6	28 ± 3b
Celery	1 L	160 ± 40	27 ± 3a
	5 L	103 ± 5	25 ± 12a
Control	1 L	134 ± 14	26 ± 1a
	5 L	95 ± 18	18 ± 1b

T-RFLP profiles of 16S rRNA bacterial and ITS fungal regions were completed. Data shown are mean ± SEM of three replicates. One-way ANOVA completed with different letters indicating differences within substrate ($p < 0.05$).

3.3.5 Leachate microbial diversity at different vermicomposting times

Leachate was collected from worm farms fed banana, green bean, rockmelon at three time points. Bacterial species richness as determined by T-RF numbers at time 0 was the same for all worm-bed substrates (Table 3.10). After 30 days, no change in bacterial species richness was observed for banana leachate. After 60 days, banana leachate showed increased bacterial species richness over the 30 day sample.

After 30 days, no change in bacterial species richness was observed for green bean leachate. After 60 days, green bean leachate showed reduced bacterial species richness compared to 30 days. No differences were observed between the 60 day sample and time 0 for green bean leachate. After 30 days, rockmelon leachate showed increased bacterial species richness than at time 0. This increase was also observed at 60 days. Control leachate showed no changes in bacterial species richness over the three time points.

After 30 days, rockmelon leachate showed increased bacterial species richness compared to banana, green bean and the control; and green bean leachate showed increased bacterial species richness compared to banana. After 60 days, rockmelon leachate showed increased bacterial species richness compared to green bean; and banana leachate showed increased bacterial species richness compared to green bean and the control. Fungal species richness as determined by T-RF numbers at time 0 was the same for all worm-bed substrates. No changes in fungal species richness were observed over time or between substrates (Table 3.10).

Table 3.10.T-RFs from leachate fed different worm-bed substrates.

Substrate	**Number of bacterial T-RFs**			**Number of fungal T-RFs**		
	0 day	30 day	60 day	0 day	30 day	60 day
Banana	66 ± 6c	57 ± 3c	118 ± 27a	31 ± 4	23 ± 0	32 ± 4
Green bean	73 ± 16bc	93 ± 2b	80 ± 5b	17 ± 8	24 ± 2	34 ± 9
Rockmelon	75 ± 2c	129 ± 10a	112 ± 12ab	16 ± 6	32 ± 4	22 ± 6
Control	69 ± 4bc	75 ± 9bc	82 ± 16b	21 ± 2	27 ± 6	18 ± 1

Leachate was harvested at three time points and T-RFLP profiles of 16S rRNA bacterial and ITS fungal regions completed. Data analysed by one-way ANOVA. Data shown are mean ± SEM of three replicates. Comparisons within substrate and harvest time are shown, with different letters indicating significant differences ($p < 0.01$).

Bacterial population diversity as determined by T-RFLP profiles are shown in Figure 3.2. Time 0 leachate, 30 day vermicomposted leachate and 60 day vermicomposted leachate all have different bacterial populations. The bacterial composition of leachates changed over time for banana (Figure 3.2a), green bean (Figure 3.2b) and rockmelon (Figure 3.2c).

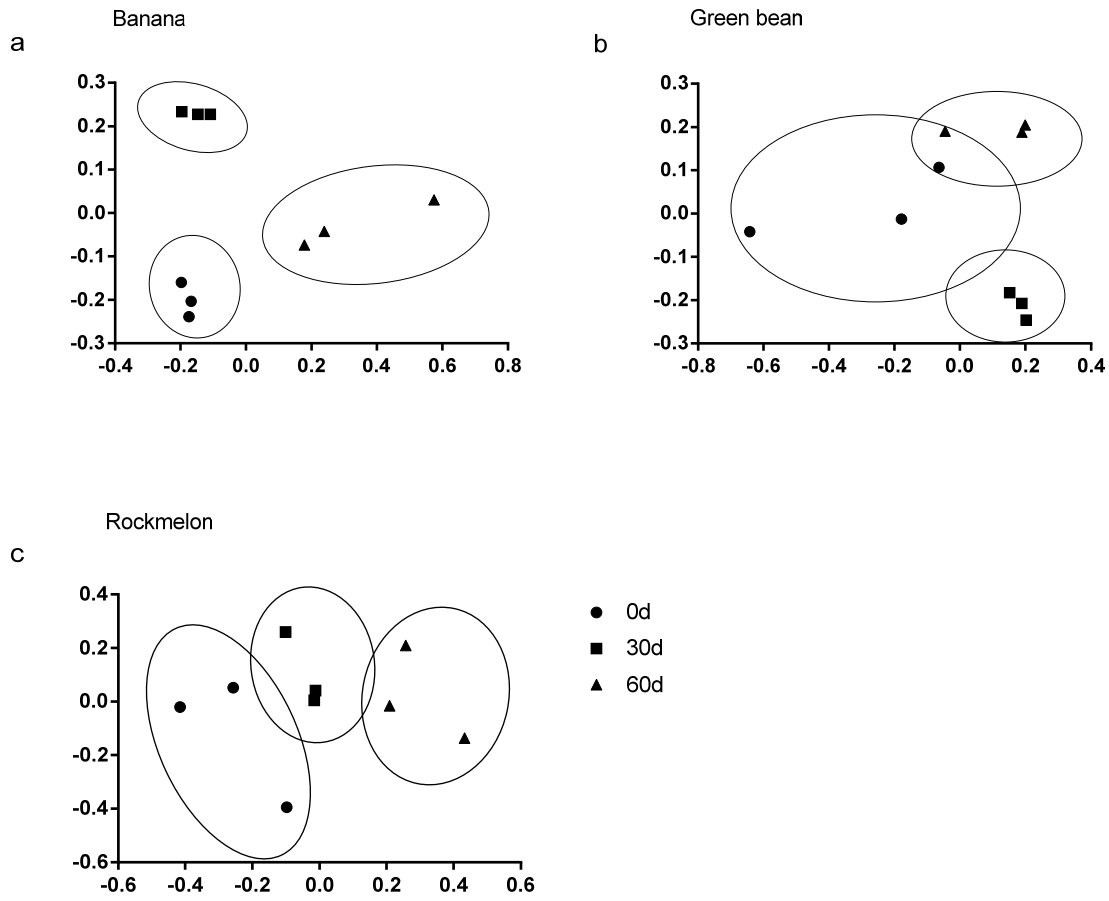

Figure 3.2. Differences in bacterial community profiles for vermicompost leachate over time. Non-metric MDS plot of T-RFLP patterns. Data normalised, converted to presence or absence points and subjected to a Bray-Curtis similarity matrix. Feed treatments shown are banana (MDS plot 3D stress (S) = 0.05982, ANOSIM p = 0.0036, ANOSIM R = 0.7778) (a), green bean (S = 0.1168, p = 0.0039, R = 0.4156) (b) and rockmelon (S = 0.09161, p = 0.0038, R = 0.5473) (c).

Fungal population diversity as determined by T-RFLP profiles are shown in Figure 3.3. Time 0 leachate, 30 day vermicomposted leachate and 60day vermicomposted leachate all have different fungal populations. The fungal composition of leachates changed over time for banana (Figure 3.3a), green bean (Figure 3.3b) and rockmelon (Figure 3.3c).

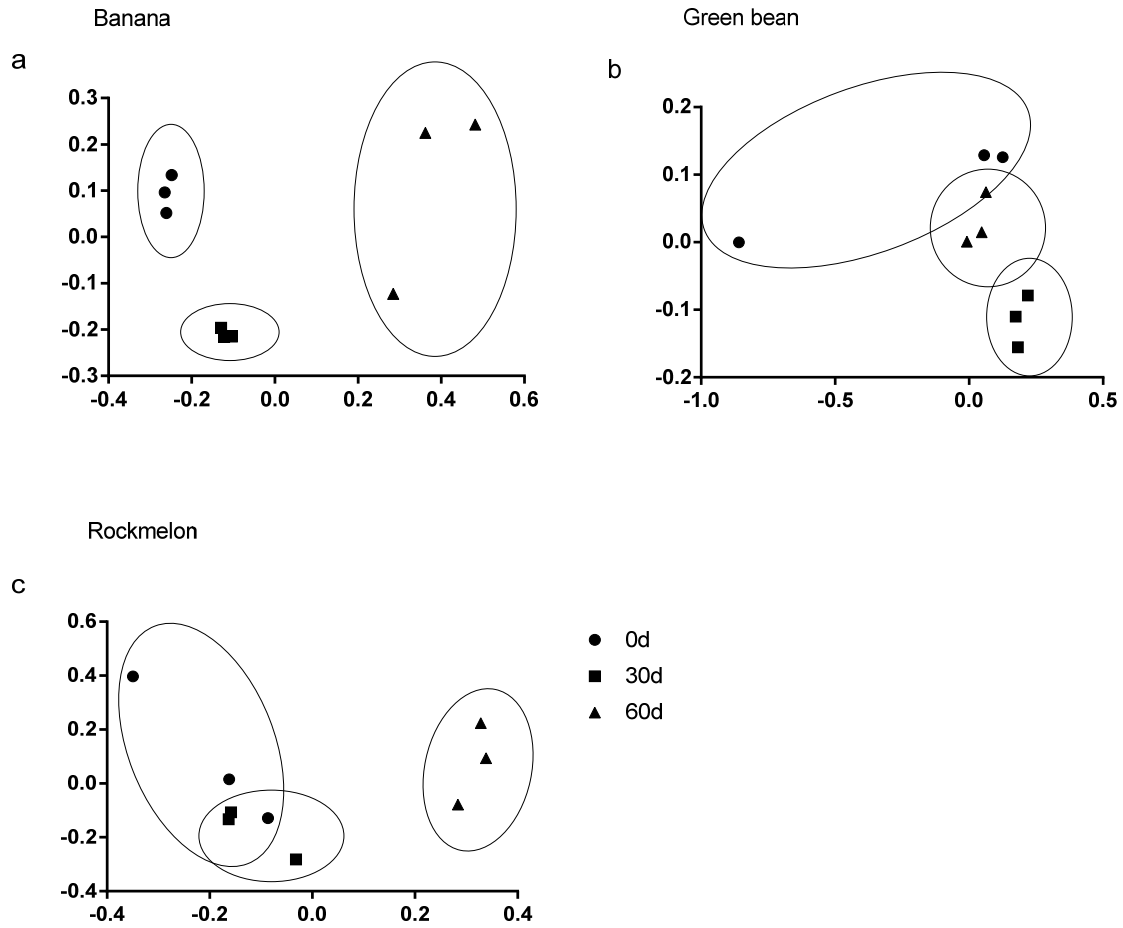

Figure 3.3. Differences in fungal community profiles for leachate samples over time. Non-metric MDS plot of T-RFLP patterns. Data normalised, converted to presence or absence points and subjected to a Bray-Curtis similarity matrix. Feed treatments shown are banana (MDS plot 3D stress (S) = 0.01111, R = 0.8436, p = 0.0036) (a), green bean (S = 0.09394, R = 0.5473, p = 0.003) (b) and rockmelon (S = 0.05982, R = 0.6008, p = 0.0038) (c).

3.3.6 Identification of leachate bacteria

Leachates from worm farms fed banana were sequenced at time 0 and 60 days, as were leachates from worm farms fed rockmelon. Relative abundance at the phylum level varied considerably across the different samples (Figure 3.4). The dominant phylum in all leachates was Proteobacteria. Banana leachate time 0 (B0) contained 55% Proteobacteria and 60 day banana leachate (B60) contained 51%. Rockmelon leachate time 0 (R0) contained 80% Proteobacteria and 60 day rockmelon leachate (R60) contained 76%.

Twelve different phyla were found in banana leachate at time 0. Samples taken after 60 days showed the presence of three additional phyla, Chlorobi, Acidobacteria and Elusimicrobia. The 60 day samples showed the absence of two phyla, SR1 and Planctomycetes. Thirteen different phyla were found in rockmelon leachate at time 0. Samples taken after 60 days showed the presence of nine additional phyla, Chlorobi, Acidobacteria and Elusimicrobia, Spirochaetes, WPS-2, Euryarchaeota, Deferribacteres, NKB19 and Synergistetes. The 60 day samples showed the absence of three phyla, Chloroflexi, TM6 and Planctomycetes. Vermicomposting increased bacterial diversity for both worm-bed substrates, to a larger degree for rockmelon.

Taxonomic profiling at genus level (Figure 3.5) indicated that *Pseudomonas*, *Azospirillum*, *Rubritale*a, *Sphingobacterium* and *Comamonas* were the five most abundant bacterial OTUs present in the time 0 leachates, comprising close to 50% of all bacterial taxa observed. Vermicomposting led to a decrease in the relative abundance of most of these taxa. The most abundant genus in both time 0 samples was *Pseudomonas*. *Pseudomonas* comprised 20% of banana time 0 and 49% of rockmelon time 0. After 60 days, the *Pseudomonas* population dropped to 0.3% for banana and 10% for rockmelon. *Rubritalea* comprised 18% of banana time 0 and 6% of rockmelon time 0. After 60 days, this genus was not reported for either worm-bed substrate. *Sphingobacterium* was found at 12% in banana time 0 and 8% in rockmelon time 0. After 60 days, *Sphingobacterium* was reduced to 0.3% for both worm-bed substrates. *Comamonas* was found at 4% in banana time 0 and 5% in rockmelon time 0. After 60 days, *Comamonas* was reduced to 0.2% in banana. After 60 days *Comamonas* in rockmelon leachate remained at 5%.

Following 60 days vermicomposting of banana, the five most abundant taxa were *Xanthobacter*, *Azospirillum*, *Parabacteroides*, *Prevotella* and unclassified *Verrucomicrobiacea*, comprising over 40% of all OTUs. Vermicomposting led to an increase in the relative abundance of these taxa.

Banana leachate contained 12% *Xanthobacter*, a taxon which was unreported at time 0. Banana leachate contained 11% *Azospirillum*, which increased from a relative abundance of 0.03% at time 0. Banana leachate contained 9% *Parabacteroidetes*, a taxon which was unreported at time 0. Banana leachate contained 7% *Prevotella,* a taxon which was unreported at time 0. Banana leachate contained 5% *Verrucomicrobiaceae*, which increased from a relative abundance of 0.13% at time 0.

Following 60 days vermicomposting of rockmelon, the five most abundant taxa were *Azospirillum*, *Pseudomonas*, *Arcobacter*, *Comamonas* and *Novispirillum*, comprising over 50% of all OTUs. Vermicomposting led to an increase in the relative abundance of these taxa. Rockmelon leachate contained 28% *Azospirillum*, which increased from a relative abundance of 0.17% at time 0. Rockmelon leachate contained 7% *Arcobacter*, a taxon which was unreported at time 0. Rockmelon leachate contained 6% *Comamonas*, which increased from a relative abundance of 5% at time 0. Rockmelon leachate contained 5% *Novispirillum*, a taxon which was unreported at time 0.

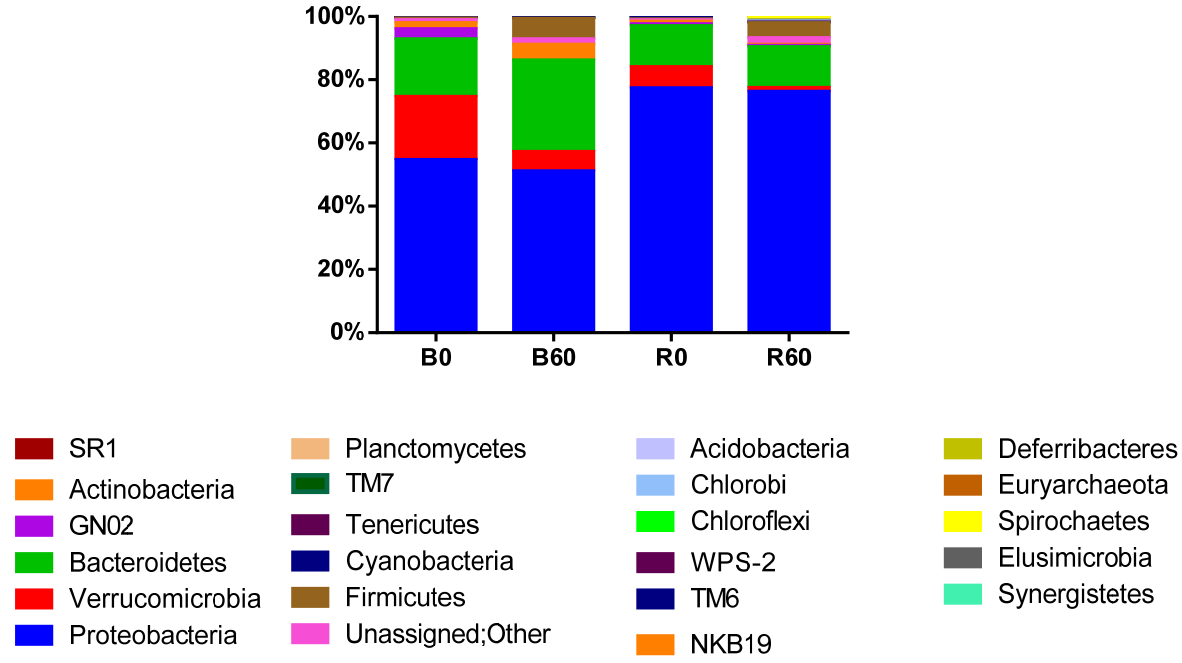

Figure 3.4. Bacterial diversity of vermicompost leachate from two worm-bed substrates over time. B0 = Banana 0 days, B60 = Banana 60 days, R0 = rockmelon 0 days. R60 = rockmelon 60 days. Y-axis represents relative OTU abundance at the phylum level. Values plotted are means of three replicates.

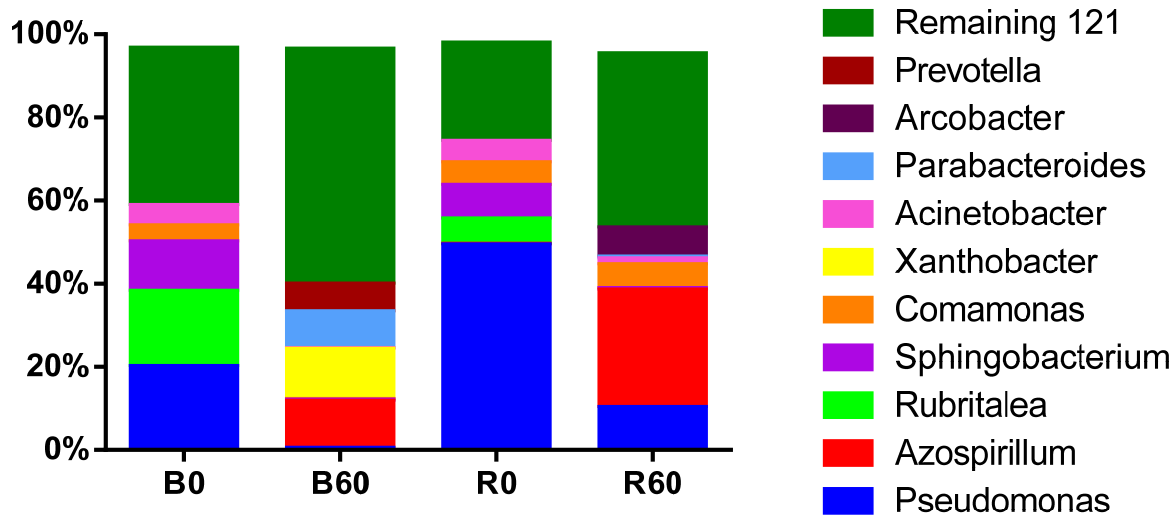

Figure 3.. Bacterial diversity of vermicompost leachate from two worm-bed substrates over time. B0 = Banana 0 days, B60 = Banana 60 days, R0 = rockmelon 0 days. R60 = rockmelon 60 days. Y-axis represents relative OTU abundance at the genus level. Values plotted are means of three replicates.

3.4 Discussion

3.4.1 Stability of leachate microbes

Stability of leachate microbes during short-term storage varied with worm-bed substrate and analysis technique. Bacterial species richness in green bean, sweet potato and control leachate as measured by T-RF number was unaffected by storage temperature. Leachates were unchanged after six weeks storage at 4°C, 25°C and -80°C. Bacterial population diversity as measured by T-RFLP profile was affected by storage temperature for sweet potato leachate. Fungal species richness as measured by T-RF number was affected by storage temperature for green bean leachate. Fungal population diversity as measured by T-RFLP profile was unaffected by storage temperature.

In this study, paper pulp was the only worm-bed substrate to produce leachate with microbes unaffected by storage temperature. As a nutrient poor worm-bed substrate, paper pulp provides less nutrients for the earthworms as discussed in Section 2.4.1. Paper pulp therefore also provides fewer nutrients to microorganisms. Paper pulp leachate being unaffected by storage may be because paper pulp introduced fewer microbes to the vermicomposting system.

The nutrient rich worm-bed substrates did show changes following storage, possibly because of a larger number of introduced microbes. Storage at 4°C led to changes in fungi in leachate from worm farms fed pureed green bean, and storage at 4°C, -80°C and 25°C led to changes in bacteria in leachate from worm farms fed sweet potato. Similar temperature effects were observed by Fritz et al. (2012) using vermicompost extract although the substrate fed to earthworms was not described. They reported no changes to bacterial communities following short-term storage of extract at 10°C, while changes were observed following storage at 22°C and 36°C. They posited that composting microbes, which are usually mesophilic, will not grow at 10°C, hence the extract population is unlikely to change at low temperature. The extract used by Fritz et al. (2012) was derived from a finished commercial vermicompost with no earthworms which likely contained a different microbial composition than the leachates used in this study.

Grantina-Ievina et al. (2013) also reported temperature effects on storage of solid cow manure vermicompost. Leachate or extract were not tested in this study. In this case, storing vermicompost at room temperature for a year resulted in lower culturable microbial and fungal populations than storage at 4°C. Grantina-Ievina et al. (2013) attributed this reduction to drying out of samples at

higher temperatures. This provides evidence that leachate is preferable to solid vermicompost in terms of storage of microbes. Refrigerating organic amendments may not be feasible due to a lack of available space or facilities. Leachate is preferable to solid matrices such as vermicompost for storage at ambient temperature as it maintains microbes in liquid suspension and is not susceptible to drying out. It is likely that soil amended with regular doses of leachate stored at ambient temperature will receive different communities of microbes each time.

Stability of leachate microbial communities from different sized worm farms varied with worm-bed substrate and analysis technique. Species richness and population diversity measurements showed differences within worm-bed substrates. In this study, celery was the only worm-bed substrate to produce leachate with microbes unaffected by the size of the worm farm. Worm farm unit size led to changes in fungal populations of green bean and control leachate. No comparative published studies could be found, however worm farm size has been shown to change vermicompost nutrients (Suthar, 2010). It may be that leachate from the different sized worm farms also contained different nutrients which contributed to changes in microbial population dynamics. The size differences used in this study were relatively small. Further increases for large scale production are likely to bring about further changes to the product. Small scale studies such as this one may not give an accurate picture of large scale leachate production.

Leachate microbial communities were stable using different starting earthworms and the same worm-bed substrate at different times. This may indicate that earthworms do not contribute significantly to the variation in leachate microbial community structure. As only one worm-bed substrate was used in this test, further examination of additional substrates is needed.

Leachate microbial populations are likely influenced by many factors. Among those are both worm-bed substrate and leachate harvest time. Different worm-bed substrates led to differences in microbial communities. Microbial communities also changed with vermicomposting time. The 60 day leachate populations differed from the 30 day populations. Conclusions about leachate microbes are difficult to make due to these changes. It is possible leachate microbes will stabilise. However, it is also possible they will continue to change as new worm-bed substrate and bedding are added and new earthworms hatch. Vermicompost addition to soil changes the microbial composition of the rhizosphere of tomato (Jack et al., 2011). No published articles on leachate addition to soil could be found but it is likely leachates produced from different substrates may have different effects on soil and plants due to their different microbial communities.

3.4.2 Identification of leachate bacteria

Banana and rockmelon leachates contained bacteria with potential benefits to plant growth. Addition of these microbes to soil through the application of leachate is likely beneficial to agriculture. As expected from T-RFLP studies, the identified leachate bacteria varied with worm-bed substrate and harvest time.

The bacterial taxa found in leachates were shown to differ between worm-bed substrates and to change markedly after vermicomposting. Results at the phylum level have been included to allow comparison with previous studies, all of which focus on this level of classification. The dominant phylum in rockmelon and banana pre-vermicomposted and vermicomposted leachates was Proteobacteria. Pre-vermicomposted leachates were harvested from worm farms immediately after the first substrate addition. These leachates likely contained microbes originating from earthworms, bedding and substrate. Vermicomposted leachates were harvested 60 days later. The relative abundance of Proteobacteria remained unchanged after vermicomposting. This is consistent with the work of Yasir et al. (2009) who observed no difference between paper and dairy waste substrate and 15 day vermicompost. In contrast, Huang et al., (2013) found Proteobacteria to dominate the substrate of cabbage, lettuce, dried cow manure vermicompost and potato peels. However, the 60 day vermicompost was dominated by Firmicutes. Proteobacteria was also found in mixed manure and crop waste vermicompost, although not among the dominant microbes (Fracchia et al., 2006). Leachate was not tested in any of these studies. Differences between this study and the findings of Huang et al. (2013) may be due to their use of dried vermicompost as a component of the substrate. Adding a product already processed by earthworms to their worm farm then allowing that product to be further processed alongside fresh food is unusual in published studies and may have led to microbial differences.

Most phyla that were found in the pre-vermicomposted leachates were also present in the vermicomposted leachates. Vermicomposted banana leachate contained three additional phyla and lost two. Vermicomposted rockmelon leachate contained nine additional phyla and lost three. Vermicomposting enhanced bacterial diversity for both worm-bed substrates, to a larger degree for rockmelon. Similar results were reported by Huang et al. (2013) who observed an increase in population diversity after vermicomposting. Huang et al. (2013) used DGGE to compare microbial diversity and sequenced dominant and representative bands. Using DNA extraction from vermicompost and sequencing of 16S rRNA genes Yasir et al. (2009) also detected additional phyla after vermicomposting, although all substrate phyla were still present.

Results at the genus level were included to provide a more detailed picture of leachate bacteria and because there is evidence that vermicompost leachate can benefit plant growth, as described in Chapter 1. The dominant genera in both vermicomposted leachates have proven benefits to plant growth. The dominant genus in vermicomposted banana leachate was *Xanthobacter*. *Xanthobacter* synthesise plant growth promoting cytokinins (Tsavkelova et al., 2006). *Xanthobacter* has been shown to increase rice growth (Gopalaswamy et al., 2000) and shoot weight and leaf number in the presence of N (Reding, Hartel, & Wiegel, 1991). The dominant genus in vermicomposted rockmelon leachate was *Azospirillum*. *Azospirillum* was the second most prevalent genus in vermicomposted banana leachate. *Azospirillum* produce plant growth promoting auxins, cytokinins and gibberellins. Inoculation with *Azospirillum* shows benefits to plant growth and yield in greenhouse and field studies (Steenhoudt & Vanderleyden, 2000) and improves root formation in many plant species, in turn improving water and nutrient uptake (Okon & Labandera-Gonzalez, 1994). Addition of *Xanthobacter* and *Azospirillum* to soil by application of leachate will provide benefits to plant growth through production of plant growth hormones.

Pseudomonas was the second most abundant genus in vermicomposted rockmelon leachate. Pathma & Sakthivel (2013) found *Pseudomonas* among the dominant microbes in vermicomposted straw and goat manure. Many species of *Pseudomonas* can solubilise P in the rhizosphere or produce cytokinins (Vessey, 2003). Addition of *Pseudomonas* to soil by application of leachate will provide benefits to plant growth through production of plant growth hormones. In addition, *Pseudomonas* addition through leachate application may reduce the volume of inorganic P required in agricultural soils.

Species of *Pseudomonas* may also be human and plant pathogens (Morris et al., 2008; Stover et al., 2000). The technique used in this study did not determine which members of the genus were present. However, it is interesting that in this study vermicomposting reduced *Pseudomonas* numbers. The gut microbes of *E. fetida* have been shown to change in response to introduction of a human pathogen *Escherichia coli*, effectively eliminating the pathogen after seven days (Zhang et al., 2013). The reduction in *Pseudomonas* may indicate that pathogens were removed by earthworm action while beneficial species persisted. Zhang et al. (2013) observed an increased Bacillus population in the earthworm gut after introduction of the pathogen. They suggested Bacillus acted as an antagonist to the pathogen, possibly through antimicrobial secretion. This may indicate a process of selection occurring in the earthworm gut. Beneficial actions of the earthworm gut were also demonstrated by Villalobos-Maldonado et al. (2015) who found that *E. fetida* consumed decachlorobiphenyl, a potential carcinogen and environmental pollutant, nearly removing it from a

worm-bed substrate of rabbit manure. Interestingly the pollutant didn't adversely effect the earthworms, as earthworm biomass and coccon production were greater than the control.

Parabacteroides was the third most prevalent genus in vermicomposted banana leachate. *Parabacteroides* have been isolated from compost shown to suppress *Pythium* disease in cucumber seedlings (Chen et al., 2012). The fourth most prevalent genus in vermicomposted rockmelon leachate was *Comomonas. Comomonas* is a plant growth promoting microbe that secretes indole-3-acetic acid (IAA) (Barazani & Friedman, 1999). It has been shown to increase root growth in kiwifruit (Erturk et al., 2010) and suppress colonisation of chrysanthemum roots by the root rot pathogen *Pythium* in hydroponic solution (Liu et al., 2007). Both *Parabacteroides* and *Comomonas* added to soil through leachate application will benefit plants, the former through suppression of pathogenic microbes, and the latter through production of plant growth hormones in addition to pathogen suppression.

Evidence of leachate microbial suppression of plant pathogens was reported by Contreras-Blancas et al. (2014). Unsterile leachate from cow manure processed by *E. fetida* inhibited growth of the pathogenic fungus *Colletotrichum gloeosporioides* on agar. In contrast, sterilised leachate had no effect on pathogen growth. Leachate microbes were not identified but Contreras-Blancas et al. (2014) speculated that these microbes either outcompeted the fungus or inhibited growth through production of secondary metabolites.

Potential benefits of the remaining dominant genera are less clear. The fourth most abundant genus in vermicomposted banana leachate was *Prevotella*. *Prevotella* is a proteolytic anaerobe generally derived from rumen (Ferme et al., 2004; Flint & Bayer, 2008). *Prevotella* has also been isolated from rice plant residue from irrigated rice field soil (Ueki et al., 2006) and from living rice roots (Ueki et al., 2007). The presence of an anaerobe may indicate suboptimal vermicomposting conditions. The fifth most prevalent genus in vermicomposted banana is *Verrucomicrobia. Verrucomicrobia* are ubiquitous in soil and found in a wide range of rhizosphere samples (Bergmann et al., 2011).

The third most prevalent genus in vermicomposted rockmelon leachate was *Arcobacter. Arcobacter* contains human and animal pathogens (Marshall et al., 1999) as well as marine sulfur oxidisers (Wirsen et al., 2002). *Arcobacter* have been identified in the rhizosphere of three species of Antarctic plant (Teixeira et al., 2010). The origin of *Arcobacter* in leachate is unclear, as are the likely effects on plants. More studies on *Arcobacter* are needed to determine whether the effect on plants

may be positive, negative or neutral. The fifth most prevalent genus in vermicomposted rockmelon leachate was *Novispirillum. Novispirillum* are found in a wide range of aquatic environments (Tranchida et al., 2012). This organism may also indicate suboptimal vermicomposting conditions, specifically high moisture content.

Of the abundant genera found prior to vermicomposting, one genus, *Rubritalea,* was not detected after vermicomposting. *Rubritalea* have been detected among the gut microbes of *E. fetida* (Zhang et al., 2013). The earthworms are the likely source of *Rubritalea* in the pre-vermicomposted leachates. These microbes probably remain in the earthworm gut during vermicomposting. Relative abundance of *Sphingobacterium* was high in pre-vermicomposted leachates. Numbers decreased but this genus was still present after vermicomposting. *Sphingobacterium* were found in *E. fetida* vermicomposted sewage sludge (Liu et al., 2012) and vegetable peels mixed with vermicompost (Huang et al., 2014). *Sphingobacterium* have been found in the gut of earthworms *Aporrectodea caliginosa* (Byzov et al., 2009). The earthworms are the likely source of *Sphingobacterium* in this study.

Other prevalent pre-vermicomposted genera were *Comamonas* and *Pseudomonas*. No published evidence was found of these microbes associated with earthworms. *Comamonas* has been isolated from banana roots (Ho et al., 2015). *Pseudomonas* have been isolated from rockmelon leaves (Morris et al., 2000) and the rhizosphere of rockmelon (Sallam et al., 2013) and banana (Saravanan, Muthusamy, & Marimuthu, 2004; Vlassak et al., 1992). The worm-bed substrates are the likely source of these genera.

Sequence identification was obtained from frozen leachates. As multiple freeze thaw cycles is a common method of cell lysis (Bej, 1995) there was concern that some of the leachate population may have been lost. Repeating this study without freezing the leachates and subjecting microbes to damage from ice crystal formation may produce different results. Many studies focus on the bacterial populations in vermicompost or leachate (Doan et al., 2013; Huang et al., 2013; Yasir et al., 2009). The current study did not identify leachate fungi but did show that leachate fungi was affected by both worm-bed substrate and leachate harvest time. Fungi are highly beneficial to agriculture, and may reduce the amount of fertiliser needed in agricultural soils for food production (American Society for Microbiology, 2011). Vermicompost has also been shown to contain fungi with potential plant-growth promoting activity and fungi antagonistic to plant pathogens (Grantina-Ievina et al., 2013). Studies of leachate fungi would be an important addition to this work.

Microbial populations of amended soil are mainly determined by the amendment used, be it vermicompost, compost or chemical fertiliser (Doan et al., 2013). Vermicompost addition increases culturable microbes in soil (Kale et al., 1992) as well as the number and diversity of culturable N fixing bacteria in soil (Sinha et al., 2010a). Vermicompost as a transplant media leads to unique rhizosphere community profiles after transplanting (Jack et al., 2011). It is unknown whether leachate similarly effects the soil microbes.

3.5 Conclusion

The worm-bed substrate used to make vermicompost changes the microbes found in the leachate. The process of vermicomposting changes the microbes found in the leachate, as does sampling time. Leachate sampled after one month contains different microbes than leachate sampled after two months. Storage temperature may change leachate microbes, as may worm farm size. These effects are variable, and depend on worm-bed substrate and analysis technique. Vermicompost leachate from banana and rockmelon contain potential plant beneficial microbes. These leachates should be trialled as soil amendments to test the benefits to plants.

Chapter 4 - Effect of long term storage on nutrient content and microbial composition of vermicompost leachate

4.1 Introduction

Vermicompost can increase soil pH, total organic carbon (TOC) (Behera, 2009), N, P (Jouquet et al., 2010) and K (Najafi-Ghiri, 2014). A correlation between increased soil N from vermicompost and plant biomass has been demonstrated by Roy et al. (2010). Vermicompost leachate application can increase soil micronutrients (Ávila-Juárez et al., 2015). Liquid extracts of vermicompost and conventional compost contain a portion of the nutrient requirements of plants, ranging from 0.1% to 70% depending on the nutrient in question. The method used to make these extracts can result in large variations in nutrient content (Marín et al., 2013) and can render some micronutrients undetectable (Pant et al., 2009).

The organic substrate fed to earthworms can create variation in vermicompost nutrient content (Arancon et al., 2004). Vermicompost leachates may contain a fraction of the nutrients recommended for plants (Preciado-Rangel et al., 2015) and supplementation with chemical fertiliser may be required to obtain adequate N, P, K for plants (Arancon, Edwards, & Bierman, 2006). When macronutrients are equal, vermicompost benefits plant growth over chemicals, suggesting that micronutrients and microbes are important (Arancon et al., 2004).

Several studies have characterised the nutrient content of leachate from animal manure (Álvarez-Solís et al., 2016; Gutiérrez-Miceli et al., 2011; Pant et al., 2009) although Pant et al. (2009) tested a liquid extract of earthworm free vermicompost. Nutrient content of vermicompost and corresponding leachate can vary. Gutiérrez-Miceli et al. (2011) found N and P were higher in sheep manure vermicompost compared to the leachate, although the difference was small. Fruit and vegetable waste are nutrient rich substrates ideal for conversion to compost (Huang et al., 2014) and are commonly used in domestic worm farms. No published nutrient analyses of leachate from such worm-bed substrates could be found.

Many commercial vermicompost liquids are available. Some are advertised as having post-harvest additives (Worms DownUnder, 2017). Some are supplied as a concentrate and provide a chemical analysis of the product (Circular Food, 2016; Ferguson, 2013). However, due to waste recycling efforts, the worm-bed substrate is not defined and the leachate may not be uniform. Others supply a typical analysis as a guide along with a list of substrates (Nutri-Health International, 2015) while

others provide no indication of substrate (Adobe Loos & Worms, n.d.). One product guarantees living microbes for four months of storage at ambient temperature (Liquid Organic Fertiliser, 2015). Most products are marketed as a source of nutrients and microbes. For the majority of these products, no nutrient analysis is available. There are no storage recommendations or indications as to whether the product is intended for immediate use (The Green Life Soil Co, n.d.; The Worm Shed, 2016; Worm Tech, n.d.; WormsRUs, 2014).

There are few studies examining the effect of storage on either nutrients or microbes in vermicompost amendments. These focus on culture techniques (Grantina-Ievina et al., 2013) or short-term storage (Fritz et al., 2012). Vermicompost storage can change the effect on plant growth parameters, a result which varies with plant species (Grantina-Ievina et al., 2013). In this study, leachates were stored for one year at two temperatures and the chemical and microbial properties compared.

T-RFLP was used to study leachate microbes in Section 3.3. This technique was not used any further to reduce the potential for errors. If restriction endonuclease digestion of PCR products is incomplete, this can lead to an increased number of T-RFs and an overestimation of community diversity (Osborn, Moore, & Timmis, 2000). Hence T-RFLP was replaced with a method of estimating community diversity which did not require digestion.

The aim of this work was to determine whether different worm-bed substrates produce leachates that vary in nutrient composition. This study also aimed to determine whether the microbial and nutritional composition of leachate changes over a growing season and whether storage temperature effects these changes. The null hypotheses for these experiments was that leachate nutrient composition will not vary with different worm-bed substrate ratios; and that leachate will not change after one year storage.

4.2 Methods

This study focused on three fresh produce substrates for in-depth analysis of leachate. The substrates chosen were banana, green bean and rockmelon. Substrate selection was based on general differences in nutritional composition of each fresh produce item and earthworm response. Banana is high in carbohydrates and K with a high C:N ratio. Green beans are low in carbohydrate, high in calcium with a low C:N ratio. Rockmelon is low in protein with an intermediate C:N ratio (Table 4.1). Earthworm growth response to substrates from previous experiments (Chapter 2) indicated banana was a poor substrate, while rockmelon was intermediate and green bean was good.

Table 4.1. Substrate nutrient composition.

Nutrient (mg/100 g)	Banana	Green bean	Rockmelon
Protein	1090	1790	840
Carbohydrate	22840	7540	8160
Sugars	12230	2210	7860
Calcium	5	42	9
Iron	0.26	0.85	0.21
Magnesium	27	22	12
Phosphorus	22	32	15
Potassium	358	186	267
Sodium	1	3	16
Zinc	0.15	0.26	0.18
Carbohydrate:Protein	21:1	4:1	10:1

Data compiled from United States Department of Agriculture (2017)

4.2.1 Leachate analysis using small scale worm farms

4.2.1.1 Vermicompost

Earthworms from Worm Affair (Midland, Australia) were separated by species and *E. fetida* stocks were maintained in a mix of fruit and vegetable waste, paper, and composted, pasteurised cow manure (ANL, Terrey Hills, Australia) until needed. Bedding (36 g) was added to 24 small scale worm farms as described in section 2.2.1 and shown in Figure 2.1. Twelve juvenile earthworms weighing

between 0.03 and 0.18 g each were added per unit. Earthworms were left overnight prior to addition of 18 g treatment substrate as listed in Table 4.2.

Table 4.2. Composition of vermicompost substrates.

Treatment	Substrate
P (Control)	Newspaper pulp, pureed
B	Banana, whole, pureed
G	Green beans, whole, pureed
R	Rockmelon, whole, pureed
B/G	1 part banana: 1 part green bean (w:w)
B/R	1 part banana: 1 part rockmelon (w:w)
G/R	1 part green bean: 1 part rockmelon (w:w)
B/G/R	1 part banana: 1 part green bean: 1 part rockmelon (w:w:w)

Substrate and bedding were prepared as described in section 2.2.1. Bedding (24 g) and substrate (12 g) were replenished weekly for 3 weeks initially and then fortnightly for the duration of the experiment. Three replicate worm farms per substrate were established. A 1:1 (w/v) mixture of substrate and water was used to determine substrate pH using a pH Cube Benchtop pH-mV-Temperature Meter (TPS, Brendale, Australia).

4.2.1.2 Leachate harvest

Leachate harvest is detailed in section 3.2.1. Leachate was harvested from three replicate worm farms per substrate. At 10 weeks, 1.2 L water was used to collect 1 L leachate. Freshly harvested leachate was stored at 4°C and subsamples were placed in defined temperature conditions within one week. Two 150 mL aliquots of leachate were transferred to sterile plastic specimen containers (Livingstone International, Rosebery, Australia). One 150 mL aliquot was stored at 4°C and the second 150 mL aliquot was stored indoors at ambient temperature for one year. Remaining leachate was stored in sterile screw capped glass bottles at 4°C for one year for pH, EC and C:N measurements. Chemical, microbiological and molecular analyses of fresh leachate and leachate stored for one year at 4°C and at 25°C were completed.

4.2.1.3 Chemical Analysis

Leachate pH was determined with a PHM210 Standard pH Meter (Radiometer Analytical, Lyon, France). Leachate electrical conductivity (EC) was determined with a PTI-18 Digital Conductivity Meter (Activon Scientific Products Co, Thornleigh, Australia). Leachate was filtered through a 0.45 µm Polyethersulfone Minisart® High Flow Syringe Filter (Sartorius, Dandenong, Australia) then acidified with 200 µL phosphoric acid (Sigma, Castle Hill, Australia). Total organic carbon (TOC) and total nitrogen (TN) measurements were determined using the TOC-V_{CSH} Analyser (Shimadzu, Kyoto, Japan). TOC and TN measurements were carried out at the Centre for Carbon Water and Food (CCWF), The University of Sydney, Cobbitty, Australia.

4.2.1.4 Cultivable microbes

Serial dilutions of leachate were made in sterile saline (0.85% NaCl, w/v)., with 100 µL of 10^{0}, 10^{-1}, 10^{-2} plated on potato dextrose agar (PDA) to enumerate fungi and 100 µL of 10^{-3}, 10^{-4}, 10^{-5} plated on R2A agar (Oxoid, Thebarton, Australia) with 500 µg/L cycloheximide (Sigma-Aldrich, Castle Hill, Australia) to enumerate bacteria. Plates were incubated at 28°C for 7 days and colonies counted to determine colony forming units (CFU) per mL. Media recipes are detailed in Appendix A.

4.2.1.5 ARISA (Automated Ribosomal Intergenic Spacer Analysis)

Cells from 150 mL of leachate were harvested at 45 000 g for 10 min in a Sorvall™ RC-5C Plus Superspeed Centrifuge (Thermo Scientific, North Ryde, Australia). Supernatant was discarded and the cell pellet stored at -80°C until processing. Cell pellets from fresh leachate were frozen on the day of leachate harvest to minimise any changes to microbial communities. The cell pellet was thawed on ice and DNA extraction carried out as described in section 3.2.1. For amplification of the 16S-23S rRNA intergenic spacer region, primers 1406F (5'-TGYACACACCGCCCGT-3') and 23SR (5'-GGGTTBCCCCATTCRG-3') were used (Fisher & Triplett, 1999). The forward primer was labelled at the 5' end with FAM. Primers were synthesised by IDT (Baulkham Hills, Australia). PCR reactions contained 20 ng DNA template, 1 X MyTaq™ Reaction Buffer, 0.4 µM of each primer, 1.25 U MyTaq™ DNA polymerase (Bioline, Eveleigh, Australia). Reaction conditions consisted of an initial 95°C for 3 min followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and a final extension of 72°C for 5 min. PCRs were run in a C1000™ Thermal Cycler (Bio-Rad, Gladesville, Australia). Amplicon visualisation and purification were carried out as described in section 3.2.1. Purified amplicons (100 g) were submitted to AGRF (Westmead, Australia) for Capillary Separation using the size standard LIZ500.

4.2.1.6 Data Analysis

Leachate pH and EC values were analysed by one-way ANOVA. Cultivable microbe numbers were log transformed and analysed by one-way ANOVA. These analyses were carried out using Genstat 17th Edition (VSN International Ltd, Hempstead, UK). Two-sided correlation analysis was performed between C:N ratio and fungal:bacterial ratio. One-way ANOVA was performed on fungal:bacterial ratio. These analyses were carried out using Genstat 18th Edition (VSN International Ltd, Hempstead, UK). Raw data from AGRF was retrieved and the size of the ARISA amplicons determined using Peak Scanner ™ v2.0 from Applied Biosystems® (Foster City, CA). T-REX (Culman et al., 2009) was used to find true peaks and eliminate background noise (Abdo et al., 2006) then align peaks (Smith et al., 2005). The diversity of the ARISA profiles was analysed by the Bray-Curtis dissimilarity index (Bray & Curtis, 1957) for presence-absence data using Past v3.02a (Hammer et al., 2001). nmMDS ordination plots, on the Bray-Curtis similarity matrix, were used to visualise dissimilarities between communities. Differences between communities were measured using one-way analysis of similarity (ANOSIM) on the Bray-Curtis distance matrices including 10 000 permutations with significant differences considered as $p < 0.01$ (Clarke, 1993).

4.2.2 Nutrient content of leachate using large scale worm farms

4.2.2.1 Vermicompost

Bedding (550 g) was added to three large scale worm farms (as described in section 2.2.3 and illustrated in Figure 2.2) and 60 clitellated earthworms each weighing between 0.26 and 0.31 g were added per unit. Earthworms were *E. fetida* from the maintained stocks described in section 4.2.1. Worm farms were left overnight then 60 g of substrate was added. A different substrate was added to each worm farm. Substrates used were banana, green bean and rockmelon (Table 4.2). Substrate was replenished weekly for 7 weeks then fortnightly for the remainder of the experiment. Bedding was replenished monthly. Worm farms were maintained at 25°C. After 27 weeks, 100 g of compost was removed from the green bean and rockmelon worm farms. The banana worm farm did not have 100 g of composted material, and 45 g was removed. Compost was removed to ensure the environment remained hospitable for earthworms. Care was taken to avoid removing earthworms and cocoons. The bedding was replenished to the starting level and 50 g of substrate was added. Substrate addition of 30 g per unit continued fortnightly.

4.2.2.2 Leachate harvest

Leachate was harvested as described in section 3.2.1. At 11 weeks, 3.2 L water was used to collect 3 L leachate. Leachate harvest was repeated at 12 weeks, 19 weeks and 21 weeks. Leachates were separated into three 1 L batches, one batch was untreated, one was filtered through a 0.2 µm cellulose acetate membrane using a polycarbonate vacuum filter (Sartorius Stedim, Dandenong, Australia) and the third batch was autoclaved for 10 min at 121°C, allowed to cool, then filtered as previously described. Leachates were stored at ambient temperature. A 45 mL aliquot of leachate was stored at -20°C in a plastic screw-capped tube for chemical analysis and 1 mL of leachate was stored at 4°C in a microcentrifuge tube for microbial analysis. At 53 weeks, 2.2 L water was used to collect 2 L of leachate. Leachate harvest was repeated at 56 weeks, 58 weeks, 60 weeks and 62 weeks. Leachates were stored at ambient temperature without any further treatment. A 45 mL aliquot of leachate was stored at -20°C in a plastic screw-capped tube for chemical analysis.

4.2.2.3 Nutrient analysis

As the techniques for nutrient analysis required samples be filtered, the difference between untreated leachate and filtered leachate from the 11, 12, 19 and 21 week harvests would not be detectable. Therefore, the untreated and filtered leachates were combined at each week of collection for this analysis. Frozen leachates were thawed, mixed, then divided into 25 mL and 10 mL aliquots. Both aliquots were filtered using a 0.22 µm Polyethersulfone Minisart® High Flow Syringe Filter (Sartorius, Dandenong, Australia) and a drop of concentrated nitric acid (Sigma, Castle Hill, Australia) was added to the 10 mL aliquot. Samples were stored at 4°C overnight. The 25 mL aliquot was acidified and used to determine TOC and TN as in section 4.2.1. The 10 mL aliquot was used to determine Boron (B), Calcium (Ca), Copper (Cu), Iron (Fe), Potassium (K), Molybdenum (Mo), Magnesium (Mg), Manganese (Mn), Phosphorus (P), Sulfur (S) and Zinc (Zn) using a 720-ES Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) with SPS3 Sample Preparation System (Varian Inc., Palo Alto, USA). ICP-OES measurements were carried out at the School of Geosciences, The University of Sydney, Camperdown, Australia.

4.2.2.4 Cultivable microbes

Cultivable bacteria and fungi were enumerated as described in section 4.2.1. Cultivable N-fixing bacteria were enumerated using the Most Probable Number (MPN) method in selective semi-solid N-free broth (NFB, 3 mL in sugar tubes) supplemented with 100 mg/L cycloheximide. Triplicate tubes were inoculated with 100 µL of 10^{0}-10^{-8} leachate serial dilutions. Tubes were incubated at 28°C for 5

days. Pellicle formation and colour change indicating alkalinisation were used to indicate a positive reaction. To remove the possibility that positive reactions were due to carry-over N from leachate, 100 µL of growth from positive tubes was used to inoculate fresh tubes. Incubation and detection were carried out as previously described. MPN was calculated using data from the second round. MPN was determined using the Most Probable Number Enumeration System (MPNES) (Woomer, Bennett, & Yost, 1990).

4.3 Results

4.3.1 Chemical properties of leachate from banana, green bean and rockmelon

Worm-bed substrate pH ranged from 4.7 to 6.8 (Table 4.3). The substrate with the lowest pH was banana (Treatment B). The substrates with the highest pH were rockmelon (Treatment R) and control. All leachates had a near neutral pH, ranging from 6.9 to 7.3. The leachate with the lowest pH was green bean (Treatment G) and the leachate with the highest pH was rockmelon. Following refrigeration for one year, no significant variation between fresh and stored leachate pH was detected for any worm-bed substrate (Table 4.4), indicating leachate pH was stable during storage.

Leachate EC ranged from 57 to 286 µS cm^{-1} (Table 4.3). Leachate from the control worm farms had the lowest EC. Rockmelon leachate recorded the highest EC, a 4.0-fold increase over the control. All worm-bed substrates except green bean produced leachate with a significantly higher EC than the control. No other significant variation in EC was observed. Following refrigeration for one year, rockmelon leachate had a significantly higher EC than banana, green bean and control. Banana and green bean leachates showed significantly higher EC than the control. No significant variation between fresh and stored leachate EC was detected for any worm-bed substrate (Table 4.4), indicating leachate EC was stable during storage.

Leachate C:N ratios ranged from 17 to 56 (Table 4.3). The control leachate showed a significantly lower C:N than the other treatments. Rockmelon showed the highest C:N, significantly greater than all other treatments. Banana and rockmelon (Treatment B/R) had significantly greater C:N than treatments B and B/G (banana and green bean mixture). No other significant variation was observed. Following refrigeration for one year, rockmelon leachate had a significantly higher C:N than banana, green bean and control. No significant variation between fresh and stored leachate C:N was detected for green bean or rockmelon (Table 4.4). Following storage, the C:N of banana and

control leachates was significantly reduced. Stability of leachate C:N ratio following storage varies with substrate.

Table 4.3. Characteristics of vermicompost leachate from different worm-bed substrates after 10 weeks.

	Substrate pH	Leachate pH	EC (µS cm^{-1})	C:N
Banana (B)	4.7	7.1 ± 0.06	231 ± 20a	27.3 ± 0.1c
Green Bean (G)	6	6.9 ± 0.02	169 ± 23ab	31.1 ± 2.8bc
Rockmelon (R)	6.8	7.3 ± 0.04	286 ± 32a	56.3 ± 4.6a
G/R	6.7	7.2 ± 0.01	282 ± 122a	30.5 ± 2.5bc
B/G	5.5	7.1 ± 0.01	214 ± 20a	28.4 ± 2.5c
B/R	5.9	7.2 ± 0.08	203 ± 19a	39.6 ± 4.2cb
B/G/R	5.9	7.3 ± 0.05	221 ± 9a	33.5 ± 4.4bc
Control (P)	6.8	7.0 ± 0.05	57 ± 12b	17.0 ± 0.7d

C:N ratio derived from TOC and TN values. One-way ANOVA performed on EC and C:N with different letters indicating significant differences ($p < 0.05$).

Table 4.4. Leachate characteristics from different worm-bed substrates comparing fresh leachate with leachate refrigerated for one year.

Substrate		pH	EC (µS cm^{-1})	C:N ratio
Banana (B)	Fresh	7.12 ± 0.06b	231 ± 20ab	27.29 ± 0.14b
	1 year storage	7.29 ± 0.09ab	197 ± 12b	24.28 ± 1.02c
Green bean (G)	Fresh	6.93 ± 0.02c	169 ± 23bc	31.11 ± 2.80b
	1 year storage	7.02 ± 0.11abc	202 ± 32c	26.22 ± 1.39bc
Rockmelon (R)	Fresh	7.29 ± 0.04a	286 ± 32a	56.26 ± 4.65a
	1 year storage	7.36 ± 0.08abc	297 ± 16a	47.66 ± 6.26a
Control (P)	Fresh	7.01 ± 0.05bc	57 ± 12d	16.97 ± 0.74d
	1 year storage	7.80 ± 0.14abc	33 ± 1.4d	14.46 ± 0.39c

Values shown are mean ± SEM for three replicates. One-way ANOVA performed with different letters indicating significant differences ($p < 0.05$).

4.3.2 Cultivable microbial content of leachate from banana, green bean and rockmelon

Leachates from large scale units were examined for cultivable microbes. Worm-bed substrate had no effect on numbers of cultivable bacteria, fungi or N-fixing bacteria (Table 4.5). Similarly, no significant variation in cultivable bacteria and fungi was observed for small scale worm farms (Table 4.6). Cultivable bacteria outnumbered cultivable fungi, as shown by the low fungal to bacterial ratios. No correlation was found between fungal to bacterial ratio and leachate C:N.

Table 4.5. Leachate microbial populations from large scale worm farms.

Worm-bed substrate	**Bacteria (cfu/mL)**	**N-fixing bacteria (cfu/mL)**	**Fungi (cfu/mL)**	**Fungal:Bacterial ratio**
Banana	3.5×10^6	9.2×10^2	1.0×10^3	0.0044
Green bean	2.2×10^7	6.3×10^2	1.4×10^2	0.0001
Rockmelon	3.8×10^6	8.0×10^3	1.8×10^3	0.0004

Values shown are means of four replicates. One-way ANOVA showed no significant differences between substrates ($p > 0.05$).

Following ambient temperature storage for one year, significant worm-bed substrate variation in cultivable bacteria and fungi was found ($p < 0.001$). Following refrigeration for one year, significant substrate variation in cultivable fungi was found ($p < 0.05$), with no variation in cultivable bacteria.

Table 4.6. Leachate microbial populations from different worm-bed substrate treatments in small scale worm farms after 10 weeks.

Substrate	Bacteria (cfu/mL)	Fungi (cfu/mL)	Fungal:Bacterial ratio
Banana (B)	1.4×10^6	2.7×10^4	0.0297
Green bean (G)	5.4×10^5	8.5×10^1	0.0013
Rockmelon (R)	2.2×10^6	7.4×10^3	0.0023
G/R	3.3×10^7	8.7×10^2	0.0003
B/G	4.1×10^6	2.7×10^4	0.0197
B/R	5.8×10^6	3.1×10^3	0.0007
B/G/R	1.8×10^6	5.5×10^3	0.0191
Control (P)	8.6×10^5	1.1×10^4	0.0078

Bacteria cultivated on R2A and fungi on PDA. One-way ANOVA showed no significant differences between substrates ($p > 0.05$). Values shown are means of three replicates.

Comparison of cultivable microbe numbers in fresh and stored leachates are shown in Table 4.7. Refrigeration for one year produced no change in cultivable bacteria in leachate from any of the substrates and no change in cultivable fungi in leachate from treatments green bean, G/R, B/G, B/R and control. However, cultivable fungi in leachates refrigerated for one year significantly reduced in treatments banana, rockmelon and B/G/R ($p < 0.05$).

Ambient temperature storage produced no change in cultivable bacteria in leachate from treatments green bean, B/R, B/G/R and control but significantly reduced cultivable bacteria in leachate from treatments banana, rockmelon, G/R and B/G. There was no change in cultivable fungi in leachate from treatments green bean, B/R, B/G, G/R and control stored at ambient temperature but cultivable fungi in leachate from treatments banana, rockmelon and B/G/R were significantly reduced ($p < 0.05$).

The fungal:bacterial ratio was unaffected by storage for most worm-bed substrates. Refrigeration of banana leachate reduced fungal:bacterial ratio because of a reduction in fungi. Storage of G/R leachate at ambient temperature increased fungal:bacterial ratio because of a reduction in bacteria.

Table 4.7. Cultivable bacterial and fungal populations of leachate from different worm-bed substrates.

Substrate		Bacteria (cfu/mL)	Fungi (cfu/mL)	F/B
Banana (B)	Fresh	1.4×10^6a	2.7×10^4a	0.03a
	1 year storage (4°C)	6.8×10^5ab	1.1×10^3b	0.003b
	1 year storage (25°C)	1.3×10^4b	4.7×10^2b	0.04a
Green bean (G)	Fresh	5.4×10^5a	8.5×10^1a	0.001a
	1 year storage (4°C)	1.1×10^6a	1.9×10^2a	0.0002a
	1 year storage (25°C)	9.2×10^4a	4.7×10^1a	0.0006a
Rockmelon (R)	Fresh	2.2×10^6a	7.4×10^3a	0.002a
	1 year storage (4°C)	9.5×10^5a	6.7×10^1b	0.0001a
	1 year storage (25°C)	1.6×10^4b	4.7×10^1b	0.006a
G/R	Fresh	3.3×10^7a	8.7×10^2a	0.0003a
	1 year storage (4°C)	9.2+ $\times 10^5$ab	1.7×10^2a	0.0002a
	1 year storage (25°C)	8.4×10^4b	1.6×10^2a	0.002b
B/G	Fresh	4.1×10^6a	2.7×10^4a	0.02a
	1 year storage (4°C)	7.9×10^5a	6.1×10^2a	0.0009a
	1 year storage (25°C)	3.5×10^4b	3.7×10^2a	0.01a
B/R	Fresh	5.8×10^6a	3.1×10^3a	0.0007a
	1 year storage (4°C)	6.3×10^5a	1.1×10^2a	0.0002a
	1 year storage (25°C)	1.7×10^4a	4.0×10^1a	0.003a
B/G/R	Fresh	1.8×10^6a	5.5×10^3a	0.02a
	1 year storage (4°C)	3.6×10^6a	1.9×10^2b	0.0001a
	1 year storage (25°C)	1.1×10^6a	4.7×10^1b	0.00004a
Control (P)	Fresh	8.6×10^5a	1.1×10^4a	0.008a
	1 year storage (4°C)	2.6×10^6a	6.0×10^1a	0.00002a
	1 year storage (25°C)	6.1×10^4ab	3.33×10^1a	0.0007a

Freshly harvested leachate and leachate stored at different temperatures are shown. Values shown are means of three replicates. One-way ANOVA performed with different letters showing significant variation within substrate ($p < 0.05$).

4.3.3 Molecular analysis of bacteria in leachate from banana, green bean and rockmelon

The rRNA PCR fingerprint spanning the variable length ITS region showed some treatment variation, indicating variation in bacterial species richness. The treatment with the most unique amplicons, rockmelon, contained significantly more than the treatment with the least, B/G/R (banana, green bean and rockmelon mixture), indicating significantly greater bacterial species richness. All treatments except B/G/R showed significantly greater species richness than the control. No other significant treatment variation was observed (Table 4.8).

Table 4.8. Leachate bacterial diversity from different worm-bed substrate treatments after 10 weeks.

Substrate	**ITS unique amplicon number**
Banana (B)	65 ± 6.4ab
Green bean (G)	57 ± 13.5ab
Rockmelon (R)	109 ± 10.1a
G/R	59 ± 25.9ab
B/G	83 ± 6.2ab
B/R	97 ± 27.8ab
B/G/R	52 ± 25.0bc
Control (P)	2 ± 0.7c

The 16S-23R rRNA internal transcribed spacer gene was amplified by PCR and the number of unique amplicons calculated. Values shown are means of three replicates ± SEM. Data analysed by LMM with different letters indicating significant differences ($p < 0.05$).

Leachates from all eight treatments were combined prior to analysis. All leachates contained different bacterial communities as indicated by ANOSIM (Table 4.9), although low R value indicates some overlap.

Table 4.9. Analysis of similarity of leachate bacterial communities from different worm-bed substrates.

		p	R
Fresh leachate		0.0001	0.4277
Leachate after one year storage	4°C	0.0189	0.2023
	Ambient temperature	0.0024	0.3786

Following one year of refrigeration, ARISA analysis of some samples could not be completed. Raw trace analysis generated no peaks for two banana leachate replicates so banana was removed from the analysis. The remaining seven treatments had significantly different leachate bacterial communities, although separation was minimal (Table 4.10).

Following one year storage at ambient temperature, ARISA analysis of some samples could not be completed. DNA could not be extracted from one banana, one B/R and one control replicate. DNA was successfully extracted but no PCR product was generated from one B/R and one B/G/R replicate. PCR was successful but raw trace analysis generated no peaks from one B/G/R replicate and two G/R replicates. G/R, B/R and B/G/R were removed from the analysis. The remaining five treatments had significantly different leachate bacterial communities, with some overlap (Table 4.10).

When comparisons were made between fresh leachate and leachate stored for one year some significant variation was observed. Green bean, rockmelon, B/G and control leachates showed significant differences in bacterial populations between fresh leachate and stored leachate at both temperatures. The degree of separation differed, with B/G communities well separated, green bean and rockmelon showing some overlap and control minimal separation (Table 4.10). For other treatments, storage had no effect on bacterial community. Fresh banana leachate and samples stored at ambient temperature for one year showed no significant differences between bacterial communities. No significant differences in bacterial communities were observed for fresh leachate and samples stored at 4°C for a year for B/R, G/R and B/G/R leachate (Table 4.10).

ARISA analysis could not be completed for some stored samples. While no statistical analyses were available, this indicates that changes occurred during storage and the bacterial populations were different from fresh leachate. Fresh banana leachate was different from leachate stored for one year at 4°C. Fresh B/R leachate was different from leachate stored for one year at ambient temperature, as was G/R leachate and B/G/R leachate.

Table 4.10. Analysis of similarity of bacterial communities from fresh and stored leachate.

	p	R
Banana (B)	0.0988	0.75
Green bean (G)	0.016	0.4486
Rockmelon (R)	0.0036	0.6914
G/R	0.1019	0.4815
B/G	0.003	0.8066
B/R	0.0981	0.7407
B/G/R	0.2011	0.2222
Control (P)	0.0346	0.2517

Storage largely had no effect on bacterial species richness as shown by number of unique amplicons. No changes were observed after storage at ambient or refrigerated temperatures for treatments green bean, rockmelon, G/R, B/G, B/G/R or control. Treatment B/R also remained unchanged after refrigeration. One year storage at ambient temperature significantly reduced bacterial species richness in banana leachate and B/R leachate and refrigeration significantly reduced bacterial species richness in banana leachate (Table 4.11).

Table 4.11. Leachate bacterial species richness from different worm-bed substrate treatments after 10 weeks.

	Number of unique ITS amplicons		
Substrate	Fresh	Stored for one year	
Banana (B)	65 ± 6.4a	4°C	15 ± 15b
		25°C	0.3 ± 0.3b
Green bean (G)	57 ± 13.5a	4°C	28 ± 23.2a
		25°C	10 ± 4.5a
Rockmelon (R)	109 ± 10.1a	4°C	25 ± 21.6a
		25°C	37 ± 29.7a
G/R	59 ± 25.9a	4°C	34 ± 13.9a
		25°C	35 ± 35a
B/G	83 ± 6.2a	4°C	32 ± 11.0a
		25°C	38 ± 25.7a
B/R	97 ± 27.8a	4°C	31 ± 21.9ab
		25°C	0.3 ± 0.3b
B/G/R	52 ± 25.0a	4°C	30 ± 21.9a
		25°C	13 ± 13a
Control (P)	2 ± 0.7a	4°C	11 ± 6.2a
		25°C	4 ± 2.0a

Freshly harvested leachate and leachate stored at different temperatures are shown. The 16S-23R rRNA internal transcribed spacer was amplified by PCR and the number of unique amplicons calculated. Values shown are mean ± SEM for three replicates. One-way ANOVA was performed and different letters show significant variation within each treatment ($p < 0.05$).

4.3.4 Nutrient content of leachate from banana, green bean and rockmelon

Macro and micro nutrient content of leachates from two leachate harvests were measured. Some significant variation between worm-bed substrates was observed. In the first harvest, the substrate used had a significant effect on the concentration of TN, TOC, Ca, K, Mg and P. Banana leachate had significantly less TOC, Ca and Mg than green bean and rockmelon leachates. Green bean leachate had significantly more TN than banana and rockmelon leachates. Rockmelon leachate had significantly more P than green bean and banana leachates. Rockmelon leachate had significantly more K than green bean leachate. These trends were found in both filtered and autoclaved leachates. Banana leachate had significantly more K than green bean leachate, a trend not seen after autoclaving (Table 4.12).

Autoclaving the leachate resulted in significantly more B than untreated leachate for all three worm-bed substrates. Autoclaving rockmelon leachate resulted in significantly more P than untreated rockmelon leachate. Autoclaved banana leachate had significantly more K than autoclaved green bean and autoclaved rockmelon leachate, a trend not seen in untreated samples.

The second harvest showed fewer differences between worm-bed substrates. Banana leachate had significantly less TN, Ca, Mg, P and S than green bean and rockmelon leachate. No significant differences were observed between green bean and rockmelon leachates.

Comparison of nutrients between the two leachate harvests showed some significant variation over time. The amount of TOC, Ca and Mg increased significantly over time for all three worm-bed substrates. The amount of TN increased over time for banana and rockmelon leachate. No change in TN was observed for green bean leachate. The amount of Mn and P increased over time for banana and green bean leachate. No change in Mn or P was observed for rockmelon leachate. The amount of S increased over time for green bean leachate. The amount of B decreased over time for green bean leachate. No changes in S or B were observed for banana or rockmelon leachate. No changes were observed for Cu, K, Fe or Zn for any worm-bed substrate (Table 4.13).

Table 4.12. Nutrient content of vermicompost leachates fed different worm-bed substrates and harvested at two time periods.

	(mg/L)	TOC	TN	P	S	Ca	Cu	K	B	Mn	Mg	Fe	Zn	Mo
11-21 weeks Untreated	Banana	38 ± 5b	1.0 ± 1.2b	0.4 ± 0.3c	6.3 ± 0.3a	8.8 ± 3.1b	0.073 ± 0.009a	92.7 1± 12.5a	0.072 ± 0.025b	0.0071 ± 0.0019a	2.07 ± 0.77b	0.040 ± 0.012a	0.020 ± 0.006a	<LOD
	Green bean	51 ± 5a	5.0 ± 1.2a	0.6 ± 0.3c	6.8 ± 0.3a	19.5 ± 3.1a	0.078 ± 0.009a	37.0 ± 12.5c	0.078 ± 0.025b	0.0070 ± 0.0019a	5.01 ± 0.77a	0.026 ± 0.012a	0.0157 ± 0.006a	<LOD
	Rockmelon	54 ± 5a	2.1 ± 1.2b	2.7 ± 0.34a	6.5 ± 0.3a	22.1 ± 3.1a	0.080 ± 0.009a	74.1 ± 12.5b	0.073 ± 0.025b	0.0091 ± 0.0019a	6.00 ± 0.77a	0.0167 ± 0.012a	0.0112 ± 0.006a	<LOD
11-21 weeks Autoclaved	Banana	41 ± 5b	1.2 ± 1.2b	0.5 ± 0.3c	6.5 ± 0.3a	10.0 ± 3.1b	0.074 ± 0.009a	96.4 ± 12.1a	0.1502 ± 0.025a	0.0063 ± 0.0019a	2.27 ± 0.77b	0.022 ± 0.012a	0.020 ± 0.006a	<LOD
	Green bean	50 ± 5a	4.7 ± 1.2a	0.8 ± 0.3c	6.4 ± 0.3a	21.1 ± 3.1a	0.088 ± 0.009a	37.6 ± 12.5c	0.1471 ± 0.025a	0.0111 ± 0.0019a	5.08 ± 0.77a	0.017 ± 0.011a	0.014 ± 0.006a	<LOD
	Rockmelon	54 ± 5a	2.5 ± 1.2b	1.8 ± 0.3b	6.9 ± 0.3a	19.5 ± 3.1a	0.081 ± 0.009a	72.9 ± 12.5ab	0.1626 ± 0.025a	0.0053 ± 0.0019a	5.74 ± 0.77a	0.018 ± 0.012a	0.009 ± 0.006a	<LOD
53-62 weeks Untreated	Banana	196 ± 50a	2.9 ± 0.7b	1.7 ± 1.0b	5.7 ± 0.2a	29.6 ± 7.9b	0.050 ± 0.015a	168.4 ± 19.1a	0.0407 ± 0.0055a	0.0201 ± 0.0102a	6.32 ± 1.31b	0.014 ± 0.014a	0.019 ± 0.004a	<LOD
	Green bean	185 ± 50a	5.1 ± 0.7a	3.9 ± 1.0a	6.2 ± 0.2b	70.4 ± 7.9a	0.031 ± 0.015a	147.5 ± 9.6a	0.0464 ± 0.0055a	0.0349 ± 0.0102a	12.25 ± 1.31a	0.0414 ± 0.014a	0.012 ± 0.005a	<LOD
	Rockmelon	303 ± 50a	5.3 ± 0.7a	4.4 ± 1.0a	6.3 ± 0.2b	64.3 ± 7.9a	0.036 ± 0.015a	194.5 ± 13.5a	0.0478 ± 0.0055a	0.0246 ± 0.0102a	13.45 ± 1.31a	0.026 ± 0.012a	0.0131 ± 0.004a	<LOD

Leachates from the 11-21 week harvest were autoclaved to assess the nutrient addition from lysed microbes. LMM analysis performed, and predicted means ± SE are shown. Values are the mean of four (11-21 weeks) or five (53-62 weeks) replicates. Different letters indicate significant differences within each harvest ($p<0.05$).

Table 4.13. Nutrient content of vermicompost leachates fed different worm-bed substrates and harvested at two time periods.

	(mg/L)	TOC	TN	P	S	Ca	Cu	K	B	Mn	Mg	Fe	Zn
Banana	11-21 weeks	38 ± 5b	1.0 ± 1.2b	0.4 ± 0.3b	6.3 ± 0.3a	8.8 ± 3.1b	0.073 ± 0.009a	92.7 1± 12.5a	0.072 ± 0.025a	0.0071 ± 0.0019b	2.07 ± 0.77b	0.040 ± 0.012a	0.020 ± 0.006a
	53-62 weeks	196 ± 50a	2.9 ± 0.7a	1.7 ± 1.0a	5.7 ± 0.2a	29.6 ± 7.9a	0.050 ± 0.015a	168.4 ± 19.1a	0.0407 ± 0.0055a	0.0201 ± 0.0102a	6.32 ± 1.31a	0.014 ± 0.014a	0.019 ± 0.004a
Green bean	11-21 weeks	51 ± 5b	5.0 ± 1.2a	0.6 ± 0.3b	6.8 ± 0.3a	19.5 ± 3.1b	0.078 ± 0.009a	37.0 ± 12.5a	0.078 ± 0.025a	0.0070 ± 0.0019b	5.01 ± 0.77b	0.026 ± 0.012a	0.0157 ± 0.006a
	53-62 weeks	185 ± 50a	5.1 ± 0.7a	3.9 ± 1.0a	6.2 ± 0.2b	70.4 ± 7.9a	0.031 ± 0.015a	147.5 ± 9.6a	0.0464 ± 0.0055b	0.0349 ± 0.0102a	12.25 ± 1.31a	0.0414 ± 0.014a	0.012 ± 0.005a
Rockmelon	11-21 weeks	54 ± 5b	2.1 ± 1.2b	2.7 ± 0.34a	6.5 ± 0.3a	22.1 ± 3.1b	0.080 ± 0.009a	74.1 ± 12.5a	0.073 ± 0.025a	0.0091 ± 0.0019a	6.00 ± 0.77b	0.0167 ± 0.012a	0.0112 ± 0.006a
	53-62 weeks	303 ± 50a	5.3 ± 0.7a	4.4 ± 1.0a	6.3 ± 0.2a	64.3 ± 7.9a	0.036 ± 0.015a	194.5 ± 13.5a	0.0478 ± 0.0055a	0.0246 ± 0.0102a	13.45 ± 1.31a	0.026 ± 0.012a	0.0131 ± 0.004a

LMM analysis performed, and predicted means ± SE are shown. Values are the mean of four (11-21 weeks) or five (53-62 weeks) replicates. Different letters indicate significant differences between time periods for each substrate ($p < 0.05$). Mo is excluded as all samples were below the limit of detection.

4.4 Discussion

4.4.1 Leachate as a soil amendment

There are several aspects of leachate that make it a candidate for soil amendment. Despite differences in worm-bed substrate pH, all substrates produced neutral leachate. Potato slurry (Frederickson, 2002), sheep manure (Gutiérrez-Miceli et al., 2011), cow manure (Bidabadi et al., 2016; León-Anzueto et al., 2011) and vegetable waste (Singh et al., 2010) have also been shown to produce near neutral leachates. Vermicompost is less predictable, as pH can be variable and reflective of substrate pH (Edwards & Burrows, 1988), pH neutral or alkaline substrates can produce acidic vermicompost (Chaudhuri et al., 2003; Domínguez, 2004). Earthworm numbers affect vermicompost pH (Suthar, 2010) as does sampling time (Huang et al., 2014). The leachates produced in this study are in the suitable pH range for plant amendments of up to 7.5 (Lake, 2000).

E. fetida inhabit natural environments with a broad pH range (Sims & Gerard, 1985) and will tolerate acidity in vermicompost beds (Windust, 1997). Hanc & Chadimova (2014) found it necessary to raise the pH of apple pomace via a two week pre-treatment prior to vermicomposting. The acidic banana worm-bed substrates used in this trial were sufficient for earthworm survival without pre-treatment. While a pH of 4.0 (Hanc & Chadimova, 2014) requires pre-treatment, in this study, banana with a pH of 4.7 did not.

pH and EC values are most frequently used to define vermicompost quality (Majlessi et al., 2012; Tognetti et al., 2005; Tognetti, Mazzarino, & Laos, 2006). This is likely because they are quick and easy measurements. Due to the high number of variables between composting systems, no recommended values exist. In this study, using different worm-bed substrates didn't significantly effect leachate EC. Leachates from this trial are well below the maximum recommended EC of 4000 µS cm^{-1} for soil additives and plant tolerance (Gupta and Garg 2009). Vermicompost, with its high soluble salt content, has been shown to inhibit seed germination (Asciutto et al., 2006) and root elongation (Pittaway, 2014). Negative effects in both these studies were attributed to high salt or pH. The reduced soluble salt in leachate may therefore remove inhibition potential as a concern when applying to soil.

The leachate EC values from this study ranged from 57 to 286 µS cm^{-1}. Differences in EC are likely related to the different nutrients in the worm-bed substrates. This is demonstrated by the lowest EC

being found in the control leachate, from the nutrient poor paper pulp. This is similar to the results of Ansari (2008) who found an EC of 200 µS cm^{-1} from cow manure and straw leachate. However, many reported values were higher. ECs from composted cow manure leachate were 1440 µS cm^{-1} (León-Anzueto et al., 2011), 1660 µS cm^{-1} (Quaik et al., 2012a) and 2600 µS cm^{-1} (Garcia-Gomez et al., 2008). EC from chicken manure leachate was 1200 µS cm^{-1} (Pant et al., 2009) and sheep manure leachate was 2600 µS cm^{-1} (Gutiérrez-Miceli et al., 2011). Frederickson (2002) reported inhibition of germination and root elongation using leachate from potato slurry with an EC of 2633 µS cm^{-1}. It is possible that the worm-bed substrates chosen contributed to these elevated EC values, although direct comparisons are difficult due to differences between vermicomposting systems. For example, Frederickson (2002) used the earthworm species *Dendrobaena veneta* while Quaik et al. (2012a) used *Eudrilus eugeniae*, Ansari (2008) *P. excavatus* and *Lampito mauritii* and the rest *E. fetida.*

In terms of vermicompost, the C:N ratio is expected to reduce over vermicomposting time, with a value below 20 used to define a stable and mature compost (Gupta & Garg, 2009). Chaudhuri et al. (2003) reported a C:N of 19.94 for leaf litter vermicomposted by *E. fetida* for 62 days. Roy et al. (2010) reported a C:N of 5.59 for cow manure and crop residue vermicomposted by *P. excavatus* for 60 days. A decrease in C:N from 30.13 to 15.40 over seven weeks was reported by Majlessi et al. (2012). Three different substrates tested by Amouei, Yousefi, & Khosravi (2017) all showed vermicompost C:N under 20. In this study, initial values were not tested, so it is unknown if a reduction in C:N occurs with leachate. All worm-bed substrate blends except the control had C:N greater than 20 after 70 days. Both freshly harvested and stored leachates had C:N over 20, while fresh and stored control leachates remained below 20. Using rockmelon as a worm-bed substrate significantly increased leachate C:N to 56. Paper was the only substrate to produce leachate with a C:N less than 20 after 70 days. This is in contrast to the findings of Gupta & Garg (2009) who reported C:N values between 11 and 35 for cow manure and paper vermicompost after 91 days, with the higher values corresponding to greater paper quantities.

There is no recommended C:N for leachate and it is unknown whether the values of vermicompost and leachate are comparable. The low vermicompost C:N values cited above may be attributed to a single addition of substrate or feed. It is possible that vermicompost C:N remains elevated with continuous feeding as carried out in this study. The drawback of single feeding to obtain the ideal C:N are the negative effects on the earthworms. Food shortage can prevent reproduction (Sheppard, 1988) and limit population size (Sims & Gerard, 1985).

There are additional complications to using C:N as a marker of leachate quality. Unuofin & Mnkeni (2014) found initial earthworm population size had a significant effect on vermicompost C:N using a single feed. It is therefore likely that ongoing population size will affect leachate C:N using repeat feedings. Earthworm population size is influenced by worm-bed substrate as demonstrated in this study (Chapter 2). The C:N values of the worm-bed substrate used in this study were not determined and may also contribute to leachate C:N.

Vermicompost addition to soil has been shown to change soil microbes (Doan et al., 2013; Parthasarathi et al., 2008; Y. Wu et al., 2013). Vermicompost from buffalo manure increased bacterial diversity and activity in soil sampled from the red river delta in Vietnam (Doan et al., 2013). Vermcompost from sugar mill waste increased total microbial activity in clay loam soil, sandy loam soil and red loam soil from India (Parthasarathi et al., 2008). Vermicompost from cattle manure increased soil microbial biomass in saline alkali soil in China (Wu et al., 2013). Vermicompost has a different effect on soil microbes than other organic amendments (Jack et al., 2011). There is no reported evidence of effects on soil microbes with leachate addition to soil. However, compost extracts increase diversity of bacteria, fungi, protozoa and nematodes in soil. Extracts supply nutrients to feed the microbes, in addition to supplying nutrients to plants (Ingham, 2005).

Leachates tested in this study are likely to have different effects on soil microbes due to their different microbial populations and nutrient content. Banana, green bean and rockmelon as solo worm-bed substrates produced different leachate microbes than when used in combination. The degree of separation varied with substrate. Banana, green bean and a mix of banana and green bean produced three well separated microbial populations. Banana, rockmelon and a mix of banana and rockmelon produced microbial populations with some overlap. It would be interesting to determine whether combining banana and rockmelon leachates post-harvest produces the same overlap.

The fungal:bacterial ratios in leachate range from 0.03 to 0.0007, with no significant worm-bed substrate variation observed. All leachates were bacterial dominated. There is no ideal fungal:bacterial ratio for compost extracts. The usefulness of the extracts depends on soil type and plant species (Ingham, 2005). According to Ingham (2005) leachate created in this study would be more useful to balance fungal-dominated soil. Bacterial dominated extracts are also ideal for foliar application (Ingham, 2005). According to Strickland & Rousk (2010), fungal/bacterial dominance in soil is dependent on the measurement method used.

Most temperate arable agricultural soils are dominated by fungi. Typical fungal:bacterial ratios range from 1.0 to 2.3, but much higher and lower ratios are well documented (Abbot, n.d.). Soil with high fungal:bacterial ratios are linked to greater C storage potential. Intensively managed soil often exhibit lower fungal:bacterial ratios (Malik et al., 2016). Soil fungal:bacterial ratio varies with factors such as humidity and pH (Ananyeva et al., 2006). Specific ratios aren't generally used to indicate soil quality, rather changes in fungal:bacterial ratio may point to adverse changes in soil (Classens, 2013). Nutrient availability may influence soil fungal:bacterial ratios (Strickland & Rousk, 2010) and addition of a source of nutrients such as leachate may alter soil fungal:bacterial ratio. Soil fungal:bacterial ratio response to leachate may depend on specific leachate fungal:bacterial ratio and nutrient content. The bacterial domination of the leachate was not affected by worm-bed substrate. This indicates that leachate from all worm-bed substrates tested would be more appropriate for application to fungal dominated soil, However, because of the lack of available data on the importance of soil fungal:bacterial ratio, this should not preclude addition of leachate from this study to bacterial dominated soil.

4.4.2 Leachate after long term storage

Vermicomposting involves distinct microbial communities, an important part of the process, believed to confer benefits to plant growth (Atiyeh et al., 2000c). In vermicomposting, the aerobic microorganisms such as fungi and bacteria principally decompose the waste (Frederickson, 2002). Most of the knowledge of vermicompost microbes comes from culture techniques (Byzov et al., 2009; Gopal et al., 2009; Pathma & Sakthivel, 2013). In some cases an increase in cultured bacteria has been observed in vermicompost when compared to the substrate (Yasir et al., 2009) but decreases in bacteria and fungi have also been observed (Morgan, 1988). Worm-bed substrate microbes were not cultured in this study so no trend was measured.

Banana, green bean and rockmelon had no effect on numbers of cultivable bacteria, fungi or free-living N-fixing bacteria. The eight worm-bed substrates tested in this study had no effect on numbers of cultivable bacteria or fungi in leachate. Grantina-Ievina (2013) studied cow manure, leaf litter, potato, grass and sewage sludge vermicompost. No worm-bed substrate effect on cultivable bacteria was found, although a substrate effect on cultivable fungi was observed. Gopal et al. (2009) studied cow manure and a mixture of cow manure and leaf litter vermicompost. No substrate effect was observed for cultivable heterotrophic bacteria, actinomycetes or spore forming bacteria. However, a substrate effect on cultivable fungi was observed, as well as a substrate effect on cultivable

pseudomonads and phosphate solubilisers. Studies using culture techniques are likely to underestimate microbial populations as a large proportion of microorganisms are non-culturable. Yasir et al. (2009) found more than half of the microorganisms from a paper and dairy waste vermicompost were omitted using culture techniques.

Molecular methods did identify differences between the leachates from different worm-bed substrates in this study. The leachate bacterial populations were different at 10 weeks for all eight substrates. Amplicon number indicates that rockmelon produced significantly more diverse leachate than all other treatments. The paper control produced leachate with a significantly less diverse bacterial population than all treatments except B/G/R. The relatively low diversity in banana and green bean leachates likely led to this reduced diversity in B/G/R leachate. It is possible that the measured diversity was present prior to vermicomposting. Additional leachate sampling is required to determine whether diversity is linked to worm-bed substrate, vermicomposting or both.

There are many commercially available vermicompost products (Circular Food, 2016; Ferguson, 2013; Nutri-Health International, 2015; Worms DownUnder, 2017). Leachates like those produced in this study can be harvested and used immediately. However, for the commercial products, as well as large-scale vermicompost, storage of the product would be reasonably common. Products would be expected to remain stable during this time. However, this study shows that is not always the case. Cultivable bacteria remained stable only when leachate was refrigerated. Refrigeration reduced cultivable fungi in some cases, lack of refrigeration reduced both bacteria and fungi and these changes varied with worm-bed substrate. Only green bean and B/R remained stable. Similar findings were reported by Grantina-Ievina et al. (2013) with fewer cultivable microbes in cow manure vermicompost stored for one year at room temperature when compared with storage at 4°C. Storage of vermicompost leachate is unlikely, especially in the large quantities required in broad acre agriculture. It is therefore likely that the leachate microbes will be different between growing seasons.

Storage recommendations of commercial products were difficult to find. Most commercial products or domestic worm farm instruction manuals don't address this question. Those that do, only consider oxygen. Ingham (2017) recommends using liquid extract within six hours of harvest to minimise detrimental effect of low oxygen on microbes. Liquid Organic Fertiliser (2015) recommend loosening the container lid to increase oxygen and prolong the microbes. Ingham (2005) recommends using unaerated extract within eight hours and aerated extract within five days. Stored leachates in this study were not supplied with additional oxygen. Leachates were left unattended in

closed containers to mimic typical storage conditions. Storage reduced cultivable microbes in most cases, although not to a statistically significant degree. In the case of green bean leachate and paper leachate, refrigeration resulted in an increase in cultivable microbes during storage, although again not to a statistically significant degree. These results show that oxygen input is not the only factor to be considered when storing organic amendments.

Molecular analysis showed fewer stable leachates than culture studies. Both techniques showed the importance of temperature. Worm-bed substrate mixtures B/R, G/R and B/G/R were stable using molecular analysis, but only at 4°C. The physical properties of leachate (pH and EC) were unaffected by storage whereas C:N was affected by storage. This is likely due to differences in microbial populations. According to the results obtained from this study, to obtain a leachate with unchanged microbial communities after one year, recommended worm-bed substrate blends are banana and rockmelon, or green bean and rockmelon and leachate should be stored at 4°C.

4.4.3 Nutrient content of leachate

Leachates were harvested and then autoclaved in borosilicate glass before being transferred to plastic tubes for storage. Borosilicate glass is a potential source of B contamination. Contamination of alkaline solutions can occur after only five minutes of exposure, but it takes over four weeks of room temperature storage for measurable B contamination of water (Green, Blincoe, & Weeth, 1976). Heat increases the potential for contamination (Dulski, 1999). Untreated leachates were not stored in glass long enough to be contaminated, but it is likely the elevated B in autoclaved leachates is due to contamination. Banana, green bean and rockmelon leachates (both autoclaved and untreated) contained 0.04-0.16 mg/L of B. Much higher B was reported by Singh et al. (2010) in leachate from cow manure, vegetable waste, and a cow manure and vegetable waste mix.

Banana, green bean and rockmelon leachates contained between 37-195 mg/L of K. K levels in this range have been reported in leachate from cow manure (Álvarez-Solís et al., 2016), composted cow manure (Gutiérrez-Miceli et al., 2008) and chicken manure (Pant et al., 2009). Much higher K levels were found in leachate from composted cow manure (Oliva-Llaven et al., 2010), cow manure, green waste (Tejada et al., 2008), vegetable waste, and a mix of vegetable waste and cow manure (Singh et al., 2010). In contrast, Garcia-Gomez et al. (2008) found no K in leachate from composted cow manure and straw.

The many variables between reported vermicomposting systems makes determining reasons for variation in nutrients difficult. The high K reported by Oliva-Llaven et al. (2010) may be due to the

large size of the worm-beds used. A total of 79m^2 was available for leachate collection. Tejada et al. (2008) and Singh et al. (2010) used 25 cm x 30 cm worm-beds, which, while comparatively small, are larger than the worm-beds used in the current study. These larger worm-beds likely contained more worm-bed substrate which resulted in more concentrated leachate with higher nutrients. In addition, Oliva-Llaven et al. (2010), Tejada et al. (2008) and Singh et al. (2010) harvested leachate only once. The multiple leachate harvests used in the current study may have depleted the nutrients.

Banana, green bean and rockmelon leachates contained between 9-70 mg/L of Ca. Ca levels in this range have been reported in leachate from chicken manure (Pant et al., 2009), cow manure (Álvarez-Solís et al., 2016) and a mix of composted cow manure and straw (Garcia-Gomez et al., 2008). Ca in this range was also found by Preciado-Rangel et al. (2015), although the substrate was not reported. Slightly higher Ca has been found in leachates from composted cow manure (Gutiérrez-Miceli et al., 2008; Oliva-Llaven et al., 2010), vegetable waste and a mix of cow manure and vegetable waste (Singh et al., 2010).

Banana, green bean and rockmelon leachates contained between 0.4-4.4 mg/L of P. P levels within this range have been reported in leachate from sheep manure (Gutiérrez-Miceli et al., 2011), cow manure (Álvarez-Solís et al., 2016) and composted cow manure (León-Anzueto et al., 2011). Slightly higher P has been reported in leachate from chicken manure (Pant et al., 2009). Much higher P has been found in leachate from cow manure, vegetable waste, cow manure and vegetable waste mix (Singh et al., 2010) and green waste (Tejada et al., 2008).

Banana, green bean and rockmelon leachates contained 1-5 mg/L of TN. This is lower than TN reported in leachate from sheep manure (Gutiérrez-Miceli et al., 2011), composted cow manure (León-Anzueto et al., 2011), chicken manure (Pant et al., 2009), cow manure, green waste (Tejada et al., 2008), vegetable waste and a cow manure and vegetable waste mix (Singh et al., 2010). This may be due to the high ammonia content of animal manure (Nahm, 2003). Banana, green bean and rockmelon leachates contained 38-303 mg/L of TOC. This is much lower than the TOC found in leachates from cow manure, vegetable waste, cow manure and vegetable waste mix (Singh et al., 2010) and green waste (Tejada et al., 2008).

Banana, green bean and rockmelon leachates contained between 2-13 mg/L of Mg. Mg levels within this range were also found by López-Martínez et al. (2016) and Preciado-Rangel et al. (2015) using unreported substrates. Much higher Mg levels have been found in leachates from composted cow

manure (Gutiérrez-Miceli et al., 2008), chicken manure (Pant et al., 2009) and a mix of composted cow manure and straw (Garcia-Gomez et al., 2008).

The high Mg reported by Gutiérrez-Miceli et al. (2008) and Garcia-Gomez et al. (2008) may be due to the large size of the worm-beds used. A total of 400m^2 was available for leachate collection. These larger worm-beds likely contained more worm-bed substrate which resulted in more concentrated leachate with higher nutrients. Pant et al. (2009) used commercial vermicompost in 19L buckets which are much larger than the worm-beds used in the current study, although the worm-bed substrate volume used is impossible to determine. The studies of Gutiérrez-Miceli et al. (2008), Garcia-Gomez et al. (2008) and Pant et al. (2009) made no mention of multiple leachate harvests. The multiple leachate harvests used in the current study may have depleted the nutrients.

Banana, green bean and rockmelon leachates contained 0.01-0.04 mg/L of Fe. Higher Fe levels have been found in leachates from cow manure (Álvarez-Solís et al., 2016), composted cow manure, and a mixture of composted barley shoots, corn and peat (Ávila-Juárez et al., 2015). Higher Fe was reported in liquid extract of crop residue vermicompost (Marín et al., 2013). In contrast, Pant et al. (2009) reported no Fe in chicken manure leachate.

Banana, green bean and rockmelon leachates contained between 0.007-0.03 mg/L of Mn. Higher Mn has been reported in cow manure leachate (Álvarez-Solís et al., 2016; Bidabadi et al., 2016), composted cow manure, and a mixture of composted barley shoots, corn and peat (Ávila-Juárez et al., 2015). Higher Mn was reported in liquid extract of crop residue vermicompost (Marín et al., 2013). Bidabadi et al. (2016) used a proportion of 40 g of worm-bed substrate per g of earthworms, while the current study used 3 g of substrate per g of earthworms. This large volume of worm-bed substrate likely produced leachate with greater Mn. Ávila-Juárez et al. (2015) used 1 m x 10 m x 0.5 m worm-beds which probably also used much greater substrate volumes. The vermicompost system used by Álvarez-Solís et al. (2016) was not described, however this study and the studies by Bidabadi et al. (2016) and Ávila-Juárez et al. (2015) made no mention of multiple leachate harvests, which likely also increased leachate nutrients.

Banana, green bean and rockmelon leachates contained between 0.01-0.02 mg/L of Zn. Higher Zn has been reported in cow manure leachate (Bidabadi et al., 2016), composted cow manure, and a mixture of composted barley shoots, corn and peat (Ávila-Juárez et al., 2015). Higher Zn was reported in liquid extract of crop residue vermicompost (Marín et al., 2013). Marín et al. (2013) soaked commercial vermicompost in water for 14 days, which likely increased the Mn and Zn levels in the extract.

Banana, green bean and rockmelon leachates contained between 0.03-0.08 mg/L of Cu. Higher Cu has been reported in leachates from cow manure (Álvarez-Solís et al., 2016; Bidabadi et al., 2016), composted cow manure, and a mixture of composted barley shoots, corn and peat (Ávila-Juárez et al., 2015). Higher Cu was reported in extracts of sewage sludge (Srivastava et al., 2005) and crop residue vermicompost (Marín et al., 2013). Srivastava et al. (2005) used 10 kg of worm-bed substrate and only one leachate harvest, which likely increased the Cu level.

Banana, green bean and rockmelon leachates contained between 5.7-6.8 mg/L of S. This is much lower than the S levels found in leachates from composted cow manure, and a mixture of composted barley shoots, corn and peat (Ávila-Juárez et al., 2015) and those reported by López-Martínez et al. (2016) and Preciado-Rangel et al. (2015) using an unreported substrate. Neither López-Martínez et al. (2016) nor Preciado-Rangel et al. (2015) described the vericomposting and leachate collection methods used so the reasons for the elevated S in these leachates can't be ascertained.

Many factors contribute to differences in leachate nutrients. Leachate nutrients are reflective of substrate nutrients for some elements but not all (Singh et al., 2010). Leachate harvest has been shown to reduce inorganic ions in vermicompost (Fornes et al., 2012; Huang et al., 2014). Leachate nutrient content is therefore directly related to nutrient content of vermicompost. Leachates harvested at 53-62 weeks showed fewer substrate related differences than the 11-21 week harvest, which may indicate a stabilisation of nutrient content over time. Vermicompost leachate nutrients may be related to worm-bed substrate nutrients but that varies with nutrients analysed and harvest time.

According to (Bidabadi et al., 2017) the nutrient content of leachate from cattle manure also varies with the food that the cattle have been fed. This is less likely to cause variation in leachate from fruit and vegetable material such as those analysed in the current study. However, increased fertilisation of the original crop may produce increased nutrient levels in the fruit and vegetable, which may be reflected in variation in leachate nutrients.

Vermicompost TOC and TN vary with harvest time when a single feed is used (Huang et al., 2014). Variation between nutrient content found in this study and those reported elsewhere may be related to harvest time in addition to substrate used and frequency of substrate addition. The presence of *E. fetida* reduces TOC compared with substrate and conventional compost (Yang et al., 2014). Relative density of earthworms and earthworm species may cause differences in nutrients between various leachates. The variation in leachate nutrients will effect their ability to support

plants. For example, supplementation with chemical fertiliser may be required to obtain adequate N, P, K for sugarcane (Gutiérrez-Miceli et al., 2017), maize (Garcia-Gomez et al., 2008) and sorghum (Gutiérrez-Miceli et al., 2008).

4.5 Conclusion

Vermicompost substrate changes leachate microbes, however, these changes are not observed using culture techniques. Molecular methods are needed to detect worm-bed substrate effect on leachate microbes. Vermicompost substrate effects leachate macronutrient content and C:N ratio but has no effect on content of most micronutrients. Macro- and micronutrient content vary with time of harvest of leachate from worm farms. According to the data from this study, banana, green bean and rockmelon, alone and in combination, produce ideal leachates for soil amendment. Leachates can be stored for a year; however, the microbial populations are likely to be different. Microbial communities were stable in leachate produced from rockmelon combined with either banana or green bean when stored at 4°C.

Chapter 5 - Effect of leachate on plants

5.1 Introduction

Vermicompost leachate from a narrow range of worm-bed substrates have been tested on a wide variety of plant species (summarised in Table 1.1). Leachate has been shown to increase shoot length and fresh and dry weight of tomato (Arthur et al., 2012) and improve shoot dry weight and oil yield of lemongrass (León-Anzueto et al., 2011). However, leachate effects were not uniformly positive. For instance, leachate improved plant height, leaf size and chlorophyll content of peppermint, but had no effect on leaf and branch numbers, plant dry weight or oil yield (Ayyobi et al., 2013). Leachate increased fruit yield but had no effect on fruit weight of strawberries (Singh et al., 2010).

Negative effects on plant growth after application of leachate have not been reported, however negative effects have been reported when leachate was applied to seeds. Several studies have concluded that the high salt content of undiluted leachate has a detrimental effect on seed germination (Garcia-Gomez et al., 2008; Gutiérrez-Miceli et al., 2008; Kandari et al., 2011) and some studies apply dilute product only (Aremu et al., 2012, 2014; Radhakrishnan & Mahendran, 2009).

Broad acre crops are generally supplied with large inputs of chemical fertiliser. The use of N and P fertilisers has increased steadily for over two decades, while K has increased more recently in response to grain quality problems (Anderson et al., 2005). Excess fertiliser application leads to environmental degradation and is not economically sustainable. In 2010, Julian Cribb wrote in a Strategic Analysis Paper that 'nutrients are the oil of the 21st century'. They are increasing in cost and demand and large volumes are wasted through transportation off-farm, consumption and eventual disposal. Nutrient recycling by composting waste and improved nutrient uptake by plants through microbial plant growth promotion may be a critical part of the solution.

Three different crops were chosen to examine the effects of vermicompost leachate, including the grass crop, wheat. Wheat (*Triticum* sp.) is the staple food of a large part of the global population and one of the most important crops in Australian agriculture. Australia consumes around 5 million tonnes annually, with the remainder exported to over 40 countries, representing around 15% of the global wheat trade (Australian Bureau of Statistics, 2007). Adequate P is essential for early growth, and soil N is the key factor determining grain protein (Hillman & Smith, 2012).

Two legume crops were chosen to examine the effects of vermicompost leachate, due to the importance of legumes in agricultural N supply. Large inputs of N can be achieved through rotation of cereal crops with legumes. Biological N_2 fixation is crucial to sustainable agriculture in soils which are frequently N deficient (M'Gee, 2011). Symbiotic N_2 fixation is dependent on effective nodulation by compatible rhizobial strains. High temperature, drought and soil acidity are detrimental to soil rhizobial populations and subsequently legume nodulation (Hungria & Vargas, 2000). Addition of chemical N is generally the only way to remedy nodulation failure, but it can be costly (Ryder, 2013). Naturalised soil rhizobia that nodulate legumes can occur in high numbers, and the poor survival of inoculant strains in soil often leads to nodulation and N-fixation by naturalised rhizobia. These naturalised rhizobia vary in efficacy, often leading to suboptimal N fixation (Drew et al., 2011).

The legume crops chosen were soybean and serradella. Globally, soybean (*Glycine max*) is one of the most significant grain industries. Australian soybeans, while comparatively small, are an important part of the $2.5 billion oilseed industry (M'Gee, 2011). Application of large quantities of chemical N to soybean is not a common practice, but it is thought that N_2 fixation does not supply enough N for maximum yield (Janagard et al., 2013). French Serradella (*Ornithopus sativus*) is a deep-rooted legume which can provide herbage for grazing animals in years of low or erratic rainfall. The hard seed cultivar Margurita is useful in crop: pasture rotations as seeds can survive a crop rotation and regenerate the following year, reducing the need for N fertiliser (Hackney, Rodham, & Piltz, 2013).

Sterilisation of both solid vermicompost and vermicompost leachate has been shown to alter the effects of the amendment, indicating that microbes play an important role in these effects. Unsterile cow manure leachate completely inhibited growth of the pathogen *C. gloeosporioides* on agar. Sterilised leachate had no effect on pathogen growth (Contreras-Blancas et al., 2014). Liquid extract of solid crop residue vermicompost completely inhibited growth of the *Botrytis cinerea*, *Sclerotinia sclerotiroum*, *Pythium aphanidermatum* and *Phytophthora parasitica* on agar. These effects were removed by sterilisation of the vermicompost (Marín et al., 2013).

The aims of the work reported in this chapter were to determine whether leachate produced using different worm-bed substrates affected seed germination and plant growth. The specific aims were to determine if the effect of leachate on plants varied with:

1. Worm-bed substrate
2. Plant species
3. Sterilisation of leachate to reduce microbial component
4. Nodulation of legumes by both introduced and naturalised rhizobia

The null hypothesis for these experiments was that leachate will have no effect on germination and growth of plants. Changing the worm-bed substrate will not change the effect of leachate on plants. Leachates will have the same effect on different species of plants. Sterilisation of leachate will not change the effect on plants. Inoculation will not change the effect of leachate on plants, nor will the presence of naturalised rhizobia.

5.2 Methods

5.2.1 Banana, green bean, rockmelon leachates with different microbial content – Effect on seed germination

5.2.1.1 Vermicompost

Vermicompost, leachate harvest and sterilisation are described in sections 4.2.2.1-2.

5.2.1.2 Germination assay

French serradella (*O. sativus* cv. Margurita) seeds were obtained from AusWest Seeds (Kellyville, Australia). Filter paper circles (100mm, Merck Millipore, Bayswater, Australia) were placed in 100 mm sterile petri dishes (Sarstedt, Mawson Lakes, Australia) and soaked with leachate. Surface sterilised seeds (100) were spread evenly on the filter paper. Seeds were incubated at 25°C in the dark for four days and the number of germinated seeds counted. Deionised water was included as a control. Diluted Hoagland's solution was included as a salt control. Hoagland's solution was diluted 1:6 to obtain an EC of 0.44 mS cm^{-1}, equal to the mean leachate EC. Treatments were established in triplicate. Nutrient solution recipes are detailed in Appendix A.

5.2.2 Effect of banana, green bean, rockmelon leachates on plants

5.2.2.1 Vermicompost

Vermicompost set up and maintenance for plant experiments 1 and 2 are described in section 4.2.2. Leachate harvest and leachate sterilisation for experiment 1 are described in section 4.2.2. Leachates for experiment 2 were harvested the day before plant application and stored at room temperature in screw capped glass bottles. Leachate harvest is detailed in section 3.2.1. At 53 weeks, 2.2 L water was used to collect 2 L leachate. Leachate harvest was repeated at 56 weeks, 58 weeks, 60 weeks and 62 weeks.

5.2.2.2 Experiment 1 – Effect of unsterilised and sterilised leachates on soybean

The soybean trial, was carried out from October 16 2015 to December 11 2015. Topsoil was harvested from lupin plots grown at Lansdowne, Cobbitty and air dried for three weeks. Soil was sieved with a 395 mm x 100 mm garden sieve with 6 mm mesh (B.M.W Plastics, Dandenong, Australia). One part soil was mixed with four parts river sand (ANL, Terrey Hills, Australia) using a cement mixer and used to fill 0.97 L capacity pots (118 mm diameter x 125 mm height, Garden City Plastics, Somersby, Australia). The pots were sterilised with household disinfectant and rinsed with distilled water before being filled with sand/soil.

The experimental design is shown in Figure 5.1 and treatments are listed in Table 5.1. The treatment design was a split-split plot design with soybeans treated with three different leachates which had been subjected to three different treatments. Each set of plants was then either inoculated with rhizobia or uninoculated. The pots were arranged in a completely randomised block design comprising four blocks with twelve replicates per treatment.

Pots were housed in a Poly house at the Plant Breeding Institute (PBI, Cobbitty, Australia). No environmental controls were in place. Monitoring equipment was unavailable for most of the trial. Temperature was recorded with a thermometer 2- 10 times per week for 12 weeks. Temperature was recorded hourly for the final 15 days with a TinyTalk II -40/75(125)°C Data Logger (Hastings Data Loggers, Port Macquarie, Australia).

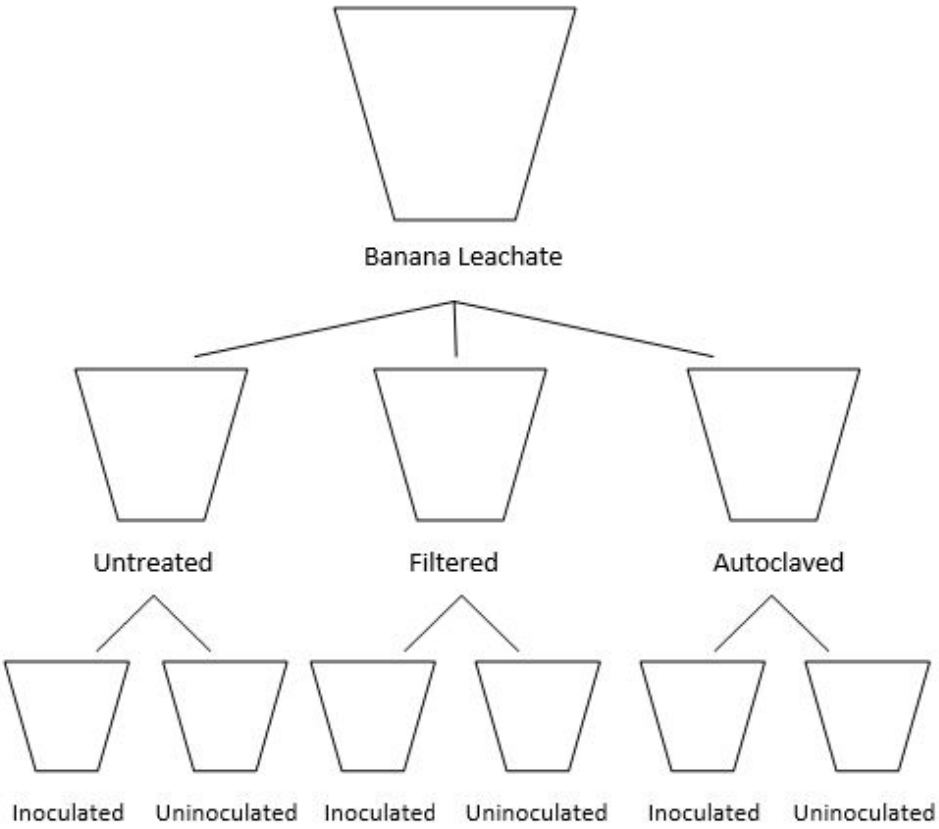

Figure 5.1. Split-split plot design of pot trial testing vermicompost leachate as a soil amendment. Leachates were subjected to three different treatments, untreated, filtered, and autoclaved then filtered. Soybean (*Glycine max*) was grown in all pots. Half the pots were inoculated with soybean-nodulating *Bradyrhizobium japonicum*. The same design was repeated with green bean leachate and rockmelon leachate.

Table 5.1. Treatments used in soybean greenhouse trial.

Vermicompost Substrate	Leachate Treatment	Inoculation
Banana	Untreated	None
		B. japonicum
	Filtered	None
		B. japonicum
	Autoclaved & Filtered	None
		B. japonicum
Green Bean	Untreated	None
		B. japonicum
	Filtered	None
		B. japonicum
	Autoclaved & Filtered	None
		B. japonicum
Rockmelon	Untreated	None
		B. japonicum
	Filtered	None
		B. japonicum
	Autoclaved & Filtered	None
		B. japonicum
Water		None
		B. japonicum

5.2.2.2.1 Leachate application

Immediately prior to plant application, EC was measured with an AS302 Pocket Conductivity Meter (Activon, Thornleigh, Australia). Leachates were diluted with tap water so they were all equal to the lowest EC. The EC values used were 0.5 mS cm^{-1} (11 week harvest), 0.2 mS cm^{-1} (12 week harvest) and 0.3 mS cm^{-1} (19 and 21 week harvest). Leachate was applied to the soil surface. The volume of diluted leachate applied was 30 mL (11 and 19 week harvest) and 35 mL (12 and 21 week harvest). An equal volume of tap water was applied to water control pots. Remaining leachate was discarded.

The first application of leachate (11 week harvest) occurred five weeks before sowing. The soil surface was then covered with low density polyethylene (LDPE) pellets (Arbee, Noble Park, Australia) to reduce contamination. The pots were also covered with round plastic containers (440 mL, 119 mm x 63 mm) (The Hospitality Store, Camperdown, Australia) to reduce moisture loss and contamination. The second application of leachate (12 week harvest) occurred three weeks before sowing.

5.2.2.2.2 Seed germination

Soybean seeds (*Glycine max*) variety SOYA 791 were sterilised in 3% (v/v) bleach (sodium hypochlorite) (Sigma-Aldrich, Castle Hill, Australia) for 5 min then rinsed six times in sterile distilled water. Seeds were wrapped in damp paper towel and placed in open zip lock bags. Seeds were germinated in the dark at room temperature for 3 days.

5.2.2.2.3 Inoculant preparation

A fresh streak plate of *Bradyrhizobium japonicum* CB1809 was made using Congo Red Yeast Mannitol Agar (CRYMA). Lawns of *B. japonicum* were made by inoculating twelve CRYMA plates with 100 µL of culture in sterile water. Plates were incubated at 28°C for 7 days. Plates were stored at 4°C until needed. On the day of inoculation, bacteria were transferred to 100 mL sterile water and mixed by vortexing. Inoculum concentration was determined by plating serial dilutions on CRYMA as described in section 4.2.1.

5.2.2.2.4 Seed sowing and leachate application

Pots were watered the day before sowing. Three holes of the same depth were made in the soil surface. Three germinated seeds were sown per pot. Seeds with the longest roots were selected. Pots were inoculated with *B. japonicum* 13 days after sowing (DAS), when cotyledons were observed. Inoculum (1 mL) was added to pots requiring inoculation and 1 mL of sterile water was added to all other pots. Inoculum concentration was 3.0×10^8 CFU/seed. Inoculum was applied by pipetting beneath the pellets, onto the soil surface. Approximately 250 µL of inoculum was applied to four evenly distributed places on the soil surface.

Excess seedlings were removed 19 DAS, leaving one seedling per pot. The third application of leachate (19 week harvest) occurred 20 DAS. The fourth application of leachate (21 week harvest) occurred 34 DAS. Plants were watered three times a week for 10 weeks then daily. Water volume used was sufficient to soak the soil while minimising drainage. Ten extra soybean plants were grown alongside the treatment plants. These plants were used to determine water volume required at each watering.

5.2.2.2.5 Plant harvest

Two replicates per treatment were harvested 55 DAS. Four replicates per treatment were harvested 56 DAS. Pots were emptied into a tray and soil shaken off the roots. Soybean roots were wrapped in damp paper towel, and sealed in zip lock bags. Plants were stored at 4°C prior to processing. Nodules were removed from roots by hand. Nodule number per plant was recorded. Nodules were washed in tap water and blotted dry with paper towel. Due to low nodule numbers, the six treatment replicates were combined and total nodule fresh weight determined. Nodules were dried at 60°C for two days. Total nodule dry weight was determined. Soybean shoots were separated from the roots. Shoots and roots were dried at 60°C for two days. Shoot dry weight and root dry weight per plant were recorded.

5.2.2.2.6 Shoot nutrient analysis

Dried shoots were placed in plastic screw capped tubes containing two 19 mm ball bearings. Shoots were ground in a flask shaker (Dynamax, Sydney, Australia) at 100 rpm for 1-2 hours until uniform. Shoots were digested using the Kjeldahl method. Ground shoots (0.8 g) was used. If not enough material was available, the entire sample was used. Shoots were placed in 300 mL glass sample tubes and two Kjeldahl Tablets ECO (BÜCHI, Flawil, Switzerland) were added per tube. ECO tablets consisted of 3.998 g K_2SO_4 and 0.002 g $CuSO_4.5H_2O$. Stearic acid (0.5 g) (Merck, Frenchs Forest, Australia) and 20 mL 98% sulfuric acid (Merck, Frenchs Forest, Australia) were added per tube. The tube contents were mixed by swirling. Nineteen plant samples were analysed along with a blank, with all reagents but no sample, to determine background N. Samples were digested using a KjelDigester K-437 (BÜCHI, Flawil, Switzerland) at 380°C for 150 min. The KjelDigester was attached to a Scrubber K-415 (BÜCHI, Flawil, Switzerland). The Scrubber neutralisation vessel was filled with 3 L 10% (w/v) sodium hydroxide (Merck, Frenchs Forest, Australia) to neutralise acid evaporation. pH

indicator (5 mL) (0.5% (w/v) bromothymol blue in 0.2 M potassium hydroxide) was added. The solution was replaced when it became pH neutral.

Digested samples were allowed to cool for 30 min. Deionised water (50 mL) was added to each tube. Samples were allowed to cool for 1 hour. Diluted digests were filtered with 110 mm Whatman Ashless Grade 42 filter paper (GE Healthcare Life Sciences, Parramatta, Australia). Filtered digests were made up to 100 mL with deionised water and mixed well. Digest (70 mL) was transferred to a sterile specimen container (Livingstone, Rosebery, Australia) and stored at room temperature until further analysis.

Digest (20 mL) was added to a 300 mL glass sample tube. Shoot Total Kjeldahl Nitrogen (TKN) was determined by distillation with a Kjelflex K-360 (BÜCHI, Flawil, Switzerland). Twelve drops of mixed indicator were added to a 250 mL receiving flask. The Kjelflex default reaction was used. The default reaction involved dispensing 60 mL 4% (w/v) boric acid (pH 4.65) into the receiving flask. The digest was diluted with 50 mL deionised water and 90 mL of 32% (w/v) sodium hydroxide was added to the digest followed by steaming with agitation for 4 min. The gaseous ammonia was condensed and dispensed into the receiving flask which contained boric acid. The distillate was back-titrated using hydrochloric acid 0.1 mol/L Titrisol® (Merck Millipore, Bayswater, Australia). The formulae used to calculate TKN are presented in Appendix B.

Shoot P was determined using a Molybdenum Blue colorimetric assay. Digest (50 µL) was transferred to a microcentrifuge tube and 50 µL deionised water, 800 µL acid diluent, 100 µL molybdate reagent and 100 µL ascorbic acid were added to the digest. The solution was vortexed then incubated at 80°C for 10 min. Absorbance at 660 nm was measured with a UVmini-1240 UV-VIS Spectrophotometer (Shimadzu, Kyoto, Japan). A P standard was made using KH_2PO_4 dried overnight at 70°C. KH_2PO_4 (0.5 g) was made up to 1 L with deionised water. Stock was diluted (10 mL made up to 100 mL) with deionised water to make a 50 mg/L standard. A 0-2.5 µg P standard curve was used to determine digest P. P concentration in the blank was subtracted from digest P to obtain shoot P. Reagent recipes are listed in Appendix A.

5.2.2.3 Experiment 2 -– Effect of banana, green bean and rockmelon leachates on serradella and wheat

The serradella and wheat trial was carried out from June 23 2016 to September 12 2016. Soil and pot preparation were carried out for experiment 1. One part soil was mixed with one part river sand (ANL, Terrey Hills, Australia). The experiment was set up in a completely randomised block design

comprising five blocks with ten replicates per treatment. The treatments are listed in Table 5.2. Pots were housed in the Controlled Environment Facility (CEF) at the CCWF (Cobbitty, Australia). Plants were grown under halogen light at 24°C from 6am to 8pm then in darkness at 18°C.

Table 5.2. Treatments used in serradella and wheat pot trial.

Plant species	Treatment solution	Inoculation
Serradella	Banana leachate	Uninoculated
		Inoculated with WSM471
	Green Bean leachate	Uninoculated
		Inoculated with WSM471
	Rockmelon leachate	Uninoculated
		Inoculated with WSM471
	Nutrient control	Uninoculated
		Inoculated with WSM471
	Water control	Uninoculated
		Inoculated with WSM471
Wheat	Banana leachate	None
	Green Bean leachate	None
	Rockmelon leachate	None
	Nutrient control	None
	Water control	None

5.2.2.3.1 Leachate application

Leachate EC was measured with an S230 SevenCompact™ conductivity meter (Mettler Toledo). Immediately prior to plant application, treatment solutions were diluted to the lowest EC with tap water. The EC values used were 0.8 mS cm^{-1} (53 week harvest), 0.9 mS cm^{-1} (56 week harvest), 0.5 mS cm^{-1} (58 and 60 week harvest) and 0.7 mS cm^{-1} (62 week harvest). Treatment solution (30 mL) was applied to the soil surface. Diluted Hoagland's solution was used as the nutrient control. Tap water was applied to water control pots. Remaining leachate was discarded.

5.2.2.3.2 Seed germination

French serradella (*O. sativus* cv. Margurita) seeds (AusWest Seeds, Kellyville, Australia) were supplied pre-coated with inoculant. Pre-coat was removed by washing three times in tap water, with agitation, for 10 min total. To confirm pre-coat removal, three sets of ten seeds were immersed in 1 mL sterile saline and vortexed. This solution was used to make streak plates on Yeast Mannitol Agar (YMA). No growth was observed following 7 days incubation at 28°C. Washed serradella seeds were sterilised in 3% (v/v) bleach (sodium hypochlorite) (Sigma-Aldrich, Castle Hill, Australia) for 5 min then rinsed ten times in sterile distilled water. Wheat (*Triticum aestivum* cv. EGA Gregory) seeds were wrapped in a single layer of muslin and sealed with a rubber band. Each muslin bundle contained approximately 20 seeds. Bundles were placed in a glass Büchner flask. The flask was attached to a second Büchner flask to act as a waste trap. The second flask was in turn attached to a water aspirator to create a vacuum. Seeds were washed by adding 2 drops TWEEN® 20 (Sigma-Aldrich, Castle Hill, Australia) then flushing 1.8 L tap water through the flask. Seeds were sterilised in 0.5% (w/v) mercuric chloride (Sigma-Aldrich, Castle Hill, Australia) for 75 seconds. Seeds were rinsed by flushing 2 L sterile deionised water through the flask. Sterile seeds were spread on water agar plates. Plates containing serradella seeds were inverted. Seeds were germinated in the dark at room temperature for 5 days.

5.2.2.3.3 Inoculant preparation

Bradyrhizobia sp. WSM471, the Australian commercial strain for serradella, was obtained from Australian Inoculants Research Group (NSW DPI, Ourimbah, Australia). Inoculum was prepared and quantified as for experiment 1. Ten CRYMA plates were used to make inoculum.

5.2.2.3.4 Seed sowing and leachate application

The first application of leachate (53 week harvest) occurred 6 days before sowing. Pots were watered the day before sowing. Six holes of the same depth were made in the soil surface. Seeds with the longest roots were selected. Six germinated seeds were sown per pot. Plastic pellets and containers were used as in experiment 1. Pots were inoculated with WSM471 7 DAS, when cotyledons were observed. Inoculation was carried out as in experiment 1. Inoculum concentration was 3.5×10^7 CFU/seed. Excess seedlings were removed 15 DAS, leaving four seedlings per pot. The second application of leachate (56 week harvest) occurred 15 DAS. The third application of leachate (58 week harvest) occurred 28 DAS. The fourth application of leachate (60 week harvest) occurred

42 DAS. The fifth application of leachate (62 week harvest) occurred 56 DAS. Plants were watered twice a week for 5 weeks then daily. Water volume used was sufficient to soak the soil while minimising drainage. Five extra serradella and wheat plants were grown alongside the treatment plants. These plants were used to determine water volume.

5.2.2.3.5 Plant harvest

Five replicates per treatment of serradella were harvested 35 DAS. Five replicates per treatment of wheat were harvested 36 DAS. Roots were wrapped in damp paper towel, and sealed in zip lock bags. Plants were stored at 4°C prior to processing. Nodules were removed from serradella roots by hand. Nodule number per pot was recorded. Nodules were washed in tap water and blotted dry with paper towel. Nodule fresh weight per pot was determined. Nodules were dried at 60°C for two days. Nodule dry weight per pot was determined. Shoots were separated from the roots. Shoots and roots were dried at 60°C for two days. Shoot dry weight and root dry weight per pot were recorded. Five replicates per treatment of inoculated serradella were harvested 70 DAS. Five replicates per treatment of uninoculated serradella were harvested 71 DAS. Five replicates per treatment of wheat were harvested 72 DAS. Plants were processed and measured as per the first harvest.

5.2.2.3.6 Data analysis

Leachate cultivable microbe numbers were analysed using Linear Mixed Model (LMM) analysis of log transformed data. Leachate EC and pH were analysed using Generalized Linear Mixed Model (GLMM) analysis. Leachate nutrients were analysed using one-way ANOVA. Germination indices were square root transformed to obtain normal distribution then analysed using one-way ANOVA. Shoot dry weight was analysed using LMM. Root dry weight, nodule number and shoot nutrients (soybean) were analysed using GLMM. To identify the significant differences LMM on square root transformed values was performed. Root dry weight (serradella and wheat), nodule number, nodule fresh and dry weight (serradella) were analysed using LMM. These analyses were completed with GenStat 17th Edition (VSN International Ltd, Hempstead, UK). Two-sided correlation analyses between nutrients applied to soil and plant growth measurements were performed using Genstat 18 (VSNi, Hempstead, UK). Correlation analyses were performed using the means of six soybean replicates and the means of five serradella and five wheat replicates. Two-sided correlation analyses were performed for three plant species combined. When a significant correlation ($p < 0.05$) was observed, individual values were plotted against nutrients applied to soil.

5.2.2.4 Leachate analyses

5.2.2.4.1 Experiment 1

On the day of leachate harvest, 50 µL of filtered and autoclaved leachates were used to make streak plates on sucrose peptone agar. Plates were incubated at 28°C for 5 days to check effectiveness of sterilisation. Growth was observed for many leachates and further microbiological analyses were undertaken for all leachates. Media recipes are detailed in Appendix A.

On the day of plant application, two 50 mL aliquots of leachate were transferred to plastic screw capped tubes for pH and nutrient measurements. On the day of plant application, a 1.5 mL aliquot of leachate was transferred to a microcentrifuge tube for microbiological analysis. Leachate aliquots were stored overnight at 4°C**.** The day after plant application, one 50 mL aliquot was used to measure leachate pH with a PHM210 Standard pH Meter (Radiometer Analytical, Lyon, France). The day after plant application, one 50 mL aliquot was transferred to -20°C storage for nutrient analysis. Nutrient analysis was carried out as described in section 4.2.2.

For microbiological analysis, leachates were diluted to the lowest EC as per plant application. The EC values were 0.5 mS cm^{-1} (11 week harvest), 0.2 mS cm^{-1} (12 week harvest) and 0.3 mS cm^{-1} (19 and 21 week harvest). Leachates were diluted in distilled water to a total volume of 1 mL. Serial dilutions were then made in sterile saline (0.85% NaCl, w/v). Diluted (100 µL) of 10^{-3}, 10^{-4}, 10^{-5} untreated leachates and 100 µL of 10^{0}, 10^{-1} and 10^{-2} filtered and autoclaved leachates were plated on R2A agar (Oxoid, Thebarton, Australia) with 500 µg/L cycloheximide (Sigma-Aldrich, Castle Hill, Australia) to enumerate bacteria. Enumeration of fungi was carried out as detailed in section 4.2.1. Enumeration of N-fixing bacteria was carried out as detailed in section 4.2.2. Diluted (100 µL) of 10^{-1}-10^{-8} untreated leachates and 100 µL of 10^{0}-10^{-6} filtered and autoclaved leachates were used to inoculate the first round of NFB media.

5.2.2.4.2 Experiment 2

Leachate pH was measured with an S220 SevenCompact™ pH/Ion meter (Mettler Toledo, Port Melbourne, Australia). Leachate (50 mL) was stored in sterile plastic screw capped tubes at -20°C for nutrient analysis. Nutrient analysis was carried out as described in section 4.2.2. Leachate (20 mL) was stored in plastic screw capped tubes at 4°C for microbial analysis. Cultivable bacteria and fungi were determined as described in section 4.2.1. Serial dilutions plated on R2A were 10^{-3} and 10^{-4}. Half strength PDA (Oxoid, Thebarton, Australia) was used and acidified as described in Appendix A.

5.3 Results

5.3.1 Banana, green bean, rockmelon leachates with different microbial content – Effect on seed germination

Leachate treatments did not inhibit serradella germination compared to the water control. Most leachate treatments significantly improved germination percentage over the salt control (filter paper soaked with dilute Hoagland's solution, $p < 0.05$). No difference between autoclaved green bean leachate and salt control was observed. Untreated and filtered green bean leachate significantly improved germination over autoclaved green bean leachate ($p < 0.05$). Filtered banana leachate improved germination over autoclaved green bean leachate. No other variation between leachate treatments was observed (Table 5.3). No correlation was observed between leachate pH or EC and germination index.

Table 5.3. Germination indices of serradella seeds treated with vermicompost leachate.

Treatment solution		**pH**	**EC (mS cm^{-1})**	**Percent germination**
Water		8.32	0.01	86.7 ± 1.8ab
Hoagland's		8.24	0.44	70.1 ± 6.1c
Banana leachate	Untreated	8.07	0.37	88.9 ± 3.6ab
	Filtered	7.91	0.35	93.2 ± 4.7a
	Autoclaved	8.90	0.42	89.0 ± 1.5ab
Green bean leachate	Untreated	7.70	0.36	91.2 ± 5.2a
	Filtered	7.67	0.34	91.6 ± 3.8a
	Autoclaved	8.88	0.34	79.5 ± 5.2bc
Rockmelon leachate	Untreated	7.49	0.52	87.3 ± 2.9ab
	Filtered	8.51	0.47	89.3 ± 2.9ab
	Autoclaved	9.05	0.46	83.6 ± 2.3ab

Leachates were subjected to two sterilisation treatments. Diluted Hoagland's solution was used as a soluble salt control. One-way ANOVA performed on transformed values with different letters indicating significant differences ($p < 0.05$). Values are back transformed means of three replicates ± SEM.

5.3.2 Experiment 1 - Effect of sterilised and unsterilised leachates on soybean

High temperatures were experienced during the trial. The maximum temperature registered during manual monitoring was 48°C. The minimum temperature was 19°C (See Appendix E, Figure E). The data logger malfunctioned so a temperature graph was unavailable for the remaining trial time. The maximum temperature registered by the data logger was 55°C. The minimum temperature was 14°C.

Neither worm-bed substrate fed to worm farms nor sterilisation techniques had an effect on leachate EC or pH (Table 5.4).

Table 5.4. pH and electrical conductivity of vermicompost leachates from different worm-bed substrates.

Substrate		Untreated	Filtered	Autoclaved
Banana	pH	7.69 ± 0.07	7.99 ± 0.05	7.98 ± 0.02
	EC (mS cm^{-1})	0.44 ± 0.11	0.45 ± 0.11	0.48 ± 0.11
Green bean	pH	7.52 ± 0.12	7.95 ± 0.11	7.66 ± 0.03
	EC (mS cm^{-1})	0.35 ± 0.06	0.35 ± 0.07	0.36 ± 0.06
Rockmelon	pH	7.73 ± 0.11	8.05 ± 0.11	7.99 ± 0.06
	EC (mS cm^{-1})	0.50 ± 0.09	0.52 ± 0.11	0.48 ± 0.09

Leachates were subjected to two sterilisation treatments. Values shown are mean ± SEM from four replicates. One-way ANOVA showed no variation due to substrate or filtration ($p > 0.05$).

Filter sterilisation, and autoclaving followed by filter sterilisation, reduced microbial populations but did not remove all microbes from leachate (Figure 5.3). Autoclaving significantly reduced cultivable bacteria in banana and rockmelon leachates by 1 – 2 $\log_{10}$ CFU/mL. Bacteria were also reduced in green bean leachate, but not to a significant degree. Filtration reduced cultivable bacteria in banana, green bean and rockmelon leachates. However, the difference was not significant.

Autoclaving significantly reduced cultivable fungi in banana, green bean and rockmelon leachates, and filtration significantly reduced cultivable fungi in banana and rockmelon leachates. Filtration reduced cultivable fungi in green bean leachate, but not to a significant degree. Neither filtration nor autoclaving had a significant effect on cultivable N-fixing bacteria.

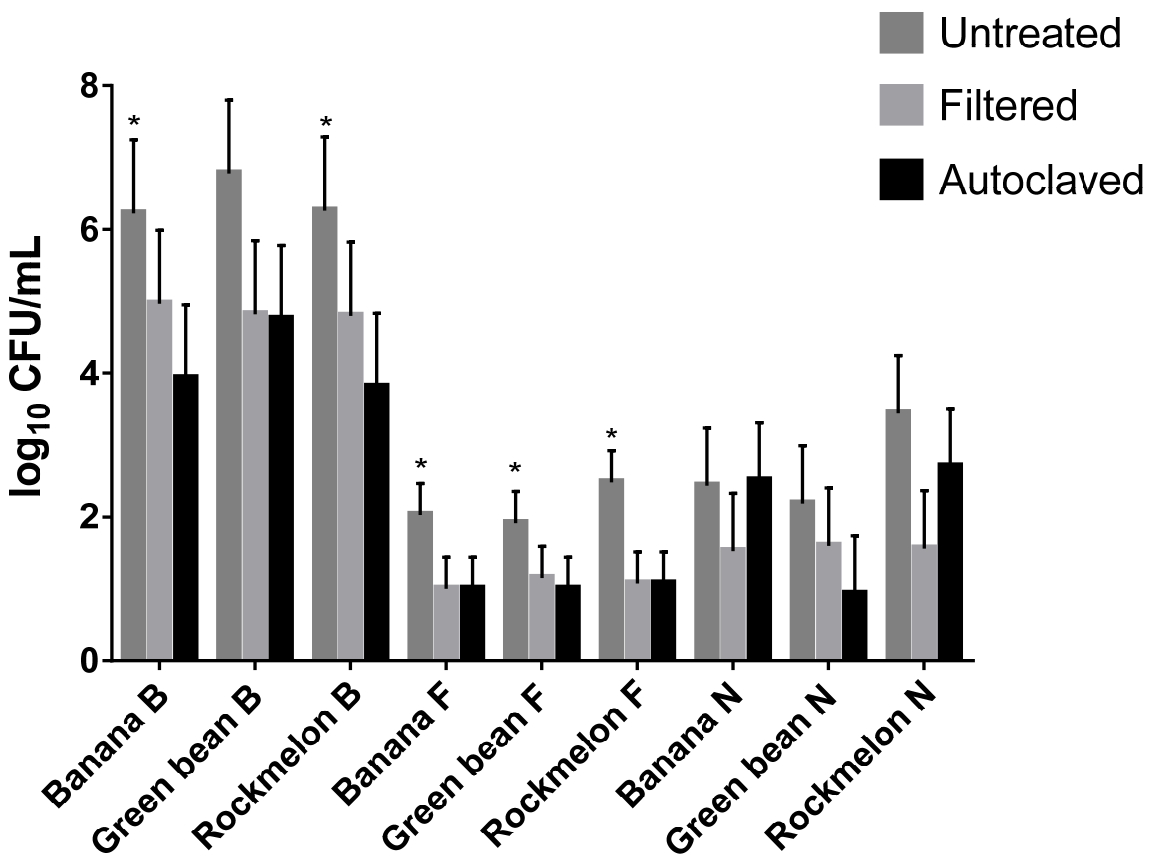

Figure 5.3. Cultivable microbes from vermicompost leachates from different substrates. Leachates were subjected to two sterilisation methods then standardised by electrical conductivity. B – bacteria grown on R2A, F -fungi grown on PDA, N - nitrogen fixing bacteria grown on NFB. Values plotted are predicted means and SE of four replicates derived from LMM Analysis of log transformed values. * indicates significant differences ($p < 0.05$).

The method used to measure leachate nutrients required filtration of samples and therefore untreated leachates and filtered leachates could not be differentiated. The substrate fed to worm farms produced leachate with significant differences in TOC, TN, Ca, K, Mg, Mn, P, S and Zn (Table 5.5). Soybean treated with green bean leachate received more TOC, TN, and S than plants treated with banana or rockmelon leachate and more K than banana leachate treated plants. Rockmelon leachate provided more P and less Zn than soybean treated with banana or green bean leachate. Soybean treated with banana leachate received less Ca and Mg than soybean treated with green bean or rockmelon leachate. These differences were observed for both untreated/filtered and autoclaved leachates.

Autoclaved green bean leachate provided more Mn than untreated and filtered green bean leachate and autoclaved banana or autoclaved rockmelon leachates. Soybean treated with autoclaved

rockmelon leachate received more Ca than soybean treated with autoclaved green bean leachate. Soybean treated with autoclaved green bean leachate received more P than soybean treated with autoclaved banana leachate.

Only B was significantly affected by both worm-bed substrate and sterilisation technique. In all cases autoclaved leachates provided more B than untreated or filtered leachate. Untreated and filtered green bean leachate provided more B than untreated and filtered rockmelon leachate.

Table 5.5. Nutrient content of leachate applied to soybean.

	(µg/pot)	TOC	TN	P	S	Ca	Cu	K	B	Mn	Mg	Fe	Zn	Mo
Untreated/ Filtered	Banana	830 ± 221b	21.8 ± 45.7b	8.4 ± 7.1c	151 ± 30b	208 ± 110c	1.6 ± 1.2a	2010 ± 425a	1.6 ± 0.6cd	0.15 ± 0.06b	47 ± 38b	0.69 ± 0.26a	0.44 ± 0.12a	<LOD
	Green bean	1545 ± 221a	147.9 ± 45.7a	18.8 ± 7.1c	205 ± 30a	605 ± 110a	2.7 ± 1.2a	1112 ± 425b	2.3 ± 0.6c	0.22 ± 0.06b	153 ± 38a	0.70 ± 0.26a	0.53 ± 0.12a	<LOD
	Rockmelon	1112 ± 221b	31.4 ± 45.7b	44.6 ± 7.1a	135 ± 30b	468 ± 110ab	1.7 ± 1.2a	1494 ± 425ab	1.3 ± 0.6d	0.17 ± 0.06b	118 ± 38a	0.34 ± 0.26a	0.25 ± 0.12b	<LOD
Autoclaved	Banana	856 ± 221b	26.6 ± 45.7b	11.4 ± 7.1c	145 ± 30b	217 ± 110c	1.4 ± 1.2a	2001 ± 425a	3.2 ± 0.6b	0.13 ± 0.06b	49 ± 38b	0.39 ± 0.26a	0.40 ± 0.12a	<LOD
	Green bean	1532 ± 221a	146.5 ± 45.7a	23.7 ± 7.1b	199 ± 30a	644 ± 110a	2.4 ± 1.2a	1162 ± 425b	5.0 ± 0.6a	0.34 ± 0.06a	156 ± 38a	0.51 ± 0.26a	0.42 ± 0.12a	<LOD
	Rockmelon	1112 ± 221b	52.7 ± 45.7b	36.4 ± 7.1ab	150 ± 30b	409 ± 110b	1.6 ± 1.2a	1486 ± 425ab	3.4 ± 0.6b	0.11 ± 0.06b	117 ± 38a	0.31 ± 0.26a	0.19 ± 0.12b	<LOD

Leachates from three substrates were diluted to the lowest EC before addition to pots. Values shown are the mean ± SEM of 4 replicates. Data analysed using one-way ANOVA, with different letters indicating significant differences ($p < 0.05$).

Analysis of soybean shoot dry weight showed that the combined effect of worm-bed substrate, sterilisation and *Bradyrhizobium* inoculation produced significant variation between treatments ($p < 0.05$) (Figure 5.4). There was no significant variation between inoculated and uninoculated plants. Untreated banana leachate showed significantly greater shoot dry weight than the water control in the absence of inoculation. No other treatments led to improvements over the water control. Untreated banana leachate showed significantly greater shoot dry weight than autoclaved banana leachate in the absence of inoculation.

Untreated and autoclaved banana leachate showed significantly greater shoot dry weight than filtered banana leachate in inoculated plants. Untreated green bean leachate showed significantly greater shoot dry weight than filtered green bean leachate in inoculated plants. Inoculated soybean plants treated with green bean leachate showed significantly greater shoot dry weight than uninoculated plants. Inoculated soybean plants treated with autoclaved banana leachate showed significantly greater shoot dry weight than uninoculated plants. The treatments had no effect on root dry weight of soybean.

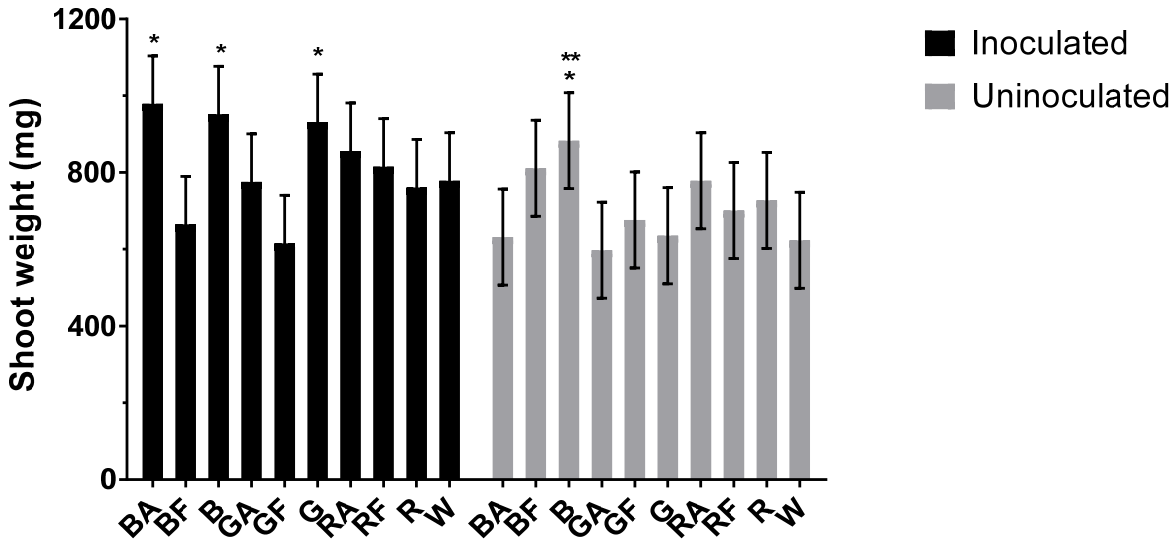

Figure 5.4. Shoot dry weight of soybean treated with vermicompost leachate from different substrates. Leachates were either untreated or sterilised. Leachates were standardised by EC and applied to soil. Plants were harvested 56 DAS. Predicted means and SE derived from Linearized Mixed Model analysis of six replicates plotted. B – banana, G – green bean, R – rockmelon, A-autoclaved, F – filtered, W – water only control. Half the plants were inoculated with soybean-nodulating *Bradyrhizobium*. * indicates significant increase over sterile leachate, ** indicates significant increase over water control ($p < 0.05$).

Analysis of soybean nodulation showed that vermicompost substrate produced significant variation between treatments ($p < 0.001$). The combination of substrate and sterilisation also produced significant variation between treatments ($p < 0.05$) as did the combination of substrate, sterilisation and *Bradyrhizobium* inoculation ($p < 0.05$) (Figure 5.5).

There was no significant variation between inoculated and uninoculated plants. Untreated green bean leachate produced more nodules per plant than all other treatments. This effect was observed in *Bradyrhizobium* inoculated plants only. Untreated and filtered green bean leachate produced more nodules in inoculated plants than uninoculated plants. Filtered and autoclaved rockmelon leachate also produced more nodules on inoculated plants than uninoculated plants.

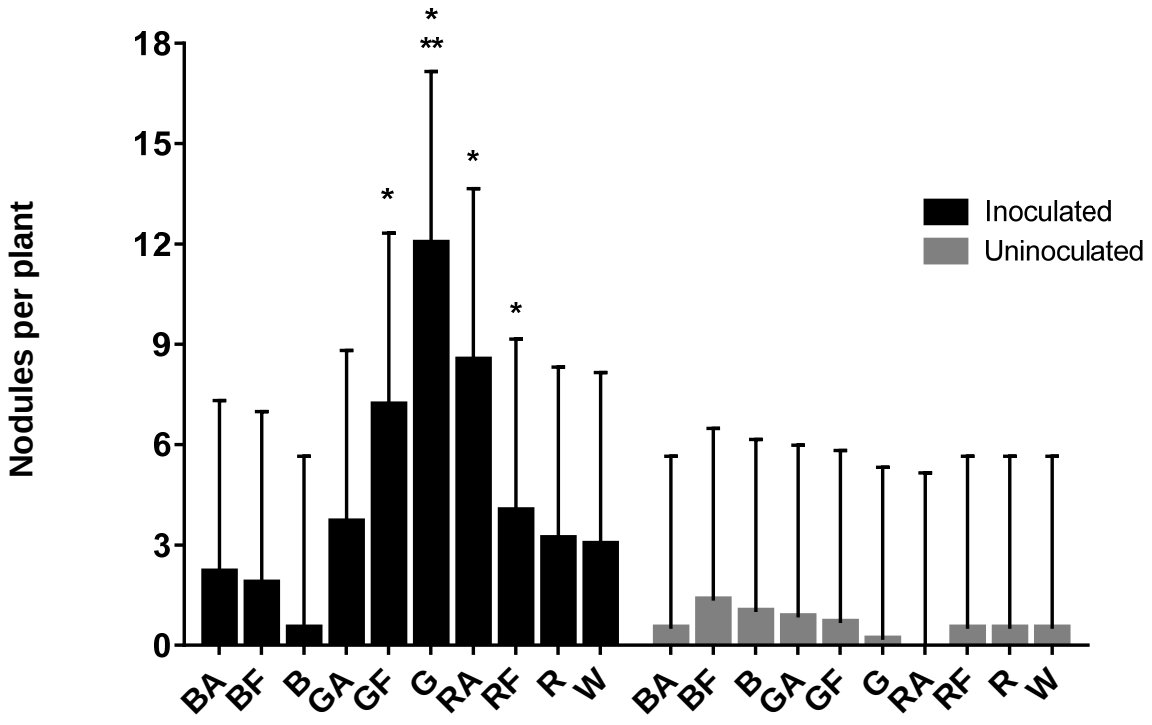

Figure 5.5. Nodule number per plant of soybean treated with vermicompost leachate from different substrates. Leachates were either untreated or sterilised. Leachates were standardised by EC and applied to soil. Plants were harvested 56 DAS. Back transformed means and SE derived from Generalized Linearized Mixed Model analysis of six replicates plotted. B – Banana, G – green bean, R – rockmelon, A- autoclaved, F – filtered, W – water only control. ** indicates significant difference within inoculated treatments ($p < 0.001$), *indicates significant difference between inoculated and uninoculated treatments ($p < 0.05$)

Variation in nodule weight was observed, but due to low nodule numbers, nodules from all replicates were combined prior to weighing. Therefore no statistical analyses were performed. The uninoculated water control produced a small number of large nodules. The inoculated green bean treatment produced a large number of small nodules. (Figures 5.6 and 5.7).

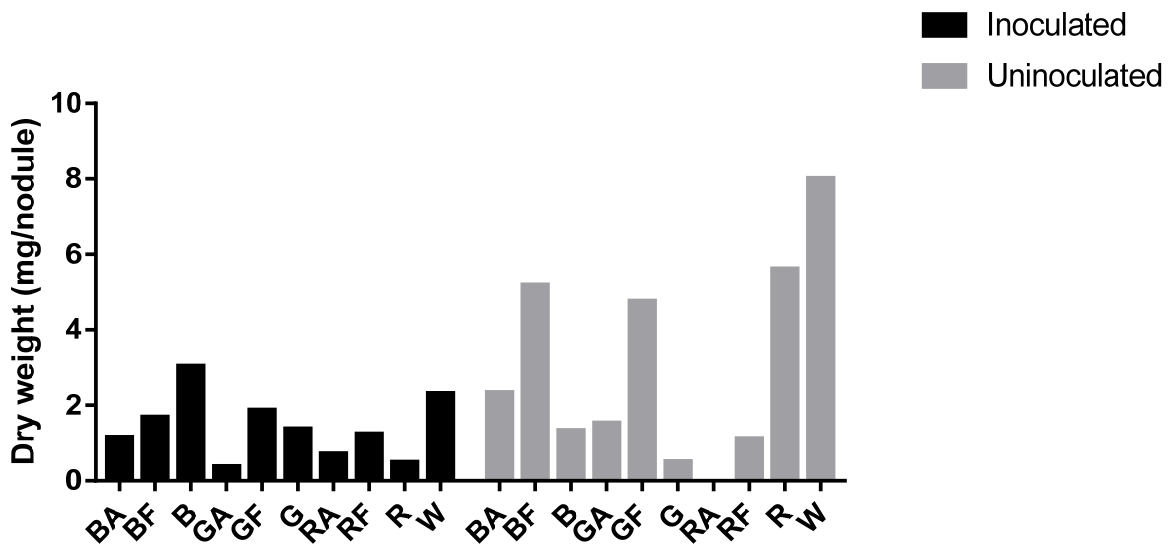

Figure 5.6. Nodule weight of soybean treated with vermicompost leachate from different substrates. Leachates were either untreated or sterilised. Leachates were standardised by EC and applied to soil. Plants were harvested 56 DAS. Nodules from six plants per treatment were combined then weighed. B – Banana, G – green bean, R – rockmelon, A- autoclaved, F – filtered, W – water only control.

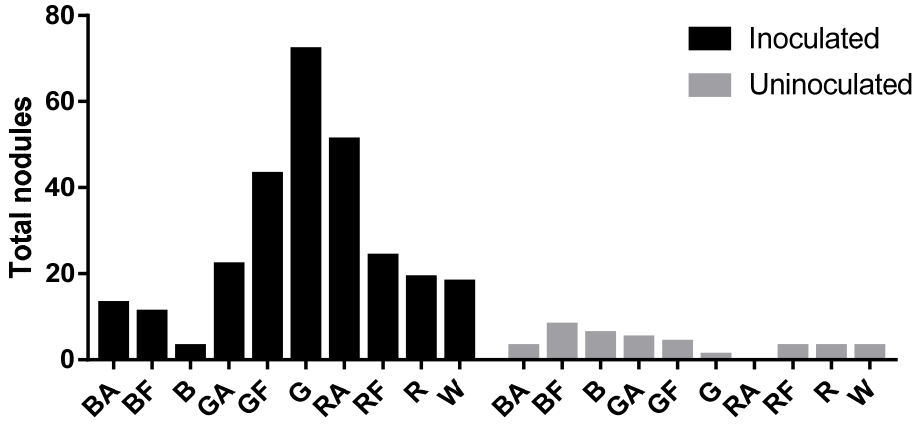

Figure 5.7. Nodulation of soybean treated with vermicompost leachate from different substrates. Leachates were either untreated or sterilised. Leachates were standardised by EC and applied to soil. Plants were harvested 56 DAS. Total nodules from six plants per treatment. B – Banana, G – green bean, R – rockmelon, A- autoclaved, F – filtered, W – water only control.

There was a moderate negative correlation between TOC applied to soil and nodule mass of soybean. This was shown in nodule dry weight ($p = 0.0456$, $R = -0.4516$) and fresh weight ($p = 0.041$, $R = -0.4606$). This correlation was not observed when analysis was performed on leachates only and was caused by the low TOC in nutrient solution.

The treatments had no effect on soybean shoot N. There was a moderate positive correlation between total N applied to soil and soybean shoot N ($p = 0.0493$, $R = 0.4696$). This correlation was observed when nutrient control was removed from the analysis. The treatments had no effect on soybean shoot P. There was a moderate positive correlation between soybean shoot P and TOC applied to soil ($p = 0.0446$, $R = 0.4748$). There was a moderate negative correlation between soybean shoot P and K applied to soil ($p = 0.0343$, $R = -0.5007$). There was a moderate positive correlation between soybean shoot P and Mg applied to soil ($p = 0.0382$, $R = 0.4918$). These correlations were observed when nutrient control was removed from the analysis.

The treatments had no effect on soybean shoot P. There was a moderate positive correlation between soybean shoot P and TOC applied to soil ($p = 0.0446$, $R = 0.4748$). There was a moderate negative correlation between soybean shoot P and K applied to soil ($p = 0.0343$, $R = -0.5007$). There was a moderate positive correlation between soybean shoot P and Mg applied to soil ($p = 0.0382$, $R = 0.4918$). These correlations were observed when nutrient control was removed from the analysis. Soybean correlation plots are shown in Appendix C, Figures C1.1-C1.3.

5.3.3 Experiment 2 - Effect of leachates on serradella and wheat

Vermicompost substrate had no effect on leachate pH. The nutrient solution applied to soil had a significantly lower pH than the leachates. Vermicompost substrate significantly affected leachate EC. Undiluted green bean leachate had a lower EC than banana or rockmelon leachate. Undiluted nutrient solution had a higher EC than the leachates (Table 5.6). EC variation was removed prior to application to soil. pH variation was not removed prior to soil application.

Table 5.6. pH and electrical conductivity of treatment solutions used in plant trial.

Treatment solution	pH	EC (mS cm^{-1})
Banana leachate	7.77 ± 0.09a	1.40 ± 0.19b
Green Bean leachate	7.60 ± 0.15a	0.69 ± 0.08c
Rockmelon leachate	7.69 ± 0.10a	1.31 ± 0.13b
Nutrient solution	5.27 ± 0.10b	2.58 ± 0.03a

Values shown are mean ± SEM from five replicates. One-way ANOVA conducted with different letters indicating significant differences ($p < 0.01$).

Worm-bed substrate had no effect on cultivable microbe numbers in leachate. All leachates were bacterial dominated and all contained living microbes at the time of application to soil (Table 5.7).

Table 5.7. Cultivable microbes in treatment solutions used in plant trial.

Treatment solution	Fungi	Bacteria
Banana leachate	5.6×10^3	2.1×10^6
Green Bean leachate	2.4×10^4	1.6×10^6
Rockmelon leachate	3.5×10^3	2.3×10^6
Nutrient solution	ND	ND

Fungi were grown on PDA and bacteria on R2A. Nutrient solution was autoclaved and numbers were not determined. Values shown are means of five replicates. LMM analysis showed no significant differences between leachates.

Soil treated with nutrient solution received more TN, Cu, Fe, Mg and Zn than soil treated with banana, green bean or rockmelon leachate. No variation between leachates was observed for TN, Cu, Fe or Zn. Soil treated with green bean leachate received more Mg than soil treated with banana leachate. Soil treated with nutrient solution received more Ca and Mn than soil treated with rockmelon or banana leachate. Soil treated with green bean leachate received more Ca and Mn than soil treated with rockmelon or banana leachate.

Soil treated with nutrient solution received less TOC, B and S than soil treated with banana, green bean or rockmelon leachate. Soil treated with green bean leachate received more TC, B and S than soil treated with banana or rockmelon leachate. Soil treated with banana leachate received more K than soil treated with the nutrient solution. Soil treated with green bean leachate received more K than soil treated with rockmelon leachate or the nutrient solution. Soil treated with green bean

leachate received more P than soil treated with banana leachate or the nutrient solution. Soil treated with rockmelon leachate received more P than soil treated with banana leachate (Table 5.8). The leachates contained three elements not found in the nutrient solution. While S, B and Mo were added (see Appendix A), they were not detected.

Table 5.8. Nutrient content of leachate applied to serradella and wheat.

(µg/pot)	TOC	TN	P	S	Ca	Cu	K	B	Mn	Mg	Fe	Zn	Mo
Banana	2917 ± 650b	43 ± 112b	25 ± 15c	89 ± 12b	442 ± 299b	0.84 ± 4.5b	3243 ± 533ab	0.61 ± 0.11b	0.32 ± 0.29c	94 ± 87c	0.27 ± 0.81b	0.33 ± 0.96b	<LOD
Green bean	5560 ± 650a	153 ± 112b	116 ± 15ab	186 ± 12a	2111 ± 299a	0.93 ± 4.5b	4426 ± 267a	1.39 ± 0.11a	1.05 ± 0.29b	368 ± 87b	1.24 ± 0.81b	0.36 ± 1.07b	<LOD
Rockmelon	4766 ± 650ab	82 ± 112b	71 ± 15bc	98 ± 12b	1015 ± 299b	0.56 ± 4.5b	2809 ± 377bc	0.73 ± 0.11b	0.4 ± 0.29c	208 ± 87bc	0.44 ± 0.71b	0.21 ± 0.96b	<LOD
Nutrient control	143 ± 21c	2009 ± 291a	55 ± 15c	<LOD	2265 ± 299a	43.80 ± 4.5a	1843 ± 238c	<LOD	1.52 ± 0.29a	798 ± 87a	6.65 ± 0.63a	12.77 ± 0.96a	<LOD

Leachate from different substrates were diluted to the lowest EC before addition to soil. Values shown are the mean ± SEM of five replicates. Data analysed with ANOVA, with different letters indicating significant differences ($p < 0.05$).

No variation in shoot dry weight of wheat was observed after five weeks. After ten weeks, the nutrient solution produced wheat with significantly greater shoot dry weight than leachates or water control. No variation between leachates was observed at either harvest (Figure 5.8).

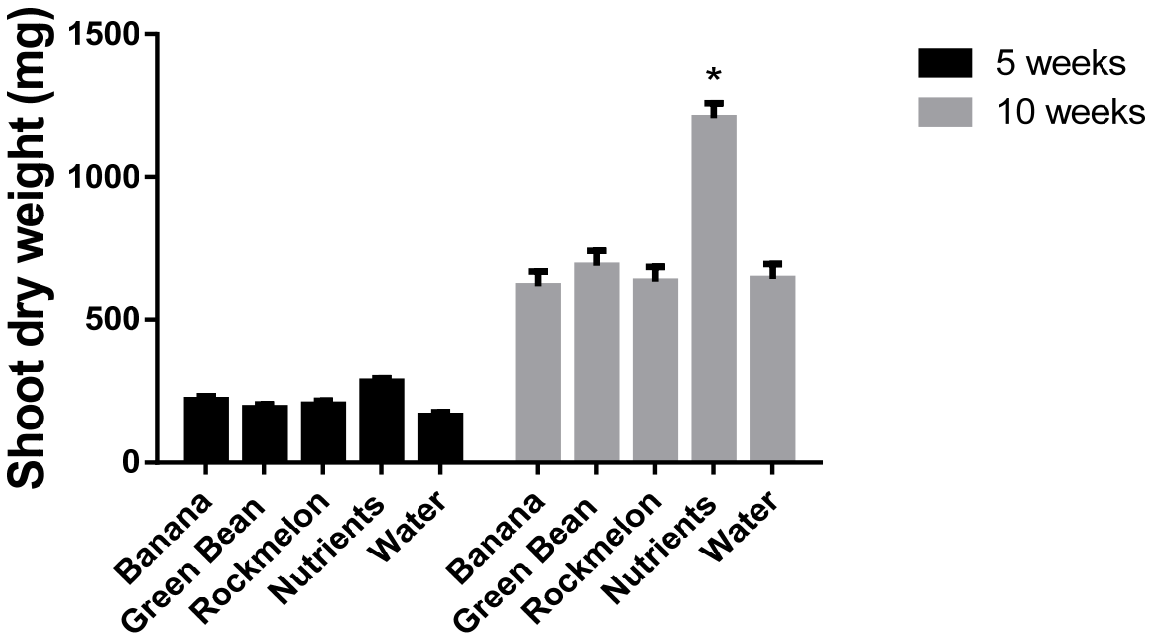

Figure 5.8. Shoot dry weight of wheat grown in soil treated with vermicompost leachate from different substrates. A nutrient solution and water control were included. Leachates and nutrient control were standardised by EC and applied to soil. Two different harvest times are shown, with data comprising predicted means and SE from Linearized Mixed Model analyses of 5 replicates. * indicates a significant difference ($p < 0.001$).

No variation in root dry weight of wheat was observed after five weeks. After ten weeks, the nutrient solution produced wheat with significantly greater shoot dry weight than leachates or water control. No variation between leachates was observed at either harvest (Figure 5.9).

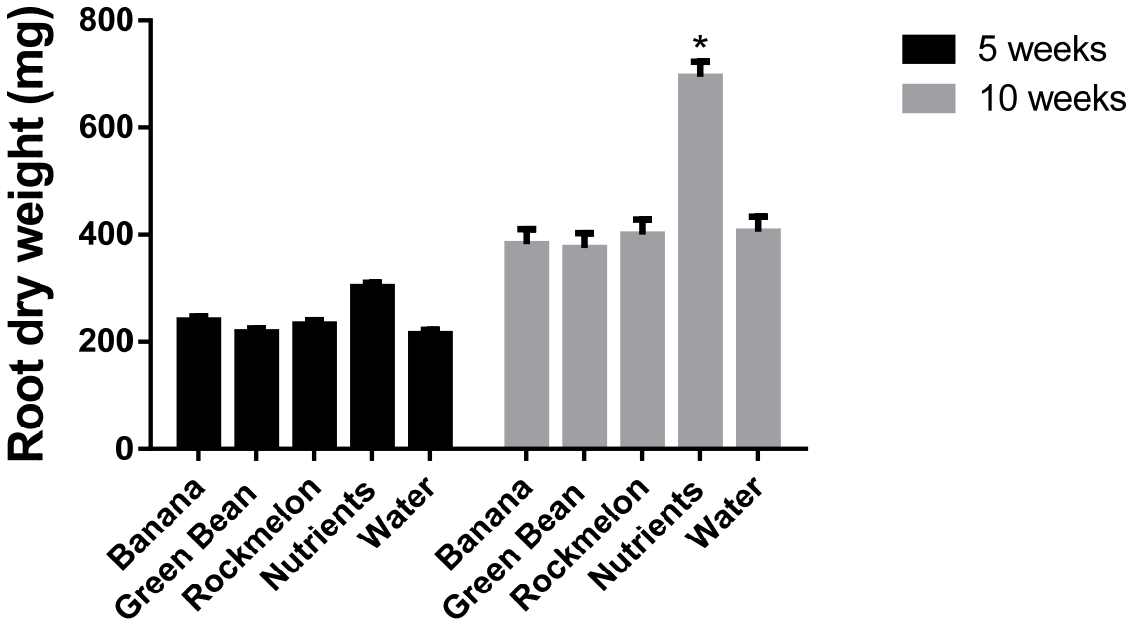

Figure 5.9. Root dry weight of wheat grown in soil treated with vermicompost leachate from different substrates. A nutrient solution and water control were included. Leachates and nutrient control were standardised by EC and applied to soil. Two different harvest times are shown, with data comprising predicted means from Linearized Mixed Model analyses of 5 replicates. * indicates a significant difference ($p < 0.001$).

No variation in wheat root length was observed after five weeks. No variation in wheat root length was observed after ten weeks (Figure 5.10).

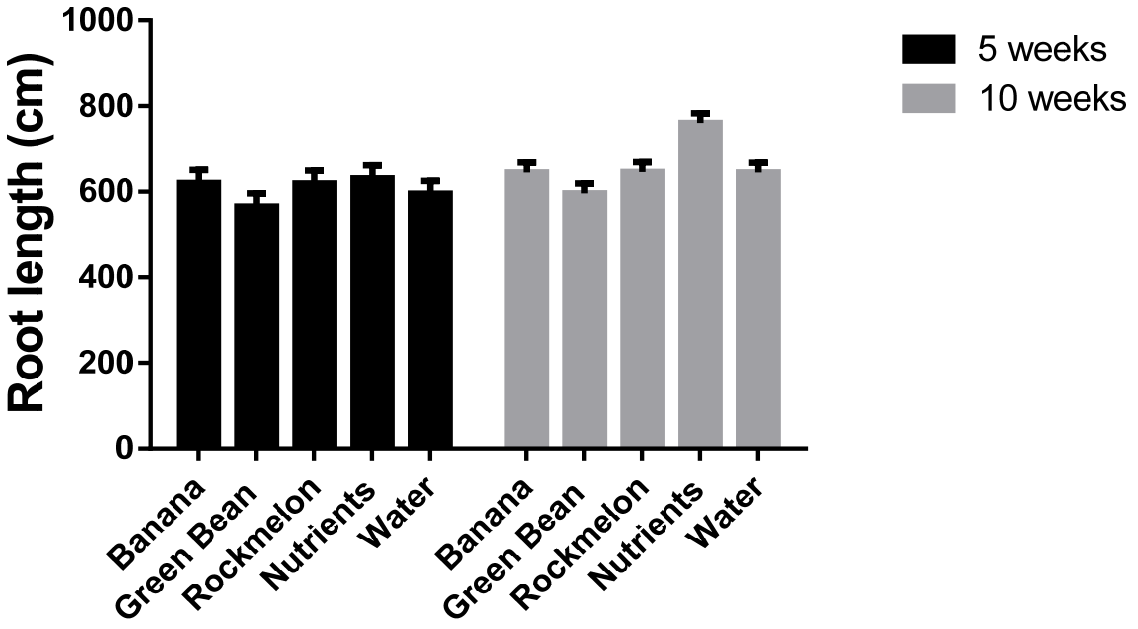

Figure 5.10. Total root length of wheat grown in soil treated with various vermicompost leachates compared with a nutrient or water only control. Two different harvest times are shown, with data comprising predicted means from Linearized Mixed Model analyses of 5 replicates.

There was a strong negative correlation between wheat root length and B, Ca, Fe, K, Mn and S applied to soil (Table 5.9). These correlations were observed when nutrient control was removed from the analysis. There was a moderate positive correlation between Zn applied to soil and wheat root length. This correlation was not observed when analysis was performed on leachates only and was caused by the high Zn in nutrient solution. Wheat correlation plots are shown in Appendix C, Figures C2.1-C2.2.

Table 5.9. Two-sided correlation analysis of soil applied nutrients and wheat root length

	B	**Ca**	**Fe**	**K**	**Mn**	**S**	**Zn**
p	0.0282	0.0465	0.029	0.0372	0.0262	0.0258	0.048
R	0.8595	0.8184	0.8575	0.8381	0.8647	0.8659	0.6363

Clear treatment variation was observed in wheat plants after 10 weeks (Figure 5.11). The nutrient control treatment produced larger plants. No differences between leachates and water control were visible. This reflects the dry weight results reported in Figure 5.8.

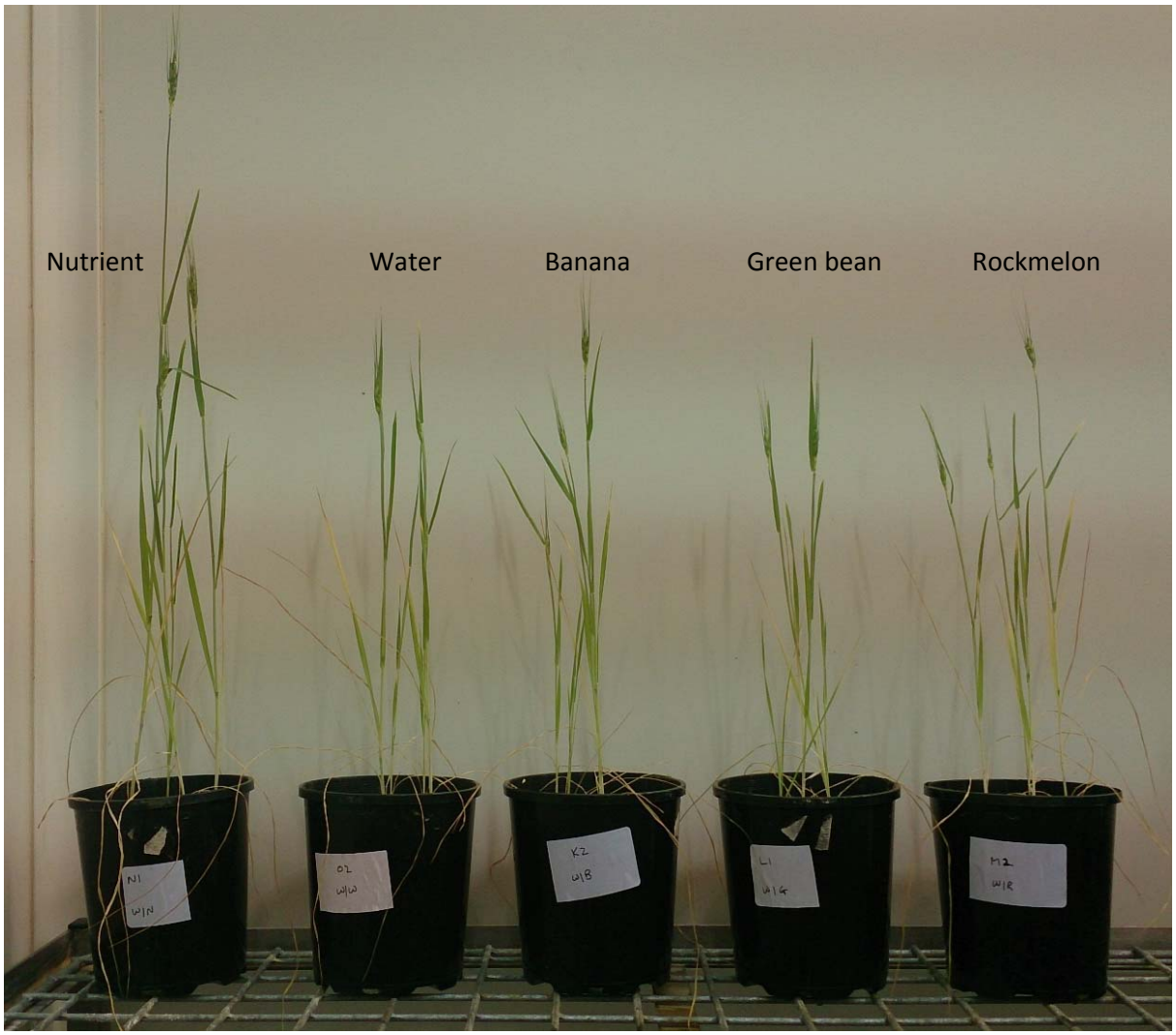

Figure 5.11. 10 week wheat plants treated with banana, green bean or rockmelon leachates compared with nutrient or water control.

After five weeks, the nutrient solution produced serradella with significantly greater shoot dry weight than leachates or water control. This difference was seen in both inoculated and uninoculated plants No variation between leachates was observed. Inoculation had no effect on shoot dry weight (Figure 5.12).

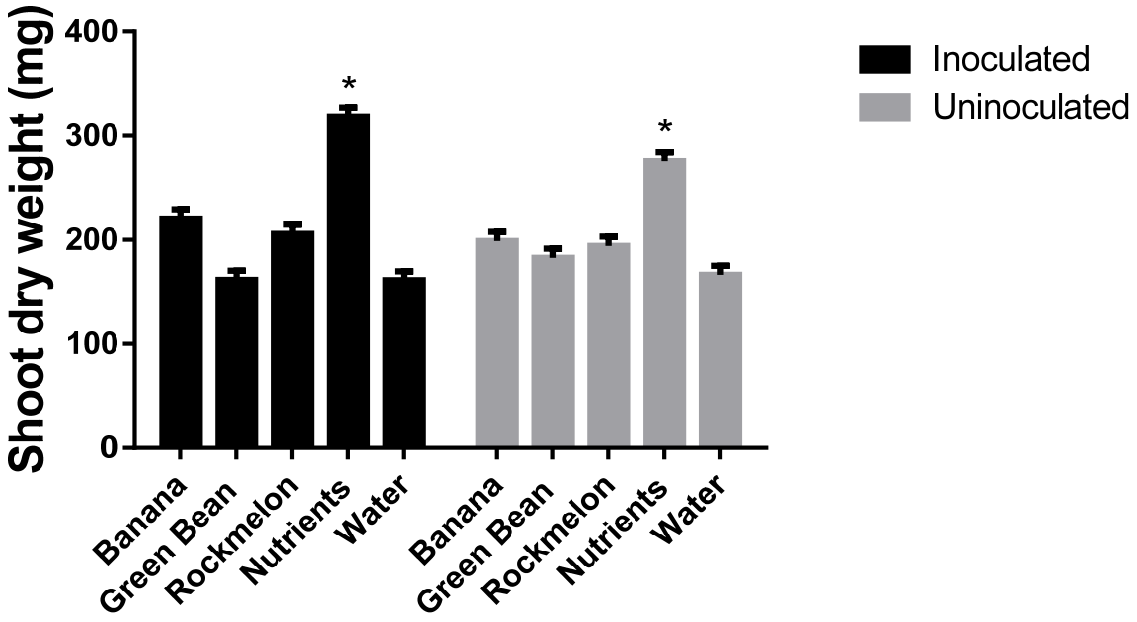

Figure 5.12. Shoot dry weight of serradella grown in soil treated with vermicompost leachate from different substrates. A nutrient solution and water control were included. Leachates and nutrient control were standardised by EC and applied to soil. Half the plants were inoculated with *WSM471*. Plants were harvested after five weeks. Values plotted are predicted means from Linearized Mixed Model analyses of 5 replicates. * indicates a significant difference ($p < 0.001$).

No variation in serradella shoot dry weight was observed after ten weeks (Figure 5.13).

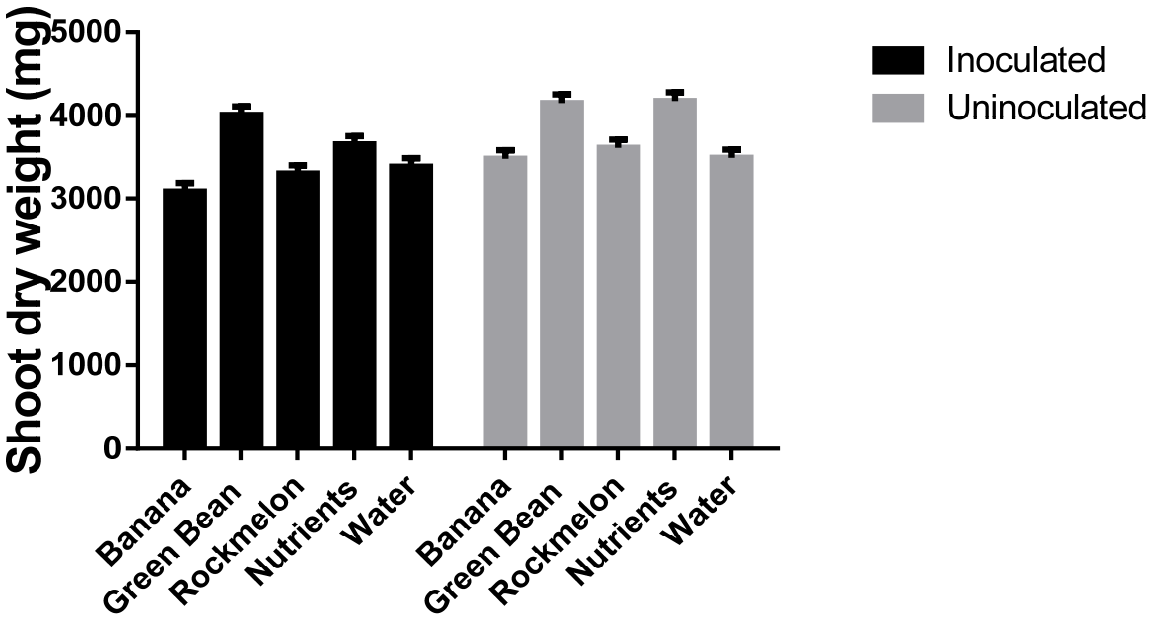

Figure 5.13. Shoot dry weight of serradella grown in soil treated with vermicompost leachate from different substrates. A nutrient solution and water control were included. Leachates and nutrient control were standardised by EC and applied to soil. Half the plants were inoculated with *WSM471*. Plants were harvested after ten weeks. Values plotted are predicted means from Linearized Mixed Model analyses of five replicates.

After five weeks, the nutrient solution combined with WSM471 inoculation produced serradella with significantly greater root dry weight than leachates or water control. In the absence of inoculation, banana leachate produced serradella with significantly greater root dry weight than green bean leachate, rockmelon leachate or water control. In the absence of inoculation, nutrient solution produced serradella with significantly greater root dry weight than green bean leachate, rockmelon leachate or water control. Soil treated with banana leachate produced plants with greater root dry weight than nutrient control, although not to a significant degree (Figure 5.14).

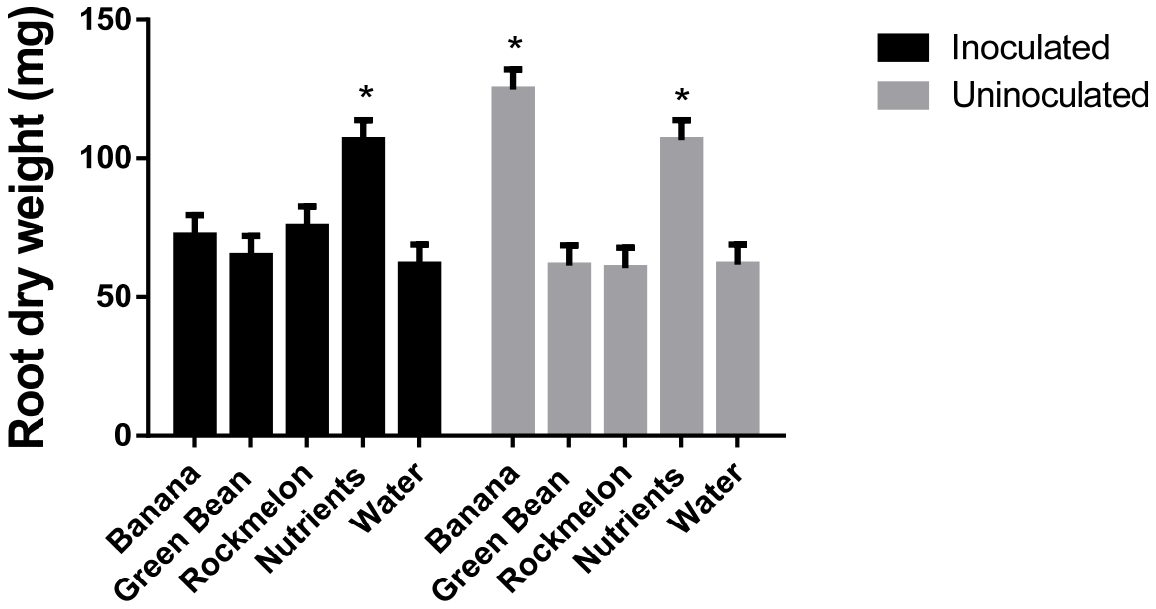

Figure 5.14. Root dry weight of serradella grown in soil treated with vermicompost leachate from different substrates. A nutrient solution and water control were included. Leachates and nutrient control were standardised by EC and applied to soil. Half the plants were inoculated with WSM471. Plants were harvested after five weeks. Values plotted are predicted means from Linearized Mixed Model analyses of five replicates. * indicates a significant difference ($p < 0.05$).

No variation in serradella root dry weight was observed after ten weeks (Figure 5.15).

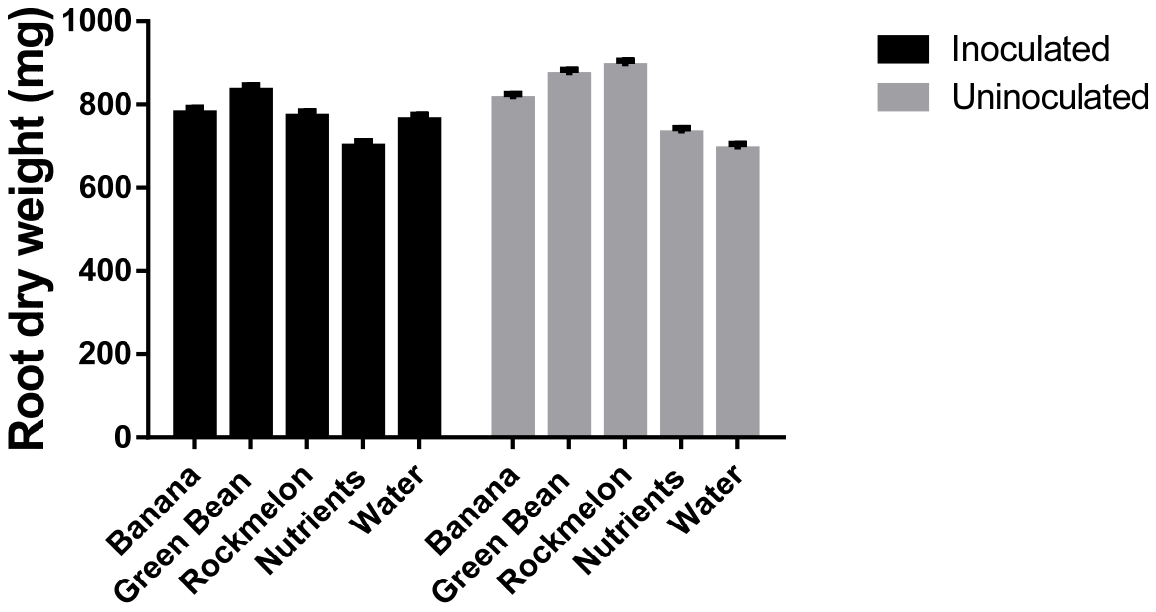

Figure 5.15. Root dry weight of serradella grown in soil treated with vermicompost leachate from different substrates. A nutrient solution and water control were included. Leachates and nutrient control were standardised by EC and applied to soil. Half the plants were inoculated with WSM471. Plants were harvested after ten weeks. Values plotted are predicted means from Linearized Mixed Model analyses of five replicates.

Clear treatment variation was not observed in serradella plants after 10 weeks (Figure 5.16). This reflects the dry weight results reported in Figure 5.13.

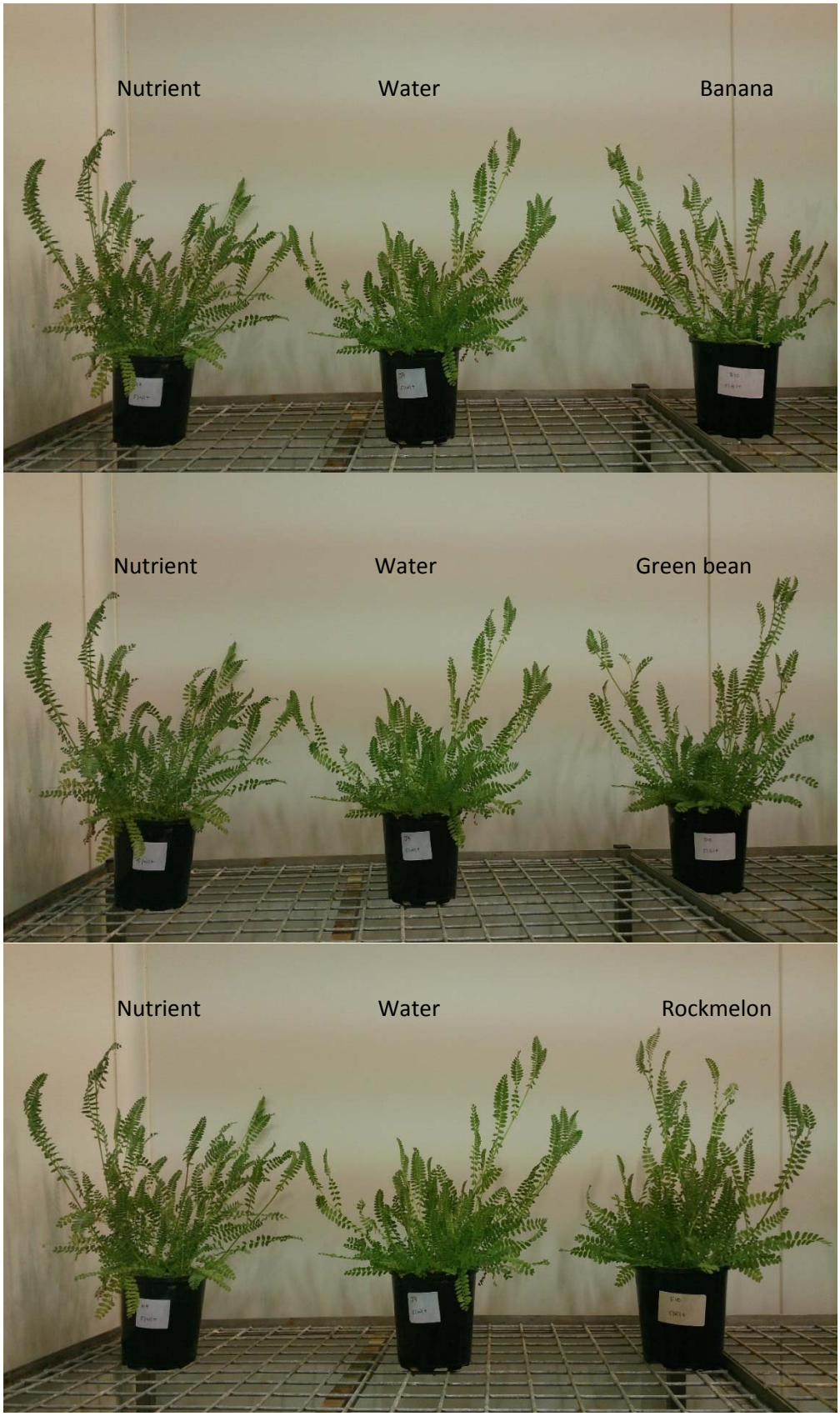

Figure 5.16. 10 week serradella plants treated with banana, green bean or rockmelon leachates compared with nutrient or water control. Plants were inoculated with WSM471.

No variation in serradella nodulation was observed after five weeks. Treatment solutions had no effect on nodule number. Inoculation also had no effect on nodule number (Figure 5.17).

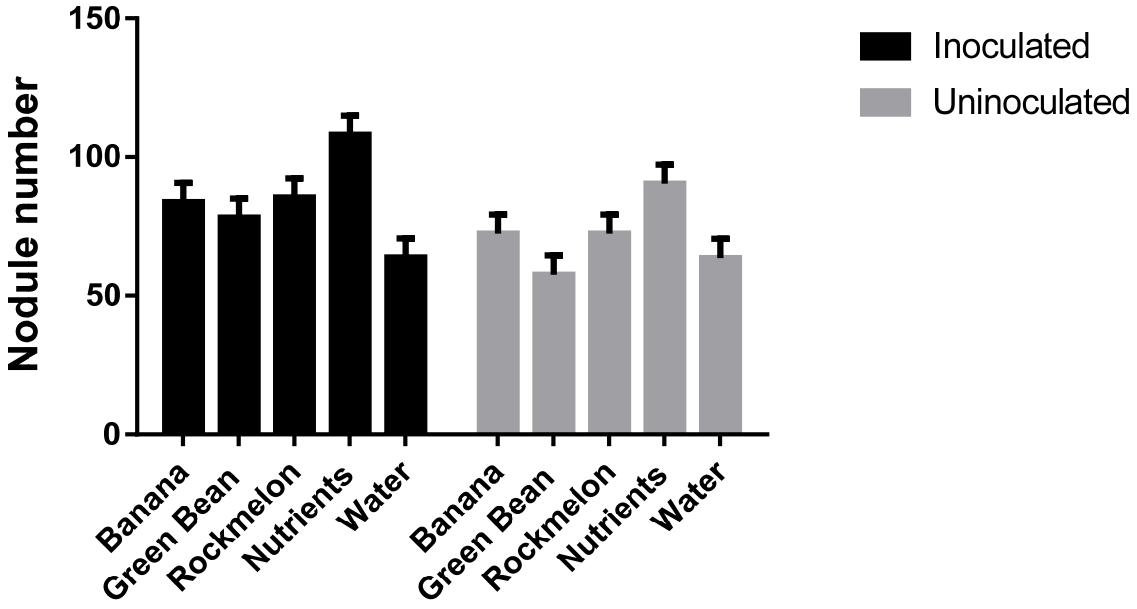

Figure 5.17. Nodulation of serradella grown in soil treated with vermicompost leachate from different substrates. A nutrient solution and water control were included. Leachates and nutrient control were standardised by EC and applied to soil containing naturalised rhizobia. Half the plants were inoculated with WSM471. Plants were harvested after five weeks and nodule number of four plants was determined. Values plotted are predicted means from Linearized Mixed Model analyses of 5 replicates.

Inoculation had no effect on serradella nodule number after ten weeks (Figure 5.18). Treatment solutions caused variation in nodulation after ten weeks. Green bean leachate combined with inoculation produced plants with more nodules than banana leachate, nutrient solution or water. No difference between green bean leachate and rockmelon leachate was observed in inoculated plants. In the absence of inoculation, green bean leachate produced plants with more nodules than all other treatments.

Banana leachate combined with inoculation produced plants with more nodules than the water control. In the absence of inoculation, banana leachate produced plants with more nodules than nutrient solution or water control. Rockmelon leachate combined with inoculation produced plants with more nodules than water control. In the absence of inoculation, rockmelon leachate produced plants with more nodules than the nutrient control. Nutrient solution combined with inoculation

produced plants with more nodules than water control. In the absence of inoculation, no difference between nutrients and water control were observed (Figure 5.18).

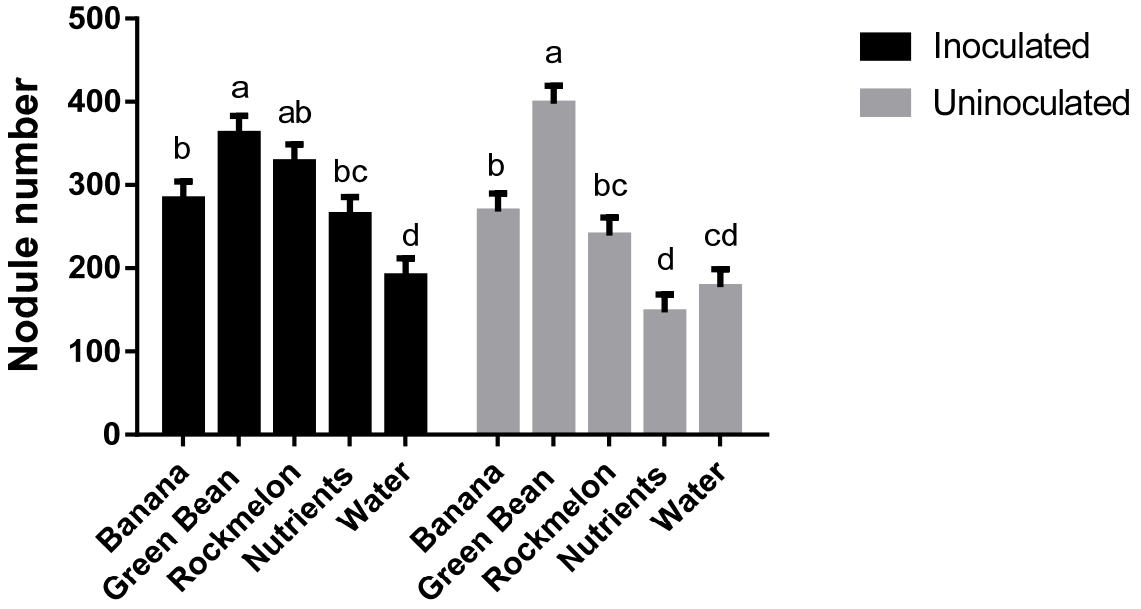

Figure 5.18. Nodulation of serradella grown in soil treated with vermicompost leachate from different substrates. A nutrient solution and water control were included. Leachates and nutrient control were standardised by EC and applied to soil containing naturalised rhizobia. Half the plants were inoculated with WSM471. Plants were harvested after ten weeks and nodule number of four plants was determined. Values plotted are predicted means from Linearized Mixed Model analyses of five replicates. Different letters indicate significant differences within inoculation treatments ($p < 0.001$). No variation between inoculation treatments was observed.

No variation in serradella nodule wet or dry weight was observed after five weeks. Treatment solutions had no effect on nodule weight. Inoculation also had no effect on nodule weight (Figure 5.19).

Inoculation significantly affected serradella nodule weight after ten weeks (Figure 5.20). Treatment with nutrient solution in the absence of inoculation produced nodules with greater fresh and dry weight than inoculated soil. Treatment with rockmelon leachate in the absence of inoculation produced nodules with greater fresh and dry weight than inoculated soil.

Treatment solutions largely had no effect on nodule weight. Banana leachate applied to inoculated serradella produced nodules with greater dry weight than nutrient solution. This difference was not observed in uninoculated plants. Nutrient solution applied to uninoculated soil produced nodules

with greater fresh weight than water control. This difference was not observed in inoculated plants. No variation between leachates was observed for inoculated or uninoculated plants. Green bean and rockmelon leachates showed no differences when compared with the control treatments.

There was no correlation between nutrient content and plant growth factors for serradella.

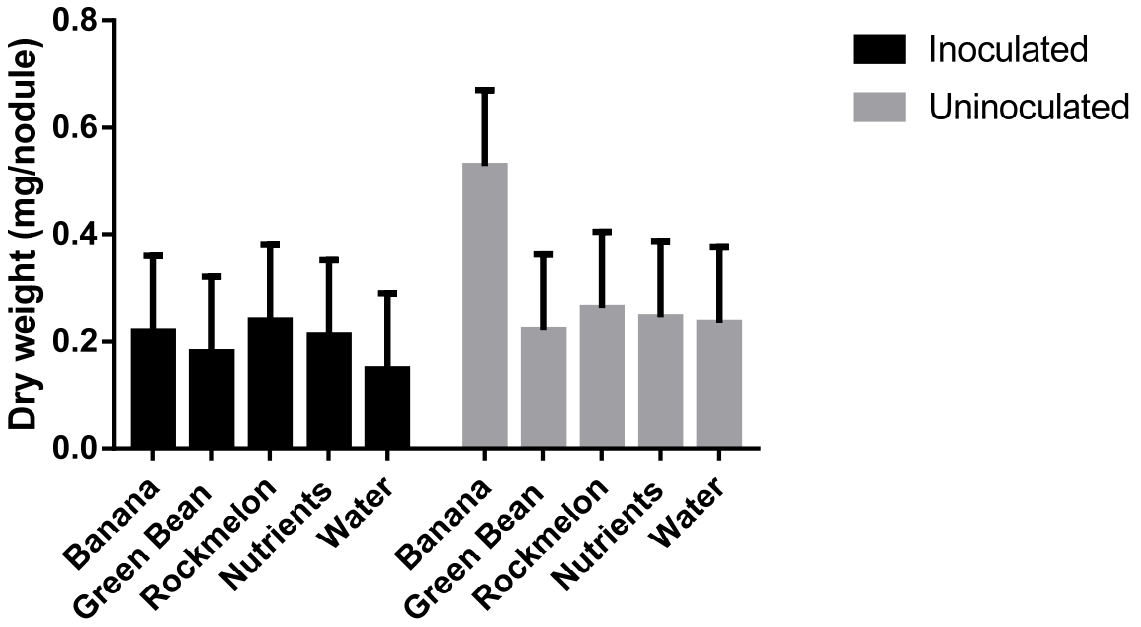

Figure 5.19. Nodule mass of serradella grown in soil treated with vermicompost leachate harvested at five weeks after sowing. A nutrient solution and water control were included. Leachates and nutrient control were standardised by EC and applied to soil containing naturalised rhizobia. Half the plants were inoculated with WSM471. Values plotted are predicted means from Linearized Mixed Model analyses of five replicates.

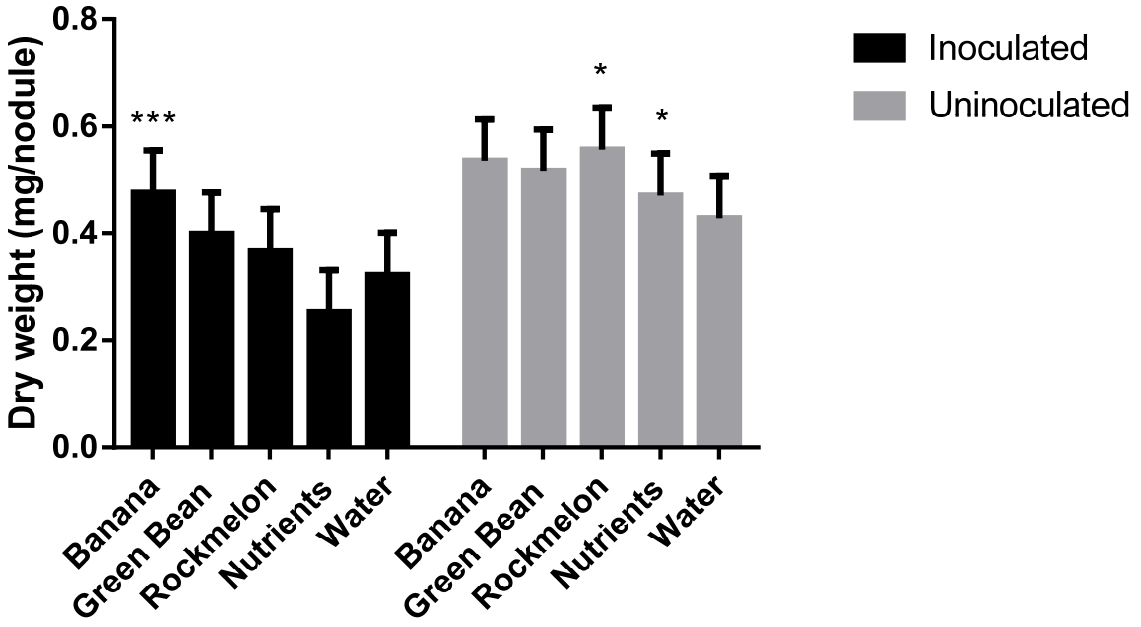

Figure 5.20. Nodule mass of serradella grown in soil treated with vermicompost leachate harvested at ten weeks after sowing. A nutrient solution and water control were included. Leachates and nutrient control were standardised by EC and applied to soil containing naturalised rhizobia. Half the plants were inoculated with WSM471. Values plotted are predicted means from Linearized Mixed Model analyses of five replicates. * indicates significant improvement over inoculated treatment ($p < 0.01$), ** indicates significant improvement over water control ($p < 0.001$), *** indicates significant improvement over nutrient control ($p < 0.01$).

5.3.4 Effect of leachate nutrients on plant growth

When correlation analyses were performed for the three plant species combined many significant trends were observed. There was a moderate positive correlation between shoot dry weight and B, Cu, Fe, S and Zn applied to soil. There was a strong positive correlation between root dry weight and B, Cu, Fe, S and Zn; a moderate positive correlation with TN; and a weak positive correlation with Mg. These correlations were observed when nutrient control was removed from the analysis (Table 5.10).

There was a moderate negative correlation between nodule number and B, K, Fe, S and Zn applied to soil. There was a moderate positive correlation between nodule dry weight and B, Cu, Fe and Zn applied to soil. These correlations were observed when nutrient control was removed from the analysis.

There was a weak positive correlation between root dry weight and TOC applied to soil. There was a weak positive correlation between K applied to soil and shoot dry weight and a moderate positive correlation with root dry weight. There was a weak positive correlation between nodule weight and B. These correlations were not observed when analysis was performed on leachates only and were caused by low TOC, B and high K in nutrient solution. Correlation plots for the three plant species combined are shown in Appendix C, Figures C3.1-C3.8.

Table 5.10. Two-sided correlation analysis of soil applied nutrients and growth factors for serradella, soybean and wheat

		TOC	TN	K	S	Mg	B	Cu	Fe	Zn
Root dry weight	p	0.0377	0.0092	< 0.001	< 0.001	0.0048	< 0.001	< 0.001	< 0.001	< 0.001
	R	0.1618	0.4283	0.5956	0.848	0.4597	0.7121	0.8241	0.7473	0.8064
Shoot dry weight	p			0.0057	< 0.001		0.0042	0.0014	0.0036	0.0019
	R			0.3852	0.5377		0.4656	0.5111	0.4727	0.4991
Nodules per plant	p			< 0.001	< 0.001		< 0.001	< 0.001	0.0021	
	R			-0.5444	-0.6849		-0.5936	-0.6724	-0.5403	
Nodule dry weight	p			< 0.001	< 0.001		0.0472	0.0033	< 0.001	< 0.001
	R			0.5593	0.5665		0.3428	0.5192	-0.6323	0.5897

5.3.5 Comparison of leachate nutrients and chemical fertiliser

The leachates contained three elements not found in the nutrient control solution, S, B and Mo. Leachates supplied 3.7-6.0-fold more TOC than the nutrient solution. The remaining nutrients were found at lower levels in the leachates. The leachates contained 0.6-1.8% of control N, 0.6-1.1% of control Zn, 1.1% of control Cu, 2.2-3.1% of control Fe, 3.3-7.7 % of control Mg, 5.2-12.0% of control Ca, 5.4-8.5% of control Mn, 11.5-39.4% of control P and 29.4-47.4% of control K.

Leachate nutrients were compared with eight commercial or recommended solutions in Table 5.11. Leachates contain more TOC than all eight, five of which contain no C. Leachates contained reduced nutrients compared to 5-11-26. Leachates contained 3-10% of N found in 5-11-26, 2-6% P, 8% S, 14-32% Ca, 44% Cu, 34-55% K, 18-19% B, 2-4% Mn, 6-14% Mg, 0.7-0.9% Fe and 7-13% Zn. Leachates contained reduced nutrients compared to 3-15-27. Leachates contained 3-10% of N found in 3-15-27, 0.3-1.2% P, 4% S, 12-28% Ca, 35% Cu, 16-26% K, 3% B, 1.1-1.8% Mn, 4-9% Mg, 0.3-0.4% Fe and 2-3% Zn.

Leachates contained 2.3-fold more Cu than Hoagland's solution. The remaining nutrients were reduced. Leachates contained 0.8-2.5% of N found in Hoagland's, 3-10% P, 10% S, 8-19% Ca, 32-51% K, 18-19% B, 2-4% Mn, 7-17% Mg, 2-3% Fe and 22-40% Zn. Green bean and rockmelon leachate contained 0.2-fold more Ca than 7-17-37. Banana leachate contained 54% of the Ca found in 7-17-37. The remaining nutrients were reduced. Leachates contained 2-7% of N found in 7-17-37 solution, 2-6% P, 12-13% S, 94% Cu, 36-57% K, 7-8% B, 1.7-2.6% Mn, 9-21% Mg, 2-3% Fe and 8-14% Zn.

Leachates contained 3-5-fold more TOC, 7-fold more Cu, 3-4-fold more B and 3-6-fold more Mn than CRS. The remaining nutrients were reduced. Leachates contained 0.1-0.4% of N found in CRS, 3-10% P, 5% S, 13-31% Ca, 1.8-2.9% K, 25-28% Mg, 0.2-0.3% Fe, and 36-64% Zn. Leachates contained 173-258-fold more TOC, 4998-14507-fold more N and 6-fold more Cu than Norris and Date solution. Banana and rockmelon leachate contained 0.5-fold more K. Green bean leachate contained 95% of the K found in Norris and Date solution. Leachates contained 3-10% P, 6-7% S, 20-46% Ca, 70-76% B, 4-7% Mn, 15-35% Mg, 5-7% Fe, and 44-79% Zn.

Leachates contained 113-169-fold more TOC, 4-13-fold more N, 4-fold more Cu and 3-fold more B than Broughton & Dilworth solution. Green bean and rockmelon leachate contained 0.2 and 0.4-fold more Mg, respectively. Banana leachate contained 58% of the Mg found in Broughton & Dilworth. Leachates contained 7-25% P, 38-41% S, 40-92% Ca, 27-42% Mn, 3-4% Fe, and 34-60% Zn.

Leachates contained higher nutrients than Jensen's nutrient solution. Leachates contained 2-10-fold more P, 205-218-fold more S, 89-195-fold more Ca, 13023-fold more Cu, 2467-3973-fold more K, 21-23-fold more B, 55-87-fold more Mn, 117-279-fold more Mg, 1.3-1.6-fold more Fe, 948-1680-fold more Zn.

To determine whether the nutrient requirements of soybean, serradella and wheat were met, comparison with reference solutions was undertaken. Table 5.12 shows the percentage of nutritional requirements covered with application of diluted leachate based on reference nutrient solutions for soybean serradella and wheat. The content of the reference nutrient solutions for soybean (Broughton & Dilworth, 1971), serradella (CRS, Yates et al., 2016) and wheat (half diluted Hoaglands, Kingsbury & Epstein, 1986), are shown in Table 5.12.

In most cases all three leachates either met requirements or only provided a proportion of required nutrient for legumes and wheat. All three leachates supplied more than the recommended amount of N, Cu and B for both legumes and only a proportion of the requirements for P, S, Ca, Mg, Fe and Zn. In addition the leachates provided only a proportion of K required for serradella and Mn for soybean.

All three leachates provided more than adequate or almost adequate Cu, K and Zn for wheat and only a proportion of N, P, S, B, Mn and Fe. Green bean and rockmelon leachates provided more than adequate Ca and Mg for wheat whereas banana leachate provided less than adequate amounts. Green bean leachate supplied most recommended wheat Zn.

Table 5.11. Nutrient content of leachate and nutrient solution control compared with some recommended solutions and commercial fertilisers.

(mg/L)	C	N	P	S	Ca	Cu	K	B	Mn	Mg	Fe	Zn	Mo
Banana	91.7	1.7	0.9	6.2	16.1	0.066	119.2	0.088	0.011	3.6	0.025	0.020	<LOD
Green bean	95.3	4.9	1.8	6.5	37.0	0.066	74.0	0.091	0.018	7.4	0.028	0.014	<LOD
Rock melon	137.0	3.3	3.0	6.6	35.3	0.066	113.8	0.094	0.013	8.4	0.020	0.011	<LOD
Nutrient control	19.6	274.2	7.5	<LOD	309.0	5.981	251.6	<LOD	0.207	108.9	0.910	1.740	<LOD
5-11-26	NA	50.0	48.0	80.0	116.0	0.150	216.0	0.500	0.500	60.0	3.000	0.150	0.1000
Hoagland's	NA	210.0	31.0	64.0	200.0	0.020	235.0	0.500	0.500	48.0	1.000	0.050	0.0107
3-15-27	NA	51.0	255.0	153.0	130.0	0.190	459.0	2.900	1.000	90.0	8.000	0.580	0.1870
7-17-37	NA	71.0	49.0	50.0	30.0	0.070	208.0	1.200	0.670	40.0	2.700	0.140	0.0700
Jensen's	NA	NA	0.3	0.03	0.2	$5x10^{-6}$	0.03	0.004	0.0002	0.03	0.011	$1x10^{-5}$	0.0110
Norris & Date	0.5	0.0003	30.9	96.3	80.1	0.010	78.1	0.125	0.250	24.3	0.409	0.025	0.0004
CRS	21.8	1390.8	30.2	120.5	120.1	0.008	4101.9	0.020	0.003	30.3	10.116	0.031	0.0018
Broughton & Dilworth	0.8	0.3	12.1	16.1	40.1	0.013	35.8	0.022	0.042	6.1	0.626	0.033	0.0095

Leachate values are means of 12 replicates. Data compiled from (Everris, n.d.; Fernandez, 2009; Hochmuth & Hochmuth, 2001; Plant Foods Inc., n.d.; Yates et al., 2016)

Table 5.12. Percentage of nutrient requirements supplied with banana (B), green bean (GB) and rockmelon (R) leachates diluted to mean EC of 0.3 (soybean) or 0.7 (serradella, wheat) mS cm^{-1}.

	Soybean			Serradella			Wheat		
	B	GB	R	B	GB	R	B	GB	R
N	>100	>100	>100	>100	>100	>100	6	10	10
P	3	5	22	6	13	15	22	50	57
S	39	42	40	5	5	5	36	39	39
Ca	22	49	55	25	59	54	59	>100	>100
Cu	>100	>100	>100	>100	>100	>100	>100	>100	>100
K	>100	>100	>100	4	4	5	>100	>100	>100
B	>100	>100	>100	>100	>100	>100	33	37	38
Mn	17	17	22	>100	>100	>100	16	28	20
Mg	34	82	99	21	40	44	53	>100	>100
Fe	6	4	3	0.1	0.4	0.3	6	17	10
Zn	61	48	34	62	39	43	>100	96	>100

Percentages were calculated based on soybean, serradella and wheat reference solutions.

Banana, green bean and rockmelon leachates supply more than the recommended amount of soybean N, Cu, K and B. Banana, green bean and rockmelon leachates supply more than the recommended amount of serradella N, Cu, B and Mn. Banana, green bean and rockmelon leachates supply more than the recommended amount of wheat Cu and K. Green bean and rockmelon leachates supply more than the recommended amount of wheat Ca and Mg. Banana and rockmelon leachates supply more than the recommended amount of wheat Zn (Table 5.13).

Table 5.13. Dilution factor required to produce leachate with recommended nutrient requirements. Banana (B), green bean (GB) and rockmelon (R) leachates were diluted to mean EC of 0.3 (soybean) or 0.7 (serradella, wheat) mS cm^{-1}.

	Soybean			Serradella			Wheat		
	B	GB	R	B	GB	R	B	GB	R
N							ND	ND	ND
P	ND	ND	ND	ND	ND	ND	ND	ND	ND
S	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ca	ND	ND	ND	ND	ND	ND	ND	0.41	0.29
Cu	4.74	5.13	5.29	5.29	2.90	3.53	9.00	5.20	6.20
K	1.59	0.03	1.07	ND	ND	ND	1.87	1.51	2.31
B	2.33	2.61	2.38	1.01	1.29	1.36	ND	ND	ND
Mn	ND	ND	ND	6.77	12.49	8.51	ND	ND	ND
Mg	ND	ND	ND	ND	ND	ND	ND	0.02	0.12
Fe	ND	ND	ND	ND	ND	ND	ND	ND	ND
Zn	ND	ND	ND	ND	ND	ND	0.52	ND	0.048

Dilution factors were calculated based on soybean, serradella and wheat reference solutions. A dilution factor of 4.74 indicates a 1:4.74 solution in water is needed to supply recommended nutrients. ND indicates no dilution required. Nutrients supplied with water have not been considered.

5.4 Discussion

5.4.1 Leachate effect on seed germination

The leachates used in this study did not inhibit seed germination when compared with water. Using similar methods, leachates from composted cow manure (Garcia-Gomez et al., 2008) and potato slurry (Frederickson, 2002) have been shown to inhibit cress germination. Pig manure leachate inhibits radish and sorghum germination (Pittaway, 2014). Potential toxicity was attributed to high salt or pH and dilutions recommended (Asciutto et al., 2006; Frederickson, 2002; Pittaway, 2014). Gutiérrez-Miceli et al. (2011) found no inhibition of radish germination using 50% and 75% dilutions of sheep manure leachate. Similarly, Quaik et al. (2012b) found no inhibition of mung bean germination using 10% cow manure leachate. Lack of standardisation between systems makes determining a recommended dose impossible.

With the exception of autoclaved green bean leachate, the leachates used in this study improved seed germination over the salt control. The salt control was diluted to the same EC and pH as the leachates and autoclaved green bean leachate did not have the highest pH or EC value. This indicates that EC or pH are not the sole causes of reduced germination.

Untreated and filtered green bean leachate significantly improved germination over autoclaved green bean leachate. There is evidence that specific microbes can increase seed germination. Soaking seeds in a suspension of *Bacillus subtilis* increased germination of *Codonopsis pilosula* (Wu et al., 2016). Soaking seeds in a mixture of *Bacillus megaterium* and *Azotobacter chroococcum* increased germination of maize (Bákonyi et al., 2013). Such a beneficial microbe present in green bean leachate may have been removed by autoclaving, but not by filtration.

Substrate fed to worm farms and leachate sterilisation treatment produced some variation in germination index. Worm-bed substrate and sterilisation treatment did not result in variation in pH or EC and there was no correlation between leachate pH or EC and germination. Additional factors have been observed to contribute to leachate effect on germination. Kandari et al. (2011) found leachate effect on eucalyptus germination varied with plant species. Majlessi et al. (2012) found vermicompost extract inhibition of cress varied with vermicompost harvest time. It should not be assumed that leachate will inhibit germination and this should be tested on a case by case basis.

The evidence of effect of vermicompost on seed germination is more comprehensive than studies with leachate. These tests generally involve soaking filter paper in aqueous extracts of vermicompost. The lack of standardisation makes determining dosage difficult. Undiluted extract of sewage sludge, cow manure and straw vermicompost inhibited cress germination (Contreras-Ramos et al., 2005) whereas a 25% extract of green waste vermicompost did not inhibit cress or lettuce germination (Morales-Corts, Gómez-Sánchez, & Pérez-Sánchez, 2014). Similarly, a 10% extract of paper waste and cow manure vermicompost did not inhibit tomato, radish or carrot germination (Unuofin & Mnkeni, 2014). Comparison between these studies is difficult because of the many different variables. However, it is apparent that extract concentration may contribute to germination inhibition, but other factors are likely to be involved.

Vermicompost has also been tested in greenhouse and field trials. Mixing vermicompost with potting mix may improve germination (Atiyeh et al., 2000a) or have no effect (Bachman & Metzger, 2008). Mixing vermicompost with soil may inhibit germination, have no effect, or improve germination. Effects vary with worm-bed substrate and vermicompost concentration (Joshi & Vig, 2010; Nadi et al., 2011). Effect of the same vermicompost varies with plant species in pot trials (Atiyeh et al., 2000b) and field trials (Roy et al., 2010). Serradella was used in this study as it was the main target for glasshouse trials. Effect on germination of soybean or wheat may be different but was not tested here.

5.4.2 Effect of sterilising leachate on soybean growth and nodulation

Soybean was grown with application of untreated and sterilised leachates. Filtration was intended to remove microbes and test leachate nutrients only whereas autoclaving was intended to augment leachate nutrients by releasing nutrients inside microbes. Untreated leachates were used to test the combination of both leachate nutrients and microbes on soybean. Neither filtration nor autoclaving removed all microbes. Therefore, leachates subjected to sterilising treatments tested the effect of reduced microbes on soybean growth and nodulation. Autoclaving had little effect on leachate nutrients except an increase in B. In this study, 10 minute autoclaving and one filtration were used in order to minimise any changes to leachate. However, effective sterilisation of vermicompost extract was achieved by Marín et al. (2013) using 30 minute autoclaving and three filtrations.

In some cases, reducing leachate microbes had a negative effect on shoot weight and nodulation. Untreated banana and green bean leachates produced significantly greater shoot weight in soybean inoculated with rhizobia than filtered banana and green bean leachates. Untreated banana leachate

produced significantly greater shoot weight in uninoculated soybean than autoclaved banana leachate. Untreated green bean leachate produced significantly more nodules in inoculated soybean than filtered green bean leachate. These differences may be attributed to significantly lower numbers of fungi in filtered banana and green bean leachate and fungi and bacteria in autoclaved banana leachate. Despite significant reductions or bacteria and fungi in sterilised rockmelon leachates, these same trends were not observed.

In contrast, Cavender et al. (2003) found steam sterilised pig manure vermicompost increased sorghum root and shoot dry weight. This was attributed to physical and chemical changes brought about by sterilisation, although measurements were not taken (Cavender et al., 2003). In this study, sterilisation of leachate did not produce chemical changes, although some changes may have occurred to factors that were not measured. Results reported in Chapter 3 indicated that leachate contains potential plant-growth promoting microbes including *Xanthobacter, Azospirillum* and *Pseudomonas*. *Xanthobacter* and *Azospirillum* have been shown to improve plant growth likely through the production of plant growth promoting hormones (Gopalaswamy et al., 2000; Steenhoudt & Vanderleyden, 2000; Tsavkelova et al., 2006), while *Pseudomonas* may solubolise P (Vessey, 2003). In this case, leachate sterilisation may have reduced or removed these microbes, resulting in reduced shoot weight and nodulation.

Increased B in autoclaved leachate had positive effects on shoot weight and nodulation of inoculated soybean. Autoclaved banana leachate produced significantly greater shoot weight than filtered banana leachate and autoclaved rockmelon leachate produced significantly more nodules than filtered rockmelon leachate. As no significant differences existed between the microbes in these leachates, the differences may be attributed to increased B. B is important in nodule development, with limited B restricting the degree of rhizobial colonisation of plant tissue (Bolanos, Brewin, & Bonilla, 1996). The amount of B supplied by untreated (43-77 µg/L) and autoclaved leachates (106-167 µg/L) in this study was within the range of 15-180 µg/L found to achieve maximum soybean nodulation (Yamagishi & Yamamoto, 1994).

Leachates were harvested and autoclaved in borosilicate glass before being transferred to plastic tubes. Borosilicate glass is a potential source of B contamination. Contamination causes serious errors in alkaline solutions, but it takes over four weeks of room temperature storage for measurable B contamination of water (Green et al., 1976). Untreated leachates were not stored in glass long enough to be contaminated although heat increases contamination potential (Dulski, 1999), indicating autoclaving induced contamination. It is likely the elevated B in autoclaved

leachates did not come from microbial biomass as intended but from borosilicate glass. There was no observed increase in other leachate nutrients from autoclaving as expected.

5.4.3 Leachate effect on plants

The effect of leachates on plant growth was minimal. Leachates had no effect on shoot dry weight of serradella or wheat. Banana leachate increased shoot dry weight of soybean in uninoculated plants. The result was questioned due to the heat stress placed on the plants. Banana leachate increased shoot dry weight of serradella at five weeks, but not at ten weeks. As an overabundance of N can stunt root growth (Mugaas, 2018) the effect of banana leachate may be due to the significantly lower N supplied by this leachate. Vermicompost leachates also contain humic and fluvic acids (Garcia-Gomez et al., 2008; Gutiérrez-Miceli et al., 2017) which may enhance both root and shoot growth (Y. Chen & Aviad, 1990).

Leachates had no effect on root length of wheat. According to Roy et al. (2010), soil amended with cow manure and crop residue vermicompost increased root length of maize, okra and *Phaseolus vulgaris*. These results may be different because compost was used rather than leachate. Vermicompost may supply more nutrients and may affect soil structure. In this study, there was some correlation between leachate nutrients and root length. There may not have been enough variation between the leachates to see an effect on the plants.

Leachates had no effect on soybean shoot N or P. Cow manure leachate has been shown to have no effect on leaf N, P, K of sorghum (Gutiérrez-Miceli et al., 2008) or tomato (Tejada et al., 2008). Similarly, green waste leachate had no effect on tomato leaf P or K, and while an increase in leaf N was shown, it was observed only in one of four harvests (Tejada et al., 2008). In this study, there was some correlation between leachate nutrients and shoot N and P.

Some studies show an effect on plant nutrients from amendment with vermicompost products. Liquid extract from earthworm free chicken manure vermicompost increased shoot N, P, K, Ca, Mg, S, Na in pak choi (Pant et al., 2009). The method of leachate harvest used by Pant et al. (2009) differed from that used in this study. It involved a week long incubation of vermicompost in water. This may explain the higher nutrient content of the extract as well as the effect on shoot nutrients. This harvest method may improve nutrients but will drown the earthworms. It therefore requires prior separation of earthworms from vermicompost and reduces the efficiency of the vermicomposting system.

Singh et al. (2010) observed increases in strawberry leaf N, P and K using leachates from three substrates (cow manure, vegetable waste and a mix of the two). These increases were not always observed, and varied with plant harvest time and substrate. The increase in shoot nutrients may be attributed to the use of foliar application by Singh et al. (2010). Foliar application may improve leaf nutrients but have no effect on soil nutrients. Foliar application may also have no effect on fruit nutrients depending on time of application. Foliar application is not guaranteed to improve shoot nutrients, as it had no effect on leaf P or K of tomato (Tejada et al., 2008).

Leachates had no effect on root dry weight of soybean or wheat. Similarly, Srivastava, Kumar, & Gupta (2005) found no effect of soil amended with a sewage sludge vermicompost extract on root dry weight of onion. Banana leachate increased serradella root dry weight in uninoculated soil at the 5 week plant harvest. Banana leachate produced more nodules than green bean leachate and the water control, although this increase was not significant. Increased root dry weight may be due to increased nodulation.

5.4.4 Leachate effect on nodulation

There is little published evidence of the effect of leachate on legumes and none could be found that examined the effect of leachate on nodulation. Cow manure leachate increased dry weight of French dwarf bean (*P. vulgaris*) when compared with a combination of cow manure vermicompost and leachate (Ayyobi et al., 2014). Vermicompost extract had no effect on *P. vulgaris* root length when compared with water (Mezbahzadeh & Astaraei, 2008). Sinha et al. (2010a) found improved growth of chickpea and pea using solid vermicompost. Effect of solid vermicompost on nodulation has been examined by Ramesh et al. (2009) who found no effect on nodulation of chickpea and soybean in the field.

In this study, green bean leachate increased nodulation of soybean and serradella. A negative correlation between applied nutrients and nodulation was found between nodule number and B, Cu, Fe, K, S and Zn provided by leachate. In contrast, Cadisch et al. (1993) found nodulation of *Centrosema* sp. was not affected by K. Green bean leachate supplied the lowest concentration of K and the highest concentration of Cu, Fe, S and Zn to soybean and the highest concentration of B, Cu, Fe, K, S and Zn to serradella. The effect of green bean leachate on soybean and serradella nodulation was therefore not likely due to nutrient supply as nodulation was highest when these nutrients were low. It is possible that the increased nodulation of inoculated soybean and both inoculated and uninoculated serradella is through a positive interaction between the rhizobial inoculant strains, CB1809 for soybean and WSM471 for serradella, and microbes present in the leachate and soil. Results reported in Chapter 3 indicate that green bean leachate contains distinctly different microbes to the banana and rockmelon leachates.

Green bean leachate increased nodulation in heat stressed soybean. High soil temperature limits nodulation and N fixation (Michiels, Verreth, & Vanderleyden, 1994). *Rhizobium* and *Bradyrhizobium* sp. have varying levels of heat tolerance (Pinto et al., 1998) and green bean leachate may favour heat tolerant species.

While the microbes in green bean leachate were not identified, *Azospirillum* was among the dominant genera in banana and rockmelon leachates (Chapter 3). Co-inoculation with *Rhizobium* and *Azospirillum* can increase nodule number over *Rhizobium* alone (Vicario et al., 2015). *Azospirillum* may be present in green bean leachate and have contributed to increased nodulation.

Inoculation did not uniformly increase nodulation. Inoculation increased soybean nodulation in conjunction with two of the green bean and two of the rockmelon leachates. Inoculation did not

increase serradella nodulation. This is due to the presence of serradella nodulating rhizobia in the soil. The soil was collected from a site where nodulated lupin was growing and the rhizobia for lupin are in the same cross-inoculation group for serradella. However, the inoculant strains are different for each legume. The commercial inoculant strain for lupin is *B. lupini* WU425. Soybean has been grown each year as demonstration plots in adjacent sites within the field and it is possible that a small population of soybean nodulating rhizobia were present from previous inoculation. Inoculant *B. japonicum* strains mostly remain at the inoculation site and most lateral root nodulation is due to indigenous soil populations (McDermott & Graham, 1989). It is therefore unsurprising that inoculation had little effect on nodulation particularly in serradella. It is of interest that inoculation combined with untreated green bean leachate and filtered green bean leachate increased soybean nodule numbers over the corresponding uninoculated treatment and that this was replicated with untreated green bean leachate applied to serradella. This provides further evidence of the positive effect of green bean leachate on nodulation and of the interaction between leachate microbes, soil microbes and rhizobial inoculant strains.

Rockmelon leachate increased serradella nodule mass in inoculated soil compared with uninoculated soil. This effect was not related to nutrients, as identical nutrients were supplied to both treatments. Increased nodule mass was likely due to an interaction between leachate microbes and WSM471 and not the lupin rhizobial strains present in the soil. Banana leachate, combined with inoculation, increased serradella nodule mass over nutrient solution. A positive correlation exists between nodule mass and supplied B, Cu, Fe, K, S and Zn. In line with this, banana leachate supplied more B, K and S than nutrient solution. Evaluating nutrient effect is complicated by the fact that the form of nutrients affects plant uptake. For example, K supplied as K_2SO_4 increased nodule mass in alfalfa over KCl (Duke, Collins, & Soberalske, 1980). It is likely that nutrients in leachate were in complex organic forms and may not be readily available to plants.

There is some evidence of microbial effects on legume nodulation. Le et al. (2016) found two of five endophytic actinobacteria isolated from wheat roots had beneficial effects on rhizobial inoculated lucerne, including increased nodule weight, plant height and dry weight. No increase in nodule numbers was observed, and two actinobacteria significantly decreased lucerne nodule number. Le et al. (2016) also found one of five endophytic actinobacteria isolated from lucerne roots or nodules increased nodule weight in rhizobial inoculated lucerne. Again, no increase in nodule numbers was found, but neither was a significant decrease. According to Le et al. (2016), this indicates that microbial benefits to lucerne began after nodule initiation. Some endophytes improved rhizobial growth on agar (Le et al., 2016) and similar microbes may be present in green bean leachate.

In a study of eight endophytic actinomycete isolates, Nimnoi, Pongsilp, & Lumyong (2014) observed significantly increased soybean nodule numbers from three isolates. Plants were grown in growth pouches treated with N-free nutrient solution and co-inoculated with *B. japonicum* USDA110. A greenhouse trial by Martínez-Hidalgo et al. (2014) found co-inoculation of rhizobia with *Micromonospora* isolated from lucerne nodules significantly increased nodule numbers in lucerne plants. Two *Micromonospora* strains were investigated and both showed increased nodulation.

Five *Streptomyces* strains isolated from herbal vermicompost were investigated in a field trial in India by Gopalakrishnan et al. (2015). Soil was not inoculated but contained naturalised rhizobia and the trial was repeated over two years. In the first year all five *Streptomyces* isolates significantly increased chickpea nodule numbers and one isolate significantly increased nodule weight. These observations varied over time, as in the second year only three isolates significantly increased nodule numbers and two increased weight. This difference was attributed to increased naturalised rhizobia in the soil in the second year. The benefits of *Streptomyces* were attributed to their ability to secrete IAA (Gopalakrishnan et al., 2015). Addition of aqueous solution of IAA increased nodule numbers of mung bean in a net house trial by Ali et al., (2007). Microbes with the ability to secrete IAA were identified in leachates in this study (see Chapter 3) and may contribute to increased nodulation. In a separate greenhouse trial, Le, Ballard, & Franco (2016) observed significantly increased lucerne nodulation resulting from co-inoculation of endophytic *Streptomyces* and the commercial strain of rhizobia. Increases varied with harvest time and supplied N.

The nutrient solution supplied significantly more N to serradella than the leachates. Increased N reduces nodulation, with maximum nodulation observed when no N is supplied (Laws & Graves, 2005). The high N in nutrient solution may have contributed to reduced nodulation at 10 weeks. However, the water control produced equal or fewer nodules at the 10 week harvest. Thus, reduced nodulation was not due solely to N suppression. The nutrient solution produced the highest number of nodules at the 5 week harvest, although the effect was not significant. Despite the high N in nutrient solution, there was no indication of N suppression of nodulation at 5 weeks. Of the three leachates, green bean supplied the highest N. This increased N did not supress nodulation.

Several studies have found a microbial effect on N suppression of nodulation. Solans, Vobis, & Wall (2009) observed inhibition of rhizobial nodulation of lucerne at high N (7 mM). Interestingly, this inhibition was removed by co-inoculation with saprophytic actinomycetes. Four actinomycete isolates were examined, and increased nodulation at high N was observed for all. An increase in nodule numbers of lucerne with increasing N was reported in a greenhouse trial by Le, Ballard, & Franco (2016). This trial involved co-inoculation of actinobacteria and rhizobia. According to Le,

Ballard, & Franco (2016), increasing N delayed nodulation thereby giving the microbial co-inoculants a chance to effect improvements to nodulation. Similar delayed nodulation effects may have been caused by green bean leachate.

5.4.5 Nutrient content of leachate

Leachates contained more TOC than chemical fertilisers and recommended nutrient solutions. The other nutrients in leachate varied with the chemical fertiliser and leachate substrate. Leachates contained reduced nutrients compared to 5-11-26, a hydroponic solution used for vegetables (Everris, n.d.) and 3-15-27, a high Mg hydroponic solution used for flowers and vegetables (Plant Foods Inc., n.d.). Leachates contained more Cu than Hoagland's solution, a hydroponic solution for a wide variety of plants (Fernandez, 2009), but the remaining nutrients were reduced.

Green bean and rockmelon leachate contained more Ca than 7-17-37, a hydroponic nutrient solution used for vegetables (Hochmuth & Hochmuth, 2001) while banana leachate contained half the recommended Ca. All remaining nutrients were reduced regardless of substrate. Leachates contained more Cu, B and Mn than CRS, a low Fe solution for plants grown in sand (Yates et al., 2016), and the remaining nutrients were reduced.

Leachates contained much more N and more Cu than Norris and Date, a solution used for tropical legumes (Yates et al., 2016), and banana and rockmelon leachate contained more K. Green bean leachate contained slightly less than the recommended K. Leachates contained more N, Cu and B than Broughton & Dilworth, a nutrient solution for legume growth (Yates et al., 2016). Green bean and rockmelon leachate contained more Mg while banana leachate contained half the recommended Mg, and all other nutrients were reduced. Leachates contained higher nutrients than Jensen's, a N-free media used for small seeds grown in test tubes (Yates et al., 2016).

Leachates are generally nutrient poor compared to commercial solutions. This varies with solution. Leachates may supply enough nutrients for some plant species. Leachates may need to be supplemented with chemicals for other plant species. Leachates may over supply some nutrients and need to be diluted. Replacing synthetic nutrients with organic amendments is complicated by this uneven supply of nutrients. It's important to remember the microbial benefits of organics such as leachate. None of the commercial solutions supply microbes. Synthetic sources don't support complex microbial communities (Jack et al., 2011).

Leachates supplied a portion of most recommended nutrients when compared with reference nutrient solutions for soybean (Broughton & Dilworth, 1971), serradella (Yates et al., 2016) and wheat (Kingsbury & Epstein, 1986). To correctly supply others, leachate should be diluted. No one leachate can be pinpointed as the most or least effective due to the variable nature of the results.

5.4.6 Leachate compared with chemical nutrients

Leachates produced lower wheat and serradella shoot and root dry weight than chemical nutrient solution. In contrast, Ayyobi et al. (2013) reported no difference between cow manure leachate and chemical fertiliser for peppermint dry weight. Similarly, Bidabadi, Afazel, & Poodeh (2016) found no difference between cow manure leachate and commercial nutrient solution for *Stevia* sp. shoot and root dry weight. The different results may be due to different plant species as well as differences in applied nutrients. As with the treatment used in this study, the nutrient solution used by Bidabadi et al. (2016) contained higher N than the leachate. Bidabadi et al. (2016) used nutrient solution with higher P and K than the leachate and lower Cu, Zn and Mn. The reverse of these trends was found in this study.

Plant harvest time affected the results. Differences were observed after the five week harvest for serradella and after the ten week harvest for wheat. In contrast, Tejada et al. (2008) reported no difference between green waste or cow manure leachates and nutrient solution for tomato height after four separate harvests. Vermicompost substrate affects leachate nutrients. Substrate can therefore also affect comparisons with nutrient solution. Nutrient solution improved tomato yield over green waste leachate, but showed no change over cow manure leachate (Tejada et al., 2008).

At five weeks, leachates were an effective substitute for nutrient solution applied to wheat. At ten weeks, leachates were an effective substitute for nutrient solution applied to serradella. Proving the efficacy of leachate is therefore dependent on plant harvest time as well as plant species. Effects of harvest time were also reported by Singh et al., (2010) who observed increased strawberry shoot dry weight due to leachate at fourteen weeks, but not at twenty-four weeks.

It is also possible that the serradella plants treated with nutrient solution suffered retarded growth due to pot size. Poorter et al. (2012) recommend a plant biomass to pot volume ratio of 2 g L^{-1} to ensure pot size doesn't retard growth. The mean pot to volume ratio of 10 week serradella was 4.6 g L^{-1}, while the mean ratio of 10 week wheat was 1.26 g L^{-1}. It is possible that in larger pots the advantage of nutrient solution would have been visible at both harvests.

Some nutrients were oversupplied with leachate. Dilutions are required to supply recommended nutrients to soybean (Broughton & Dilworth, 1971), serradella (Yates et al., 2016) and wheat (Kingsbury & Epstein, 1986). This differs from the findings of Marín et al. (2013) who did not find the recommended amount of tomato nutrients from four different compost extracts, including crop residue vermicompost.

Diluted leachates supplied more Cu than plants required. Cu becomes toxic at elevated levels (Flemming & Trevors, 1989) but levels vary with plant species and soil type (Rooney, Zhao, & McGrath, 2006). Symptoms of Cu toxicity include reduced shoot and root weight, increased permeability of root cells and necrosis. Symptom onset in hydroponic systems can begin at 0.002 mg/L Cu (Lidon & Henriques, 1993; Lombardi & Sebastiani, 2005; Mocquot et al., 1996). The Cu supplied by leachate is within the range for potential toxicity. However, hydroponic systems may not be comparable to plants grown in soil. Cu levels in soil causing 50% root and shoot inhibition began at 36 mg/L for tomato and 22 mg/L for barley (Rooney et al., 2006). These levels were much higher than those supplied by leachate, but Cu already present in soil or water has not been considered.

Diluted leachates supplied more Mn than required by serradella. Nodulated legumes are more sensitive to the toxic effect of high Mn (Hungria & Vargas, 2000). Toxic levels vary among and within plant species (El-Jaoual & Cox, 1998). Increased soybean leaf browning and decreased root length were observed at 0.0014 mg/L Mn, while reduction in root and shoot fresh weight were observed at 0.0028 mg/L Mn (Chen et al., 2016). The Mn supplied by leachate is within the range for potential toxicity. However, the values reported by Chen et al. (2016) was from hydroponics which may not be comparable to soil.

The diluted leachates applied to soil used to grow plants in this study supplied more B than required by soybean or serradella. The line between deficiency and toxicity is narrow and toxic levels vary with plant species (Gupta et al., 1985). Oversupply of nutrients is a potential drawback of organic amendments. Diluted leachates also supplied N, which may be a drawback when growing nodulated legumes.

5.5 Conclusion

Green bean leachate increased nodulation of soybean and serradella. The mechanism of this increase is potentially microbial through an interaction with the rhizobial inoculant strain, legume host plant and/or nodulation process. This effect varies with vermicompost substrate, which is likely because of the different microbes found in different leachates. Leachates largely had no effect on plant growth factors including shoot dry weight and nutrients, root dry weight and length, which would be largely influenced by nutrient content. Leachate nutrients varied with substrate, with green bean leachate generally supplying more nutrients. Leachates undersupplied some nutrients but oversupplied others and this varied with substrate and plant species. The variable nature of leachates makes it difficult to satisfy optimum plant growth requirements. It may be possible to achieve this through a combination of substrates fed to earthworm-beds. The potential benefit from microbial communities in leachate is clearly complex and likely to vary with plant species and soil microbial community.

Chapter 6 - Effect of green bean leachate nutrients and microbes on growth and nodulation of serradella

6.1 Introduction

Vermicompost leachate has proven beneficial effects to plants (Table 1.1) although research on leachate is limited in comparison with solid vermicompost (Figure 1.1). Vermicompost is also an excellent food waste processing system. Food waste is estimated to cost Australian households more than $8 billion per year, with 361 kg of food waste generated per person. The Australian government lacks the infrastructure or technology for effective waste management. Disposal of food waste into landfill by businesses is being discouraged with levies and there is increasing interest in placing a monetary value on this waste (Australian Government Department of the Environment, 2016). The majority of information about Australian food waste concerns individual households and consumers. There is a large gap in data between the farm gate and the consumer (Mason et al., 2011), suggesting that national food waste is actually a lot higher.

Limited data exists on the benefits of vermicompost leachate from green bean worm-bed substrate but the importance of vermicomposting as a method of disposing of green bean waste shouldn't be discounted. Green beans (also known as French beans) are grown year round, mostly in Queensland. In 2009, Queensland produced over 14 000 tonnes of green beans, worth over $50 million (Department of Agriculture and Fisheries, 2014). National production of French and runner beans in 2009 totalled 27 779 tonnes worth $72.7 million (AUSVEG, 2011). Most beans are sold on the fresh market, where beans must be at least 3 inches, or 7.62 cm, in length. This leads to a large amount of waste. For example, Hughes Farms and Produce in Tennessee discards 3 million pounds, or 1361 tonnes, of green beans per year as they are snapped or too short for retail standards (Basten, 2016; Department of Agriculture and Fisheries, 2014).

Limited data exists on the benefits of vermicompost leachate from banana and rockmelon worm-bed substrates, but waste recycling is a crucial consideration. The quantity of rockmelons grown per year across mainland Australia is 210 000 tonnes. These are mostly grown in Queensland and 95% of these are sold on the fresh market. Retailers often sell cut rockmelons, displayed at ambient temperature due to lack of refrigeration space. Supermarkets discard unsold fruit at the end of the day (Department of Primary Industries Food Authority, 2017; Keogh, Mullins, & Robinson, 2008). Over 310 000 tonnes of bananas are grown in Australia each year, the majority in North Queensland

(Clarke, Jensen, & Hardin, 2008). On North Queensland farms, 30 000 tonnes of bananas are discarded per year for cosmetic reasons (White, Gallegos, & Hundloe, 2011).

Vermicomposting is an efficient method of processing green bean waste. Green bean vermicompost leachate contains macro and micronutrients and benefits to plant growth were demonstrated in Chapter 5. Soil application of green bean leachate improved nodulation of soybean and serradella. The mechanism of increased nodulation is unknown. Vermicomposting is also an efficient method of processing rockmelon and banana waste. Banana and rockmelon leachates improved serradella nodulation, to a lesser extent than green bean leachate. It is possible that the nodulation benefits of banana and rockmelon leachates could be increased by mixing the worm-bed substrates with green bean.

N and P are among the most limiting nutrients for plant growth. Replenishment is usually achieved by adding chemical fertiliser to the soil. Improper chemical fertiliser use is economically and environmentally detrimental and has created a multitude of health problems. As a result, there is increasing interest in microorganisms with the ability to fix N or solubolise P (Berg, 2009; Korir et al., 2017). When macronutrients are equal, vermicompost benefits plant growth over chemical fertiliser. This indicates that micronutrients and microbes play an important role in the action of organic amendments (Arancon et al., 2004). To study is designed to separate the microbial component of vermicompost leachate from the nutrient component and, by applying these components separately to plants, determine the mechanism of leachate effect on nodulation.

The aims of the work presented in this chapter are:

1. To determine whether plant growth benefits are a result of the nutrient or microbial component of green bean leachate and determine which component affects nodulation in serradella
2. To determine whether leachate from a mixed worm-bed substrate of banana, green bean and rockmelon improved plant response over leachate from a single substrate of either banana, green bean or rockmelon
3. To measure leachate nutrients and determine whether nutrients in worm beds changed over time

The null hypothesis for these experiments was that leachate components will not have a differential effect on growth and nodulation of serradella. In addition, that leachate nutrients will not change with time or worm-bed substrate.

6.2 Methods

6.2.1 Vermicompost and leachate harvest

Worm farms (Figure 2.2) were sterilised with ethanol prior to use. Four containers were filled with 72 g shredded newspaper. Enough tap water was added to cover the newspaper before being drained. This liquid (40 mL) was collected in plastic screw capped tubes as time 0 leachate. Leachates were stored at 4°C for nutrient analysis.

Earthworm source and species are described in section 2.2.1. Earthworms (80), weighing a mean of 0.5 g each, were added per unit. Worm farms were covered as in section 2.2.3 and maintained under controlled conditions as described in section 5.2.3. Worm farms were left overnight then 40 g of worm-bed substrate was added. Substrates were banana, green bean, rockmelon and B/G/R as detailed in Table 4.2. No immediate drainage of rockmelon through the units was observed, so rockmelon was not filtered. Substrate (80 g) was added weekly on top of the unit contents. Dry bedding (20 g) was added fortnightly beneath the unit contents to soak up excess moisture. Leachate harvest is detailed in section 3.2.1. Prior to commencement of the pot trial, leachate harvest was carried out weekly, with 60 mL water used to collect 40 mL leachate. This leachate was stored in screw capped plastic tubes at 4°C for nutrient analysis. Leachate harvest for plant application commenced at 8 weeks. The volume of leachate needed for the plant trial was harvested, plus an additional 10%. Therefore, 2L water was used to collect 1600 mL green bean leachate, 750 mL water was used to collect 600 mL B/G/R leachate, 150 mL water was used to collect 125 mL banana and rockmelon leachates. Following the first plant harvest, the volume of leachate needed for plant application was reduced at 12 weeks. Water (1L) was used to collect 800 mL green bean leachate, 375 mL water was used to collect 300ml B/G/R leachate.

6.2.2 Application of leachate components to serradella grown in sand

The pot trial was carried out from March 18 2017 to April 27 2017. Pots used in section 5.2.2 were sterilised with bleach (0.4% sodium hypochlorite, Coles, Glen Iris, Australia), rinsed in tap water and left to dry overnight under conditions described in section 5.2.3. Pots were filled with river sand (Australian Builders, Port Melbourne, Australia) and sterilised by air drying for two weeks.

Treatments involved separation of green bean leachate into nutrient and microbial fractions. Green bean and B/G/R leachates were applied along with two nutrient solutions. This experiment was set

up in a completely randomised block design comprising five blocks with ten replicates per treatment. The experimental design is shown in Figure 6.1 and treatments are listed in Table 6.1.

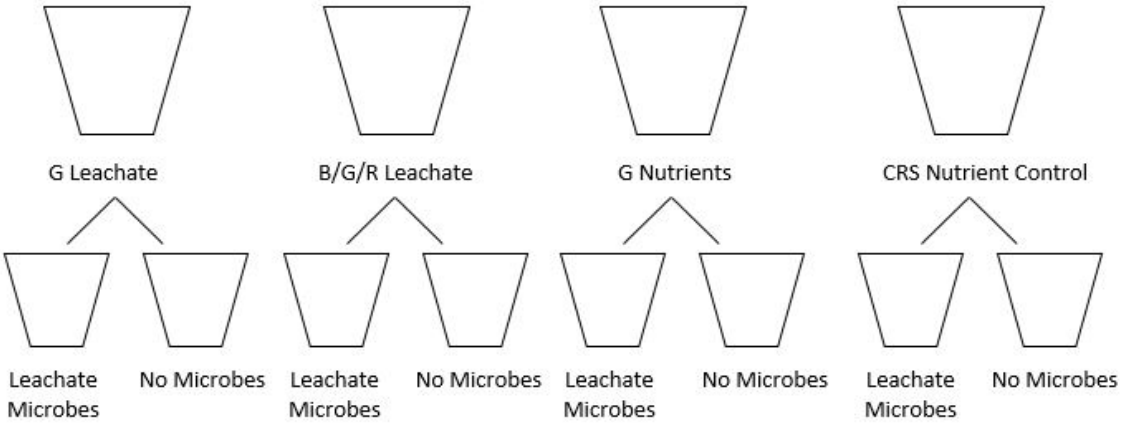

Figure 6.1. Design of pot trial testing vermicompost leachate as an organic amendment for serradella grown in sand. Two vermicompost substrates were used. G – green bean, B/G/R – an equal mixture of banana, green bean and rockmelon. Serradella plants were grown in sand treated with G leachate, B/G/R leachate, G nutrients (a nutrient solution based on macro- and micronutrient analysis of G leachate from a previous pot trial), or CRS nutrient solution containing the recommended requirements for legumes grown in sand. Half the pots were inoculated with the cell pellet harvested from G leachate. All pots were inoculated with serradella inoculant strain WSM471.

Table 6.1. Treatments used in serradella pot trial.

Treatment solution	Leachate microbes	Inoculation
Green bean leachate	Green bean	Serradella commercial inoculant strain WSM471
	None	
B/G/R leachate	Green bean	
	None	
Nutrient solution (G)	Green bean	
	None	
CRS Nutrient control	Green bean	
	None	

B/G/R contained an equal amount by weight of banana, green bean and rockmelon. Nutrient solution (G) contained the nutrient content of diluted G leachate from a previous trial. CRS nutrient control contained recommended nutrients for legumes grown in sand.

6.2.3 Seed germination and inoculant preparation

French serradella (*O. sativus* cv. Margurita) seeds were washed, sterilised and germinated as described in section 5.2.3. Pre-diluted bleach (2%, White King, Shepparton, Australia) was used. Rhizobial inoculant strain WSM471 was prepared as in section 5.2.3 using ten YMA plates incubated at 25°C for nine days.

6.2.4 Seed sowing and inoculation

Four germinated seeds were sown per pot and plastic covers were used as described in section 5.2.2. Plants were watered twice a week for five weeks then five times a week. Pots were inoculated with WSM471 6 DAS, when cotyledons were observed. Inoculation was carried out as described in section 5.2.2. Inoculum concentration was 4.3×10^5 CFU/seed. Excess seedlings were removed 18 DAS leaving three seedlings remaining in each pot.

6.2.5 Application of treatment solutions

Due to time constraints, a pre-sowing treatment was not used in this trial and weekly application of treatment solutions began 11 DAS. Green bean nutrient solution and control nutrient solution were made the day before the first application. Solutions were stored at room temperature. N-free CRS was used as the control solution. The nutrients added per pot to soybean in a previous pot trial

(Table 5.5) was used as the green bean nutrient solution. Nutrients in the soybean trial were chosen as the leachate harvest times were closer to those used in the current trial. Nutrient solution recipes are detailed in Appendix A. Nutrient solutions were sterilised by autoclaving at 121°C for 20 min.

Leachate was harvested immediately prior to plant application and EC measured as described in section 5.2.2. Green bean and B/G/R leachates were diluted with tap water to the lowest measured EC at each harvest. The EC values used were 0.8 mS cm^{-1} (8 week harvest), 0.9 mS cm^{-1} (9 week harvest), 0.7 mS cm^{-1} (10 week harvest), 0.6 mS cm^{-1} (11 and 12 week harvests), 0.7 mS cm^{-1} (13 week harvest), 0.5 mS cm^{-1} (14 week harvest), 0.4 mS cm^{-1} (15 week harvest). The EC values of the nutrient solutions were not measured. Diluted green bean and B/G/R leachates (40 mL) were stored in plastic screw capped tubes at 4°C for nutrient analysis.

Leachate pellets were obtained by centrifuging 20 mL leachate at 2700 g for 5 min. Supernatant was discarded and cell pellet washed in 20 mL deionised water. Centrifugation was repeated and cell pellet resuspended in 20 mL deionised water. Treatment solutions (20 mL) were added to the soil surface. Resuspended cell pellet (20 mL) was added to the soil surface. An equal volume of tap water was added to all other pots.

6.2.6 Plant harvest

Serradella plants were harvested at two time points. Five replicates per treatment were harvested 39 DAS. Plant height was determined by measuring from the soil surface to the highest point of the tallest of the three plants per pot. Roots were wrapped in damp paper towel and sealed in zip lock bags. Plants were stored at 4°C prior to processing. Nodules were removed from serradella roots by hand. Nodule number per pot was recorded. Nodules were washed in tap water and blotted dry with paper towel. Nodule fresh weight per pot was determined. Nodules were dried at 60°C for two days. Nodule dry weight per pot was determined. Shoots were separated from the roots. Shoots and roots were dried at 60°C for two days. Shoot dry weight and root dry weight per pot were recorded. Remaining plants were harvested 68 DAS. Measurements were taken as per the first harvest. Nodules were smaller than at the first harvest and nodules per treatment were combined. Nodule fresh and dry weight per treatment were recorded. Due to a malfunctioning oven, samples were dried at 65°C.

6.2.7 Nutrient analysis

Leachates were filtered through a 0.2 µm cellulose acetate membrane using a polycarbonate filter holder (Sartorius Stedim, Dandenong, Australia) attached to a vacuum. Leachates were divided into a 10 mL and a 25 mL aliquot. The 25 mL aliquot was acidified and used to determine TOC and TN as

described in section 4.2.1. The 10 mL aliquot was acidified and used to determine Ca, Cu, Fe, K, Mg, Mn, P, S, Zn, Barium (Ba), Sodium (Na), Lead (Pb) and Strontium (Sr) using the ICP-OES as described in section 4.2.2.

6.2.8 Data analysis

Leachate nutrients and EC were analysed with one-way ANOVA. Plant growth data were analysed with LMM. Nutrients and plant growth were compared with two-sided correlation analyses. All analyses were carried out with GenStat 18th Edition (VSN International Ltd, Hempstead, UK).

6.3 Results

6.3.1 Effect of green bean nutrients and microbes on serradella

Worm-bed substrate led to significant variation in EC of undiluted leachate (Table 6.2). Rockmelon had a significantly higher EC than all other leachates and B/G/R had a significantly higher EC than green bean leachate. There was no difference between B/G/R and banana leachate ECs or banana and green bean leachate ECs. Harvest time had no effect on leachate EC. Statistical comparisons were restricted to leachate samples collected during the first 8 weeks of the duration of the worm farms. Later leachate samples used different volumes of water to harvest different volumes of leachate which likely resulted in variation in EC.

Table 6.2. Electrical conductivity of vermicompost leachate from different worm-bed substrates.

	EC (mS cm^{-1})	
Substrate	Undiluted	Diluted
Banana	0.89 ± 0.15bc	
Green bean	0.64 ± 0.06c	0.64 ± 0.06
Rockmelon	1.81 ± 0.15a	
B/G/R	0.99 ± 0.08b	0.67 ± 0.05

Values shown are mean ± SEM of eight replicates. Two leachates were diluted for soil amendment. One-way ANOVA performed with different letters indicating significant differences ($p < 0.05$).

Green bean leachate supplied significantly more TOC, TN, Ca, Fe, Na, P, Sr and Zn than B/G/R leachate. Both leachates supplied equal amounts of Cu, K, Mg, Mn and Na. The synthetic green bean nutrients differed from green bean leachate. Leachate supplied more TOC, Ca, Cu, K, Mg, Na and Sr. Green bean nutrients supplied more TN, Fe and S. Both solutions supplied equal amounts of Mn and

Zn. B/G/R leachate supplied more TOC, Ca, Cu, Fe, K, Na and Sr than green bean nutrients. Green bean nutrients supplied more TN, Fe and S than B/G/R leachate. Both solutions supplied equal amounts of Mg, Mn, P and Zn (Table 6.3).

CRS nutrient solution supplied more Ca, K, Mg, P, S and Zn than all other treatment solutions and more Fe than both leachates. It supplied less TOC, Cu, Na and Sr than both leachates and more Sr than green bean nutrients. CRS and green bean nutrients supplied equal amounts of TOC and Cu but CRS nutrients supplied less Fe and Na than green bean nutrients. All treatment solutions supplied an equal amount of Mn. CRS nutrient solution contained measurable TN, although no N was added (see Appendix A). The N source is unknown. CRS nutrients supplied less TN than green bean nutrients. CRS nutrients supplied an equal amount of TN as green bean leachate and more TN than B/G/R leachate (Table 6.3).

CRS nutrient solution produced significantly taller plants than all other treatments at both 5 and 10 weeks (Figure 6.2). At 10 weeks, green bean leachate produced taller plants than B/G/R leachate or green bean nutrients, although the difference was not significant. Addition of harvested microbial cells from green bean leachate had no effect on serradella height at either harvest.

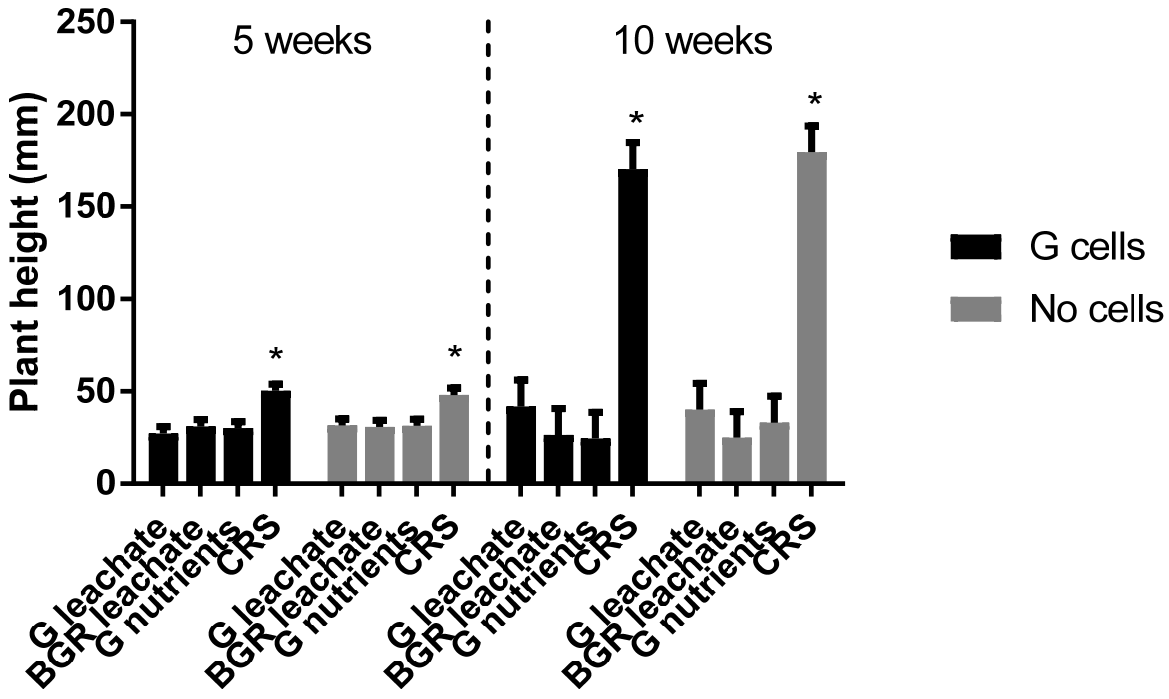

Figure 6.2. Serradella height in response to soil treatments five weeks and ten weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Controls were green bean nutrient solution and recommended nutrient solution (CRS). Half the plants were inoculated with microbial cells harvested by centrifugation from green bean leachate. Values plotted are predicted means from LMM analysis of five replicates. * indicates a significant difference ($p < 0.001$).

Table 6.3. Nutrient content of leachate applied to serradella.

mg/L	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Sr	Zn	TOC	TN	Ba	Pb
BGR	22.8 ± 2.0c	0.26 ± 0.02a	0.009 ± 0.001d	120.1 ± 15.6b	7.1 ± 0.5bc	0.007 ± 0.002a	18.5 ± 1.3b	1.1 ± 0.2c	1.9 ± 0.2c	0.049 ± 0.004b	0.03 ± 0.003c	60.4 ± 6.2b	3.1 ± 0.3c	<LOD	<LOD
GB	40.5 ± 3.6b	0.26 ± 0.02a	0.017 ± 0.002c	111.4 ± 18.1b	7.9 ± 0.6b	0.007 ± 0.002a	22.0 ± 1.9a	2.1 ± 0.3b	2.1 ± 0.2c	0.077 ± 0.005a	0.06 ± 0.01b	77.4 ± 6.5a	6.0 ± 1.1b	<LOD	<LOD
CRS	121.9a	0.15b	0.053b	183.8a	29.0a	0.0037a	7.3c	30.1a	214.8a	0.030c	0.23a	18.1c	7.5b	<LOD	<LOD
SGB	16.6d	0.11b	0.070a	60.0c	6.3c	0.0021a	0.4d	1.4c	13.5b	0.004d	0.05bc	19.7c	36.1a	<LOD	<LOD

Leachate from different substrates was diluted to the lowest EC before addition to soil. Substrates were green bean (GB) and a combination of banana, green bean and rockmelon (BGR). Values shown are the mean ± SEM of eight replicates. One-way ANOVA performed, with different letters indicating significant differences ($p < 0.001$). Leachates were compared with recommended nutrients (CRS) and synthetic green bean nutrients (SGB).

When CRS nutrients were removed from the analysis, no variation was observed after 5 weeks; however, significant variation was observed after 10 weeks. Green bean leachate produced taller plants than B/G/R leachate and green bean nutrients in combination with harvested microbial cells from green bean leachate. Without green bean microorganisms, green bean leachate produced taller plants than B/G/R leachate (Figure 6.3).

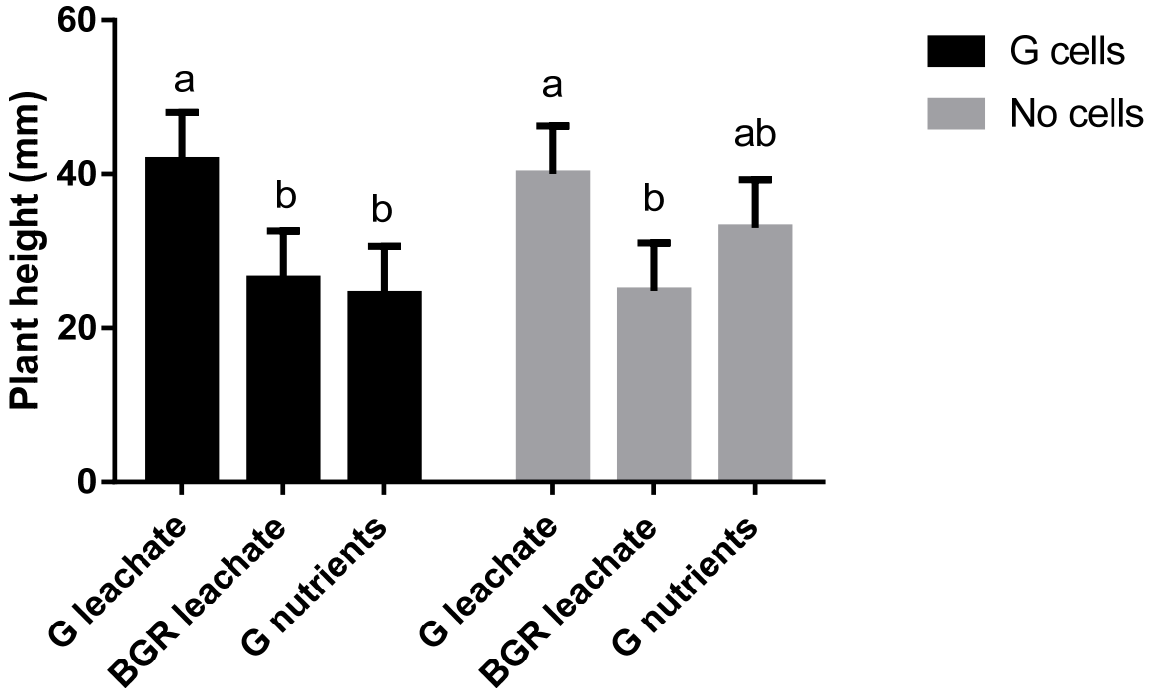

Figure 6.3. Serradella height in response to soil treatments 10 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Control was green bean nutrient solution. Half the plants were inoculated with cells from green bean leachate. Values plotted are predicted means from LMM analysis of five replicates. Different letters indicate significant differences ($p < 0.01$).

CRS nutrient solution produced plants with significantly greater shoot dry weight than all other treatments at both 5 and 10 weeks (Figures 6.4 and 6.5). At 10 weeks, green bean leachate produced plants with greater shoot dry weight than B/G/R leachate or green bean nutrients, although the difference was not significant (Figure 6.5). Addition of green bean microbial cells had no effect on shoot dry weight at either harvest.

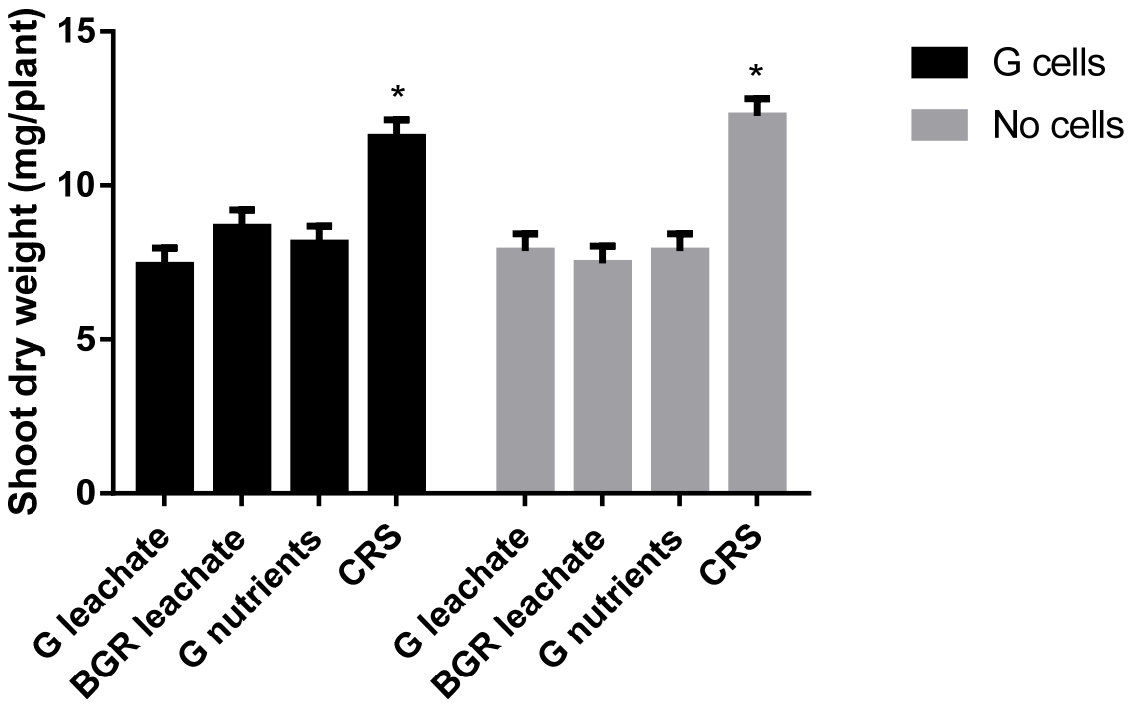

Figure 6.4. Serradella shoot dry weight in response to soil treatments 5 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Controls were synthetic green bean nutrient solution and recommended nutrients (CRS). Half the plants were inoculated with cells from green bean leachate. Values plotted are predicted means from LMM analysis of five replicates. * indicates a significant difference ($p < 0.001$).

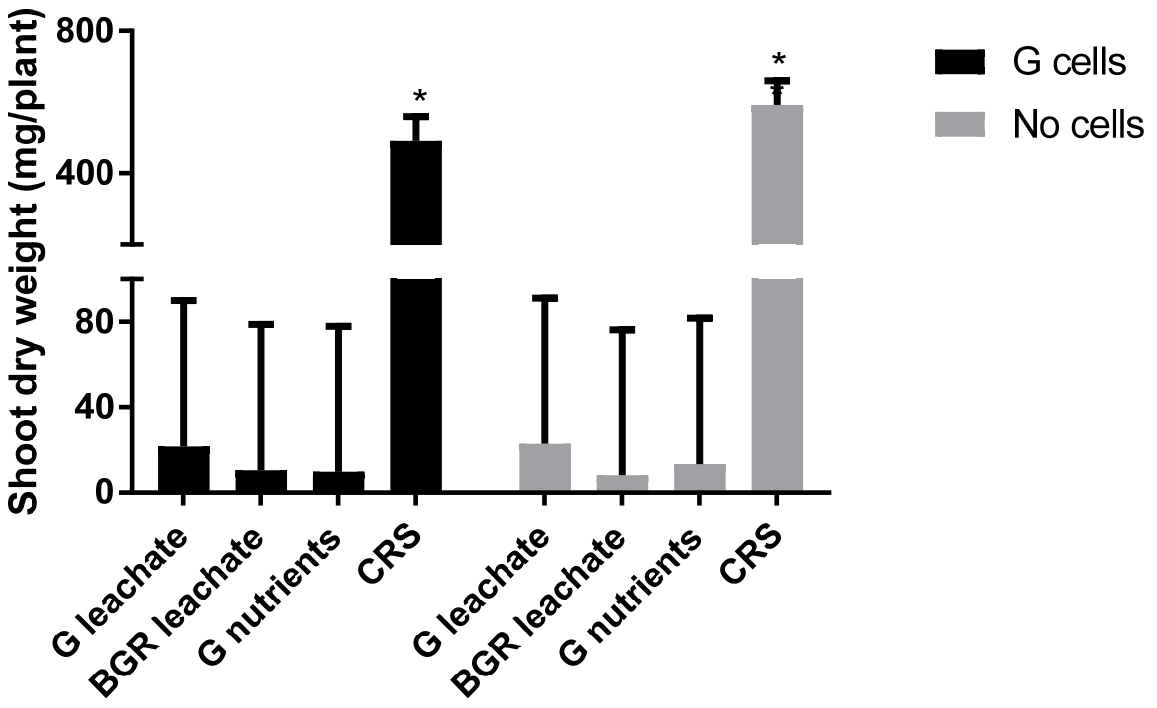

Figure 6.5. Serradella shoot dry weight in response to soil treatments 10 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Controls were green bean nutrient solution and recommended nutrients (CRS). Half the plants were inoculated with cells from green bean leachate. Values plotted are predicted means from LMM analysis of five replicates. * indicates a significant difference ($p < 0.001$).

When CRS nutrients were removed from the analysis, no variation was observed after 5 weeks. Significant variation was observed after 10 weeks. Green bean leachate produced plants with greater shoot dry weight than B/G/R leachate and green bean nutrients. Without green bean cells, green bean nutrients produced plants with greater shoot dry weight than B/G/R leachate (Figure 6.6).

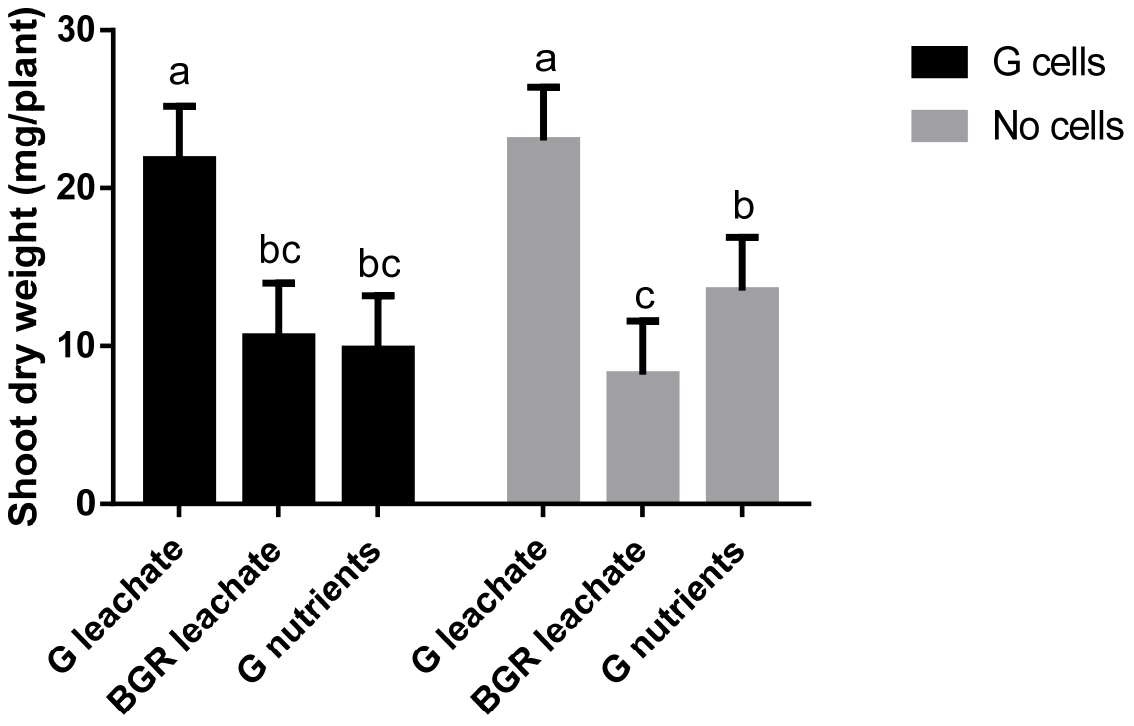

Figure 6.6. Serradella shoot dry weight in response to soil treatments 10 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Control was green bean nutrient solution. Half the plants were inoculated with cells from green bean leachate. Values plotted are predicted means from LMM analysis of five replicates. Different letters indicate significant differences ($p < 0.01$).

After 5 weeks, green bean nutrients produced the greatest root dry weight, significantly greater than CRS nutrients both with and without green bean cells. B/G/R leachate resulted in significantly greater root dry weight then CRS nutrients both with and without green bean cells. Green bean leachate produced greater root dry weight than CRS nutrients. This difference was statistically significant when no green bean cells were added (Figure 6.7a).

After 10 weeks, CRS nutrients produced greater root dry weight than all other treatments. Green bean leachate produced greater root dry weight than B/G/R leachate and green bean nutrients, but this difference wasn't significant. The presence of green bean cells had no effect on root dry weight (Figure 6.7b).

When CRS nutrients were removed from the analysis, variation was observed after 10 weeks. Green bean leachate produced greater root dry weight than B/G/R leachate and green bean nutrients (Figure 6.7c).

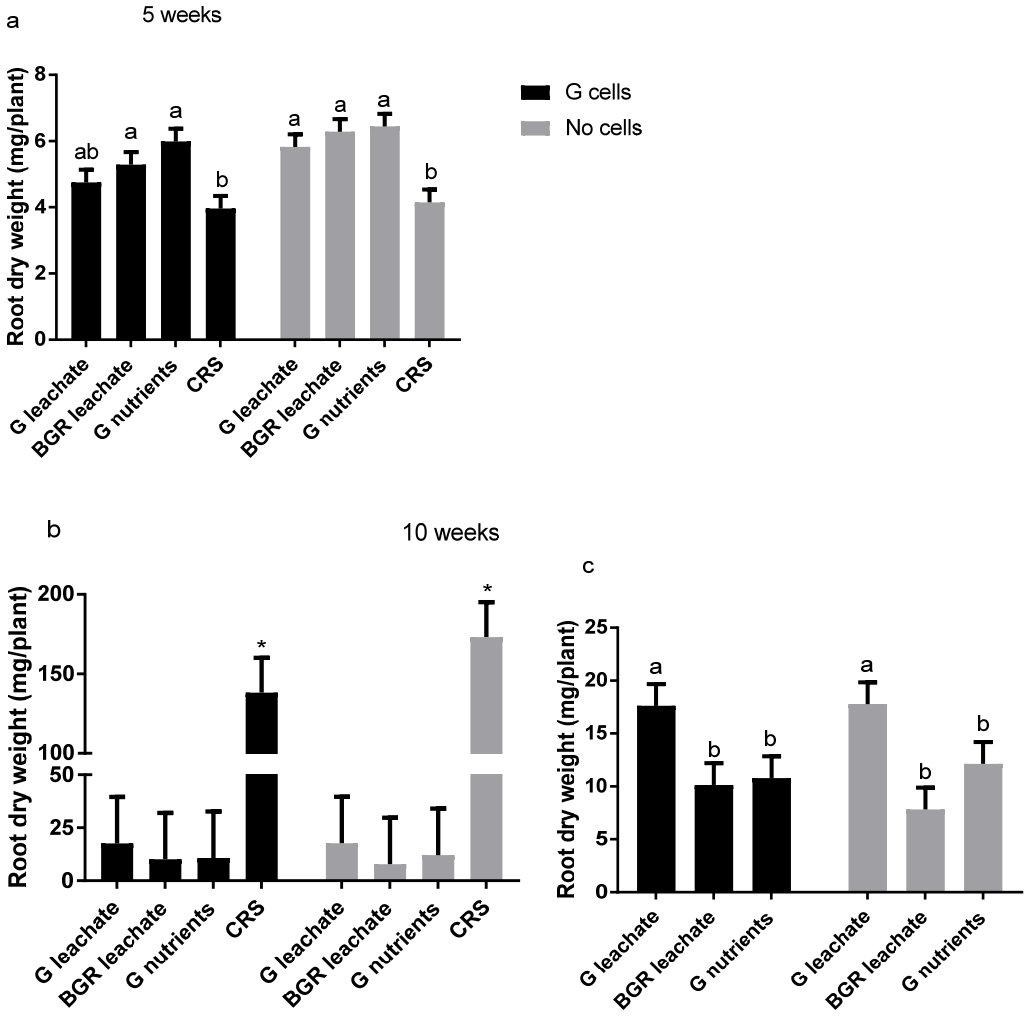

Figure 6.7. Serradella root dry weight in response to soil treatments 5 and 10 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Controls were synthetic green bean nutrient solution and recommended nutrients (CRS). Half the plants were inoculated with cells from green bean leachate. Values plotted are predicted means from LMM analysis of five replicates. 5 week harvest (a) Different letters indicate significant differences ($p < 0.01$). 10 week harvest (b) * indicates significant difference ($p < 0.001$). 10 week harvest without CRS (c) different letters indicate significant differences ($p = 0.001$).

At 5 weeks, B/G/R leachate produced the most nodules (Figure 6.8). B/G/R leachate produced significantly more nodules than green bean leachate and green bean nutrients. B/G/R leachate produced more nodules than CRS nutrients in the presence of green bean microbial cells, although the difference was not significant. CRS nutrients produced more nodules than green bean nutrients. CRS nutrients produced more nodules than green bean leachate in the absence of green bean microbial cells. With the addition of green bean microbial cells, no difference between green bean leachate and CRS nutrients was observed. Green bean leachate produced significantly more nodules than green bean nutrients in the absence of green bean microbial cells. When green bean microbial cells were added, this difference was not significant.

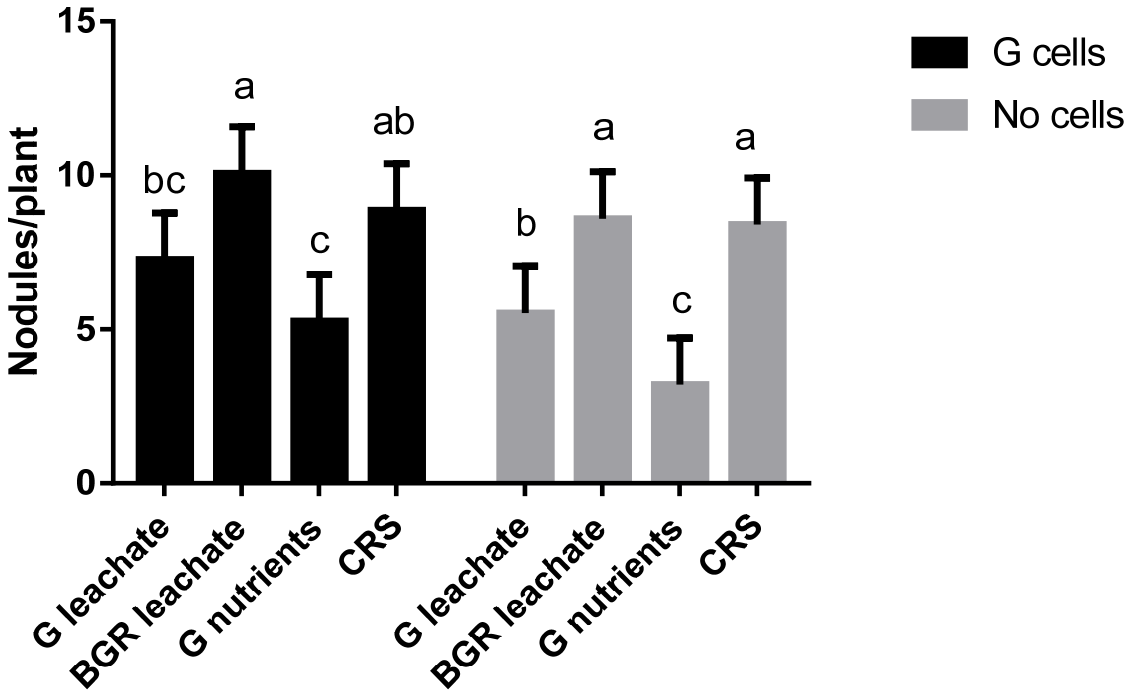

Figure 6.8. Serradella nodulation in response to soil treatments 5 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Controls were green bean nutrient solution and recommended nutrients (CRS). Half the plants were inoculated with cells from green bean leachate. All plants were inoculated with rhizobia WSM471. Values plotted are predicted means from LMM analysis of five replicates. Different letters indicate significant differences ($p < 0.001$).

At 10 weeks, CRS produced the most nodules, significantly more than all other treatments. Green bean leachate produced more nodules than B/G/R leachate and green bean nutrients, although the difference wasn't significant. B/G/R leachate produced more nodules than green bean nutrients, although the difference wasn't significant. CRS nutrients combined with green bean cells produced more nodules than CRS nutrients alone, although the difference wasn't significant (Figure 6.9).

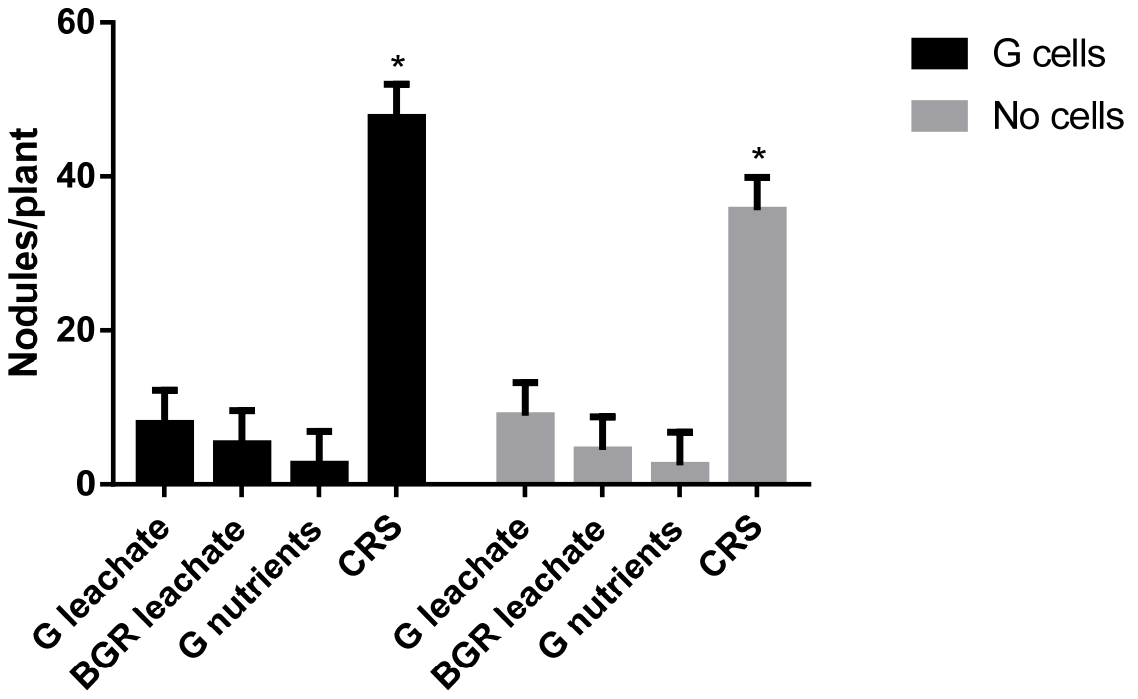

Figure 6.9. Serradella nodulation in response to soil treatments 10 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Controls were green bean nutrient solution and recommended nutrients (CRS). Half the plants were inoculated with cells from green bean leachate. All plants were inoculated with rhizobia WSM471. Values plotted are predicted means from LMM analysis of five replicates. * indicates significant difference ($p < 0.001$).

When CRS nutrients were removed from the analysis, variation was observed after 10 weeks. Green bean leachate produced significantly more nodules than B/G/R leachate and green bean nutrients. B/G/R leachate produced significantly more nodules than green bean nutrients (Figure 6.10).

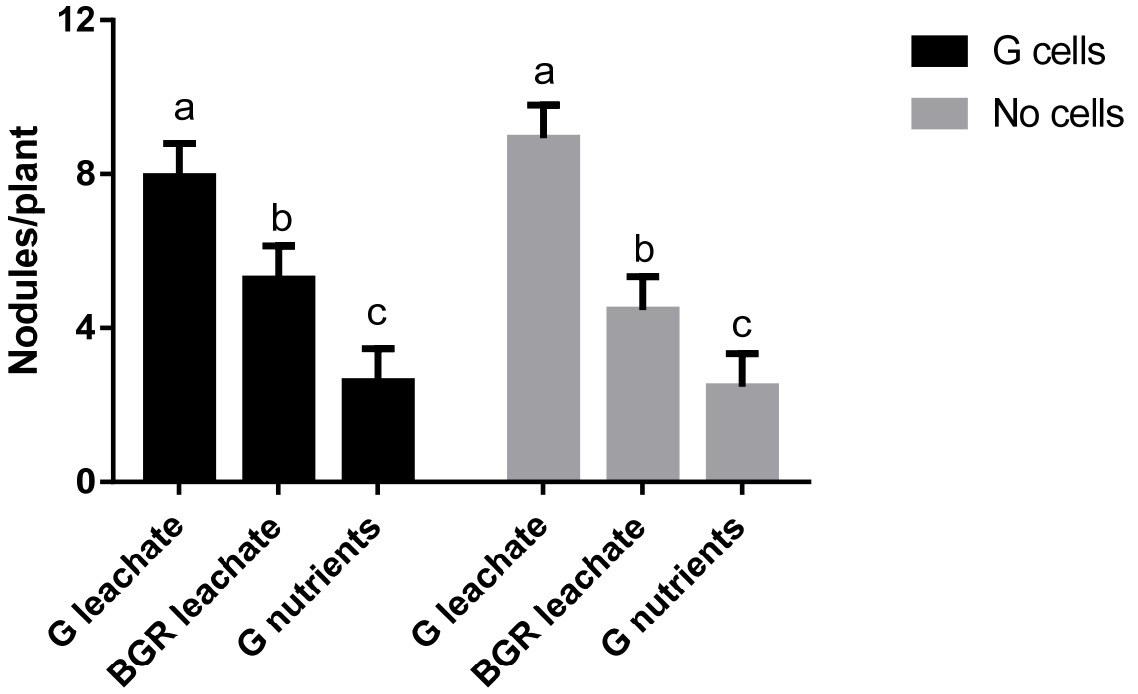

Figure 6.10. Serradella nodulation in response to soil treatments 10 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Control was green bean nutrient solution. Half the plants were inoculated with cells from green bean leachate. All plants were inoculated with rhizobia WSM471. Values plotted are predicted means from LMM analysis of five replicates. Different letters indicate significant differences ($p < 0.001$).

When CRS recommended nutrients were supplied, nodule numbers increased at the second harvest. Nodules increased 4.4-fold in the presence of green bean microbial cells. Nodules increased 3.2-fold without cells. Green bean leachate also led to an increase in nodulation between harvests, 0.08-fold with cells and 0.38-fold without cells. B/G/R leachate and green bean nutrients led to a reduction in nodules between the 5 week and 10 week harvests. B/G/R leachate reduced nodules between five and ten week harvests by 48% both with and without green bean cells. Green bean nutrients reduced nodules by 51% with cells and 23% without cells.

CRS nutrients inoculated with green bean cells produced nodules with significantly greater fresh weight than all other treatments (Figure 6.11). CRS nutrients without cells produced nodules with greater fresh weight than green bean leachate, B/G/R leachate and green bean nutrients. Green bean leachate produced significantly larger nodules than green bean nutrients. Green bean leachate produced larger nodules than B/G/R leachate. This difference was significant in the absence of green bean cells. B/G/R leachate produced larger nodules than green bean nutrients. This difference was significant in soil treated with green bean cells.

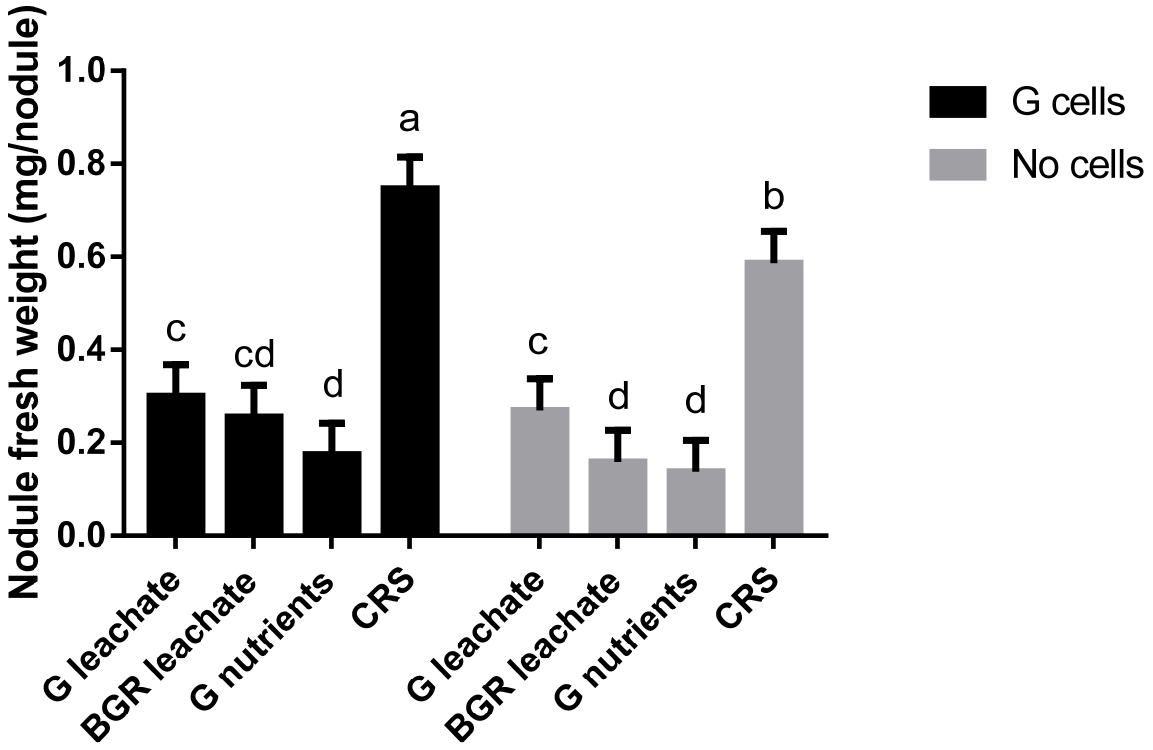

Figure 6.11. Serradella nodule weight in response to soil treatments 5 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Controls were green bean nutrient solution and recommended nutrients (CRS). Half the plants were inoculated with cells from green bean leachate. All plants were inoculated with rhizobia WSM471. Values plotted are predicted means from LMM analysis of five replicates. Different letters indicate significant differences ($p < 0.001$).

CRS nutrients produced larger nodules at 10 weeks than all other treatments (Figure 6.12). Green bean leachate produced larger nodules than B/G/R leachate and green bean nutrients. Nodules from five replicates were combined prior to weighing and statistical analysis were not performed.

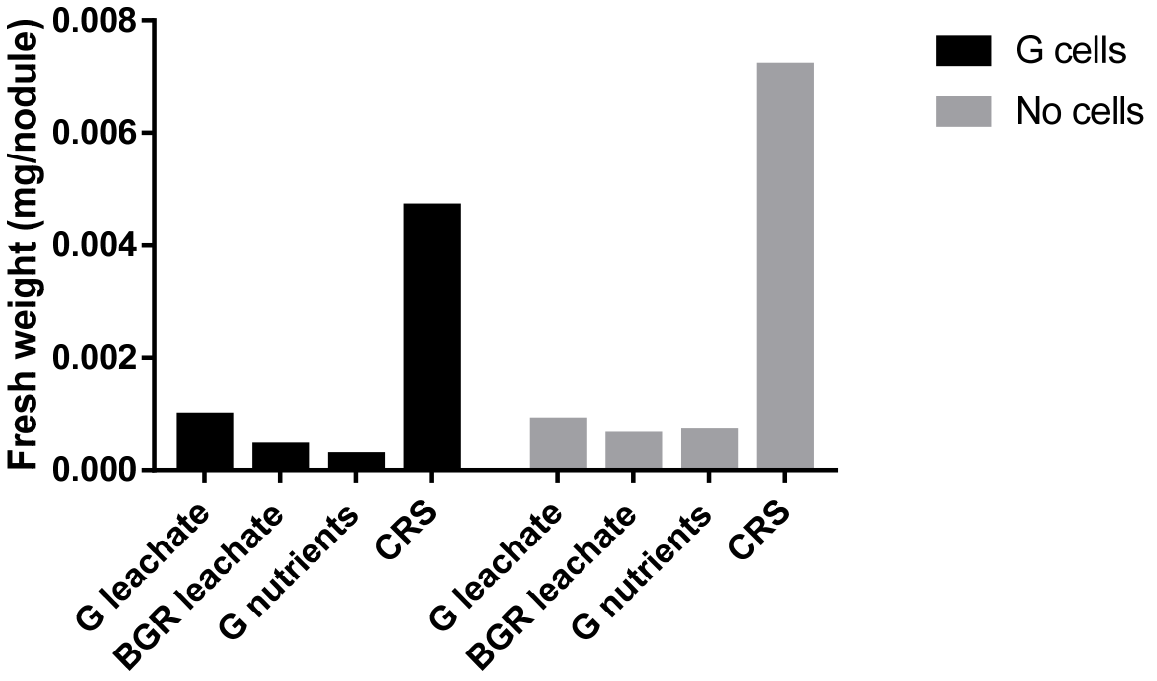

Figure 6.12. Serradella nodule weight in response to soil treatments 10 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Controls were green bean nutrient solution and recommended nutrients (CRS). Half the plants were inoculated with cells from green bean leachate. All plants were inoculated with rhizobia WSM471. Plants were harvested after 10 weeks. Replicate nodules per treatment were combined and no statistical analyses were performed.

CRS nutrients produced nodules with greater dry weight than all other treatments at 5 and 10 weeks (Figures 6.13 and 6.14). At 5 weeks, green bean leachate produced nodules with greater dry weight than B/G/R leachate and green bean nutrients in the absence of green bean cells. At 5 weeks, B/G/R leachate produced nodules with greater dry weight than green bean nutrients in the absence of green bean cells. At 10 weeks, green bean leachate produced nodules with greater dry weight than B/G/R leachate and green bean nutrients both with and without green bean cells. Nodules from five replicates were combined prior to weighing and statistical analyses were not performed.

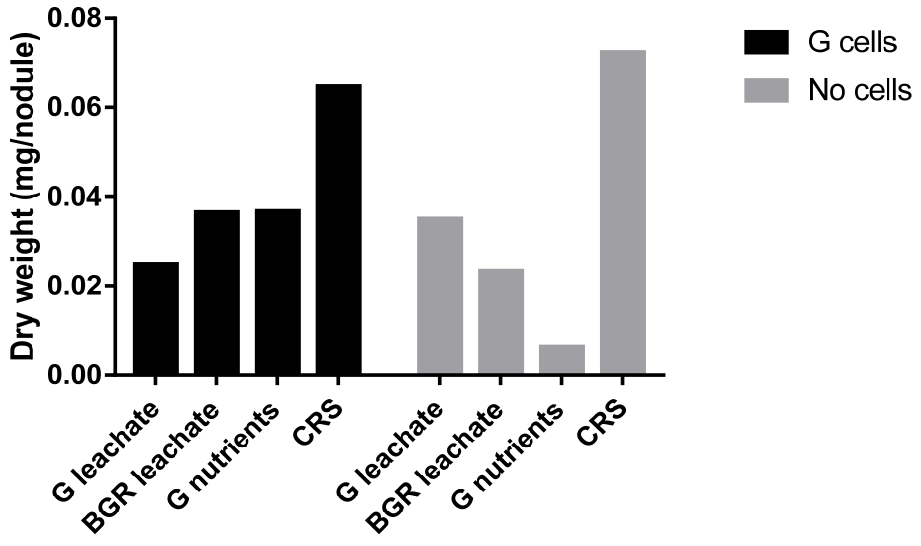

Figure 6.13. Serradella nodule weight in response to soil treatments 5 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Controls were green bean nutrient solution and recommended nutrients (CRS). Half the plants were inoculated with cells from green bean leachate. All plants were inoculated with rhizobia WSM471.

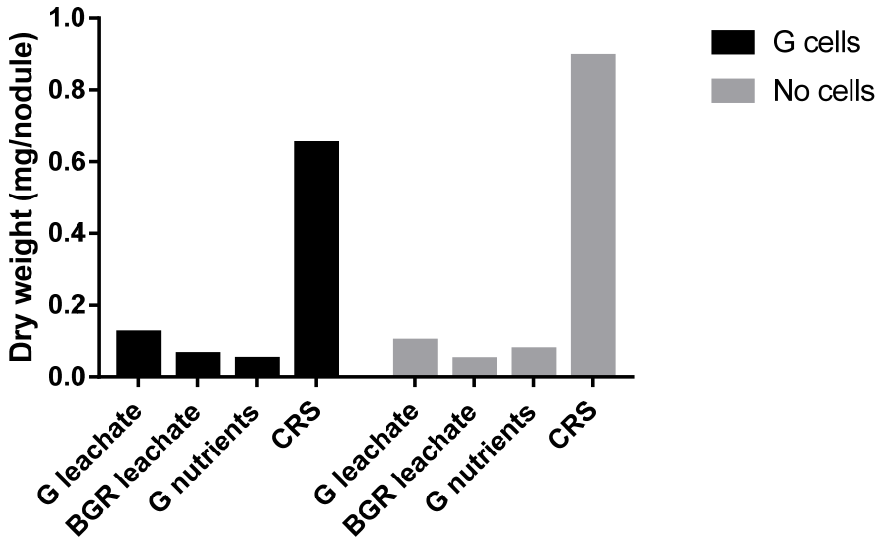

Figure 6.14. Serradella nodule weight in response to soil treatments 10 weeks after sowing. Treatments were vermicompost leachate from green bean (G) and a combination of banana, green bean and rockmelon (BGR). Controls were green bean nutrient solution and recommended nutrients (CRS). Half the plants were inoculated with cells from green bean leachate. All plants were inoculated with rhizobia WSM471.

6.3.3 Effect of leachate nutrients on serradella

There was a positive correlation between P supplied by treatment solutions and serradella nodule weight. When CRS nutrient solution was included, there was a strong positive correlation between P and nodule fresh weight (Figure 6.15a, $p < 0.001$, $R = 0.955$). There was a strong positive correlation between P supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule fresh weight (Figure 6.15b, $p = 0.0481$, $R = 0.8151$). This trend was not observed when green bean nutrients were removed from the analysis. The trends observed in Figure 6.15 were due to high P in CRS and green bean nutrients.

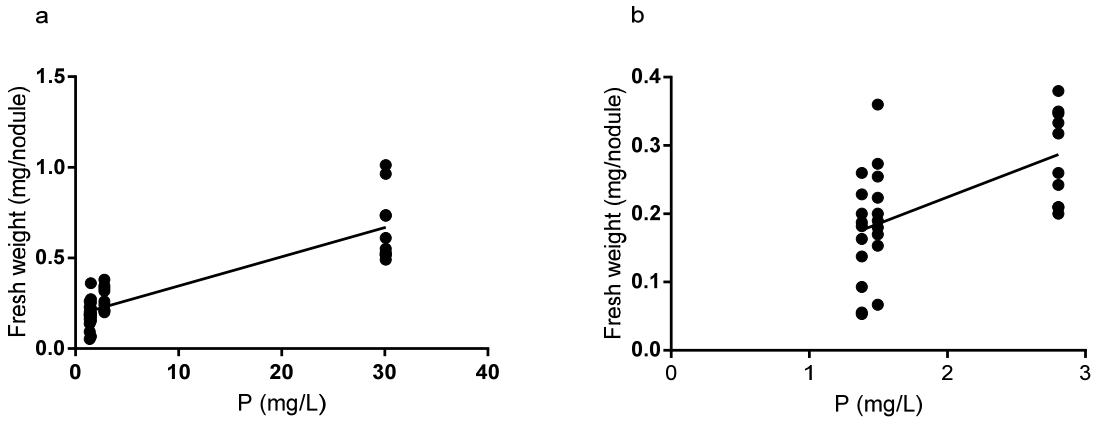

Figure 6.15. Correlation between phosphorus applied to soil and serradella nodule weight. P sources were vermicompost leachate, leachate nutrients and recommended nutrients (CRS). With CRS (a) and without (b).

There was a positive correlation between Ca supplied by treatment solutions and serradella nodulation. When CRS nutrient solution was included, there was a moderate positive correlation between Ca and nodule numbers (Figure 6.16a, $p = 0.0027$, $R = 0.6962$). There was a moderate positive correlation between Ca supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule numbers (Figure 6.16b, $p = 0.0202$, $R = 0.6573$). When CRS nutrient solution was included, there was a strong positive correlation between Ca and nodule weight (Figure 6.16c, $p < 0.001$, $R = 0.9709$). There was a strong positive correlation between Ca supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule weight (Figure 6.16d, $p = 0.0274$, $R = 0.8615$).

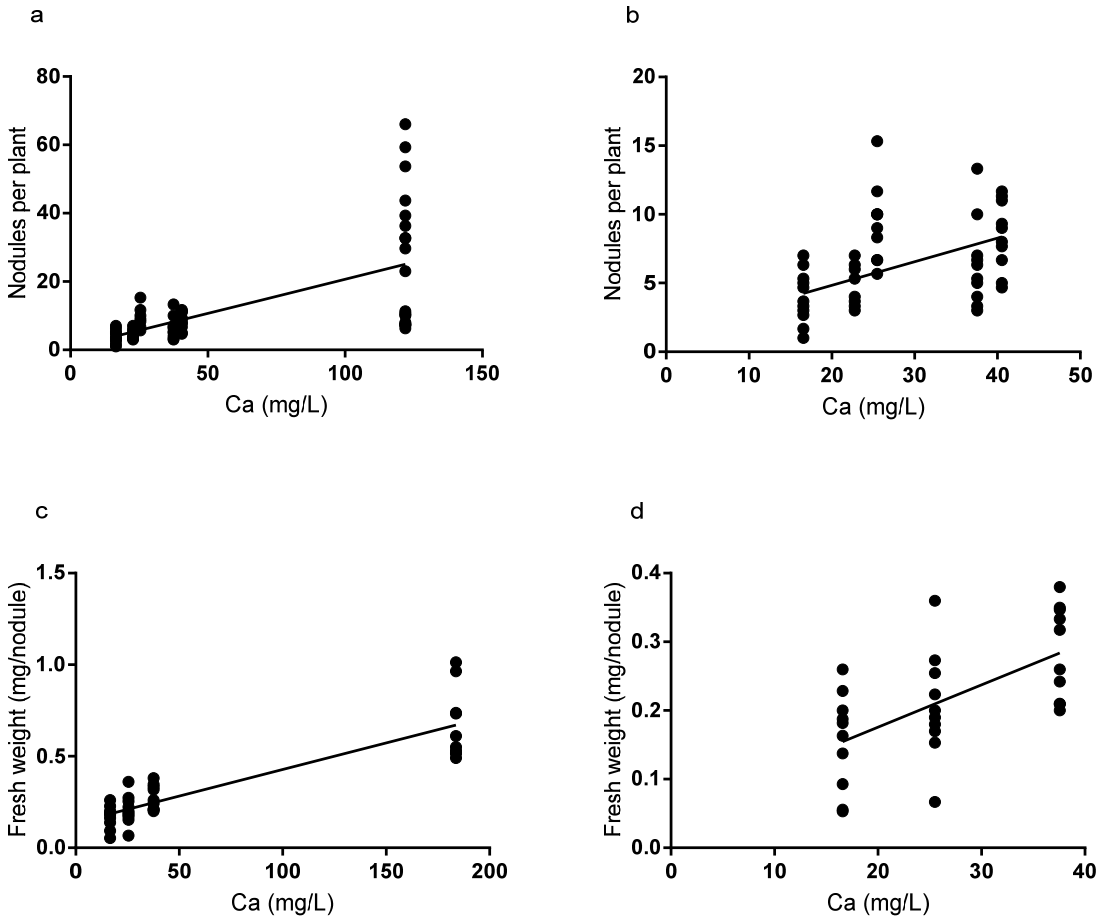

Figure 6.16. Correlation between calcium applied to soil and serradella nodules. Ca sources were vermicompost leachate, leachate nutrients and recommended nutrients (CRS). Nodule numbers with CRS (a) and without (b) and nodule weight with CRS (c) and without (d).

There was a positive correlation between Mg supplied by treatment solutions and serradella nodulation. There was a moderate positive correlation between Mg supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule numbers (Figure 6.17a, $p = 0.0097$, $R = 0.7102$). This trend was not observed when CRS nutrients were included in the analysis. There was a strong positive correlation between Mg supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule weight (Figure 6.17b, $p = 0.00416$, $R = 0.8285$). When CRS nutrients were included, there was a strong positive correlation between Mg and nodule weight (Figure 6.17c, $p < 0.001$, $R = 0.9624$).

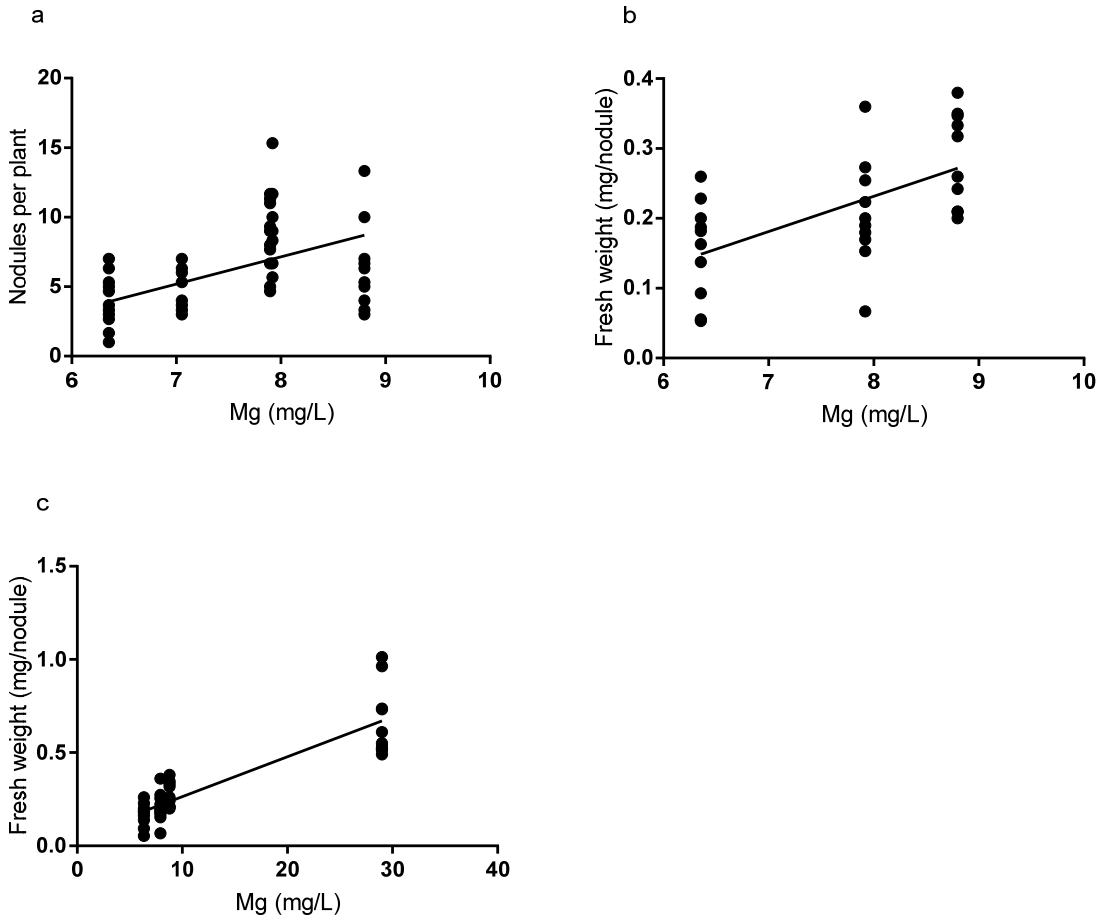

Figure 6.17. Correlation between magnesium applied to soil and serradella nodules. Mg sources were vermicompost leachate, leachate nutrients and recommended nutrients (CRS). Nodule numbers (a) and weight (b) without CRS. Nodule weight with CRS (c).

There was a positive correlation between K supplied by treatment solutions and serradella nodulation. When CRS nutrient solution was included, there was a moderate positive correlation between K and nodule numbers (Figure 6.18a, p = 0.0185, R = 0.5799). There was a moderate positive correlation between K supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule numbers (Figure 6.18b, p = 0.007, R = 0.7304).

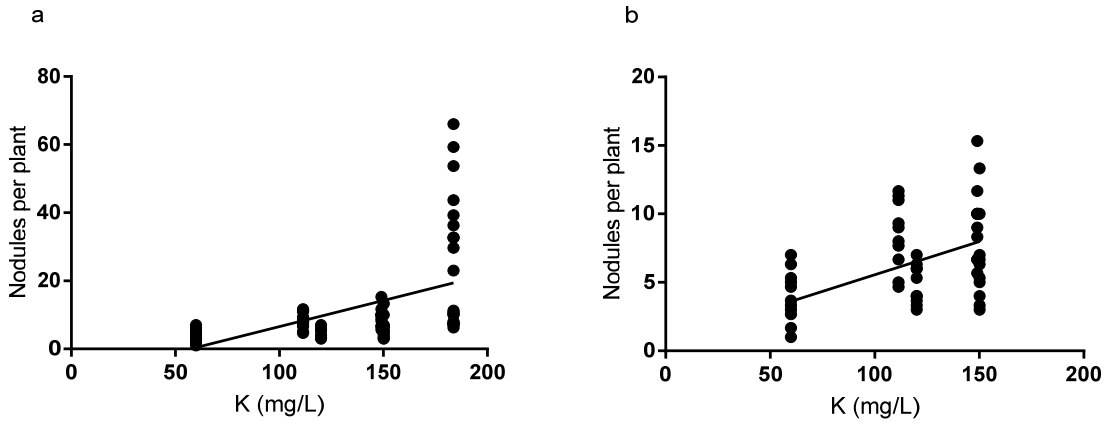

Figure 6.18. Correlation between potassium applied to soil and serradella nodulation. Mg sources were vermicompost leachate, leachate nutrients and recommended nutrients (CRS). Nodule numbers with CRS (a) and without (b).

There was a positive correlation between Zn supplied by treatment solutions and serradella growth. When CRS nutrient solution was included, there was a moderate positive correlation between Zn and plant height (Figure 6.19a, p < 0.001, R = 0.7432). There was a moderate positive correlation between Zn supplied by green bean leachate, B/G/R leachate, green bean nutrients and plant height (Figure 6.19b, p = 0.0293, R = 0.6263). When CRS nutrient solution was included, there was a moderate positive correlation between Zn and shoot dry weight (Figure 6.19c, p = 0.0064, R = 0.6506). There was a moderate positive correlation between Zn supplied by green bean leachate, B/G/R leachate, green bean nutrients and shoot dry weight (Figure 6.19d, p = 0.0162, R = 0.6741). When CRS nutrient solution was included, there was a moderate positive correlation between Zn and shoot dry weight (Figure 6.19e, p = 0.0099, R = 0.6232). There was a moderate positive correlation between Zn supplied by green bean leachate, B/G/R leachate, green bean nutrients and root dry weight (Figure 6.19f, p = 0.0156, R = 0.6769).

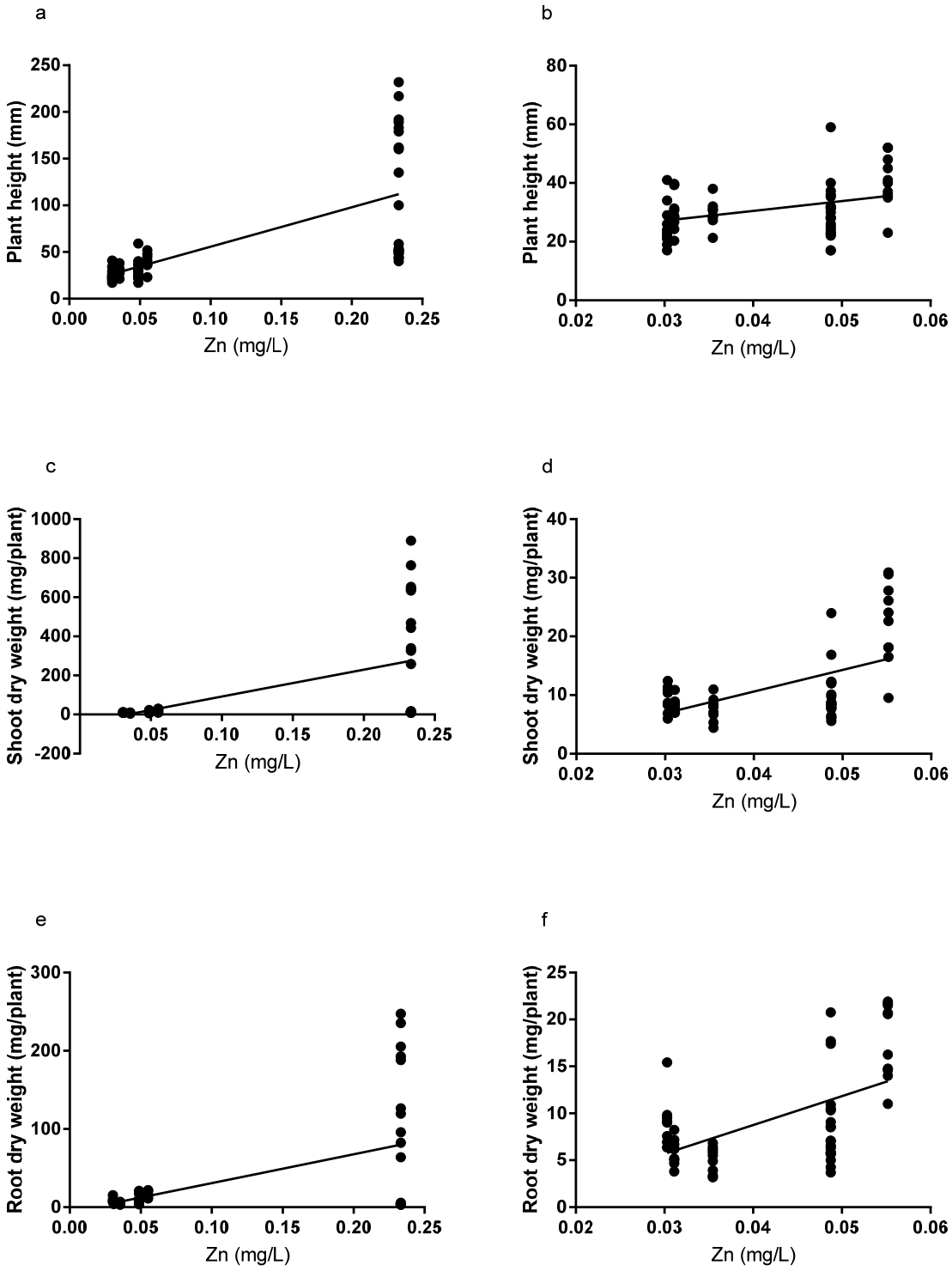

Figure 6.19. Correlation between zinc applied to soil and serradella growth. Zn sources were vermicompost leachate, leachate nutrients and recommended nutrients (CRS). Plant height with CRS (a) and without (b). Shoot dry weight with CRS (c) and without (d). Root dry weight with CRS (e) and without (f).

There was a strong positive correlation between Cu supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule number (Figure 6.20a, p = 0.0021, R = 0.7926). This trend was not observed when CRS nutrients were included in the analysis. This trend was not observed when green bean nutrients were removed from the analysis. The trend in Figure 6.20a is due to low Cu in synthetic green bean leachate. There was a strong negative correlation between Fe supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule number (Figure 6.20b, p = 0.0072, R = -0.7287). This trend was not observed when CRS nutrients were included in the analysis. This trend was not observed when green bean nutrients were removed from the analysis. The trend in Figure 6.20b was due to high Fe in green bean nutrients.

There was a moderate positive correlation between TOC supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule number (Figure 6.20c, p = 0.0066, R = 0.7341). This trend was not observed when CRS nutrients were included in the analysis. This trend was not observed when green bean nutrients were removed from the analysis. The trend in Figure 6.20c is due to low TOC in synthetic green bean leachate. There was a strong negative correlation between TN supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule number (Figure 6.20d, p = 0.0062, R = -0.7374). This trend was not observed when CRS nutrients were included in the analysis. This trend was not observed when green bean nutrients were removed from the analysis. The trend in Figure 6.35d was due to high TN in green bean nutrients.

There was a strong negative correlation between TN supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule number (Figure 6.20d, p = 0.0062, R = -0.7374). This trend was not observed when CRS nutrients were included in the analysis. This trend was not observed when green bean nutrients were removed from the analysis. The trend in Figure 6.20d was due to high TN in synthetic green bean leachate. There was a strong positive correlation between Sr supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule number (Figure 6.20e, p = 0.0041, R = 0.76). This trend was not observed when CRS nutrients were included in the analysis. This trend was not observed when green bean nutrients were removed from the analysis. The trend in Figure 6.20e was due to low Sr in green bean nutrients.

When CRS nutrient solution was included in the analysis, there was a moderate positive correlation between S and nodule number (Figure 6.20f, p = 0.0038, R = 0.6792). There was a moderate negative correlation between S supplied by green bean leachate, B/G/R leachate, green bean nutrients and nodule number (Figure 6.20g, p = 0.0065, R = -0.7345). This trend was not observed when green bean nutrients were removed from the analysis. The trend in Figure 6.20f was due to high S in CRS. The trend in Figure 6.20g was due to low S in green bean nutrients.

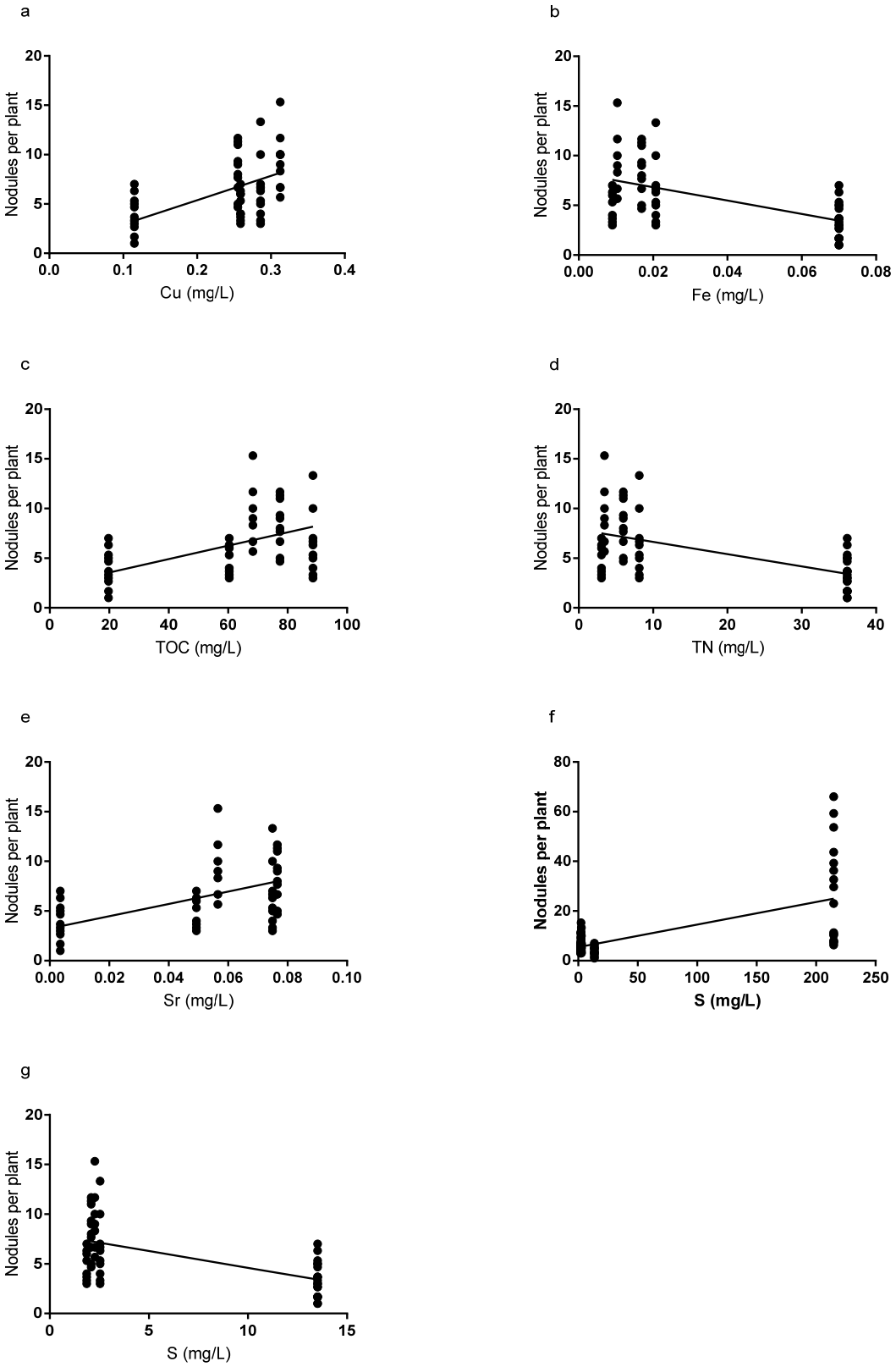

Figure 6.20. Correlation between nutrients applied to soil and serradella nodulation. Nutrient sources were vermicompost leachate, leachate nutrients and recommended nutrients (CRS). Plots without CRS are Cu (a), Fe (b), TOC (c), TN (d), Sr (e) and S (g). Plots with CRS are S (f).

6.3.4 Leachate nutrients over time

Changes in leachate nutrients over time are shown in Appendix D. Leachate nutrients varied with time and worm-bed substrate. In most cases, substrates supplied additional nutrients to those provided by newspaper bedding and tap water which are indicated by horizontal dotted lines on Figures D1.1-D1.13.

Ca levels in green bean leachate increased over the first two weeks before dropping. Levels increased again at weeks 4 and 6. The highest Ca level was reached at week 4, the lowest at week 8. Ca levels remained above background at all time points. Ca levels in B/G/R leachate dropped in the first week, then increased from weeks 2 to 4. The highest Ca level was reached at week 7, the lowest at week 14. Ca levels dropped below background at weeks 1, 8, 14 and 15. Ca levels in banana leachate dropped in the first week, then increased from weeks 2 to 4. The highest Ca level was reached at week 6, the lowest at week 1. Ca levels dropped below background at weeks 1, 8, 10, 11 and 12. Ca levels in rockmelon leachate dropped in the first week, then increased from weeks 2 to 5. The highest Ca level was reached at week 7, the lowest at week 1. Ca levels remained above background from weeks 2 to 15. The highest Ca was obtained from B/G/R leachate at week 7 (Figure D1.1).

Cu levels in green bean leachate increased over the first two weeks before dropping. Levels increased again at weeks 4 to 6. The highest Cu level was reached at week 8, the lowest at week 3. Cu levels remained above background at all time points. Cu levels in B/G/R leachate increased in the first week before dropping. Levels increased again at weeks 3, 4, 6 and 8. The highest Cu level was reached at week 8, the lowest at week 7. Cu levels remained above background at all time points. Cu levels in banana leachate increased in the first week before dropping. Levels increased again at weeks 5, 8 and 14. The highest Cu level was reached at week 8, the lowest at week 3. Cu levels dropped below background at weeks 2 to 4. Cu levels in rockmelon leachate increased in the first week before dropping. Levels increased again at weeks 7, 8, 10, 11, 13 and 15. The highest Cu level was reached at week 15, the lowest at week 4. Cu levels dropped below background at weeks 3 to 5. The highest Cu was obtained from B/G/R leachate at week 8 (Figure D1.2).

Fe levels in green bean leachate dropped in the first week then increased in weeks 2 and 4 to 6. The highest Fe level was reached at week 6, the lowest at week 15. Fe levels remained above background from weeks 1 to 14. Fe levels in B/G/R leachate dropped in the first week then increased in weeks 2, 3 and 7. The highest Fe level was reached at week 7, the lowest at week 11. Fe levels dropped below background at weeks 1 and 9 to 15. Fe levels in banana leachate increased in the first

two weeks before dropping. The highest Fe level was reached at week 2, the lowest at week 13. Fe levels dropped below background at weeks 8, 10 and 12 to 15. Fe levels in rockmelon leachate increased in the first two weeks before dropping. Levels increased again at weeks 5 and 7. The highest Fe level was reached at week 7, the lowest at week 15. Fe levels remained above background at all time points. The highest Fe was obtained from banana leachate at week 2 (Figure D1.3).

K levels in green bean leachate increased over the first six weeks, then dropped over the remaining weeks. The highest K level was reached at week 6, the lowest at week 1. K levels remained above background at all time points. K levels in B/G/R leachate increased over the first six weeks before dropping. Levels increased again at week 9 then dropped over the remaining weeks. The highest K level was reached at week 6, the lowest at week 1. K levels remained above background at all time points. K levels in banana leachate increased in the first three weeks before dropping. Levels increased again at weeks 5, 6, 9, 11 and 14. The highest K level was reached at week 14, the lowest at week 1. K levels remained above background at all time points. K levels in rockmelon leachate increased in the first six weeks. Levels dropped at weeks 8 and 9 and increased again at weeks 10, 12 and 14. The highest K level was reached at week 14, the lowest at week 1. K levels remained above background at all time points. The highest K was obtained from green bean leachate at week 6 (Figure D1.4).

Mg levels in green bean leachate increased over the first six weeks before dropping. The highest Mg level was reached at week 6, the lowest at week 1. Mg levels remained above background at all time points. Mg levels in B/G/R leachate dropped in the first week then increased over weeks 2 to 4. Levels dropped at week 5 then increased at week 7. The highest Mg level was reached at week 7, the lowest at week 1. Mg levels remained above background from weeks 2 to 15. Mg levels in banana leachate increased in the first six weeks before dropping. Levels increased again at weeks 12 and 13. The highest Mg level was reached at week 14, the lowest at week 12. Mg levels dropped below background at weeks 8 and 13. Mg levels in rockmelon leachate dropped in the first week before increasing in weeks 2 to 6. Levels dropped at weeks 7 to 9 then increased at weeks 10, 12 and 14. The highest Mg level was reached at week 6, the lowest at week 1. Mg levels remained above background from weeks 2 to 15. The highest Mg was obtained from green bean leachate at week 6 (Figure D1.5).

Mn levels in green bean leachate dropped and remained below background at all time points. Mn levels in B/G/R leachate dropped and remained below background at all time points except weeks 6 and 7. The highest Mn level was reached at week 7. Mn levels in banana leachate dropped and

remained below background at all time points except week 2. Mn levels in rockmelon leachate increased at weeks 3, 4, 5 and 6 before dropping. The highest Mn level was reached at week 7, the lowest at week 9. Mn levels dropped below background at weeks 1, 2 and 9 to 15 The highest Mn was obtained from rockmelon leachate at week 7 (Figure D1.6).

Na levels in green bean leachate increased over the first two weeks before dropping. Levels increased again at weeks 4 and 6. The highest Na level was reached at week 2, the lowest at week 13. Na levels dropped below background at weeks 7 to 15. Na levels in B/G/R leachate dropped in the first week then increased at weeks 2, 4 and 7. The highest Na level was reached at week 2, the lowest at week 15. Na levels dropped below background at weeks 8 to 15. Na levels in banana leachate dropped in the first week before increasing at weeks 2, 4, 5 and 11. The highest Na level was reached at week 2, the lowest at week 12. Na levels dropped below background at weeks 8 to 10 and 12 to 15. Na levels in rockmelon leachate increased in the first two weeks before dropping. Levels increased again at weeks 4, 5, 8, 10, 12 and 14. The highest Na level was reached at week 2, the lowest at week 15. Na levels remained above background for all time points. The highest Na was obtained from banana leachate at week 2 (Figure D1.7).

P levels in green bean leachate increased over the first six weeks before dropping. The highest P level was reached at week 6, the lowest at week 15. P levels remained above background for all time points. P levels in B/G/R leachate increased in week 2 before dropping. Levels increased again in week 7 before dropping. The highest P level was reached at week 7, the lowest at week 15. P levels remained above background for all time points. P levels in banana leachate increased in the first week before dropping. Levels increased again in weeks 3, 4, 9, 11, 13 and 14. The highest P level was reached at week 4, the lowest at week 12. P levels dropped below background at weeks 8, 10, 12 and 15. P levels in rockmelon leachate increased in the first four weeks before dropping. Levels increased again at weeks 6, 8, 10, 12 and 14. The highest P level was reached at week 4, the lowest at week 1. P levels remained above background for all time points. The highest P was obtained from rockmelon leachate at week 4 (Figure D1.8).

S levels in green bean leachate increased over the first two weeks before dropping. Levels increased again at weeks 4 and 6 before dropping. The highest S level was reached at week 2, the lowest at week 12. S levels dropped below background at weeks 8 to 15. S levels in B/G/R leachate dropped in the first week before increasing. Levels then dropped and increased again at week 6. The highest S level was reached at week 2, the lowest at week 13. S levels dropped below background at week 1 and 8 to 15. S levels in banana leachate dropped in the first week before increasing. Levels increased again in weeks 5 and 11. The highest S level was reached at week 2, the lowest at week 12. S levels

dropped below background at weeks 3, 6 to 10 and 12 to 15. S levels in rockmelon leachate increased in the first two weeks before dropping. Levels increased again at weeks 5 and 7. The highest S level was reached at week 2, the lowest at week 12. S levels dropped below background at weeks 11 to 13. The highest S was obtained from rockmelon leachate at week 2 (Figure D1.9).

Sr levels in green bean leachate increased over the first two weeks before dropping. Levels increased again at weeks 4, 6 and 13. The highest Sr level was reached at week 6, the lowest at week 15. Sr levels remained above background for all time points. Sr levels in B/G/R leachate dropped in the first week before increasing over weeks 2 to 5. Levels then dropped and increased again at week 7. The highest Sr level was reached at week 7, the lowest at week 14. Sr levels dropped below background at week 1 and 11 to 15. Sr levels in banana leachate dropped in the first week before increasing over weeks 2 to 5. Levels dropped again then increased over weeks 13 to 15. The highest Sr level was reached at week 4, the lowest at week 12. Sr levels dropped below background at weeks 1 and 8 to 12. Sr levels in rockmelon leachate dropped in the first week before increasing over weeks 2 to 5. Levels then dropped and increased again at weeks 7, 10, 12 and 14. The highest Sr level was reached at week 7, the lowest at week 1. Sr levels remained above background at weeks 2 to 15. The highest Sr was obtained from B/G/R leachate at week 7 (Figure D1.10).

Zn levels in green bean leachate increased over the first week before dropping. Levels increased again over weeks 4 to 6 before dropping. Levels increased again at weeks 14 and 15. The highest Zn level was reached at week 15, the lowest at week 3. Zn levels remained above background for all time points. Zn levels in B/G/R leachate increased over the first week before dropping. Levels increased again at weeks 4 and 7. The highest Zn level was reached at week 7, the lowest at week 11. Zn levels remained above background for all time points. Zn levels in banana leachate increased over the first two weeks before dropping. Levels increased again at weeks 5, 6, 9 and 14. The highest Zn level was reached at week 6, the lowest at week 3. Zn levels remained above background for all time points. Zn levels in rockmelon leachate increased over the first two weeks before dropping. Levels increased again at weeks 5, 7, 13 and 15. The highest Zn level was reached at week 7, the lowest at week 4. Zn levels remained above background for all time points. The highest Zn was obtained from rockmelon leachate at week 7 (Figure D1.11).

TOC levels in green bean leachate increased over the first two weeks before dropping. Levels increased again over weeks 4 to 6 before dropping. The highest TOC level was reached at week 6, the lowest at week 15. TOC levels dropped below background at weeks 14 and 15. TOC levels in B/G/R leachate increased in week 2 before dropping at week 5 then increasing again at weeks 6 and 7. The highest TOC level was reached at week 7, the lowest at week 15. TOC levels dropped below

background at weeks 1, 8, 10 to 12, 14 and 15. TOC levels in banana leachate increased in week 2 before dropping in weeks 6 and 7. Levels increased again at week 14. The highest TOC level was reached at week 2, the lowest at week 8. TOC levels dropped below background at weeks 1, 8, 12 and 13. TOC levels in rockmelon leachate increased in week 2 before dropping. Levels increased again at weeks 4 and 7. The highest TOC level was reached at week 7, the lowest at week 1. TOC levels remained above background between weeks 2 to 15. The highest TOC was obtained from rockmelon leachate at week 7 (Figure D1.12).

TN levels in green bean leachate increased over the first seven weeks before dropping. The highest TN level was reached at week 7, the lowest at week 15. TN levels remained above background for all time points. TN levels in B/G/R leachate increased in weeks 2 to 4. The highest TN level was reached at week 4, the lowest at week 1. TN levels remained above background for all time points. TN levels in banana leachate increased in the first week before dropping at week 8. Levels increased again at week 11 and 14. The highest TN level was reached at week 5, the lowest at week 15. TN levels remained above background for all time points. TN levels in rockmelon leachate increased in the first four weeks before dropping. Levels increased again at weeks 7, 8 and 14. The highest TN level was reached at week 8, the lowest at week 1. TN levels remained above background for all time points. The highest TN was obtained from green bean leachate at week 6 (Figure D1.13).

Many samples exhibited a nutrient spike at six or seven weeks. Ca in B/G/R leachate increased at seven weeks. Ca in green bean leachate increased at six weeks. Cu in B/G/R and green bean leachate increased at seven weeks. Fe in B/G/R and rockmelon leachate increased at seven weeks. K in green bean leachate increased at six weeks. Mg in B/G/R leachate increased at seven weeks. Mg in green bean leachate increased at six weeks. Mn in B/G/R and rockmelon leachate increased at seven weeks. P in B/G/R leachate increased at six weeks. P in green bean leachate increased at seven weeks. S in B/G/R leachate increased at seven weeks. S in green bean leachate increased at six weeks. Sr in B/G/R leachate increased at seven weeks. Sr in green bean leachate increased at six weeks. Zn in green bean leachate increased at six weeks. Zn in rockmelon leachate increased at seven weeks. TOC in rockmelon leachate increased at seven weeks (Figure 6.13). TN in green bean leachate increased at six weeks.

Green bean provided the most consistent nutrient supply, with eight nutrients above background over 15 weeks. Rockmelon supplied six nutrients and B/G/R supplied five. Banana provided the least consistent supply, with only three nutrients above background for 15 weeks. The nutrients supplied by all four substrates for the entire time course were TN, K and Zn. The harvest time needed for maximum nutrients varied. Of the thirteen measured nutrients, six peaked at week 7, three peaked

at week 2, two peaked at week 6, one peaked at week 4 and one peaked at week 8. Ba and Pb were below the limits of detection for all samples.

Mean nutrient content of undiluted leachates from the first seven weeks are given in Table 6.4. All leachates contained an equal amount of TOC, Ca, Fe, K, Mg, Sr and Zn. Rockmelon leachate contained more Mn, Na, P and S than banana, green bean and B/G/R leachates. Banana, green bean and B/G/R leachates contained the same amount of Mn, Na, P and S. Green bean leachate contained more Cu than banana, rockmelon and B/G/R leachates. Banana, rockmelon and B/G/R leachates contained the same amount of Cu. Green bean leachate contained more TN than banana and B/G/R leachates. Green bean leachate and rockmelon leachate contained the same amount of TN. Rockmelon, banana and B/G/R leachates contained the same amount of TN.

Some banana and rockmelon nutrients were reduced after eight weeks. Mean nutrients of all banana and rockmelon leachates are given in Table 6.5. Rockmelon contained more TOC, TN, Ca, K, Mg, Na, P and S than banana leachate. Banana and rockmelon leachates contained an equal amount of Cu, Fe, Mn, Sr and Zn.

Table 6.4. Nutrient content of vermicompost leachate from different substrates.

	TOC	TN	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Sr	Zn	Ba	Pb
Banana	115 ± 11a	5 ± 0.5b	43.5 ± 4.8a	0.08 ± 0.02b	0.04 ± 0.017a	129 ± 28a	7 ± 0.7a	0.02 ± 0.007b	49 ± 4b	1 ± 0.2b	4 ± 0.4b	0.09 ± 0.009a	0.04 ± 0.009a	<LOD	<LOD
Green bean	232 ± 33a	29 ± 9.5a	51.8 ± 6.5a	0.20 ± 0.03a	0.03 ± 0.005a	247 ± 80a	13 ± 3.7a	0.02 ± 0.002b	65 ± 9b	5 ± 1.0b	7 ± 0.9b	0.10 ± 0.012a	0.06 ± 0.011a	<LOD	<LOD
Rockmelon	344 ± 149a	19 ± 3.5ab	48.6 ± 9.2a	0.08 ± 0.02b	0.05 ± 0.010a	252 ± 54a	17 ± 4.1a	0.07 ± 0.024a	94 ± 12a	13 ± 2.9a	14 ± 3a	0.09 ± 0.015a	0.10 ± 0.051a	<LOD	<LOD
B/G/R	215 ± 35a	10 ± 1.5b	60.0 ± 10.3a	0.11 ± 0.02b	0.02 ± 0.005a	216 ± 41a	14 ± 2.9a	0.03 ± 0.007b	45 ± 6b	4 ± 0.7b	5 ± 0.8b	0.12 ± 0.019a	0.05 ± 0.006a	<LOD	<LOD

B/G/R – equal mix of banana, green bean and rockmelon. Values shown are the mean ± SEM of seven replicates. One-way ANOVA performed, with different letters indicating significant differences ($p < 0.05$).

Table 6.5. Nutrient content of rockmelon and banana vermicompost leachate.

	TOC	TN	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Sr	Zn
Banana	93.8 ± 8.8b	4.2 ± 0.5b	36 ± 4b	0.14 ± 0.02a	0.025 ± 0.009a	131 ± 17b	6.7 ± 0.6b	0.013 ± 0.004a	36.0 ± 3.8b	1.1 ± 0.2b	2.8 ± 0.3b	0.076 ± 0.008a	0.040 ± 0.005a
Rockmelon	269.5 ± 70.4a	15.2 ± 2.5a	51 ± 5a	0.14 ± 0.02a	0.036 ± 0.005a	273 ± 29a	17.1 ± 2.1a	0.039 ± 0.014a	74.8 ± 7.7a	10.7 ± 1.6a	8.4 ± 1.9a	0.088 ± 0.007a	0.093 ± 0.027a

Values shown are the mean ± SEM of fifteen replicates. One-way ANOVA performed, with different letters indicating significant differences ($p < 0.05$). Ba and Pb were below limit of detection.

6.4 Discussion

6.4.1 Comparison of leachates and chemical nutrients on growth of serradella

6.4.1.1 Recommended nutrients

Chemical fertiliser in the form of CRS generally outperformed leachate. CRS produced taller plants with greater shoot dry weight. This is due to higher nutrient content. For example, CRS supplied more Fe than leachates. Fe affects crop yield and human nutrition in terms of edible Fe. Chemical form is important, as some compounds may be insoluble (Morrissey & Guerinot, 2009). A disadvantage of leachate is unknown nutrient forms.

An advantage of leachate is the supply of TOC and, although they were not measured in this study, humic and fluvic acids (Garcia-Gomez et al., 2008; Gutiérrez-Miceli et al., 2017) which may enhance plant growth (Chen & Aviad, 1990). CRS contained measurable TOC in the form of EDTA. EDTA doesn't degrade in natural environments (Oviedo & Rodríguez, 2003). CRS contained no available C, which is needed for soil microbes (Grayston, Vaughan, & Jones, 1996). Although CRS had more positive short-term effects, in the long-term leachate is preferable. No long-term studies of leachate exist. Residual benefits of vermicompost were reported in increased second year grain yield (Rathod et al., 2013).

Effects varied with plant harvest time. Variation with harvest time has been reported in other studies (Gutiérrez-Miceli et al., 2008; Singh et al., 2010). Leachates increased root dry weight at five weeks. This effect is likely related to nutrients. Leachates supplied significantly less Mg and P than CRS. Previous studies have associated low Mg and P with reduced root dry weight of legumes. Reducing Mg increased root dry weight of common bean and cowpea (Fageria & Moreira, 2011). Reducing P increased root dry weight of Velvet bean and Pigeon pea (Fageria, He, & Baligar, 2017).

6.4.1.2 Synthetic green bean leachate

Chemical fertiliser in the form of green bean nutrients had no effect on plant height or shoot dry weight compared to B/G/R leachate. Similarly, Ayyobi, Peyvast, & Olfati (2013) reported no difference between cow manure leachate and chemical fertiliser on peppermint height or dry weight. Worm-bed substrate variation was found. Green bean leachate increased plant height and dry weight over green bean nutrients, while B/G/R leachate had no effect. Substrate variation on plant growth has been reported using vermicompost (Nadi et al., 2011) and liquid extracts (Contreras-Ramos et al., 2005; Grantina-Ievina et al., 2013).

Green bean nutrients represent a diluted form of nutrients. As expected, diluted nutrients performed poorly compared to recommended nutrients. The exception was root dry weight at five weeks. Green bean nutrients showed a significant improvement over CRS. Green bean nutrients supplied significantly less Mg and P than CRS, further evidence that root dry weight at 5 weeks is affected by these nutrients.

Effect of green bean leachate may also be due to nutrients. Green bean leachate supplied more Zn than green bean nutrients, although the difference was not significant. A positive correlation was observed between applied Zn and plant height and dry weight. Increasing Zn has been shown to increase plant height and dry weight of cowpea (Upadhyay & Singh, 2016). Zn levels have an important consideration for human health, as increasing shoot Zn leads to an increase in legume grain Zn (Xue et al., 2016).

6.4.2 Effect of combining worm-bed substrates

Combining worm-bed substrates did not increase leachate nutrients. Green bean and rockmelon leachates contained higher nutrients than B/G/R leachate. B/G/R leachate contained higher nutrients than banana leachate, though these differences weren't significant. Banana did not produce K rich leachate and likely reduced available nutrients in B/G/R leachate. Reducing proportions of worm-bed substrate banana is recommended.

Combining worm-bed substrates increased early nodulation but decreased later nodulation. The change in nodulation at the second harvest is likely due to build-up of nutrients. Green bean leachate supplied more nutrients than B/G/R leachate. The accumulation of these nutrients increased nodulation in the second harvest.

Combining worm-bed substrates reduced height and dry weight of shoots compared to green bean leachate. In contrast, in a trial by Singh et al. (2010), leachates from combined worm-bed substrates outperformed leachates from single substrates. This trial compared three leachates; cow manure leachate, green waste leachate, and leachate from a substrate mixture of cow manure and green waste. Leachate from a substrate mixture of cow manure and green waste increased strawberry shoot dry weight compared with single substrate leachates. The differences observed between this study and the study by Singh et al. (2010) may be due to the latter study using foliar application of leachate.

6.4.3 Leachate effect on nodulation

The first harvest assessed early nodulation. Early nodulation is critical for symbiosis and N_2 fixation, particularly in legumes with short growth cycles (Chibeba et al., 2015). Nodulation initiation occurs within hours of *Rhizobium*-legume interaction (Lohar et al., 2006). Combining worm-bed substrates increased nodulation over single substrate leachate. This was not a nutrient effect, as green bean leachate supplied more nutrients. B/G/R leachate increased nodulation over chemical fertilisers and is therefore an effective substitute for recommended nutrients. All treatments supplied N, which has been shown to reduce nodulation (Laws & Graves, 2005). B/G/R leachate supplied the lowest N, which may contribute to increased nodulation. Reduced N in B/G/R leachate is likely due to the reduced amount of worm-bed substrate green bean. B/G/R leachate is therefore recommended as a treatment for legumes.

Nodulation effect was different at the second harvest. Combining worm-bed substrates reduced nodulation compared to single substrate leachate. Reduced nodulation is likely due to the dilution of B/G/R leachate microbes. Both green bean and B/G/R leachate increased nodulation over synthetic green bean nutrients, which indicates that leachate microbes contributed to increased nodulation. There is no evidence of N suppression of nodulation at ten weeks as CRS both supplied more N and produced more nodules than leachates.

To determine whether the treatment effect was due to nutrient content, correlation between nutrients and plant growth parameters was carried out. CRS produced significantly more nodules per plant at ten weeks, but not at five weeks. This increase in nodulation is likely due to nutrient accumulation over the ten weeks. There was a positive correlation observed between applied Mg and P concentration, and nodule number and weight. The CRS treatment supplied significantly more P and Mg than the other treatment solutions. In addition, CRS supplied more K and significantly more Ca, and Zn. Although soil nutrients were not tested, it is likely the soil treated with CRS retained had more of these nutrients. There is evidence that high quantities of these nutrients effect nodulation. Ca accumulates at Rhizobial infection sites and plays a role in nodule formation (Prell & Poole, 2006; Sprent, 1989). K and P increased soybean nodule numbers (Jones, Lutz, & Smith, 1977) and P increased nodule weight (Graham & Rosas, 1979; Ribet & Drevon, 1995). Mg and Zn increased nodule numbers of pea (Kiss, Stefanovits-Bányai, & Takács-Hájos, 2004) and chickpea (Yadav & Shukla, 1983).

There was a positive correlation between nodule numbers and applied Cu, but Cu was supplied in lower quantities in the CRS treatment. Tindwa, Semu, & Msumali (2014) found that increased Cu

inhibited nodulation, N fixation and rhizobia numbers in soil. The low Cu supplied by CRS may have removed this inhibition. The relationship between Cu and nodulation for the leachate treatments was likely affected by the presence of microbes in the treatments. There was a positive correlation between TOC and nodule numbers. Unsurprisingly, the leachates supplied the largest amount of TOC. TOC will benefit the rhizobia (Libault, 2014) and it is likely that the relationship between TOC and nodulation for the leachate treatments was also affected by the presence of microbes in the treatments.

6.4.4 Plant-microbe interactions

Green bean leachate microbes were harvested. Microbe application had no effect on plant height or dry weight. Microbe application increased nodule number and weight, though most differences weren't significant. Microbes combined with recommended nutrients increased nodule number and significantly increased nodule fresh weight. Microbes are needed for optimum nodulation and N_2 fixation.

Increased early nodulation indicates interaction between serradella and B/G/R microbes. These microbes weren't identified but likely contain some microorganisms described in Chapter 3. Banana and rockmelon leachates contain *Azospirillum* sp. *Azospirillum* is a well-studied co-inoculant (Abou-Aly, 2001; Askary et al., 2009; Hungria, Nogueira, & Araujo, 2013; Vicario et al., 2015). Co-inoculation of *Bradyrhizobium* and *A. brasilense* increased early soybean nodulation (Cerezini et al., 2016) and reduced time to nodulation (Chibeba et al., 2015). Co-inoculation of rhizobia and *Azospirillum* increased nodule numbers of bean (Peres et al., 2016) and soybean (Aung et al., 2013). *Azospirillum* enhances root growth and root hairs, creating more potential rhizobia infection sites (Chibeba et al., 2015). *Azospirillum* may be among the green bean microbes that increased nodule weight. Chibeba et al. (2015) observed *B. japonicum* and *A. brasilense* increased nodule dry weight of soybean. Unlike the current study, Chibeba et al. (2015) observed a corresponding plant biomass increase. Increased biomass was also found in other studies (Aung et al., 2013).

Many microorganisms with disease control or plant growth promoting properties have been isolated and many are available commercially. In 2004, the global biocontrol market was $588 million. A major drawback of microbial inoculants is plant species specificity (Berg, 2009). Another drawback is difficulty with rhizosphere colonisation. Endophytes such as *Azospirillum* are of interest in overcoming this difficulty (Franco et al., 2007). Endophytes belonging to *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* can enter and colonise plants. Some actinobacterial endophytes are potentially beneficial, with the ability to fix N_2 and supress plant disease (Swarnalakshmi,

Senthilkumar, & Ramakrishnan, 2016). Leachates contain members of these phyla (Chapter 3) and many potential co-inoculants. Multiple microbes in leachate will be beneficial to a range of plant species.

There is increasing evidence of the benefits of co-inoculation with endophytes. Co-inoculation of rhizobia with *Bacillus megaterium* increased shoot dry weight, nodule weight and fixed N_2 in *P. vulgaris* (Korir et al., 2017). *B. japonicum* and *Bacillus amyloliquefaciens* increased soybean nodule number (Masciarelli, Llanes, & Luna, 2014). Co-inoculation with *Streptomyces* spp. increased soybean yield (Le, Ballard, & Franco, 2017). In the paddock, rhizobial inoculation may not be necessary. *Bacillus simplex* increased nodule weight and fixed N_2 in field pea in soil with indigenous rhizobia (Barnett et al., 2017). The results of Korir et al. (2017) were strain specific, with several combinations of endophyte and rhizobia showing no improvement. Using leachate, with its suite of microbes, is preferable to single strain co-inoculation.

The mechanisms of endophyte action are not understood. Increasing this difficulty, plant growth promotion can be difficult to distinguish from disease suppression (Berg, 2009). Co-inoculants have been shown to increase *in vitro* rhizobial growth (Le et al., 2016), bacteroid numbers and lifespan (Tokala et al., 2002). Root-colonising *Streptomyces lydicus* increased nodule numbers by forming clusters of nodules from the same node (Tokala et al., 2002).

Co-inoculation with leachate is largely untested. *Glomus etunicatum* is a plant beneficial mycorrhizal fungus. *Pseudomonas fluorescens* can suppress soil-borne pathogens. These microbes had no effect on tomato in combination with cow manure leachate (Oliva-Llaven et al., 2010). This study didn't include microbe-only controls. It is possible that the microbes were equally ineffective without leachate. Green waste leachate increased ghost pepper dry weight and shoot nutrients in plants colonised by arbuscular mycorrhizal fungi. Improvements varied with fungus species (Khan et al., 2014).

Microbial inoculants have other drawbacks. They are subject to technical issues including upscaling production, inadequate or variable shelf-life. Plant-associated microbes can become opportunistic human pathogens (Berg, 2009). They may be subject to legislation depending on the country. The Australian government may require registration. Requirements depend on many factors, including potential pathogenicity and potential to disrupt existing organisms (Australian Pesticides and Veterinary Medicines Authority, 2014). The answers to many requirements are impossible to know with any certainty. Using leachate overcomes many of these drawbacks.

It is of interest that the most effective leachates contain legume worm-bed substrates. In addition to microbes these legume substrates likely secreted other chemicals. Legumes produce a wide range of secondary metabolites, some of which serve to attract rhizobia (Wink, 2013). Flavonoids induce expression of rhizobial *nod* genes which are necessary for symbiosis. Many legumes produce flavonoids for specific rhizobia (Cooper, 2007; Liu & Murray, 2016). Nine flavonoids from black bean (*P. vulgaris*) seeds and roots induced *nod* gene expression in *R. leguminosarum* (Hungria, Johnston, & Phillips, 1992). Further study is needed to determine whether similar compounds were secreted from green beans into leachate which may have contributed to increased nodulation.

Two additional legume substrates were tested in Chapter 1. These were garden pea and lucerne. Garden pea was a lethal worm-bed substrate, and is therefore inappropriate for further study. Lucerne supported earthworms, although it was a fairly moderately performing substrate. However, lucerne, as a common crop fed to livestock (Agriculture Victoria, 2017) is not a useful worm-bed substrate in terms of waste recycling efforts.

There is evidence of interaction between legume secondary metabolites and microbes. Addition of plant growth promoting bacteria *Chryseobacterium balustinum* changed the flavonoids in bean root exudate (Dardanelli et al., 2012). No other published studies were found using legume worm-bed substrates. Further investigation is needed to determine whether there is a correlation between legume substrate and legume symbiosis.

6.4.5 Leachate nutrient content

The leachates in this trial are suitable for soil amendment, as they have an EC below the maximum recommended value of 4000 µS cm^{-1} for soil additives and plant tolerance (Gupta and Garg 2009). Leachates are a source of macro and micronutrients. Leachate nutrient content is highly variable, both with worm-bed substrate and time. The variation in nutrient content over time is likely due to the changing nature of the vermicompost system, with uptake of nutrients by earthworms and microbes, removal of nutrients in leachate harvest, and addition of nutrients in the form of new worm-bed substrate. Many leachates experienced a nutrient spike at six or seven weeks. A seven week spike in green bean or B/G/R leachate may be due to dilutions for soil application commencing at eight weeks. Dilutions do not explain six week spikes in these leachates. Spikes also occurred in undiluted rockmelon leachate. The best leachate harvest time is six or seven weeks after first substrate addition.

The variation in leachate nutrients will effect their ability to support plants. This will also be effected by the nutrient requirements of plant species. For example, supplementation with chemical fertiliser may be required to obtain adequate N, P, K for sugarcane (Gutiérrez-Miceli et al., 2017), maize (Garcia-Gomez et al., 2008) and sorghum (Gutiérrez-Miceli et al., 2008).

Rockmelon supplied more P on average than other leachates. P is essential for plants and vital for food production. P is largely obtained from mined rock phosphate, a non-renewable resource facing depletion (Cordell, Drangert, & White, 2009). Rockmelon supplied more S than other leachates. S is essential for protein and chlorophyll synthesis. Most water sources and chemical fertilisers don't supply adequate S (McCauley, Jones, & Jacobsen, 2011; Pro-Mix, 2016). Maximum P and S are desirable and rockmelon leachate the best option.

Rockmelon supplied significantly more Mn than other leachates. Banana, green bean and B/G/R leachates largely supplied no additional Mn compared to tap water. There are advantages and disadvantages to both outcomes. Mn is required for photosynthesis but is toxic in excess (Millaleo et al., 2010). Required Mn likely varies with plant species. It is difficult to choose an ideal leachate for Mn supply.

Differences between leachates varied with harvest time. Late harvest banana leachate supplied low Ca, Fe, Na, S and Sr. Because of this, greater variation was observed between banana and rockmelon leachate than between all four leachates. Ca is an essential nutrient, involved in most aspects of plant development (Hepler, 2005). Fe is another essential nutrient. Plant available Fe in the rhizosphere is very low (Morrissey & Guerinot, 2009) making leachate an important source of Fe. Sr is found in most soils and plants (Isermann, 1981) but may not be essential. Banana is not recommended as a worm-bed substrate due to unreliable supply of essential micronutrients.

Rockmelon supplied significantly more Na than other leachates. The form of Na is unknown. Excess Na in the form of NaCl can restrict plant growth (Tank & Saraf, 2010). An advantage of chemical fertilisers is known nutrient forms. Unknown nutrient forms increases the difficulty of replacing chemicals with organics. It should be noted that diluted green bean and B/G/R leachates supplied less Na than tap water. Excess Na was therefore not applied to serradella. Soil applied commercial leachate improves growth of salt stressed tomato (Chinsamy et al., 2013) and the leachates in the current study may have a similar effect. NaCl supplied by Chinsamy et al. (2013) to induce stress was much higher than Na supplied by undiluted rockmelon leachate. It is therefore unlikely that undiluted rockmelon leachate will stress plants. Salt tolerance also varies with plant species (Zahran, 1999).

Green bean leachate contained more TN than other leachates, although the difference between green bean and rockmelon was not significant. This is probably due to higher protein in green beans as reported by United States Department of Agriculture (2017). N is the nutrient plants require the most of, except in the case of nodulated legumes (Crawford, 1995; Laws & Graves, 2005). There is no evidence that leachate N negatively effects nodulation (Chapter 5). Rockmelon leachate contained the highest nutrient content. The seven week harvest contained peak TOC, Ca, Fe, Mn, Na, Sr and Zn. Recommended conditions for maximum nutrients are weekly additions of rockmelon for seven weeks.

6.5 Conclusion

Vermicompost is an ideal method for reducing banana, green bean and rockmelon waste in landfill. Selected organic substrates may provide different benefits for plant growth. For example, banana could be mixed with other substrates to increase leachate nutrients and green bean could be mixed with other substrates to increase nodulation in legumes. Leachates had no effect on plant growth compared to recommended nutrient concentrations. However, leachates improved plant growth over inorganic chemical solution with comparable nutrient concentrations included in this study as synthetic green bean nutrients. Addition of microbial cells from green bean leachate increased nodulation of plants treated with recommended plant nutrient solution. Clearly, vermicompost leachates may promote beneficial plant-microbe interactions but this needs to be defined for different conditions. Leachates may also benefit from supplementation with chemicals to amend nutrient deficiencies for maximum plant growth.

Chapter 7 – Final discussion

Major findings from this study

Vermicompost substrate fed to worm beds is an essential component in producing a useful organic amendment. Careful consideration should be paid to substrate choice as substrate can influence many factors including earthworm health, leachate microbial communities, and leachate nutrients. Vermicompost substrate supplies nutrients to earthworms, and forms part of the environment in which the earthworms live. The findings in Sections 2.4.1 and 2.4.2 show that a poor worm-bed substrate can drastically reduce earthworm reproduction, and even stop it altogether, in the case of newspaper pulp. A poorly chosen worm-bed substrate can also reduce the amount of weight gained by juvenile earthworms. As earthworm weight is directly related to the volume of substrate consumed (Munroe, 2007) small earthworms will consume much less substrate, which will drastically reduce the volume of waste that the vermicompost system can recycle. As these small earthworms will consume much of your poorly chosen worm-bed substrate, this substrate may begin to rot. Rotting food can attract pests (Ciavarella, 2013) and lead to anaerobic conditions (Growing and Gathering, 2014) and odours (Eurobodalla Shire Council, n.d.).

This study confirmed that most fruit and vegetable based worm-bed substrates will support earthworm species *E. fetida*, *E. andreii* and *P. excavatus*. This study also confirmed that banana, green bean and rockmelon, alone and as mixtures, can be used in vermicompost. Few other published studies use fruit and vegetable waste as a substrate. Composted apple waste, manure and straw supported *Eisenia* sp. (Hanc & Chadimova, 2014), potato waste supported *E. fetida* (Frederickson, 2002) as did ground corn cobs (Neuhauser et al., 1980). This study demonstrates the potential of vermicompost to recycle the vast quantities of fruit and vegetable waste generated globally, the majority of which is sent to landfills and incinerators (Plazzotta, Manzocco, & Nicoli, 2017).

The worm-bed substrate directly affects leachate microbes by acting as a source of microbes, as discussed in Section 3.4.2. The substrate also supplies nutrients for microbes, so a poor substrate may lead to reduced microbe numbers. In a study by Ji et al. (2017) a high nutrient diet significantly changed the gut microbial community of pigs. Available nutrients affect the microbial community in other ways. Microbial activity in rice field soils was positively correlated with available potassium (Luo et al., 2016). A poorly chosen substrate may therefore reduce the activity and change the population of the leachate microbes.

This study shows that worm-bed substrate significantly changes leachate microbial communities. Banana, green bean, rockmelon, celery, newspaper pulp, sweet potato, and a mixture of sweet corn and celery all produced leachates with different microbial communities. . Fritz et al. (2012) applied eight different substrates to commercial vermicompost made using a green waste and cattle manure worm-bed substrate. These substrates included polenta, wheat flour, oat bran, sunflower press cake, citric acid, silica, green leaf compost and compost leachate and were added after earthworm processing. They made liquid extracts of these mixtures and found differences in numbers of cultivable bacteria and fungi. While Fritz et al. (2012) demonstrated different substrates changed liquid extract microbes, the substrates were not processed by earthworms. The current study also shows that leachate harvest time significantly changed leachate microbial communities. Banana, green bean and rockmelon leachates had different microbial communities at 0 days, 30 days and 60 days.

Bacteria with potential benefits to plants such as *Xanthobacter* and *Azospirillum* were identified in banana and rockmelon leachates. Green bean leachate was not tested but consistently increased nodulation in legumes indicates it also contains potentially beneficial microorganisms. No published studies involving identification of leachate microbes were found. However, Pathma & Sakthivel (2013) studied 193 bacterial isolates from solid straw and goat manure vermicompost. Many (51) of these isolates produced IAA, indicating a possible benefit to plants. Gopal et al. (2009) used culture methods to identify *Azospirillum* in solid vermicompost from cow manure and from a mixture of coconut leaves and cow manure. *Azospirilum* was also identified in the worm-bed substrates. Interestingly, the numbers of cultivable *Azospirillum* significantly increased after vermicomposting for the cow manure and coconut leaf mixture. The numbers of cultivable *Azospirillum* significantly decreased after vermicomposting for the cow manure worm-bed substrate. This was attributed by Gopal et al. (2009) to the lower nutrient content in the cow manure worm-bed substrate. The use of nutritious fruit and vegetable waste in this study may have increased the presence of beneficial microbes.

Leachates from this study had little effect on plant growth parameters such as shot and root dry weight, likely due to the low levels of most nutrients contained in the leachate. This study shows that leachate can improve legume nodulation. Application of green bean leachate to soil significantly increased nodulation of soybean and serradella. This increase in nodulation likely occurred through microbial interaction as discussed in Section 6.4.4. Addition of the microbial component extracted from green bean leachate significantly increased serradella nodulation when the plants were fertilised with the recommended supply of plant nutrients. This demonstrates that green bean

microbes were responsible for the increase in nodulation. The different microbial communities found in rockmelon and banana leachates did not have the same effect of increased nodulation.

Green bean leachate increased serradella nodulation over B/G/R (a mixture of banana, green bean and rockmelon) leachate at the ten week plant harvest. At the five week plant harvest, B/G/R leachate increased serradella nodulation over green bean leachate. Leachate harvest time has been shown to change the leachate microbial community. Thus the different leachate microbial communities applied to soil resulted in different effects on legume nodulation.

Leachates from this study contain macro and micronutrients, the levels of which vary with substrate and leachate harvest time. Similar levels of macronutrients were reported in leachates from cow manure (Álvarez-Solís et al., 2016; Gutiérrez-Miceli et al., 2008), sheep manure (Gutiérrez-Miceli et al., 2011) and a liquid extract of chicken manure vermicompost (Pant et al., 2009). Animal manures are used for many vermicompost studies. The current study shows that fruit and vegetable waste such as green bean improves earthworm growth and reproduction over cow manure. This study also shows that fruit and vegetable waste such as green bean supplies just as much nutritional value as animal manures. The use of fruit and vegetable waste removes the potential for pathogens to be present in the leachate, which is a concern with cow manure (Aira et al., 2011). Most of the plant essential nutrients are not completely supplied by leachates. This is evidenced by the improvements in serradella plant height and dry weight when the recommended dose of chemical fertiliser was used.

Banana, green bean and rockmelon leachates largely had no effect on plant growth factors such as shoot dry weight, due to undersupply of many nutrients. Arthur et al. (2012) found that commercial leachate improved shoot length, leaf number and fresh weight of tomatoes only when a source of chemical N was also supplied. Applied leachate nutrients varied with worm-bed substrate, with green bean leachate generally supplying higher quantities. Leachates generally supplied less than the recommended nutrients for soybean, serradella and wheat. Green bean and B/G/R leachates reduced serradella growth compared to recommended nutrient concentrations. Green bean and B/G/R leachates improved serradella growth over inorganic plant nutrients at comparable nutrient concentrations and this may have been because of beneficial plant-microbe interactions. This study shows that leachate use is essential for maximum nodulation due to microbial interaction, but supplementation with chemical fertiliser is necessary for maximum plant growth.

Does worm-bed substrate change the properties of vermicompost leachate?

The worm farm system is affected by worm-bed substrate, and substrate is a fundamental part of worm farms. Worm-bed substrate provides nutrition for earthworms and has variable effects on growth and survival of earthworms. A nutritious worm-bed substrate will increase earthworm mass, allowing individual earthworms to consume more substrate (Edwards, 1988). A nutritious worm-bed substrate will also promote reproduction, ensuring a large earthworm population that is being continuously repopulated. This study demonstrated that worm-bed substrates such as paper or banana do not have these effects on earthworms. Poor substrates can therefore reduce the efficiency of vermicompost production through their effect on earthworms. It is unknown whether any links can be drawn between human nutrition and earthworm nutrition. For example, bananas are very nutritious for humans (Szalay, 2017) but this effect did not extend to earthworms.

Substrate will have structural effects on earthworm beds, effects that weren't tested in this study due to pureed worm-bed substrates being used. The moisture level, particle size and rate of decomposition will likely effect aeration, which will in turn effect earthworm survival and microbial communities. For example, increased aeration from substrates with larger particles should reduce populations of anaerobic microorganisms. Some worm-bed substrates tested in this study (rockmelon and celery) were filtered to reduce moisture. High moisture substrates may effect leachate microbes, particularly in terms of increasing anaerobic conditions. Overall, the use of purees may have contributed to high moisture content. Many published leachate studies used animal manure as a substrate (Ansari, 2008; Ayyobi et al., 2014, 2013; Bidabadi et al., 2016). Ayyobi et al. (2013) cited the large amount of animal manure produced in Iran as the reason they chose cattle manure. However, most studies do not indicate the reasons behind their choice of animal manure substrate. An advantage of animal manure substrate over the fruit and vegetable purees used in this study may be a reduced moisture content.

Worm-bed substrates are a source of microbes, and have a direct effect on leachate microbial communities. For this reason, sources of potential pathogens, both plant and human, are best avoided. Aira et al. (2011) detected the presence of levels of *Clostridium* sp. and total coliforms above the Environmental Protection Agency standard in solid cow manure vermicompost processed using *E. Andrei*. Worm-bed substrate pH affects the earthworms, as do measures taken to neutralise substrate pH. As soil pH has a strong influence on soil microbial communities (Rousk, Brookes, & Bååth, 2009) substrate pH will likely directly effect leachate microbial communities.

Worm-bed substrate effects the concentration of some nutrients in leachate. This in turn effects the species of plants that leachates are useful for. For example, wheat has a lower requirement for Ca than soybean and serradella. Both green bean and rockmelon leachates satisfied this requirement, while banana leachate did not. None of the tested leachates satisfied the Ca requirements of soybean or serradella. Marín et al. (2013) found that a liquid extract of solid crop residue vermicompost did not satisfy any of the nutritional requirements of tomato. Worm-bed substrates effect the microbes in leachate, which in turn effects the potential plant-microbe interactions.

Is the effect on plants from application of leachate chemical or microbiological?

The findings from this study support the theory that microbiological interaction occurs following leachate application. When serradella is supplied with recommended nutrients in the form of N-free CRS, application of a pellet of microorganisms from green bean leachate increased nodulation. This pellet was added weekly. As leachate harvest time effects leachate microbial communities, the microbes in each pellet would probably have been different. The microbes in the pellets supplied to plants would also differ from the microbes studied in the population experiments using T-RFLP and ARISA. Therefore, drawing conclusions about the identity and effect of the microbes supplied to plants is difficult.

Application of unsterilised green bean leachate increased soybean nodulation over application of sterile green bean leachate. This seems to indicate that leachate microbes were responsible for increased nodulation. However, sterilisation did not remove all microbes, only reduced their numbers. It is possible that sterilisation removed beneficial microbes which increased nodulation, but further investigation is needed to confirm this. Sterilisation may also have brought about chemical changes in the leachate which contributed to reduced nodulation. Cavender et al. (2003) believed that steam sterilisation of solid pig manure vermicompost produced physical and chemical changes that resulted in increased dry weight of sorghum.

Green bean and B/G/R leachates improved plant growth and nodulation over diluted chemical fertiliser in the form of sterile synthetic green bean nutrients. This finding indicates that removing microbes from leachate and supplying only the nutrients removed beneficial effects. Synthetic green bean solution was designed to mimic green bean leachate. However, further analysis showed some significant nutritional differences between these solutions. For example, green bean leachate contained significantly more P and Ca than synthetic green bean solution. The separation of green bean microbial component and nutrient component provided some evidence for microbial action.

However, due to other chemical differences between the treatment solutions, this cannot be definitively stated.

Evidence exists of a chemical effect of green bean leachate on nodulation. In many of the plant application studies, green bean leachate supplied higher concentrations of important nutrients. These increased nutrients may have contributed to increased nodulation. For example, green bean leachate supplied significantly more Ca than banana, rockmelon and B/G/R leachate. Increased Ca increases nodulation (*nod*) gene expression in *R. leguminosarum* (Richardson et al., 1988).

Higher green bean leachate P may also indicate a chemical effect. Increased P is associated with increased nodulation and N_2 fixation in soybean (Israel, 1987) and cowpea (Kyei-Boahen et al., 2017). However, this evidence is complicated by variation between leachate batches. In the soybean trial, applied green bean leachate contained more P than banana leachate but significantly less than rockmelon leachate. In the first serradella trial, green bean leachate supplied significantly more P than both banana and rockmelon leachate and the chemical nutrient solution. In the second serradella trial, green bean leachate supplied significantly more P than B/G/R leachate and synthetic green bean nutrients but significantly less than CRS nutrient solution. Despite these differences, green bean leachate led to the highest nodulation in all studies.

Is leachate a stable product? Is the effect on plants likely to be sustained over a shelf life?

Leachate is chemically stable over one year. EC and pH remained unchanged regardless of leachate storage temperature. A limited number of measurements were used, with further analysis needed to declare leachate a stable product. Leachate is not stable microbiologically. Leachate C:N ratio changes after one year, which is likely a reflection of changes in microorganisms. Leachate was more stable following refrigeration. However, refrigeration is an impractical method of storing amendments, particularly in large quantities. Leachate was less microbiologically stable following indoor ambient temperature storage, which is more representative of real-world storage conditions. Grantina-Ievina et al. (2013) also found refrigeration to be preferable to ambient temperature storage, as one year refrigeration resulted in increased culturable microbial and fungal populations in solid cow manure vermicompost.

Microbial properties of leachate are unlikely to be sustained over a growing season. This presents difficulties as the microorganisms are probably the component of leachate responsible for benefits to plants. Increased nodulation may occur across two growing seasons, but this is unlikely. Stored green bean leachate should be tested across two growing seasons to determine if increased

nodulation occurs after storage. If increased nodulation is not found after storage, this would seem to indicate that the mechanism for increased nodulation is microbial.

Leachate microbes following storage were not identified. It is unknown whether a new microbial community is formed or whether a reduction in numbers of the current microbial community occurs. Similarly, microbial identification was not performed by Grantina-Ievina et al. (2013). Identification would be useful for determining the effect of storage on leachate microbes. This would allow further conclusions to be drawn regarding the mechanism of increased nodulation.

Could leachate be custom designed for improved plant growth?

Green bean leachate increased nodulation of - soybean grown in a 1 part sand, 4 parts soil without environmental controls; serradella grown in 1 part sand, 1 part soil under controlled conditions; and serradella grown in sand under controlled conditions. Green bean leachate is effective across multiple plant species and a range of growth conditions. Using green bean as a component of a mixed worm-bed substrate also improved nodulation, as B/G/R leachate increased nodulation of serradella grown in sand. It is recommended that green bean leachate be applied to legumes.

Other worm-bed substrate mixtures should be tested to determine if this result is restricted to green bean or whether it extends to other legume substrates. Three other legume worm-bed substrates were used in this study. Lucerne provided good earthworm support, while garden pea and a combination of rockmelon and garden pea were lethal to earthworms. Lucerne leachate should be tested for effects on legume nodulation. No published studies were found using legumes as a vermicompost substrate. Additional legume substrates should be tested for their usefulness as worm-bed substrates.

Recommendations from this study

Selecting a worm-bed substrate

1. Select a pH neutral or alkaline worm-bed substrate. Modifications to pH can create hostile environmental conditions for earthworms and reduce vermicompost efficiency.
2. Select a worm-bed substrate free from potential pathogens. Do not use animal manures.
3. Examine the effects of substrate on *E. fetida* on a small scale under controlled conditions. Earthworms will generally respond quickly to poor worm-bed substrates.
4. Banana should be mixed with other substrate components. Banana alone will reduce vermicomposting efficiency and produce a low nutrient leachate.
5. Green bean should be used as a worm-bed substrate for legume application. Green bean can be mixed with other substrate components.

Harvesting and storing leachate

1. Wait at least seven weeks after setting up worm farms before harvesting leachate. In the short-term, leachate nutrient levels increased over the first six weeks.
2. Wait at least one year before harvesting leachate for plant application. Many nutrients significantly increased between five months and a year. One year will increase leachate nutrients, providing worm-bed substrate is renewed regularly.
3. Store leachate at room temperature in the short-term (six weeks) and at 4°C in the long-term.
4. Do not assume that leachate will be the same following long-term storage. Test fresh and stored leachate for effect on plants.

Applying leachate to plants

1. Do not filter or sterilise leachate prior to plant application.
2. Shake or stir leachate well to ensure particles containing microorganisms are suspended in liquid and will be applied to plants.
3. Apply leachate to the soil to improve soil microbial communities.
4. Apply leachate from green bean worm-bed substrate to soil used to grow legumes.
5. Foliar application of leachate is unlikely to increase legume nodulation. Test both application methods to confirm this.
6. Augment leachate with chemical fertiliser to maximise plant growth and yield.
7. Apply leachate alone to obtain genuine organic produce.

Organic amendments have been in use for decades but interest is increasing due to consumer demand for organic produce (St Martin, 2015). Certified organic growers have access to growing local and international markets for genuine organic produce and premium prices (NASAA Organic, 2016; The Organic Food Chain, 2016). While studies on residual effects of leachate are lacking, it is likely leachate will add soil microbes and be beneficial long-term. There are many benefits to vermicompost leachate use. These include less organic waste in landfill, creation of a useful commercial product, increased soil N through increased nodulation, increased soil microbes, and access to higher prices for organic produce.

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Appendix A

Culture media and nutrient solutions

CRS Plant Growth Nutrient Solution (Yates et al., 2016)

Magnesium sulphate heptahydrate	12.3g
Potassium di-hydrogen phosphate	6.8g
Potassium sulphate	17.5g
Iron chelate	2.5g

1. Dissolve each reagent in 1 L deionised water to make four stock solutions
2. Combine 50 mL of each stock
3. Add 0.5 mL trace element solution
4. Make up to 1.6 L with deionised water
5. Autoclave 121°C 20 min and allow to cool
6. Add 400 mL well agitated calcium sulphate solution

Trace element solution

Boric acid	0.464g
Sodium molybdate	0.018g
Zinc sulphate	0.539g
Manganese sulphate	0.042g
Cobalt sulphate	0.141g
Copper sulphate	0.125g

1. Make up to 1 L with deionised water
2. Store at 4°C

Calcium sulphate 2.04g

1. Make up to 1 L with deionised water
2. Autoclave 121°C 20 min and allow to cool

Green Bean Nutrient Solution

Ammonium dihydrogen orthophosphate (1M)	20.23µL
Potassium nitrate (1M)	1.3mL
Calcium nitrate tetrahydrate (1M)	251.6µL
Magnesium sulphate heptahydrate (1M)	209.83µL
Iron chelate (0.5% (w/v))	2.88µL
Glucose	0.05g

1. Combine the five stock solutions in 900ml deionised water
2. Dissolve glucose

Boric acid	0.44g (1)
Copper sulphate heptahydrate	0.4g (2)
Zinc sulphate heptahydrate	0.1g (3)
Sulphur	0.2g (4)
Sodium molybdate	0.04g (5)
Manganese sulphate tetrahydrate	0.06g (6)

1. Dissolve each reagent in 1 L deionised water to make six stock solutions
2. Add 1 mL of solutions 1-4
3. Add 0.1 mL of solution 5
4. Dilute solution 6 1000 X. Add 1 mL of dilution
5. Make up to 1 L with deionised water
6. Autoclave 121°C 20 min

Hoagland's Nutrient Solution No. 2

Ammonium dihydrogen orthophosphate (1M)	1mL
Potassium nitrate (1M)	6mL
Calcium nitrate tetrahydrate (1M)	4mL
Magnesium sulphate heptahydrate (1M)	2mL
Iron chelate (0.5% (w/v))	2mL
Micronutrient solution	1mL

1. Autoclave solutions 1-4 separately (121°C 15 min)
2. Combine all solutions and make up to 1 L with deionised water

<u>Micronutrient solution</u>

Boric acid	2.86g
Manganese chloride tetrahydrate	1.81g
Zinc sulphate pentahydrate	0.22g
Copper sulphate pentahydrate	0.08g
Molybdic acid hydrate	0.02g

1. Make up to 1 L with deionised water
2. Store at 4°C

Mixed Indicator

Methyl Red	0.2g
Methylene Blue	0.1g

1. Dissolve in 150 mL 90% ethanol
2. Store at room temperature in the dark

Molybdenum Blue Assay Reagents

Acid diluent

Boric acid 36g

Tartaric acid 2.4g

Make up to 1 L with deionised water

Molybdate reagent

1. Dissolve 8.5 g ammonium molybdate in 600 mL deionised water
2. Add 99.2 mL concentrated sulfuric acid
3. Dissolve 0.196 g potassium antimony tartrate in 100 mL deionised water
4. Combine both solutions
5. Make up to 1 L with deionised water
6. Cover contents with foil to prevent light exposure
7. Store at 4°C

Ascorbic acid

1. Dissolve 0.875 g ascorbic acid in 80ml deionised water
2. Make up to 100 mL with deionised water
3. Discard unused solution. Make fresh on the day of use

Nitrogen Free Broth

L -(-)- Malic acid	5g
Di-Potassium hydrogen orthophosphate	0.5g
Magnesium sulphate heptahydrate	0.2g
Sodium chloride	0.1g
Calcium chloride	0.02g
Iron chelate (1.64% (w/v))	4mL
Trace element solution	2mL
Bromothymol blue solution	4mL

Agar 1.8g

1. Make up to 900 mL with deionised water
2. Adjust pH to 6.8 using 80% (w/v) Potassium hydroxide
3. Adjust volume to 1 L with deionised water
4. Bring to a boil to dissolve agar
5. Dispense 3 mL into test tubes and cover with caps
6. Autoclave 121°C 15 min then allow to cool
7. Add 30 µL vitamin solution per tube
8. Add 30 µL filtered 1% (w/v) Cycloheximide per tube
9. Store at 4°C

<u>Trace element solution</u>

Sodium molybdate dihydrate	0.2g
Manganese sulphate monohydrate	0.235g
Boric acid	0.28g
Copper sulphate pentahydrate	0.008g
Zinc sulphate heptahydrate	0.024g

Make up to 1 L with deionised water

Bromothymol blue solution

Dissolve 0.5 g Bromothymol blue in 100 mL 0.2 M Potassium hydroxide

Vitamin solution

Biotin	0.01g
Pyridoxin	0.02g

1. Make up to 20 mL with deionised water
2. Store at -20°C
3. Prior to use, add 1 mL solution to 49ml deionised water
4. Filter sterilise and add to media
5. Store unused dilution at -20°C

Potato Dextrose Agar

Dextrose	20g
Potato puree	500g
Agar	15g

1. Make up to 1 L with deionised water
2. Autoclave 121°C 15 min
3. Add 1 drop Lactic acid

Potato puree

1. Place 250 g peeled and diced potatoes in a pot.
2. Add 1 L deionised water. Boil for 20 mins
3. Strain through two layers of muslin
4. Return potatoes to pot. Add 1 L deionised water. Boil for 10 mins
5. Repeat step 3
6. Adjust volume to 2 L with deionised water
7. Store unused puree at -20°C

Sucrose Peptone Agar

Sucrose	20g
Peptone	5g
Di-Potassium hydrogen orthophosphate	0.5g
Magnesium sulphate heptahydrate	0.25g
Agar	15g

1. Make up to 1 L with deionised water
2. Autoclave 121°C 15 min

Water Agar

1. Add 12 g agar to a 1 L deionised water
2. Autoclave 121°C 15 min
3. Store plates right way up at 4°C so the agar surface remains moist

Yeast Mannitol Agar

Mannitol 10g

Di-Potassium hydrogen orthophosphate 0.5g

Magnesium sulphate heptahydrate 0.2g

Yeast extract 0.5g

Agar 15g

1. Make up to 1 L with deionised water
2. To make CRYMA, add 15 mL 0.25% Congo Red (w/v)
3. Autoclave 121°C 15 min

Appendix B

Total Kjeldahl Nitrogen (TKN) formulae

N in digest (mg) = (T-B) x 14.0067 x 0.1

T = mL HCl added to the sample digest

B = mL HCl added to the blank digest

14.0067 = N atomic weight

0.1 = HCl concentration

% N in shoot sample = mg N x 100mL/ 20mL/ S x 100

100mL = total digest volume

20mL = digest volume distilled

S = shoot weight used in digest (mg)

Appendix C

Two-sided correlation analysis plots

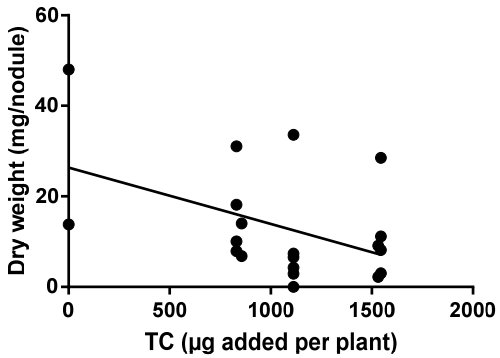


Figure C1.1 Correlation between soybean nodule dry weight and total carbon supplied to plants by vermicompost leachate and nutrient solution.

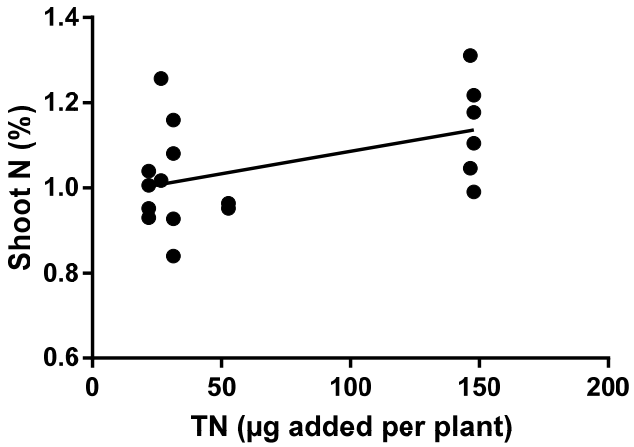


Figure C1.2 Correlation between soybean shoot nitrogen and total nitrogen supplied to plants by vermicompost leachate.

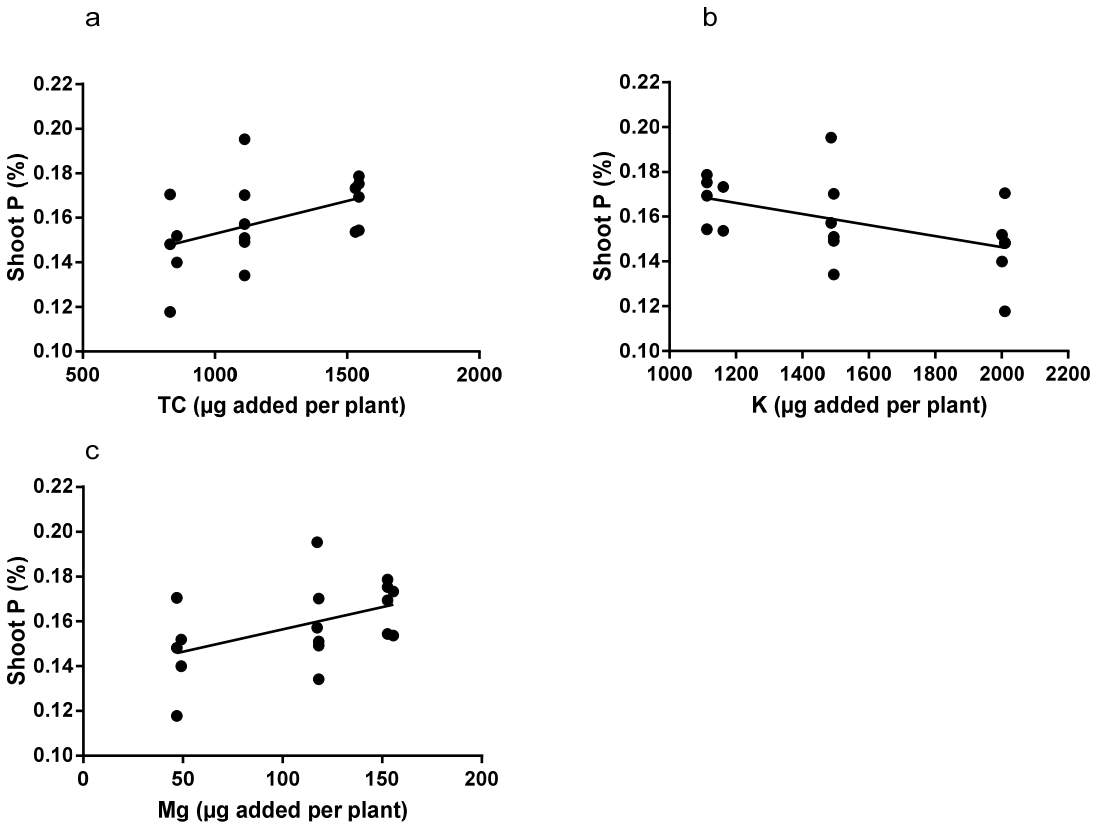


Figure C1.3 Correlation between soybean shoot phosphorus and total carbon (a) potassium (b) or magnesium (c) supplied to plants by vermicompost leachate.

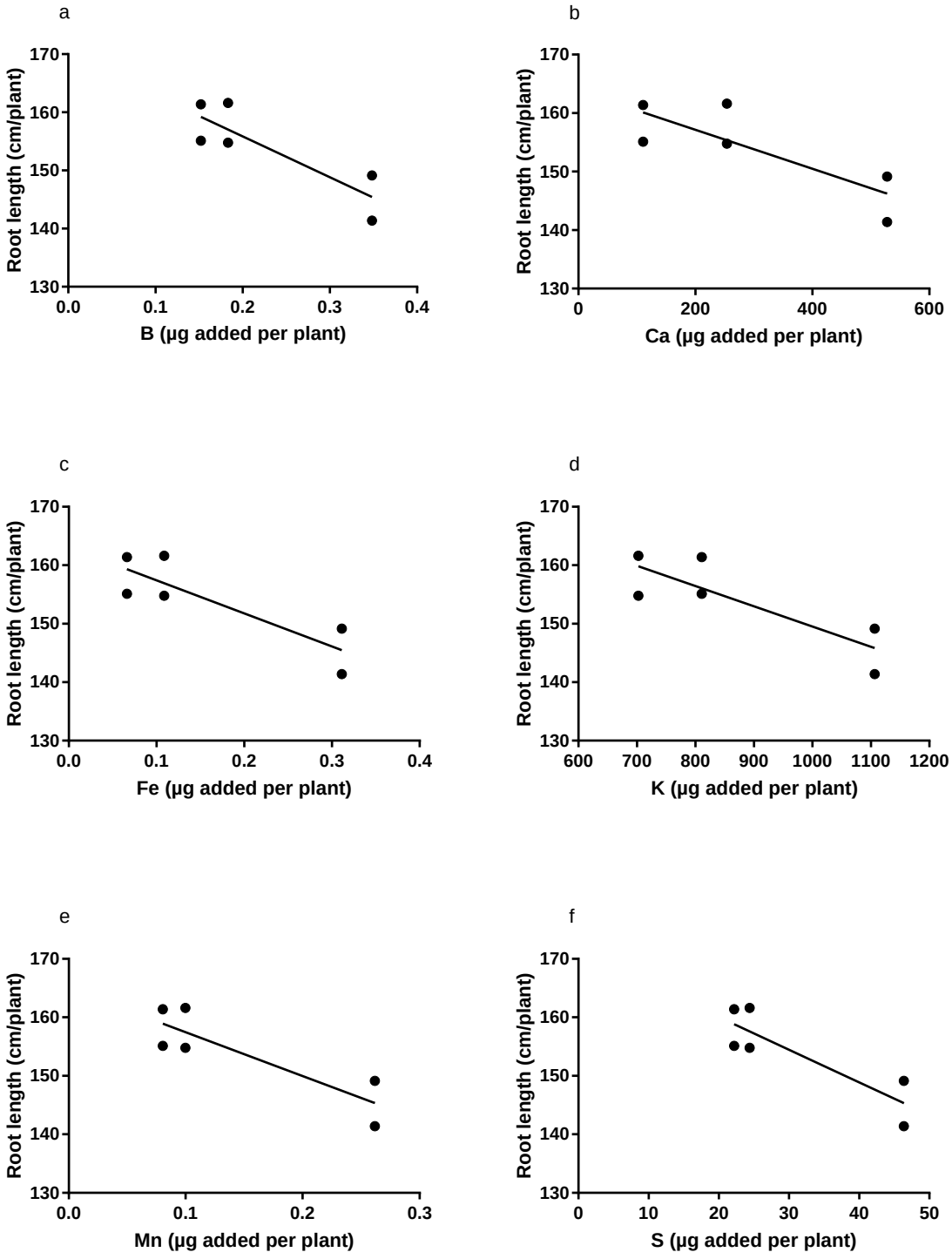


Figure C2.1 Correlation between wheat root length and Boron (a) Calcium (b) Iron (c) Potassium (d) Manganese (e) or Sulfur (f) supplied to plants by vermicompost leachate.

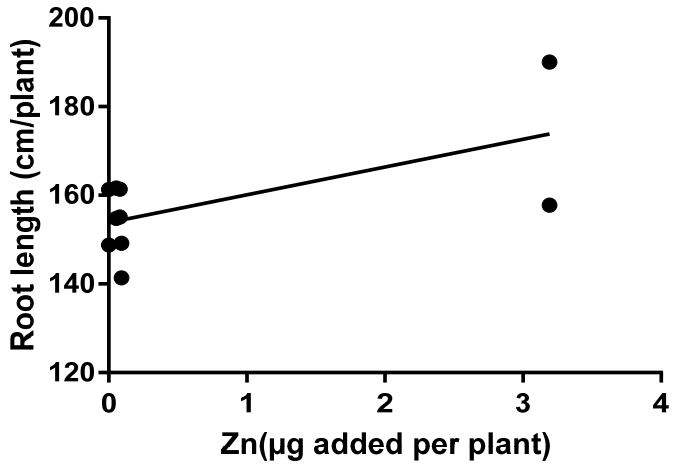


Figure C2.2 Correlation between total root length of wheat and zinc supplied to plants by vermicompost leachate and nutrient solution.

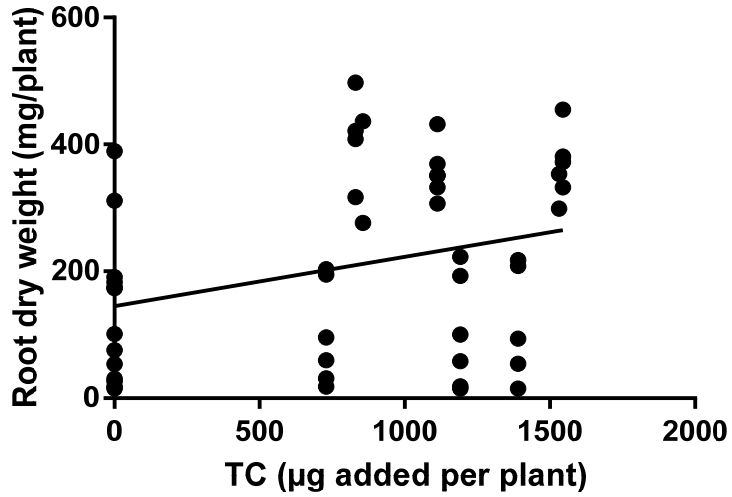


Figure C3.1 Correlation between total carbon and root dry weight. Data plotted are 20 replicates of serradella, 20 of soybean and 10 of wheat. Nutrients supplied by vermicompost leachate or nutrient solution.

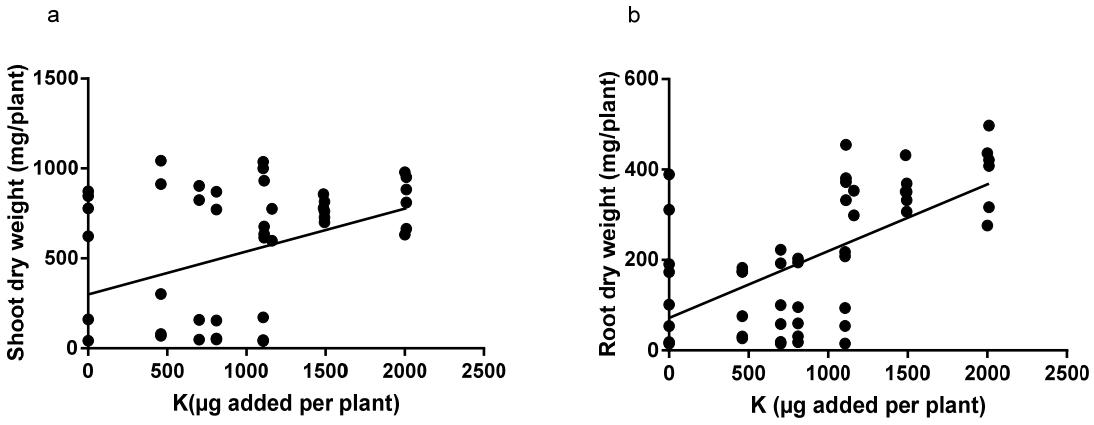


Figure C3.2 Correlation between potassium and dry weight of shoots (a) and roots (b). Data plotted are 20 replicates of serradella, 20 of soybean and 10 of wheat. Nutrients supplied by vermicompost leachate or nutrient solution.

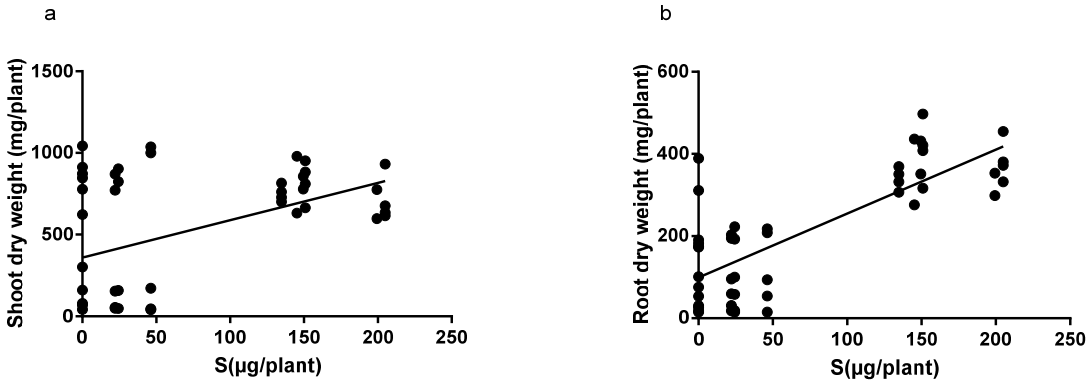


Figure C3.3 Correlation between sulphur and dry weight of shoots (a) and roots (b). Data plotted are 20 replicates of serradella, 20 of soybean and 10 of wheat. Nutrients supplied by vermicompost leachate or nutrient solution.

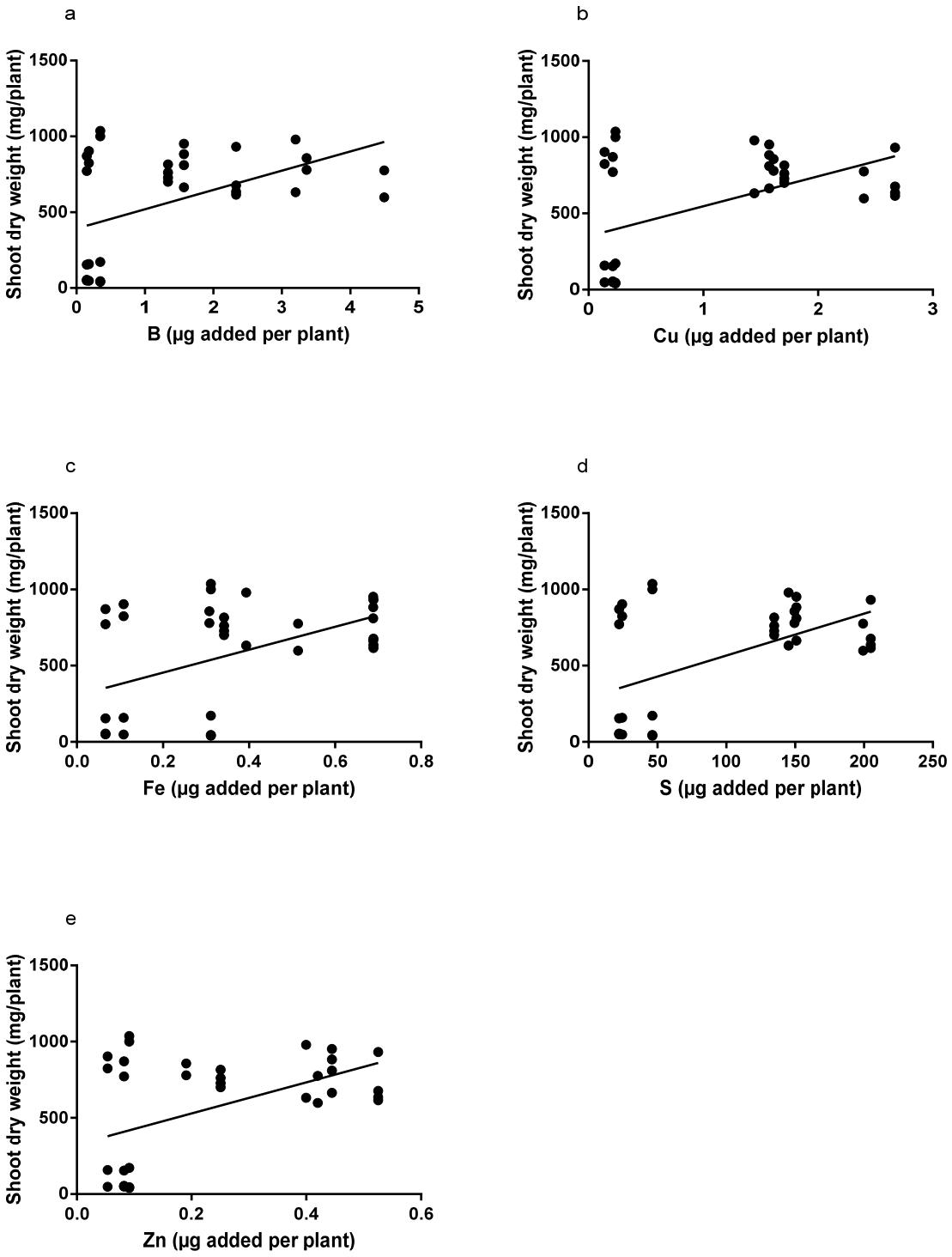


Figure C3.4 Correlation between shoot dry weight and Boron (a) Copper (b) Iron (c) Sulfur (d) or Zinc (e) supplied to plants by vermicompost leachate. Data plotted are 12 replicates of serradella, 18 of soybean and 6 of wheat.

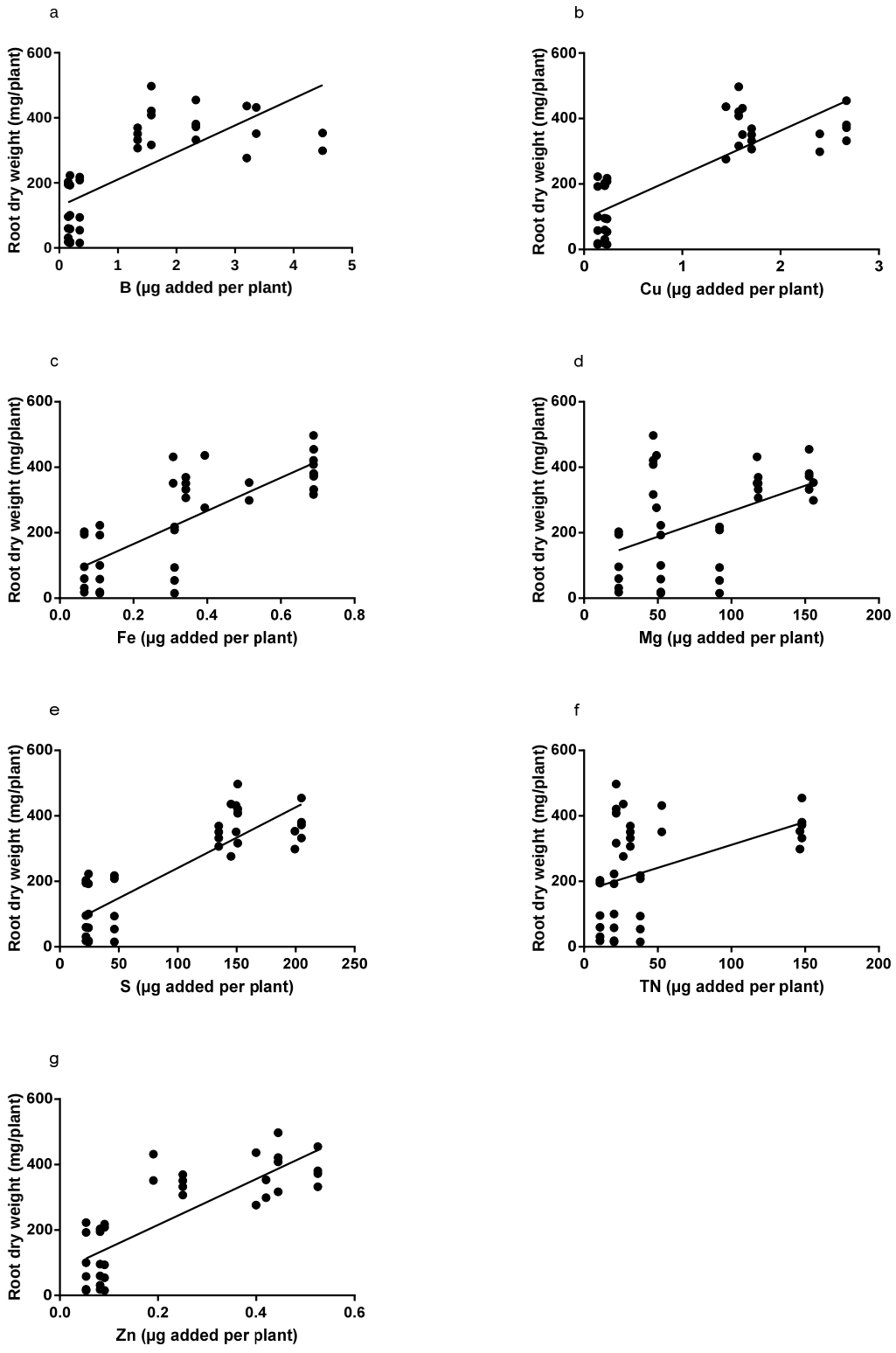


Figure C3.5 Correlation between root dry weight and Boron (a) Copper (b) Iron (c) Magnesium (d) Sulfur (e) total Nitrogen (f) or Zinc (g) supplied to plants by vermicompost leachate. Data plotted are 12 replicates of serradella, 18 of soybean and 6 of wheat.

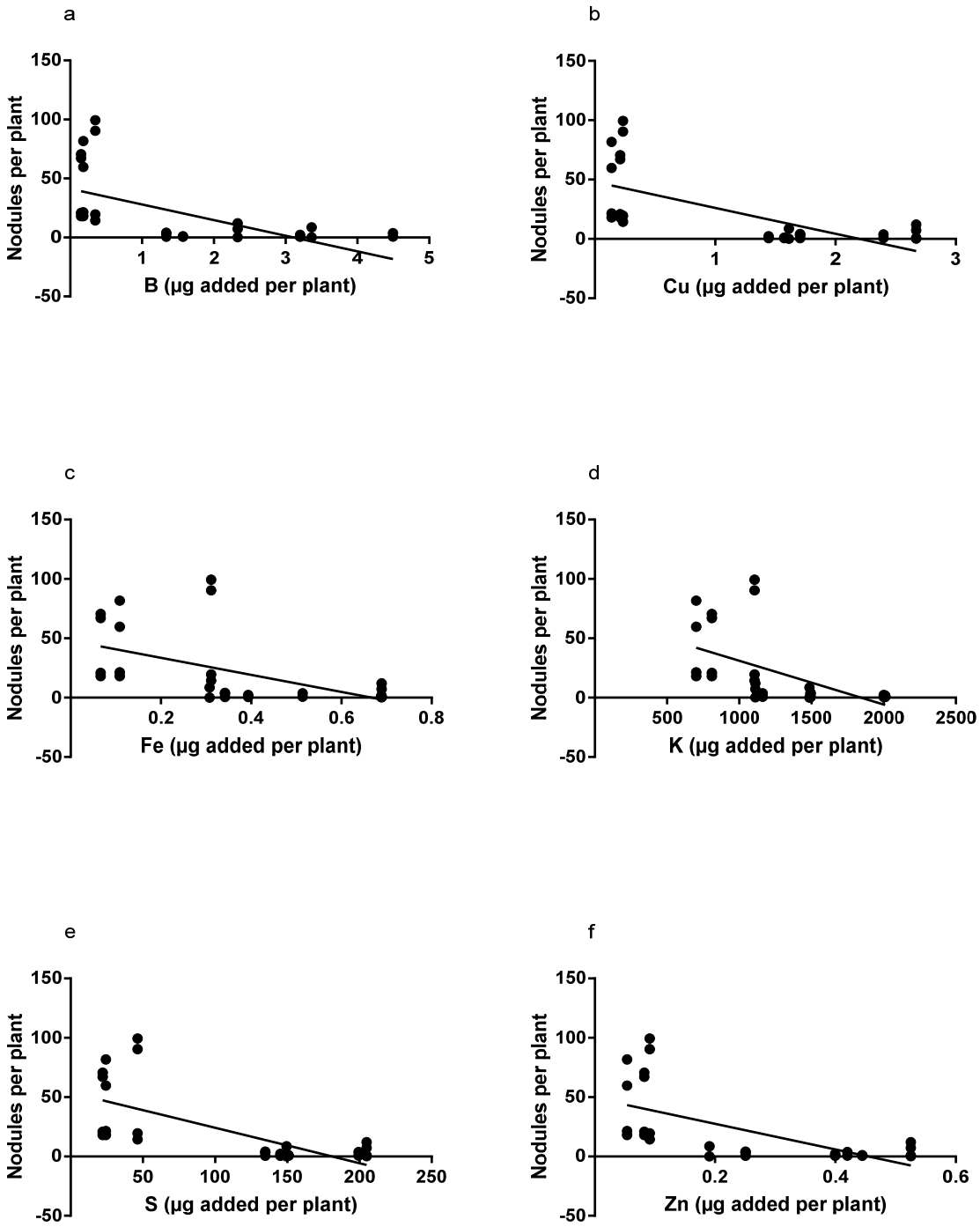


Figure C3.6 Correlation between nodule number and Boron (a) Copper (b) Iron (c) Potassium (d) Sulfur (e) or Zinc (f) supplied to plants by vermicompost leachate. Data plotted are 12 replicates of serradella and 18 of soybean.

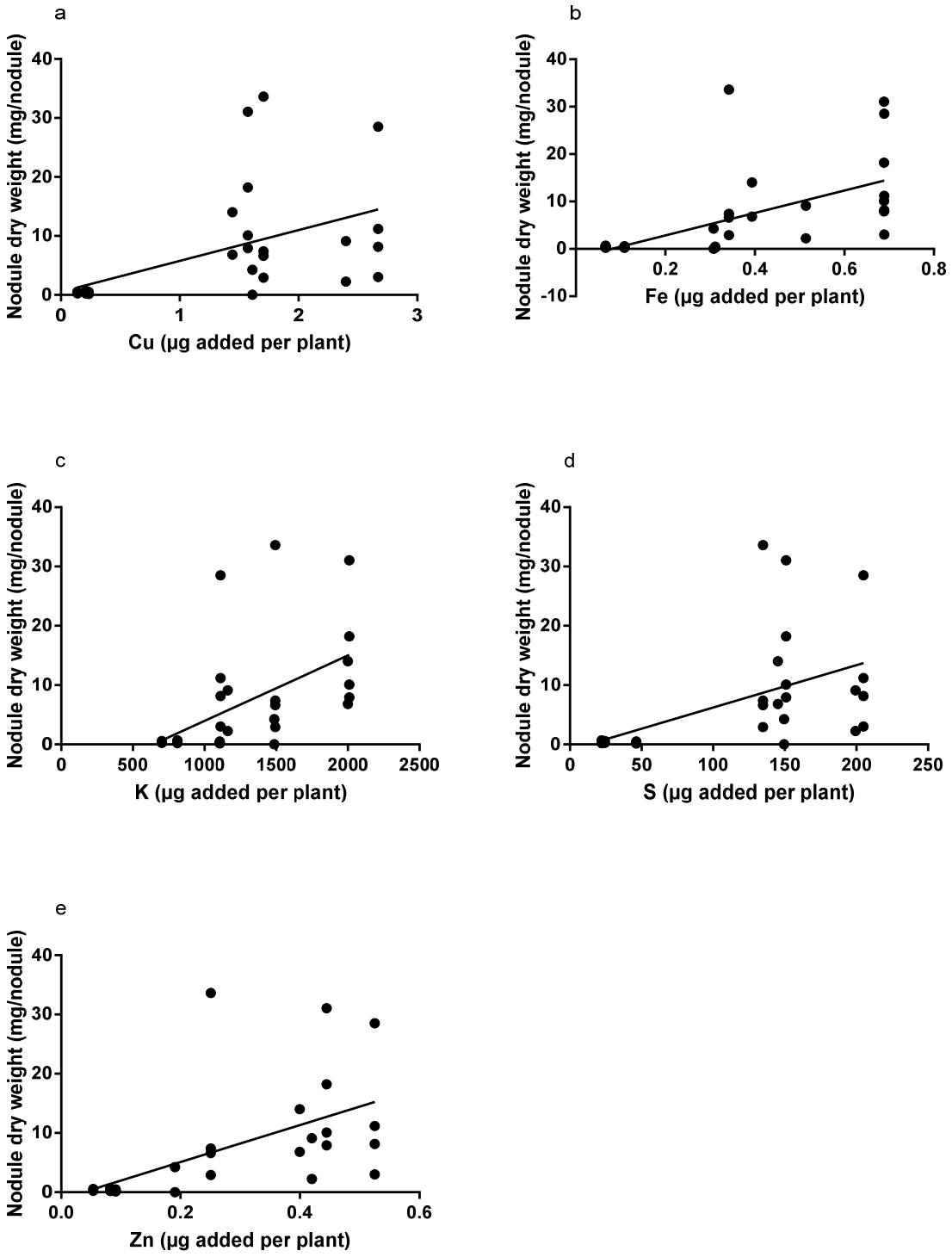


Figure C3.7 Correlation between nodule dry weight and Copper (a) Iron (b) Potassium (c) Sulfur (d) or Zinc (e) supplied to plants by vermicompost leachate. Data plotted are 12 replicates of serradella and 18 of soybean.

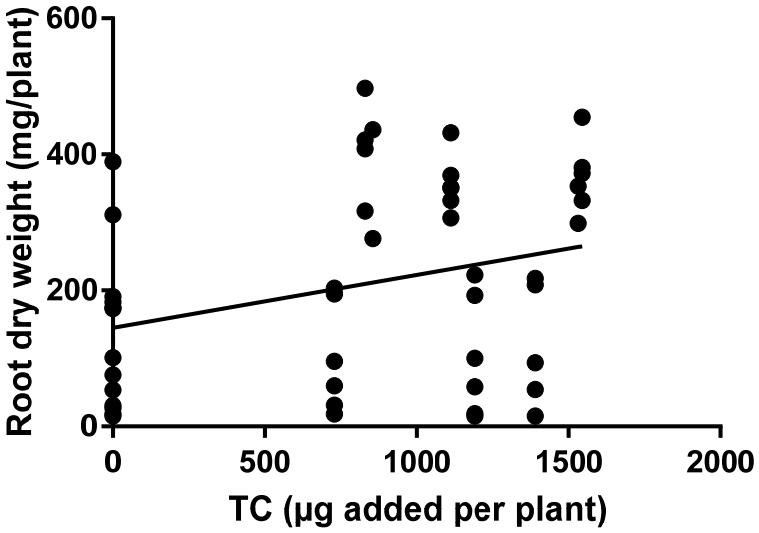


Figure C3.8 Correlation between total carbon and root dry weight. Data plotted are 20 replicates of serradella, 20 of soybean and 10 of wheat. Nutrients supplied by vermicompost leachate or nutrient solution.

Appendix D

Leachate nutrient contents over time

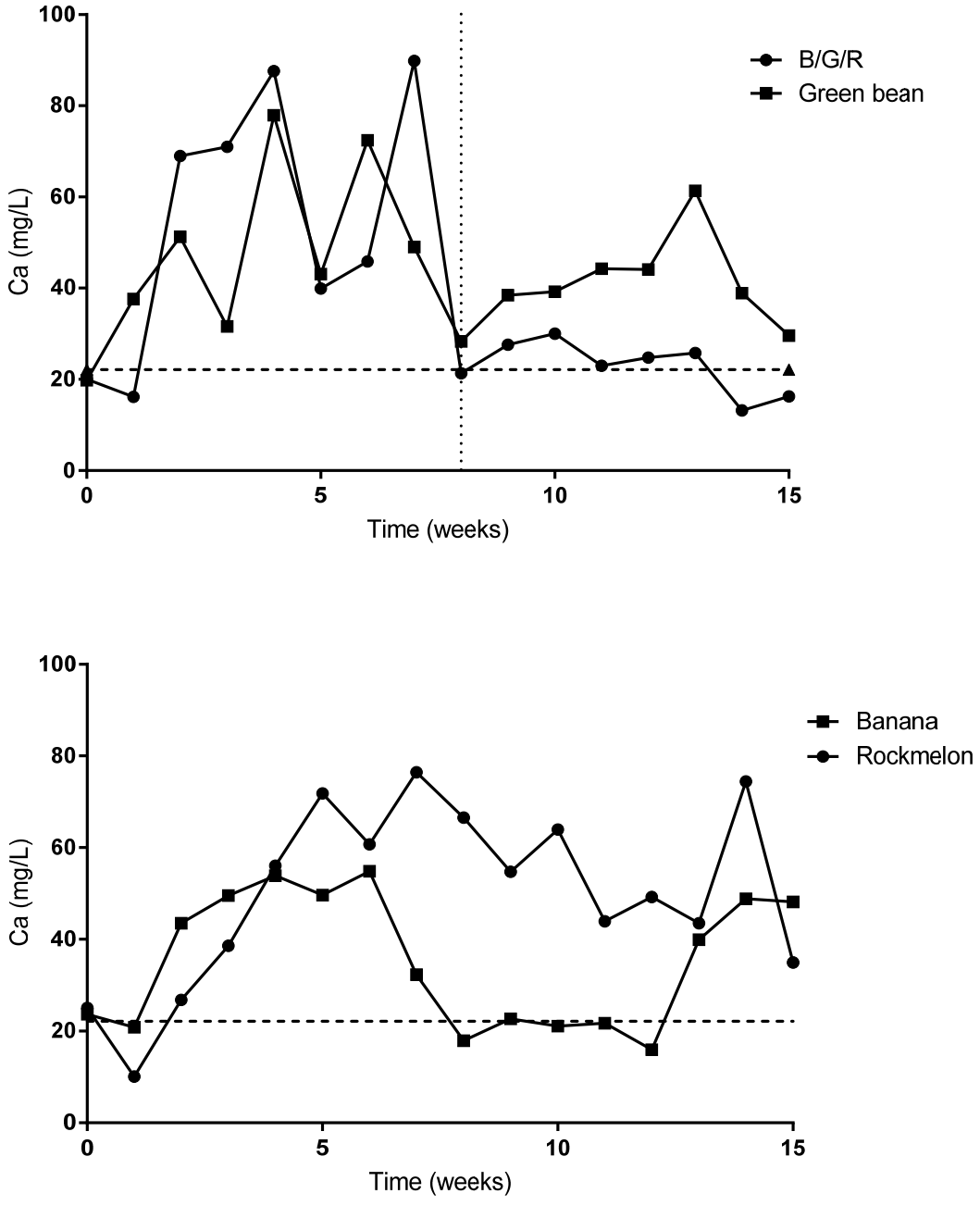


Figure D1.1 Calcium content of vermicompost leachate from different substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.

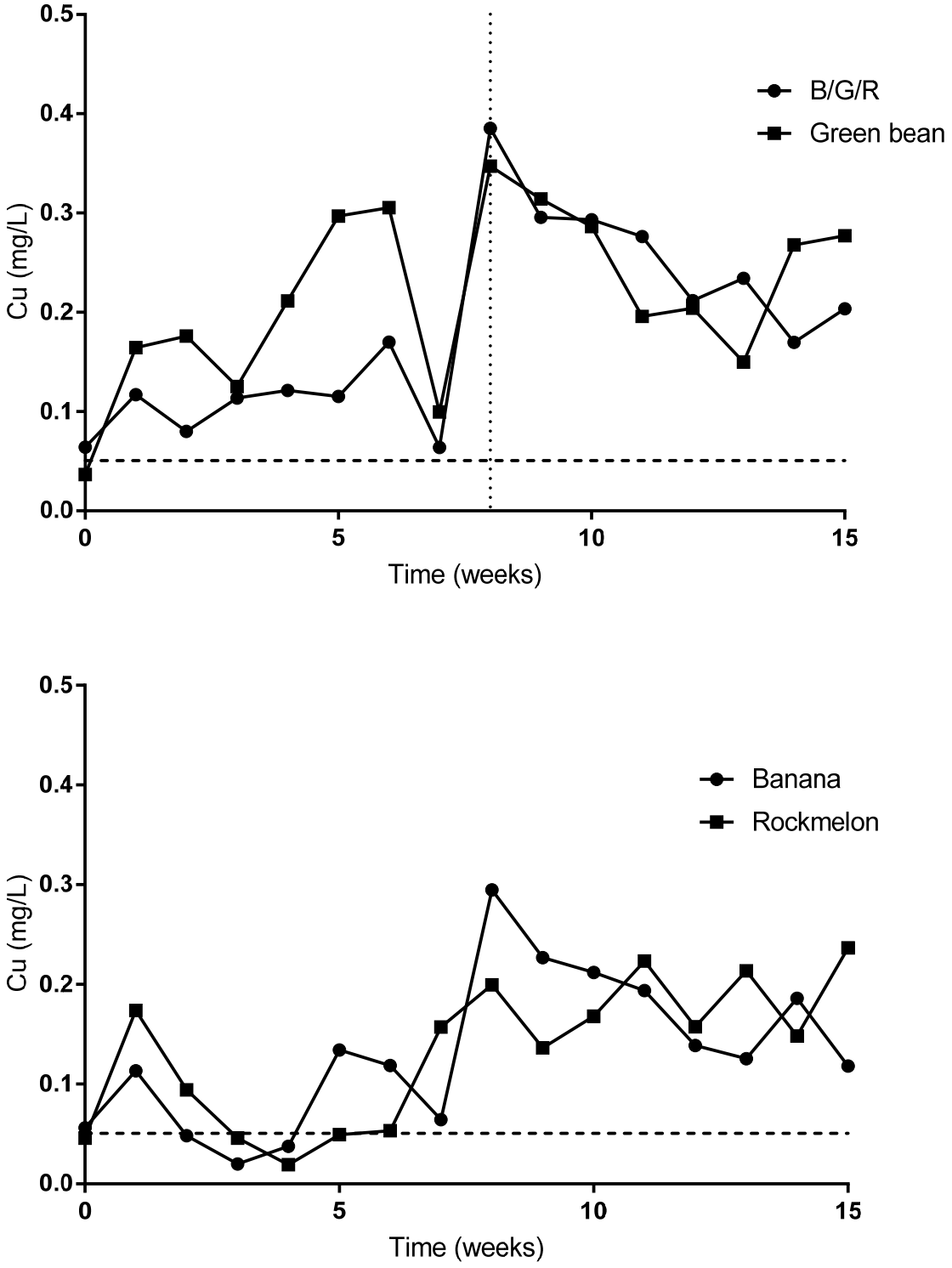
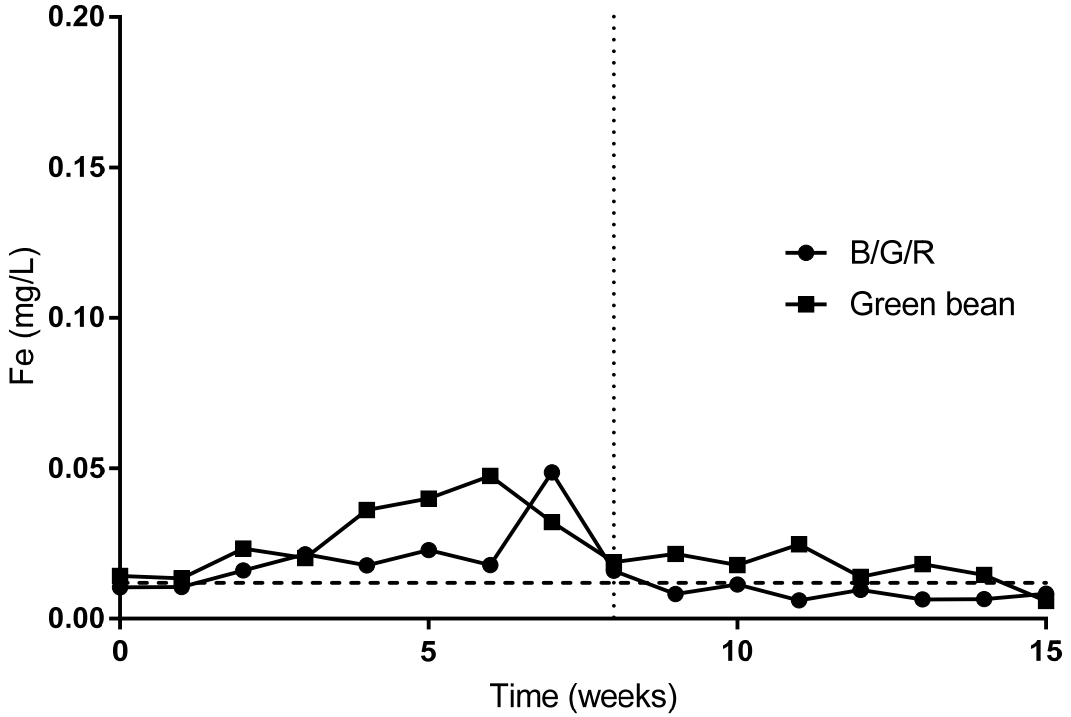


Figure D1.2 Copper content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.



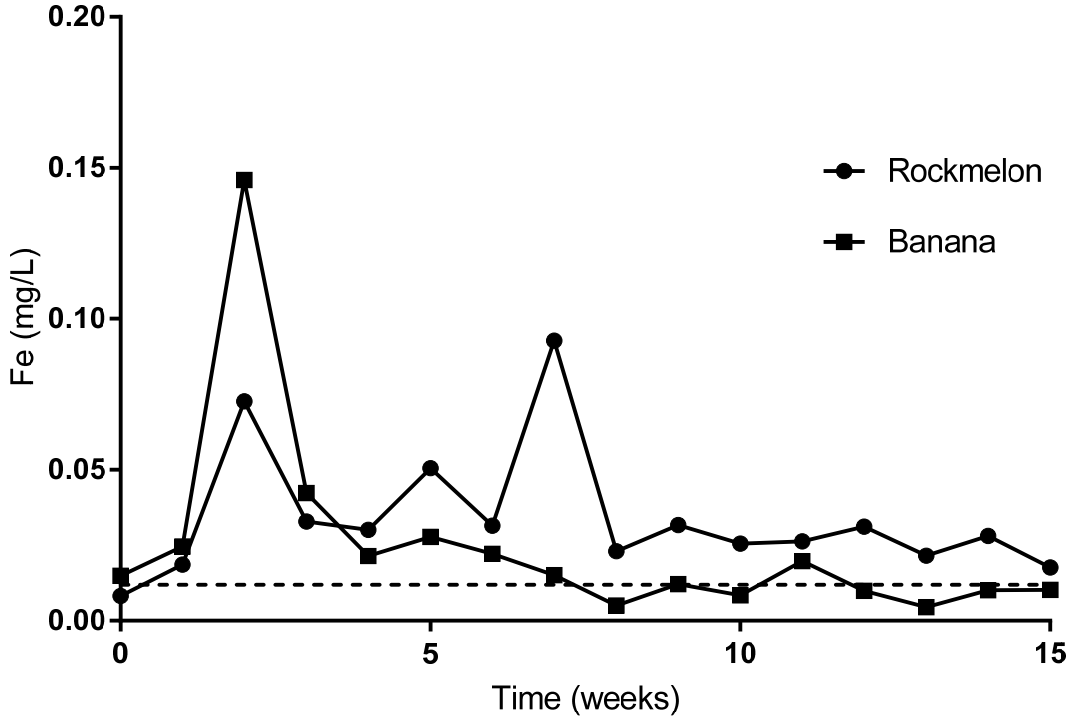


Figure D1.3 Iron content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.

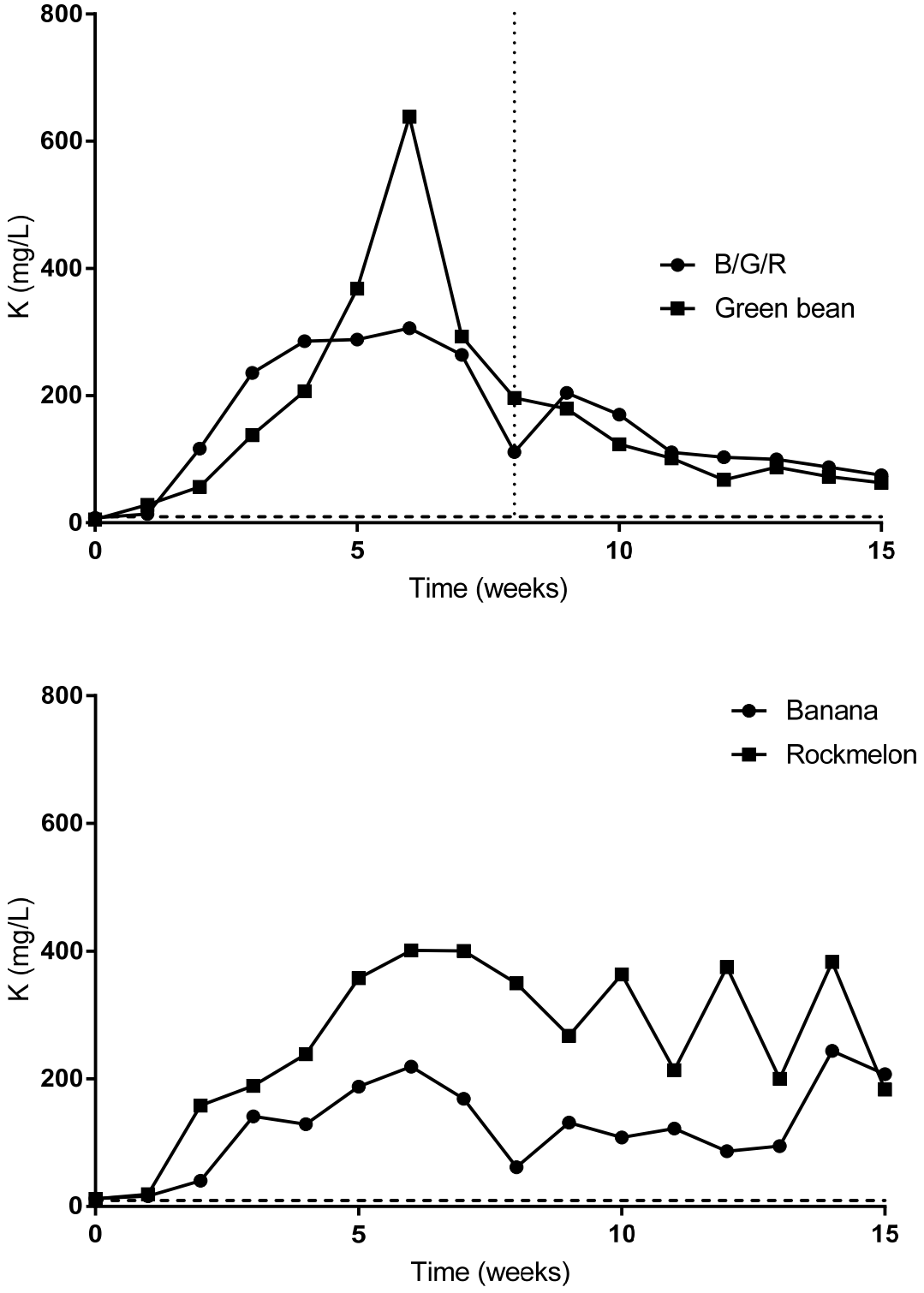
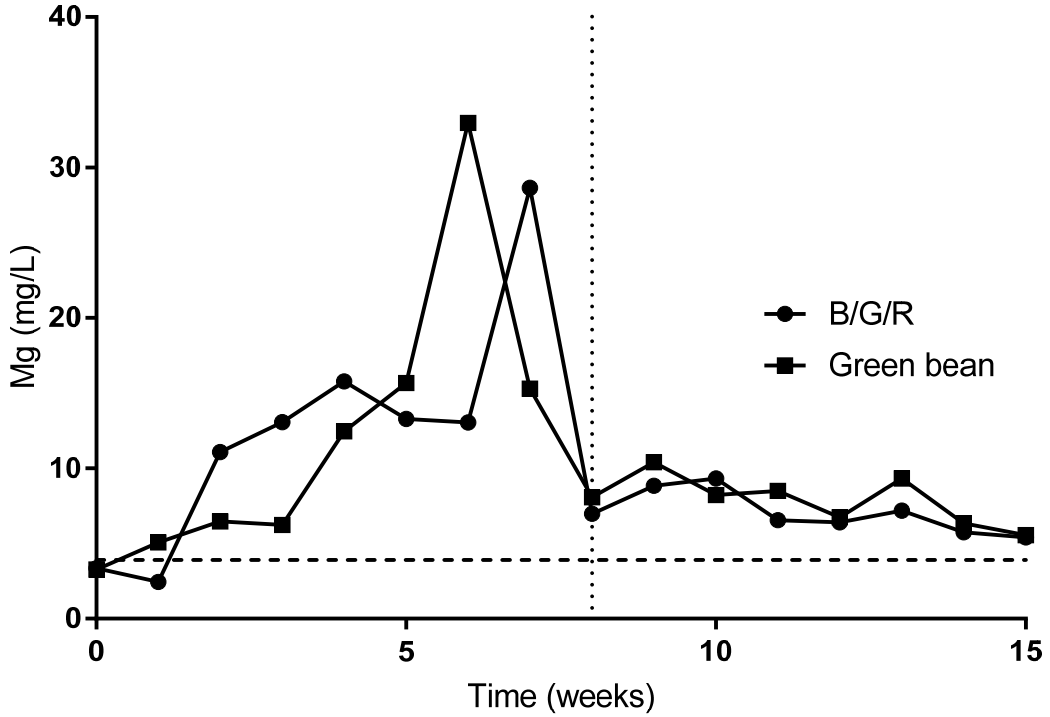


Figure D1.4 Potassium content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.



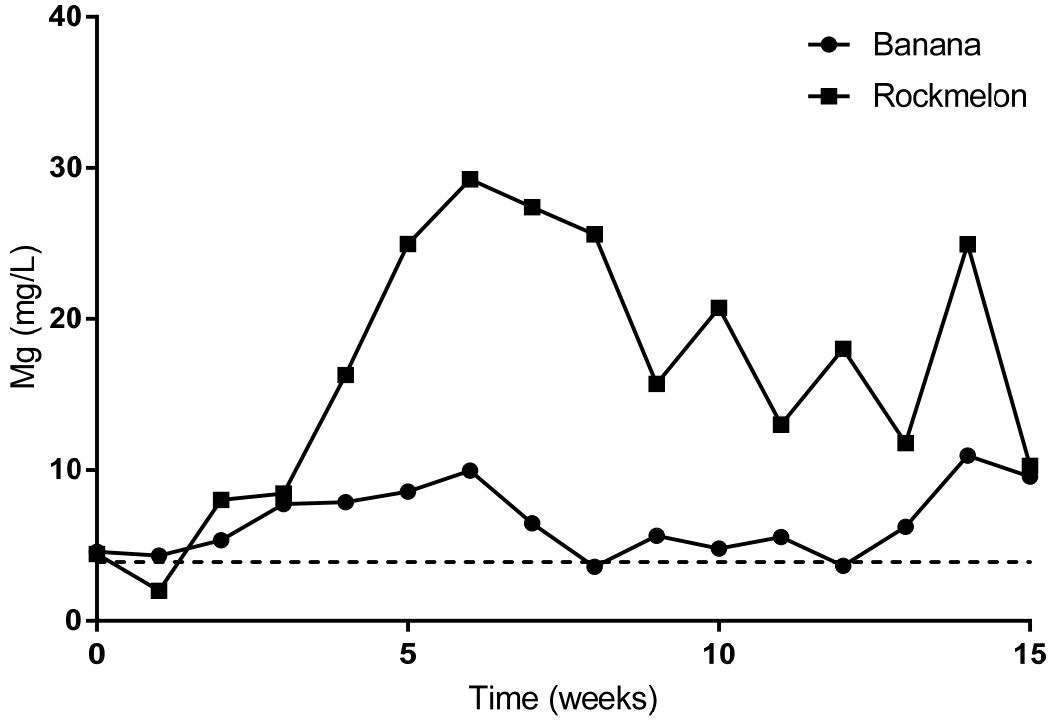


Figure D1.5 Magnesium content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.

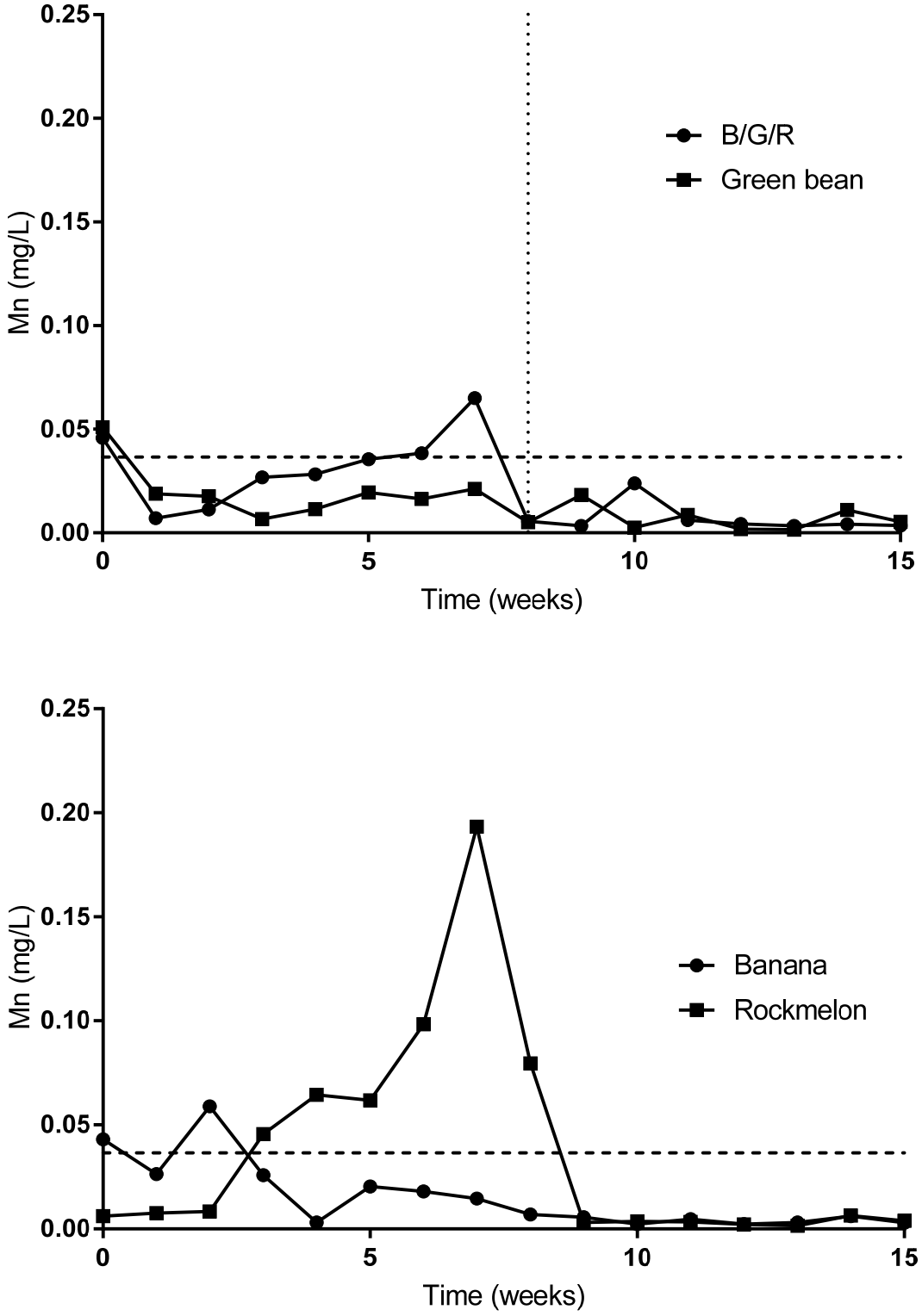


Figure D1.6 Manganese content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.

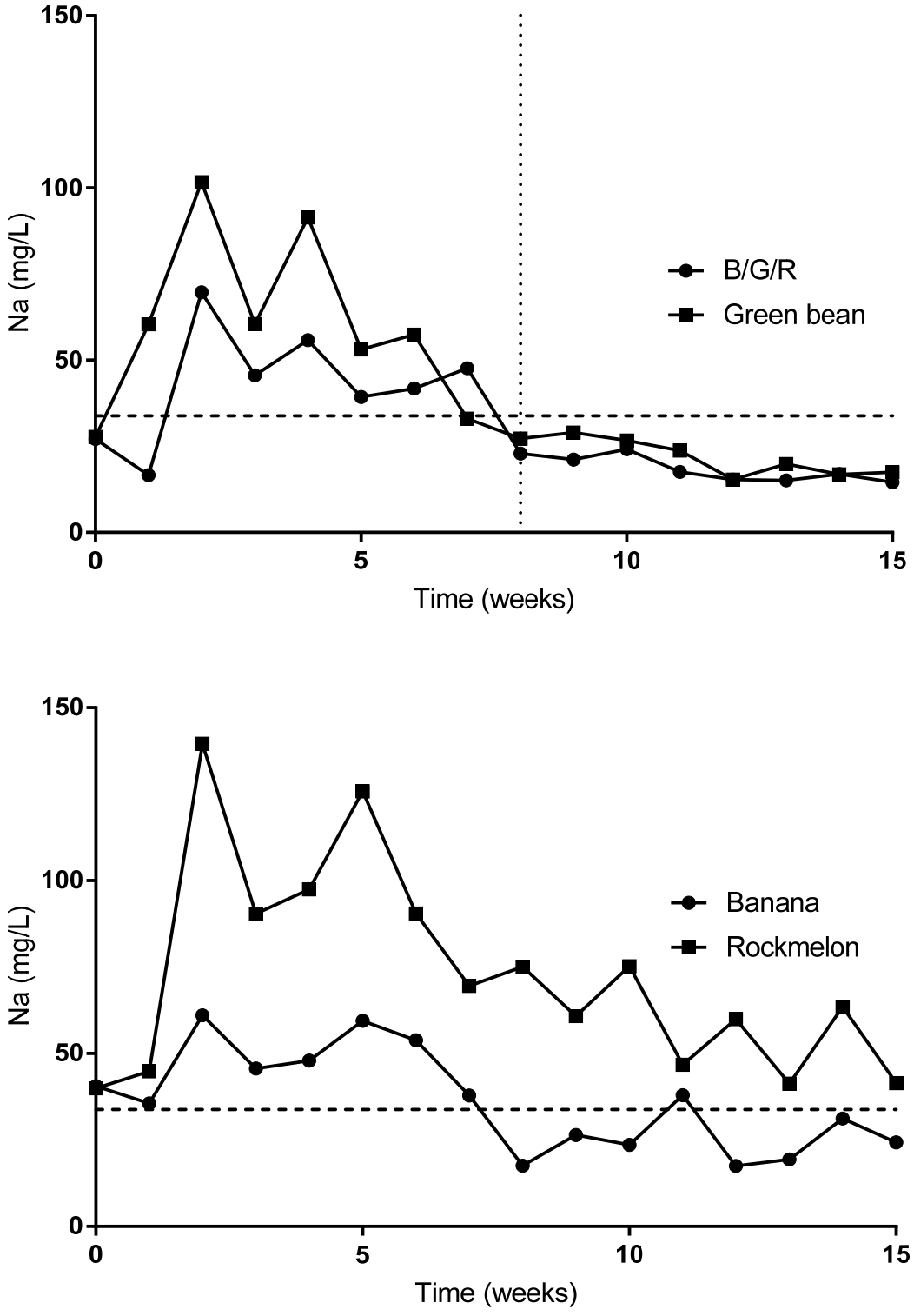


Figure D1.7 Sodium content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.

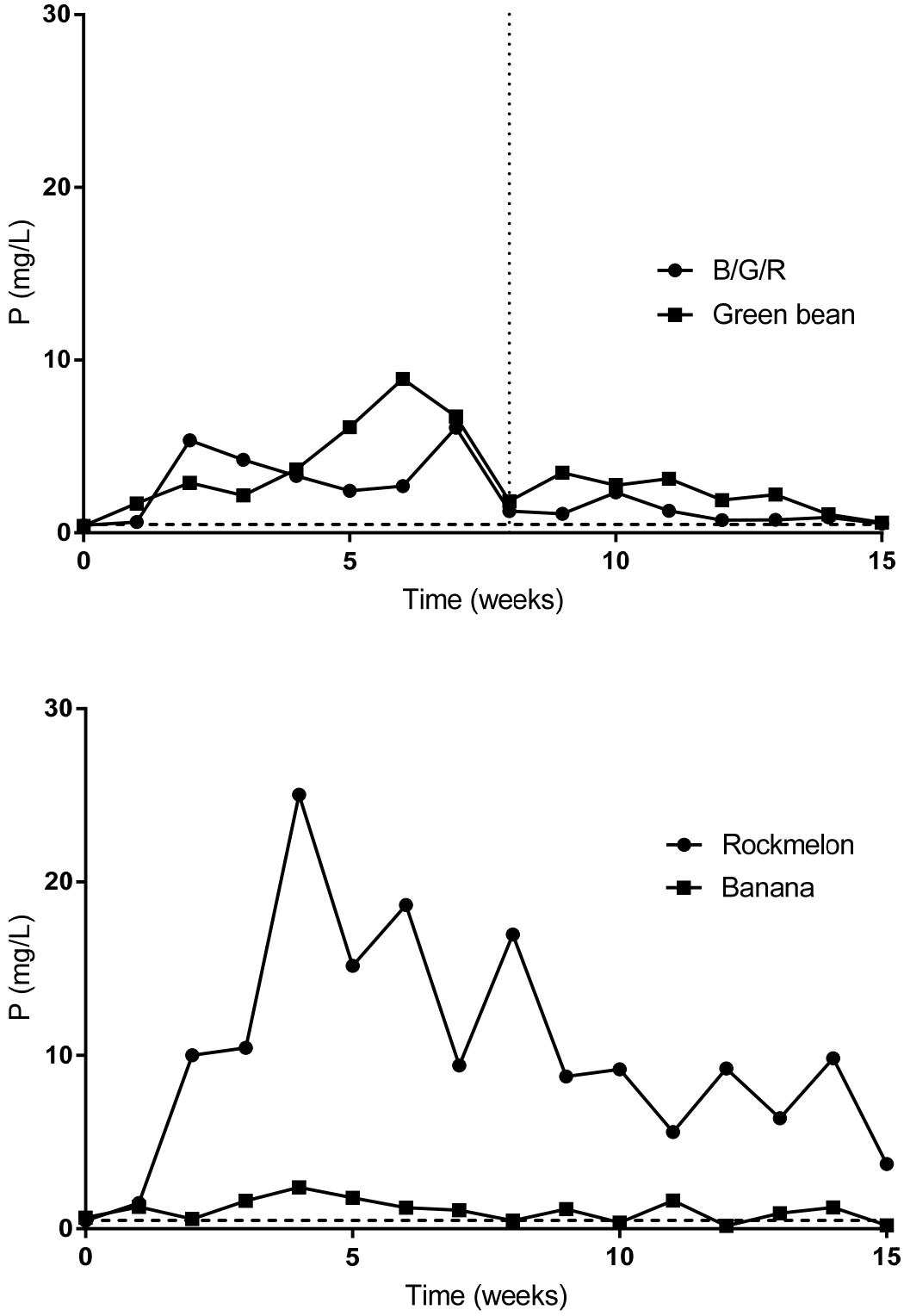


Figure D1.8 Phosphorus content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.

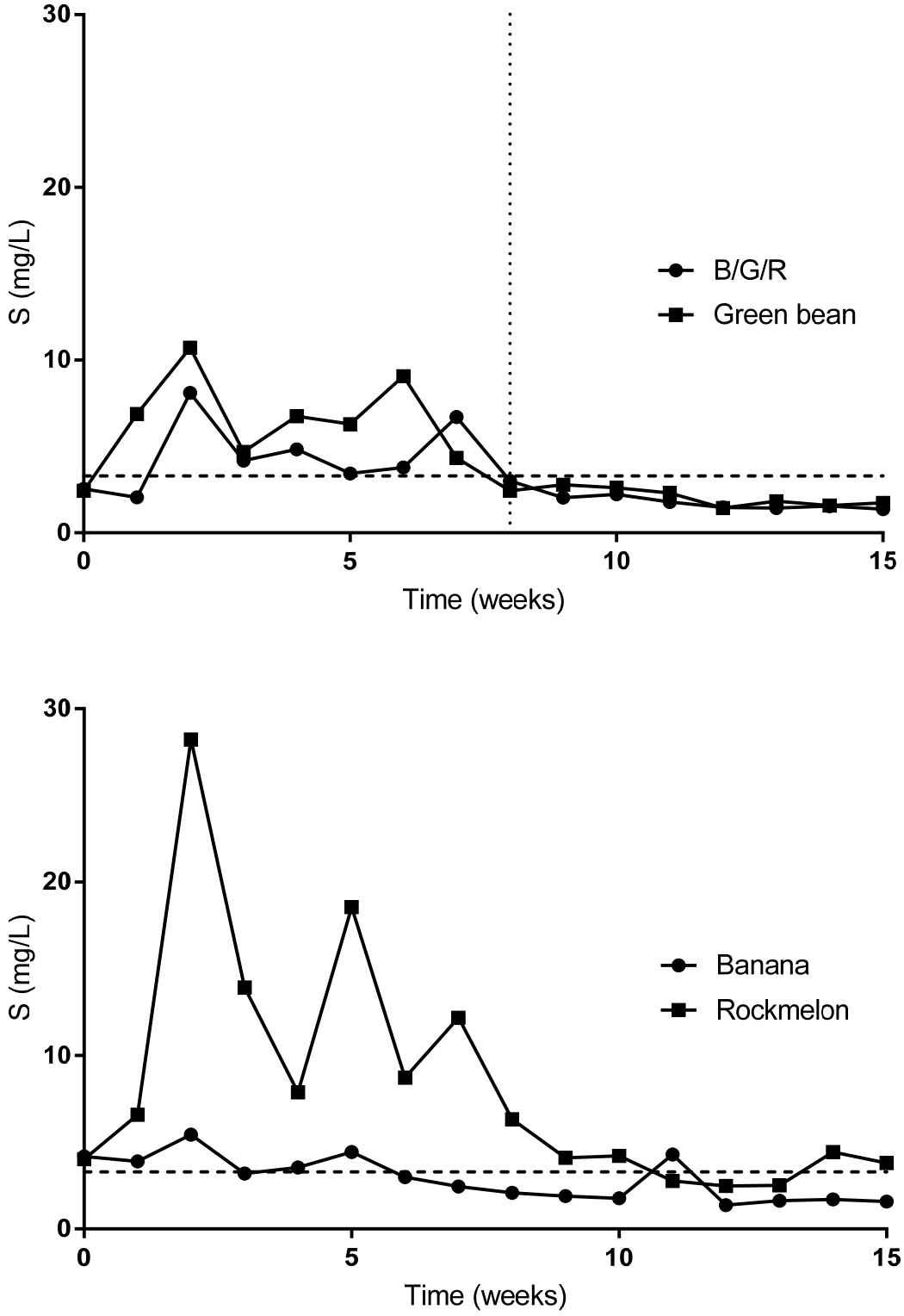
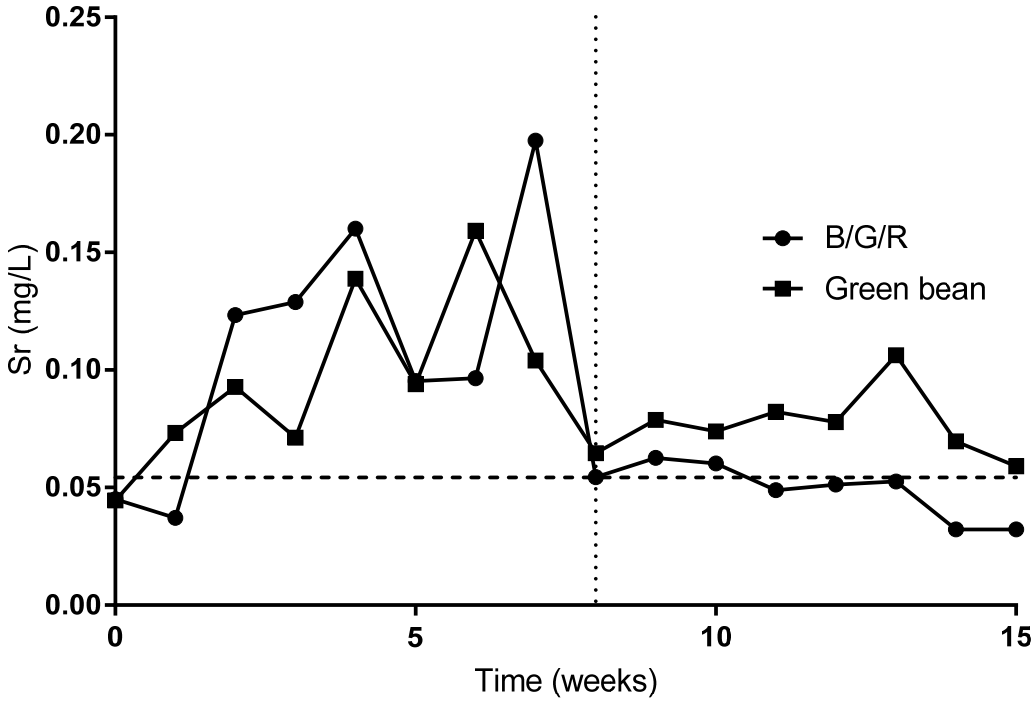


Figure D1.9 Sulphur content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.



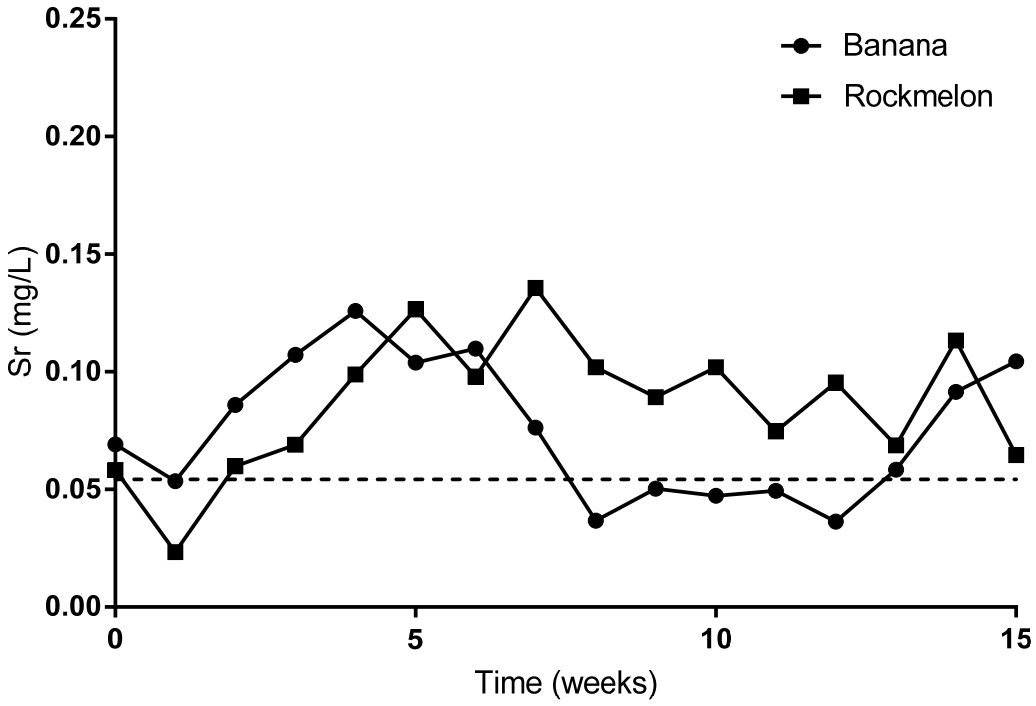


Figure D1.10 Strontium content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.

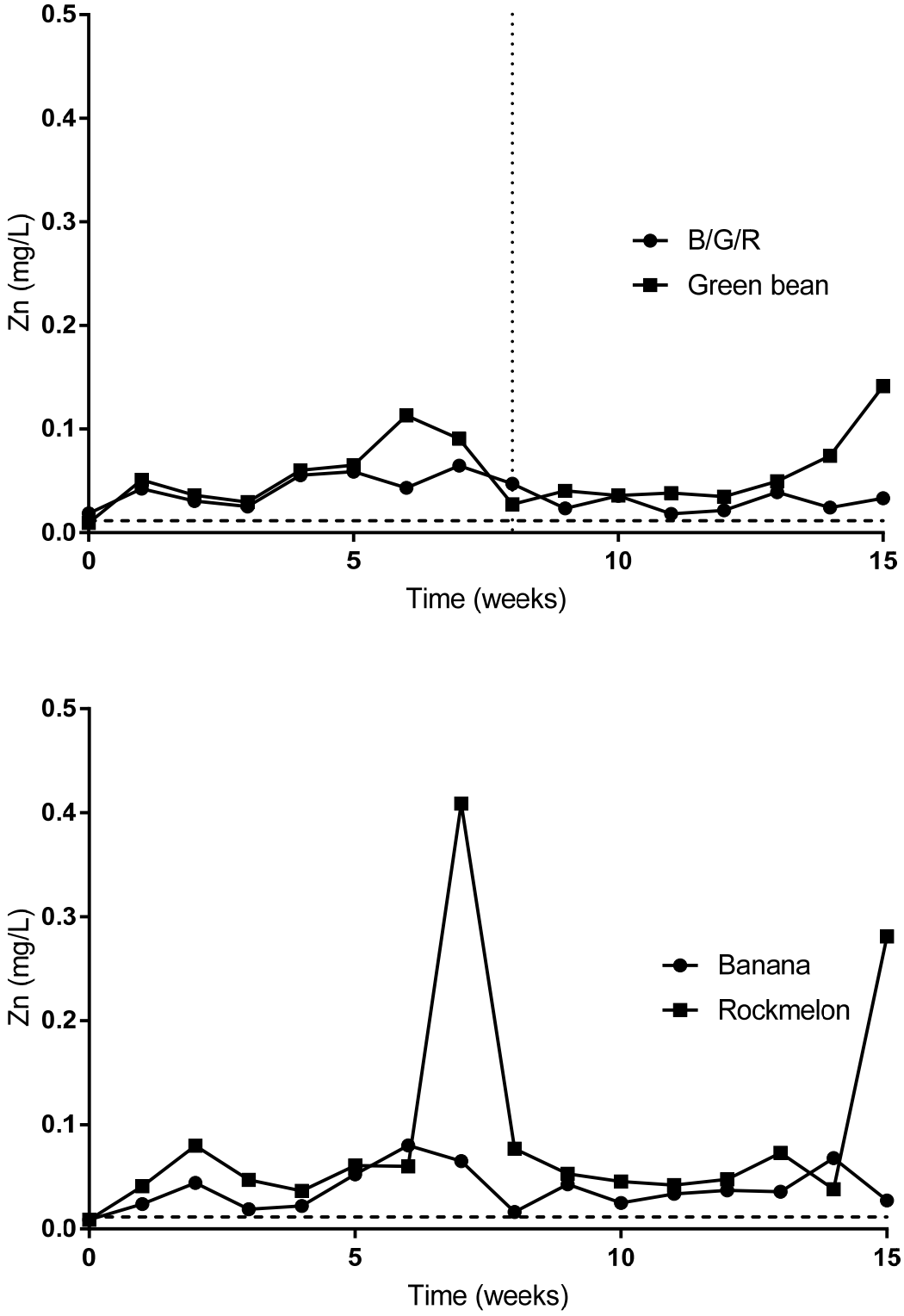


Figure D1.11 Zinc content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.

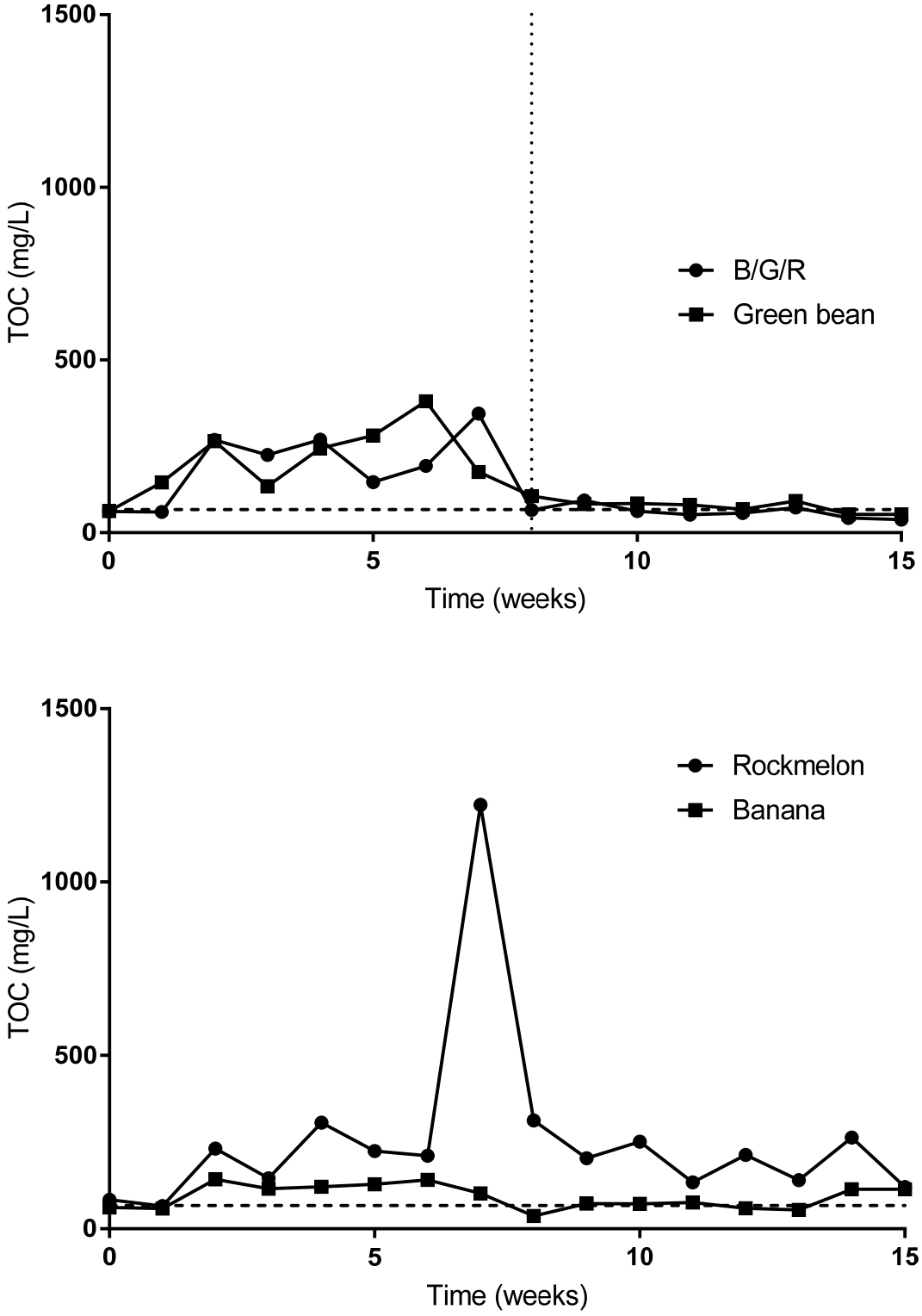


Figure D1.12 Total organic carbon content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.

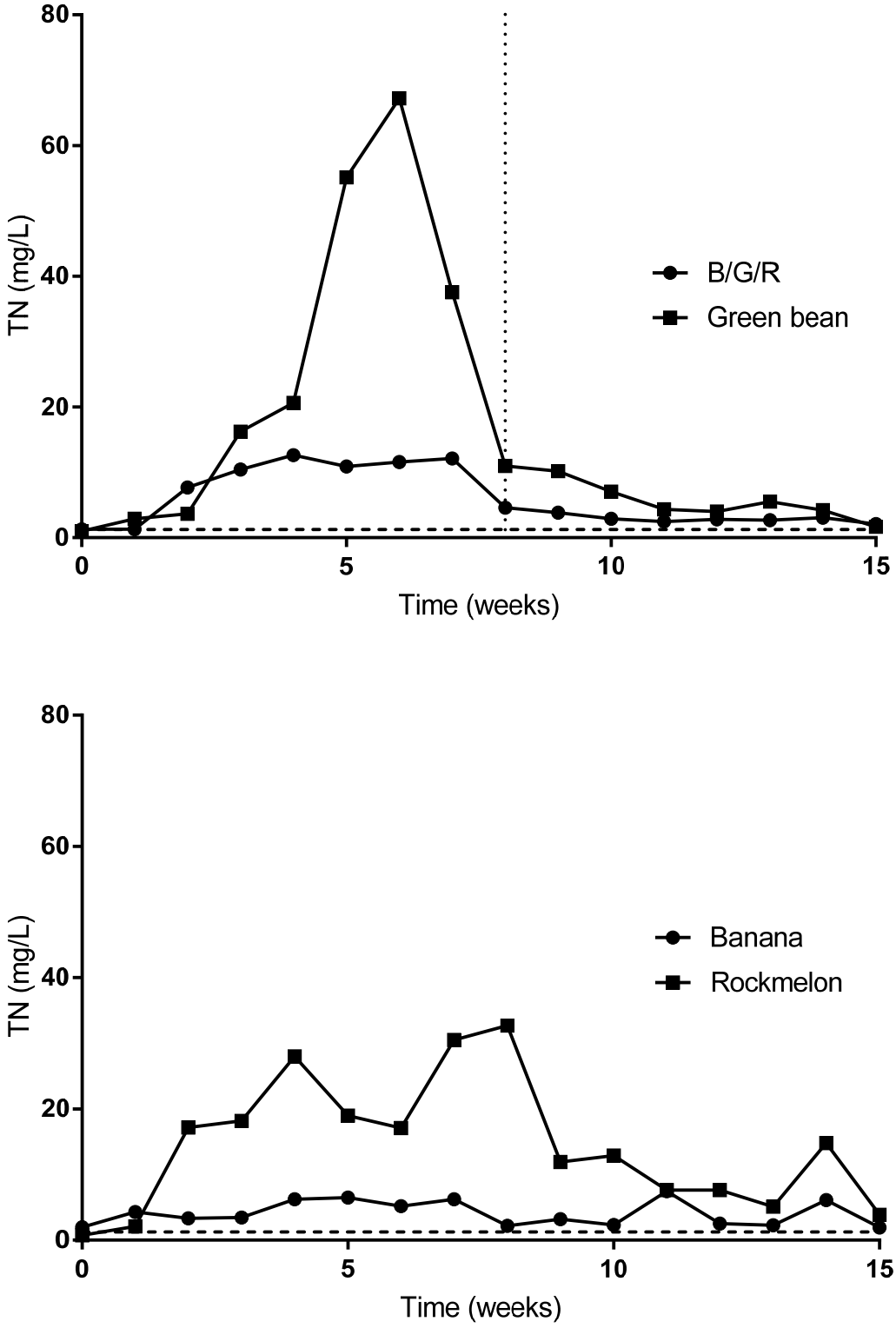


Figure D1.13 Total nitrogen content of vermicompost leachate from different worm-bed substrates. Green bean and B/G/R leachates were diluted to the same EC from 8 weeks, as indicated by the vertical line. The horizontal line indicates mean nutrient content prior to substrate addition. B/G/R = banana, green bean and rockmelon.

Appendix E

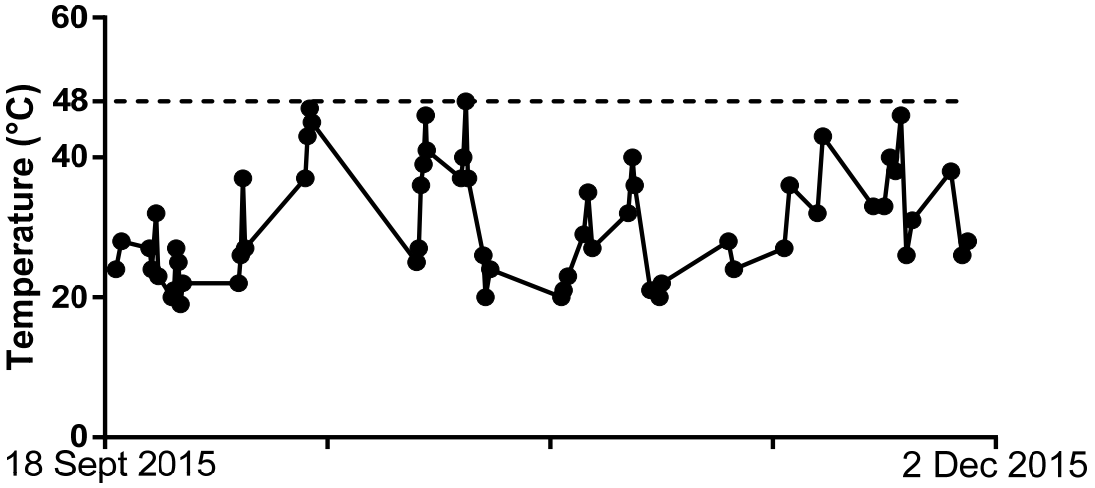


Figure E. Temperature in poly house during soybean trial. Temperature was monitored at irregular intervals. Dotted line indicates the maximum temperature.