PRODUCTION OF A GRANULAR COMPOST CARRIER FOR BACTERIAL INOCULANTS

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ABSTRACT

Bacterial inoculants play an important role in both agriculture and bioremediation. The long-term use of *Rhizobium* inoculants in agriculture has resulted in increased demands on natural peat reserves (the primary carrier material for these inoculants) and environmental concerns regarding the sustainable use of our natural resources. Methods of application of other bacterial inoculants, such as those used in the bioremediation of petroleum oil-contaminated soil, have been relatively unsuccessful. It is with consideration of these concerns that this study was undertaken to identify and develop compost as an alternative carrier material for *Rhizobium* and as an alternative to liquid bacterial inoculants for bioremediation technologies.

Composted manure was selected as the carrier material in both studies. The survival of *Rhizobium* and petroleum-hydrocarbon (phenanthrene)-degrading bacteria on granular compost was examined. In addition, the ability of these newly developed inoculants to fulfill their expected functions was assessed. For example, delivery of *Rhizobium* to leguminous roots and the degradation of phenanthrene in soil by phenanthrene-degrading bacteria.

Rhizobium leguminosarum bv. *viciae* bacteria were not detected via standard plating of non-sterile compost granules after ~30 days of storage for the composted Saskatoon Zoo waste (SZ) and ~70 days for the composted cattle manures (designated as EA, PA) at 4°C. However, survival of *R. leguminosarum* bv. *viciae* (9.28×10^7 CFU g^{-1} compost inoculant) inoculated onto sterile granular composted zoo waste was 5.36×10^5

CFU g^{-1} compost inoculant after 224 days of storage at 4°C. The populations of rhizobia present on the sterile granular composted zoo waste after 224 days of storage consistently nodulated field pea (*Pisum sativum*) roots under sterile conditions. However, variable nodulation occurred when either non-sterile or sterile, freshly-inoculated compost granules were added to soil prior to seeding pots with peas.

The sterile granulated composts in this study, due to their many similarities to inoculant-quality peat and their capability of promoting the survival of *R. leguminosarum* bv. *viciae* for an extended period of time, should be further examined as potential carriers for *Rhizobium*.

The survival of phenanthrene-degrading strains of *Arthrobacter globiformis* and *Rahnella aquatilis*, inoculated onto granular composted manure, was generally one log unit higher when stored at 4°C as opposed to 22°C after 240 days of storage. Survival was relatively consistent between the different composts. When the *A. globiformis*-inoculated compost granules were added to 1.0% phenanthrene-spiked soil no significant phenanthrene degradation occurred, possibly in part due to the lack of bacterial movement from the granules into the spiked soil environment. However, when crushed granules inoculated with phenanthrene-degrading *A. globiformis*, or a liquid broth of the *A. globiformis* were added to phenanthrene-spiked soil, a decrease in measurable volatile phenanthrene was seen.

The inoculant/contaminant contact is a very important aspect of bioremediation technologies. The use of PAH-degrading bacteria inoculated onto crushed granules by way of direct incorporation into contaminated soil or application by coating seeds used to promote phytoremediation should be investigated further.

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1.0 INTRODUCTION

Microbial inoculants contain microorganisms that are capable of performing various tasks. Some of the more popular and commonly used inoculants are those used in the inoculation of legumes with rhizobia that fix atmospheric dinitrogen and those used, to a lesser extent, as microbial inoculants applied to contaminated soil for remediation purposes.

Legumes are being cropped in many different parts of the world. It is estimated that around 1.3 – 1.5 million km^2 of land are cultivated to legumes, on average resulting in roughly 20 million tonnes of seed (Hansen, 1994). Legumes can be used for food, animal feeds, and in some tree legumes, production of antibacterial and antifungal agents, thus making them an important crop for some regions (Hansen, 1994; Roughley, 1976).

Rhizobia are a unique type of bacteria that are capable of converting atmospheric dinitrogen into ammonia that is available for plant use. These bacteria invade the roots of legumes and form a growth called the nodule, in which N_2-fixation occurs. One of the first documented uses of *Rhizobium* inoculants was the transfer of soil from a legume stand to a new site in hopes of relocating the rhizobia thought to be in the soil (Roughley, 1976). In later years, the use of pure *Rhizobium* cultures to inoculate legumes was commonly performed (Roughley, 1976; Strijdom and Deschodt, 1976).

Today, *Rhizobium* inoculants are used in all parts of the world and are applied via several different methods. In some regions the successful application of these

inoculants is essential for legume crop production (Hansen, 1994). Liquid *Rhizobium* formulations applied to seed, coating of seeds with the inoculant and the application of granular formulations at time of seeding are some of the more common application methods (Roughley, 1976). One widely used and easy mode of application is the addition of *Rhizobium* to the soil at time of seeding via a granular carrier. Over the past decade, different carrier materials have been examined for this purpose. However, the most outstanding is natural peat. Peat holds various characteristics that make it an excellent carrier material for *Rhizobium*, including high moisture holding capacity and excellent nutritional value (for the *Rhizobium*). Despite its excellent reputation as a carrier for rhizobia, peat has some shortcomings. In some parts of the world, inoculant-quality peat is unavailable making it very uneconomical to use due to the expense of importing it (Corby, 1976). In addition, the increased demand for inoculant-quality peat has resulted in peat bogs that are unable to regenerate proportionally to meet the industrial demands for production. Also, peat bog mining presents various environmental concerns and is a very expensive and labour-intensive procedure. Based on these concerns industry is currently investigating the use of alternative materials to produce a carrier for rhizobia.

Aside from *Rhizobium*-inoculant production, the production of bacterial inoculants used to remediate petroleum-contaminated soil is also of interest to scientists and industry. Petroleum oil spills date back to when drilling for oil was first performed. When spilled in terrestrial and aquatic environments, oil has the potential to harm the well-being of organisms in contact with it for an extended period of time (Edwards, 1983). Two "natural" technologies are currently being developed to aid in the reclamation of petroleum-contaminated soil and water. Bioremediation (the use of

bacteria) and phytoremedation (the use of a combination of plant and bacterial interactions) have been implemented in soil reclamation. Some of the first documented cases of successful bioremediation of oil-contaminated beaches date back to the Exxon Valdez spill (Atlas, 1995a). Various combinations of nutrients were applied to the beaches in an attempt to induce the growth and proliferation of native bacteria. This was successful in stimulating bacterial growth, and as a result, the level of contaminant left on the beaches was significantly reduced (Atlas, 1995a). Phytoremediation has also been shown to have some success in the reclamation of hydrocarbon-contaminated soil.

Some of the obstacles scientists face with the application of this technology are all of the variables involved with its application, such as environmental conditions, soil conditions and nutrient limitations (Barriault and Sylvestre, 1993). New application techniques should be examined such as coating seeds used in phytoremediation programs with hydrocarbon-degrading bacteria and the addition of hydrocarbon-degrading bacteria to contaminated soil via a granular carrier material. Further investigations into the production of a more consistent and successful delivery system for this type of inoculant, such as using solid carrier materials for bacterial delivery, could benefit the industry substantially by resulting in better degradation outcomes.

The work presented in this thesis examined two areas of inoculant formulation. Firstly, to develop a granular compost formulation that could be used as an alternative delivery system to granular peat in the inoculation of legumes with *R. leguminosarum* bv. *viciae*. Secondly, to examine the ability of granular compost to deliver hydrocarbon-degrading bacteria in a phenanthrene-(petroleum-hydrocarbon) contaminated soil environment resulting in contaminant degradation.

It was hypothesized that granular compost would perform well as an alternative granular carrier for peat and that it would also be successful in increasing remediation rates in contaminated soils by successfully delivering petroleum-hydrocarbon-degrading bacteria to phenanthrene-spiked soil.

2.0 LITERATURE REVIEW

2.1 *Rhizobium* inoculant production

2.1.1 *Rhizobium* and symbiotic nitrogen fixation

Rhizobia are Gram-negative bacteria, part of the family *Rhizobiaceae* and are naturally present in soil (Date and Halliday, 1987; Graham, 1976). These bacteria are capable of forming a symbiosis with legume roots and are important to legume production because they fix atmospheric N_2 and convert it to a usable form for plants (Appelbaum, 1990).

The formation of this symbiotic relationship is dependent on a number of factors including the type of legume and the species of invading *Rhizobium*. For example *R. leguminosarum* bv. *viciae* is host-specific for a number of plants including field pea (*Pisum sativum*), lentil and vetch (Graham, 1976). The series of events that occurs in the formation of a root nodule begins with the exudation of root signal molecules that stimulate the attachment of rhizobia to the root hairs. Following attachment the root hair curls to surround the infecting bacteria and an infection thread forms. The infection thread is a tubular passageway through the root hair that leads to the interior of the root cortical cell. As time goes on the bacteria differentiate into bacteroids, within the developing nodule, and nitrogen fixation occurs shortly thereafter (Hansen, 1994).

Aside from plant/rhizobia specificity, the soil N concentration also affects the formation of this symbiotic relationship (Date, 1976). Nitrate is the form of soil N

that is readily available for plant use (Carroll and Mathews, 1990). The amount of nitrate present in the soil is important because in most cases a legume will preferentially use the available nitrate rather than proceed with nodule production (Carroll and Mathews, 1990; Toro, 1996).

2.1.2 *Rhizobium* inoculant production: industry status

Rhizobium inoculants are one of the most widely used bacterial inoculants in agriculture (Denardin and Freire, 2000; Einarsson *et al.*, 1993). Their increased use stems from a realization of the importance of rhizobia to legume production via their role as N suppliers (Appelbaum, 1990; Hansen, 1994). The application of *Rhizobium* inoculants is generally required when rhizobia present in the soil are not compatible with the legume being cropped, the natural population of compatible rhizobia is too small to produce sufficient nodulation or if the native rhizobia have very poor nodulation capabilities (Brockwell and Bottomley, 1995; Hansen, 1994).

Today, *Rhizobium* inoculant production is a very successful industry. Viable and effective inoculants are currently in use throughout the world. Three successful forms of *Rhizobium* inoculants have been developed. The first involves the *Rhizobium* in liquid culture, applied at time of legume seeding. The second form is the application of rhizobia to a carrier material that is applied to the soil at seeding (i.e. granular inoculant). The third form is a combination of *Rhizobium* and carrier material that is applied directly to seeds as a seed coat prior to seeding (Burton, 1976). These inoculant formulations have in one way or another successfully overcome some of the obstacles involved in inoculant production such as lack of

Rhizobium survival due to desiccation and competition and ease of inoculant application.

Carrier materials are used as both a method of storage and transport. An inoculant carrier is a solid or liquid compound that can sustain live bacterial cells for an extended period of time. A number of different carriers have been developed. However, the most successful, widely accepted and commonly used is peat (Einarsson *et al.*, 1993; Hansen, 1994; Sparrow and Ham, 1983).

A concentration of 10^3-10^5 rhizobia per gram of carrier is generally required to meet Canadian industrial inoculant standards. Peat has qualities that promote the survival of *Rhizobium* cells. Such qualities include high water holding capacity, high nutritional value and protection from the environment (Biederbeck and Geissler, 1993). These qualities are crucial to the survival of rhizobia on the carrier. Despite the ability of peat to provide superior survival of rhizobia, some concerns about its use remain. For instance, a shortage of inoculant-quality peat is projected, as there are increased demands on industry for *Rhizobium* inoculants (Hafeez *et al.*, 1989). For this and other environmental and economic reasons a number of different materials have been tested for their ability to perform like peat carriers. Some of these substances include peanut hulls, corncobs, vermiculite, charcoal, pumice, wheat straw, manure, clay and compost (Chao and Alexander, 1984; Einarsson *et al.*, 1993; Sparrow and Ham, 1983). To date these alternative carriers have varied in their success and none have proven to be as successful as peat-based carriers.

One of the newer alternatives for peat-based inoculants is granular carrier material inoculated with rhizobia. *Rhizobium*-inoculated granular carriers are of

interest because they can be applied directly to the soil at time of seeding and in turn successfully deliver rhizobia to the developing legume roots. In addition, these carriers provide protection against desiccation, chemical damage and act to slowly release rhizobia throughout the primary stages of seed germination and seedling development (Hansen, 1994; Kutcher *et al.*, 2002). Survival of rhizobia on carrier materials is obviously a very important aspect of inoculant production. Survival on carriers relies on factors such as the water moisture holding capacity of the carrier, competing (indigenous) microorganisms, nutritional value and storage temperature.

Rhizobia are susceptible to desiccation and as a result the water content of the carrier material is crucial for survival (Bushby and Marshall, 1977a). For example, Strijdom and Deschodt (1976) reported reduced viability when appropriate moisture levels (~40-55% moisture) were not met in the preparation of carrier materials. Other studies also suggest a moisture level between 40 and 50% to ensure survival (Einarsson *et al.*, 1993; Rodriguez-Navarro *et al.*, 1991; Van Schreven, 1970). Conversely, Abd-Alla and Wahab (1995) indicated that a water content as low as 20% provides conditions conducive to *Rhizobium* survival. Moisture content may also influence survival in combination with other factors such as sterility.

The sterilization of carrier materials for *Rhizobium* inoculants has long been debated. Some sterilization processes include gamma irradiation, steam sterilization, and ethylene oxide treatment (Roughley and Vincent, 1967; Strijdom and Deschodt, 1976). Interest in sterilization of carrier material prior to inoculation with rhizobia is a result of inferior survival of *Rhizobium* obtained on non-sterile versus sterile carrier materials (Roughley and Vincent, 1967; Vincent, 1958). For example, non-sterile

inoculants were, in some cases, shown to contain 100-fold fewer rhizobia than sterilized inoculants (Brockwell and Bottomley, 1995). Similarly, contaminating strains of bacteria present in inoculants could include plant pathogens and other deleterious bacteria (Olsen *et al.*, 1994). Competition with indigenous bacteria, as well as indigenous populations of rhizobia present in carrier materials can negatively influence the survival of introduced rhizobia and can also decrease the ability of introduced rhizobia to nodulate target legumes (Brockwell and Bottomley, 1995; Van Schreven, 1970). Interestingly, a study by Santamaria and colleagues (2002) indicated that a non-sterile form of peat used in combination with high moisture resulted in poor survival of rhizobia. Therefore, it is apparent that many factors can simultaneously contribute to the survival of successful populations of rhizobia.

Due to peat's nutrient rich qualities, it is successful in promoting the growth of rhizobia. Unlike some other carrier materials it does not require additional nutrient supplementation in order to meet the nutritional requirements of rhizobia. However, in most cases peat does require the addition of calcium carbonate to neutralize the pH (Biederbeck and Geissler, 1993; Roughley, 1976). In addition, it is commonly found that peat can vary greatly, from batch to batch, in its nutritional characteristics (Kremer and Peterson, 1983; Sparrow and Ham, 1983). In order to select an alternative carrier material, the alternative must demonstrate the ability to supply the bacteria with all of their nutritional requirements, preferably without further nutritional inputs (Hafeez *et al.*, 1989). Sparrow and Ham (1983) tested the ability of peanut hulls, ground corncobs, charcoal, and vermiculite to support survival of *Rhizobium phaseoli* in comparison to peat. Although not superior, some

degree of success was seen with the charcoal and vermiculite. Vermiculite is also a successful medium for direct fermentation of *Rhizobium* when supplemented with additional nutrients (Graham-Weiss *et al.*, 1987).

Storage temperature of inoculants also influences the survival of *Rhizobium*. For example, survival appeared to fluctuate less when the inoculants were stored at sub-zero temperatures (Biederbeck and Giessler, 1993; Santamaria *et al.*, 2002). In addition, survival tended to decrease with increased storage temperatures (Biederbeck and Giessler, 1993; Hafeez *et al.*, 1989; Kremer and Peterson, 1983). However, in these studies the strains of *Rhizobium* and the type of carriers used varied greatly.

Compost is a nutrient-rich medium and can supply sufficient nutrition to *Rhizobium*. Little work has been done on examining compost materials as carriers for *Rhizobium* inoculants. Due to its similarity to inoculant quality peat, however, it is thought that compost could be a successful carrier.

2.2 Inoculants for remediation of hydrocarbon-contaminated soil

2.2.1 Polycyclic aromatic hydrocarbons (PAHs) in soil

Polycyclic aromatic hydrocarbons (PAHs) occur naturally in the environment and are formed when organic substances are exposed to extremely high temperatures. For example, volcanoes, forest fires and prairie fires result in the release of PAHs. Polycyclic aromatic hydrocarbons also may originate from sewage sludge, wood treatment processes and are present in petroleum oil (Edwards, 1983; Goodin and Webber, 1995; Henner *et al.*, 1997; Juhasz *et al.*, 1996).

Polycyclic aromatic hydrocarbons are compounds made of two or more fused benzene rings, consisting of carbon and hydrogen, that can be oriented in either a linear, angular or cluster arrangement (Edwards, 1983; Reilley *et al.*, 1996). These structures are hydrophobic (Reilley *et al.*, 1996) and the ring size and arrangement are related to the biodegradability of the PAHs (Henner *et al.*, 1997). For example, PAHs with four or more aromatic rings fused together tend to be more resistant to microbial degradation than PAHs with structures containing less than four aromatic rings due to their increased insolubility (Li *et al.*, 1996; Trzesicka-Mlynarz and Ward, 1995).

The bioavailability of PAHs is a very important factor in the successful remediation of these compounds (de Jong *et al.*, 1997). Polycyclic aromatic hydrocarbons may react differently when spilled on soil due to the soil's physical, chemical and biological characteristics (Carmichael and Pfaender, 1997). For instance, PAHs will adhere strongly onto the surface of clay minerals, soil humus and other organic solids, thus making them relatively unavailable (Madsen and Kristensen, 1997). The nutrient status of the soil may also contribute to the successful biodegradation of PAHs. For example, a soil that is low in nitrogen, phosphorus and inorganic nutrients may exhibit delayed rates of PAH biodegradation (Steffensen and Alexander, 1995). In addition, the microbial diversity of the soil, as well as physical factors including moisture and oxygen content, temperature and pH all affect the fate of PAHs in soil (Margesin *et al.*, 2000; Sztompka, 1999; Whyte *et al.*, 1998).

2.2.2 Bioremediation

Bioremediation is considered to be one of the most important processes for the natural removal of PAHs from soil (Madsen and Kristensen, 1997; Margesin and Schinner, 1999a; Trzesicka-Mlynarz and Ward, 1995). Bioremediation is a technology that involves the natural breakdown of toxic substances in the environment by microbial processes such as mineralization, detoxication and cometabolism (Atlas, 1995a; Bollag *et al.*, 1994; Riser-Roberts, 1998).

In general, heterotrophic bacteria and fungi (microbes that obtain energy and carbon for growth from the decomposition of organic matter) are the key organisms in the soil environment with relation to bioremediation. Some common genera of soil bacteria implicated in the degradation of petroleum hydrocarbons include *Acinetobacter* sp., *Arthrobacter* sp., *Bacillus* sp., *Corynebacterium* sp., *Flavobacterium* sp., *Micrococcus* sp., *Nocardia* and *Pseudomonas* sp. (Chaineau *et al.*, 1999; Reilley *et al.*, 1996; Riser-Roberts, 1998; Whyte *et al.*, 1998). As well, white rot fungus (*Phanerochaete chrysosporium)* has been implicated in the bioremediation of PAHs (Bogan and Lamar, 1996; Brodkorb and Leege, 1992).

As previously mentioned, a number of factors influence the fate of PAHs in soil. Many of these same factors contribute to the overall success of bioremediation. Soil moisture, temperature, pH, oxygen supply, nutrient status, organic matter and soil structure can influence the rate of PAH biodegradation in soil (Ellis *et al.*, 1990; Riser-Roberts, 1998).

Moisture is needed for most processes in nature, including microbial growth. A highly saturated soil will produce an anaerobic environment and will reduce the extent of bioremediation due to changed nutrient status and oxidation-reduction potentials (Riser-Roberts, 1998). Conversely, extremely low amounts of water in soil will greatly retard microbial growth and metabolism as well as biodegradation rates (Riser-Roberts, 1998).

All natural processes have an optimum temperature. Soil temperature plays an important role in regulating these processes as well as influencing microbial growth. Thus, the optimal temperature requirements exhibited by various soil bacteria may be of use in applying bioremediation technologies to different parts of the world that have vastly different climates. For instance, cold adapted microorganisms have demonstrated the ability to degrade diesel oil under temperature conditions as low as 10°C (Margesin and Schinner, 1999b).

The influence of soil pH is similar to that of soil temperature in that soil microorganisms have an optimum pH at which they can grow and survive. A slight fluctuation in this optimum pH could end or subdue the growth of the microorganisms. The optimum soil pH for degradation of PAHs is in the range of 6.5-8.5 (Riser-Roberts, 1998), which encompasses the optimum pH range for many soil bacteria.

Oil provides a barrier through which oxygen cannot diffuse. This can lead to anaerobic conditions in the soil environment, limiting the processes and growth that may otherwise occur. Many soil microorganisms use oxygen as an electron carrier and thus require it for growth (Riser-Roberts, 1998). Some microorganisms,

however, have evolved to use other sources of electron carriers, such as nitrate in the case of denitrifying bacteria (Ivanov *et al.*, 1995).

The soil structure (pore spaces) has a large influence on the nutrient status of a soil and is influenced by the organic matter content of the soil. For example, soil pores provide microhabitats in which soil microorganisms live. In the microhabitats, the microorganisms find nutrients, water, a means of transportation and protection from predators. If the volume of soil occupied by pores is reduced or if the pores are saturated with water, the flow of nutrients is greatly decreased. This may influence the ability of non-motile microorganisms to access the nutrients essential for their growth. Similarly, if there is too little moisture in the soil pores the mobile microorganisms will be unable to relocate to sources where nutrients are abundant, for instance where PAH contaminants may be deposited. Consideration of all the environmental properties that affect bioremediation is an essential step in the development of a successful bioremediation strategy.

2.2.3 Current status of bioremediation technology

Several different approaches have been taken to develop bioremediation programs. These include the addition of nutrients to contaminated soil, the addition of pure cultures of PAH-degrading microorganisms and the addition of consortia of PAH-degrading microorganisms.

In the first approach to bioremediation, nutrients are added to the contaminated soil. One of the first applications of this bioremediatory technology came with the Exxon Valdez spill (Atlas, 1995b). The general approaches taken in

this massive clean-up project were the application of oleophilic, water soluble, and slow release fertilizers. Different combinations of these fertilizers were applied and resulted in a visible reduction in the amount of oil on the contaminated beaches as compared to untreated beaches (Atlas, 1995b). In most cases, nutrient application increased the rates of degradation to 3-5 times that of the natural rates present in untreated beaches (Atlas, 1995b). Concerns about the environment, such as eutrophication (formation of algal blooms in the treated areas) and acute toxicity to sensitive fish and invertebrates, were resolved when numerous tests indicated no such environmental effects. This approach to bioremediation over came one of the major obstacles in this area of study, specifically, nutrient availability.

Steffensen and Alexander (1995) demonstrated that the availability of nutrients, such as N and P, is very important for ensuring contaminant degradation. This importance relates to nutrient competition between microbes. For example, non-degrading microorganisms will compete with degrading microorganisms for nutrients in a contaminated area and as a result, the degrading bacteria may be out-competed, resulting in less degradation. In another bench-scale investigation, Taylor and Viragaghavan (1999) found that the greatest degradation of diesel-contaminated soil was seen in the soils amended with nutrients as compared to non-amended soils.

The second approach to bioremediation is through the addition of pure cultures of contaminant-degrading microorganisms. A large number of bench-scale investigations support the successful degradation of PAHs using pure cultures of bacteria and fungi (Boyle *et al.*, 1998; Brodkorb and Leege, 1992; Juhasz *et al.*, 1996; Madsen and Kristensen, 1997; Martin *et al.*, 1995; Trzesicka-Mlynarz and

Ward, 1995; Whyte *et al.*, 1998). However, in many of these studies only the degradation of PAHs in solution was examined.

To date, there has been little work done to assess the degradation of petroleum PAHs in soil by consortia of microorganisms. However, one study by Trzesicka-Mlynarz and Ward (1995) involved assessing the ability of both pure cultures and a consortium of bacteria to degrade PAHs in aqueous media. In this case, the consortia were able to degrade a wider range of PAHs to a fuller extent than the purified cultures. In order to fully understand the mechanisms behind the use of pure cultures and consortia of microorganisms for bioremediatory purposes, more research is needed.

2.2.4 Phytoremediation

Phytoremediation is a remediation technology that implements the use of plants (natural or genetically modified) for the reclamation of land contaminated with numerous kinds of toxic substances (Flathman and Lanza, 1998; Nyer and Gatliff, 1996). Plants help to remediate contaminated soil by transpiration of volatile hydrocarbons out of the soil, accumulation within plant tissues, adsorption onto the plant's roots, stabilization of the hydrocarbons and the stimulation of microorganisms in the rhizosphere of the plants (Cunningham and Ow, 1996; Flathman and Lanza, 1998; Raskin *et al.*, 1998; Watanabe, 1997).

The volume of soil that is under direct influence of the plant root is called the rhizosphere (Keister and Cregan, 1989). The microorganisms associated with the rhizosphere are called rhizobacteria. The exudation of compounds from the roots

stimulates the activity of the rhizobacterial communities (Tate, 1995). This effect is known as the rhizosphere effect.

Microorganisms in the soil are subject to a number of pressures that affect their ability to survive and thrive. The factor that most limits their growth is substrate depletion (Burges and Raw, 1967). Therefore, plant roots are important because they function to provide the rhizobacteria with exudates important for growth and also provide a unique environment for microbial communities. Furthermore, roots also provide a surface for the bacteria to colonize, as well as function to increase the contact area between the bacteria, soil and PAHs. In addition, it has been reported that roots may function to increase rates of degradation by releasing compounds that are analogs of the contaminants and, as a result, induce production of enzymes capable of degrading the contaminants (Reynolds *et al.*, 1999).

Many different types of plants have been used in phytoremediation studies. Some plant species used are grasses such as tall fescue, sudan grass, switch grass, rye grass, alpine pennygrass, and other plants such as alfalfa, and duck weed to name a few (Aprill and Sims, 1990; Comis, 1996; Gunther *et al.*, 1996; Huang *et al.*, 1993; Schwab and Banks, 1994).

The use of plants to increase rates of petroleum-hydrocarbon degradation has had some success. Studies indicate that the addition of plants to a contaminated site greatly increase the rate and degree of degradation (Aprill and Sims, 1990; Gunther *et al.*, 1996; Schwab and Banks, 1994). These studies investigated using different prairie grasses and other plant species to degrade PAHs in soil. The addition of

plants to the contaminated soil had a significant effect on the degradation of the PAHs in the soil.

Currently, little research has focused on the coating of seeds with PAH degrading microorganisms for use in phytoremediation strategies. In addition more research is needed in methods of applying the PAH-degrading microorganisms to soil.

3.0 PRODUCTION OF A GRANULAR COMPOST CARRIER FOR *RHIZOBIUM* INOCULANTS

3.1 INTRODUCTION

Rhizobia are a very important part of legume production in many parts of the world. It is estimated that these bacteria provide 70 million metric tonnes of N globally by way of atmospheric N_2-fixation (Brockwell and Bottomley, 1995). *Rhizobium leguminosarum* bv. *viciae* forms a symbiotic relationship with the root systems of field pea (*Pisum sativum*). Due to the efficiency of this bacterium, and similar species, to provide N to legumes via methods other than chemical application, the demand for *Rhizobium* inoculants has escalated. This increased demand is also a result of increased legume cropping throughout different parts of the world (Graham-Weiss *et al.*, 1987).

Rhizobium inoculants can be applied in a number of ways. Two of the most commonly used *Rhizobium* inoculants are granular inoculants and the seed-coat powdered inoculants (Bashan, 1986; Bezdicek *et al.*, 1978; Denardin and Freire, 2000; Olsen *et al.*, 1994). Two popular formulations of granular Rhizobium inoculants include carrier materials being composed of clay and also peat. Clay based inoculants are very successful when supplemented with nutrients because of their excellent water holding capacity. One clay that has been examined as a carrier material is montmorillonite, supplemented with nutrients. Bushby and Marshall (1997a, 1997b) found that when supplemented with nutrients, montmorillonite promoted the survival of high populations of rhizobia due to the excellent water

holding capacity of the carrier. Peat-based inoculants are produced from peat harvested from natural peat reserves. Depending on where these reserves are located, this procedure can be very expensive and labour intensive. In addition, increased legume production has increased the demands on peat bogs and, in some cases the demand for inoculant-quality peat is greater than the time needed for the bogs to regenerate. These concerns, as well as other environmental concerns have prompted the scientific community to research alternative carrier materials for *Rhizobium*.

Alternative carrier materials that have been evaluated include corncobs, charcoal, vermiculite, manure, wheat straw, and to a lesser extent, compost (Hafeez *et al.*, 1989; Sparrow and Ham, 1983). In general, these carrier materials were tested for their ability to support the long-term survival of *Rhizobium* cultures. Thus far, none of these materials has proven to be superior to inoculant-quality peat. However, alternatives such as vermiculite, vermiculite supplemented with nutrients and charcoal show potential (Sparrow and Ham, 1983). Little work has been done investigating different composted materials as an alternative carrier for *R. leguminosarum* bv. *viciae*.

Compost is generally high in nutrients and holds many similarities to inoculant-quality peat. If successful, *Rhizobium*-inoculated granulated compost could provide a successful and easy method for the application of this type of inoculant. To investigate the ability of compost to deliver *R. leguminosarum* bv. *viciae* to pea plants (*Pisum sativum*) the following objectives were investigated:

1. To quantify the survival of *Rhizobium leguminosarum* bv. *viciae* on sterile

and non-sterile compost granules; and

2. To assess the ability of the granular inoculant to deliver nodulating rhizobia in sterile and non-sterile growth chamber conditions.

3.2 MATERIALS AND METHODS

3.2.1 Compost descriptions

Three different composts were obtained for potential use as carriers for *Rhizobium leguminosarum* bv. *viciae*, and are described in Table A1 (Appendix A). Two inoculant-quality peat products were also included in the examinations as a means of comparison to a model carrier material (peat). An assessment of the physical characteristics was completed for the composts and two powdered peat samples prior to this study (Table 3.2.1). Similarly, an evaluation of the chemical characteristics of the composts was performed and is summarized in Table A2 (Appendix A). A series of microbiological tests were also performed to assess the bacterial, fungal and pathogenic bacterial populations present in the non-sterile composts and peat samples. This data is presented in Table 3.2.2. Based on the results of these preliminary screenings it was concluded that the three composts were very similar to the inoculant quality peat samples and consequently the composts were chosen for further study. The three composts were designated PA, EA and SZ. The PA compost was obtained from Corcan Agribusiness, Prince Albert SK and consisted of composted cattle manure mixed with straw. The EA compost was obtained from EcoAg Initiatives, Calgary AB and consisted of horticultural grade compost from cattle manure and animal bedding. The SZ compost was obtained from the Forestry Farm Park Saskatoon Zoo, Saskatoon SK and consisted of composted animal waste and bedding material mixed with horticultural waste, grass clippings and leaves. Two commercial granular inoculants (BU and LT) were selected

Table 3.2.1. Physical characteristics of non-sterile raw compost[†].

Compost	Characteristic										
	pH	EC (dS m-1)	TOC (%)	TN (%)	TS (%)	C:N ratio	C:S ratio	N:S ratio	Total solids (%)	Ash content (%)[‡]	Water holding capacity (%)
EA	7.00	5.12	20	1.66	0.41	12:1	49:1	4:1	55.6	56	62
PA	7.13	1.84	12	1.36	0.3	9:1	40:1	5:1	54.9	73.9	69
SZ	7.31	2.58	5.97	0.48	0.044	12:1	136:1	11:1	68.7	77.8	41
Peat 1	6.63	1.40	32.9	2.07	0.49	16:1	67:1	4:1	96.7	44.7	>100
Peat 2	4.48	0.65	30.9	1.37	0.32	23:1	97:1	4:1	56	36.8	81

[†] The raw products were supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; Peat 1 from Becker Underwood, Saskatoon, SK; and Peat 2 from LiphaTech, Milwaukee, WI.

[‡] Calculated as a percentage of the total solids.

Table 3.2.2. Microbiological analysis of non-sterile raw compost and peat materials.

Compost†	TSA‡	MCA‡	MC-ISA‡	CDA‡	AA‡
	Colony forming units (CFU) g^{-1} compost				
EA	2.0×10^7	5.5×10^7	1.0×10^5	3.5×10^3	1.4×10^6
PA	5.2×10^7	4.5×10^4	1.5×10^5	3.5×10^3	1.5×10^6
SZ	3.5×10^8	5.0×10^2	1.0×10^3	2.5×10^3	1.5×10^4
Peat 1	5.0×10^5	0	0	6.5×10^4	1.5×10^3
Peat 2	1.5×10^8	6.5×10^3	2.5×10^2	6.0×10^5	6.9×10^5

† The raw products were supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; Peat 1 from Becker Underwood, Saskatoon, SK; and Peat 2 from LiphaTech, Milwaukee, WI.

‡ Media used include Tryptic Soy Agar (TSA) for enumeration of total heterotrophic bacteria; MacConkey's Agar (MCA) for enumeration of total *Enterobacteriaceae*; MacConkey's-Inositol-Carbenicillin Agar (MC-ISA) for separation of presumptive Enterobacteriaceae and non-Enterobacteriaceae; Czapek-Dox Agar (CDA) for enumeration of fungi; and Actinomycete Agar (AA) for enumeration of actinomycete populations.

as commercial standards for comparison. The commercial granular inoculant obtained from MicroBio RhizoGen Corp., Saskatoon, SK (BU) (referred to as Becker Underwood in this thesis) consisted of a clay-based rather than a peat-based granule formulation.

Aside from their similarity to peat with regards to their microbiological, physical and chemical attributes, these composts were of the type A classification, indicating restricted use in horticulture and agriculture. In addition the three composts came from sources that have repeatedly produced uniform compost lots and have high quantities available for commercial sale. Finally, the absence of pathogens was also a key factor in the selection of these three composts (Table 3.2.2).

3.2.2 Raw material preparation and granulation

The raw powdered forms of the EA, PA and SZ composts were air-dried (3 days, 25°C) and sieved (2 mm). The sieved compost was finely ground and stored at 22°C until

processed into granules. The fine grinding was necessary to assist in granule production. Granules were formed using a roller drum obtained from PCS Potash Corporation (Saskatoon, SK) and was operated at a setting of 8. Finely ground compost (~200-400 g) was added to the operating drum and immediately sprayed with distilled water (~500 mL total) via a plastic bottle with an adjustable misting head. Additional amounts of compost and water, respectively, were added to the moistened compost until granules formed. The granules were dried (24 hours, 22°C). The dried granules (varying in size) were sieved (1.168 - 2 mm) and collected. This size was of interest because of its similarity to the commercial granular inoculants. The dry granules were packaged in polyethylene bags and stored (4°C) until inoculated.

3.2.3 Survival studies

Survival of *Rhizobium leguminosarum* bv. *viciae* on non-sterile EA, PA and SZ compost granules

Experimental parameters

The treatments included in this study were non-sterile peat granules and the EA, PA, SZ composts, all of which were freshly inoculated with a commercial strain of *R. leguminosarum* bv. *viciae* (M1) supplied by Becker Underwood, Saskatoon, SK. The peat granules were prepared using the same technique as the compost granules using finely ground peat, supplied by Becker Underwood, as the raw material. The BU and LT commercial inoculants were included as positive controls. The treatments were stored at 4°C and enumerated weekly for a period of 77 days, after which time enumerations ceased based on obtainment of acceptable enumeration lower limit (10^3 rhizobia g^{-1} compost). A low limit of detection (10^3 rhizobia g^{-1} compost) was set to

indicate a point at which the composts no longer retained *Rhizobium* populations acceptable for industry approval standards. After this point, composts were no longer enumerated for their *Rhizobium* populations.

Culture preparation and carrier inoculation

A commercial strain of *R. leguminosarum* bv. *viciae* (M1) was grown in Yeast Extract Mannitol broth (5 days) on a rotary shaker (160 rpm, 22°C) (Siciliano and Germida, 1997). The culture was then centrifuged in sterile tubes (20 minutes, 5,000 g at 4°C) and re-suspended in sterile distilled water to a concentration of 10^{10} cells/mL (Nijhuis *et al.*, 1993).

Inoculation was completed in a laminar flow hood to prevent airborne contamination. The compost granules were spread out over a sheet of sterile Kraft paper. *Rhizobium* cells were applied to the granules using a sterile water bottle with an adjustable misting head. A consistent inoculation of the granules was obtained by ensuring that all sides of the granules were inoculated through rolling and mixing the granules during the inoculation. The low percent of recovery after inoculation (~ 0.6% of the original inoculum) may in part be due to heat created by rehydration of the compost granules, a common occurrence in inoculant production. The final moisture content of the granules was adjusted to 20% (Abd-Alla and Wahab, 1995).

The number of the *Rhizobium* cells present in the commercial inoculants and compost and peat granules on Day 0 of the experiment is presented in Table 3.2.3. All treatments were replicated four times.

Table 3.2.3. Enumeration of rhizobia present in commercial inoculants, inoculated peat granules, and non-sterile compost granules on Day 0 of the survival monitoring using standard plating experiment[†].

Inoculant[‡]	Rhizobia CFU g^{-1}
EA	9.25×10^7 (1.71×10^7)[§]
PA	7.88×10^7 (1.75×10^7)
SZ	2.50×10^7 (1.47×10^7)
Peat	3.73×10^7 (2.12×10^6)
BU	2.49×10^7 (6.42×10^6)
LT	7.50×10^4 (1.00×10^4)

[†] Enumeration was performed immediately following granule inoculation.
[‡] The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; Peat granules prepared from peat obtained from Becker Underwood, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech; Milwaukee, WI.
[§] Number in brackets is the standard deviation of the mean of 4 replicates.

Survival monitoring via standard plating

Sub-samples (4.5 g) of the inoculated compost and commercial inoculants were aseptically weighed out and transferred to sterile polyethylene Whirlpac® bags and sealed.

All samples were placed in a larger polyethylene bag, sealed and placed in a cardboard box (in order to remove samples from possible exposure to light) and stored in a refrigerator (4°C) for the incubation period (77 days). Four degrees Celsius was chosen as the storage temperature because literature supports less fluctuation in *Rhizobium* populations when inoculants are stored around sub-zero temperatures (Sanatamaria *et al.*, 2002).

Yeast Extract Mannitol Agar with Congo Red indicator dye (YEM) has long been used as a selective medium for growing *Rhizobium* (Vincent, 1970) and was the enumeration medium of choice in this experiment. On the sampling day, one Whirlpac® bag from each treatment was removed from cold storage. Four sub-samples (1.0 g) of each inoculant were aseptically diluted in 99 mL of sterile phosphate buffered saline (PBS) containing 1.2 g Na_2HPO_4, 0.18 g NaH_2PO_4, and 8.5 g NaCl per Litre of distilled water, pH adjusted to 7.6. The dilution was hand shaken vigorously for 30 seconds, allowed to rest for 30 seconds and then shaken for an additional 30 seconds. This shaking scheme ensured that the granules were broken-up and well dispersed. Serial dilutions (1:10) in the range of 10^5 to 10^7 were made into blanks of sterile PBS. Aliquots (0.1 mL) of the dilutions were plated on YEM and incubated (6 days, 27°C). Colonies exhibiting the common morphological characteristics of *R. leguminosarum* bv. *viciae* (off-white colonies covered with a gelatinous polysaccharide material) were distinguished from non-*Rhizobium* colonies that appeared dark red because they lacked the polysaccharide material and absorbed the red dye.

Survival of *Rhizobium leguminosarum* bv. *viciae* on sterile EA, PA and SZ compost granules

Experimental parameters

The treatments included in this study were sterile granular EA, PA and SZ granular composts freshly inoculated with a commercial strain of *R. leguminosarum* bv. *viciae* (M1), and BU and LT commercial inoculants.

Culture preparation and carrier inoculation

The compost granules used in this experiment were sterilized by Becker Underwood using gamma irradiation (20 kgrads).

Table 3.2.4. Enumeration of rhizobia present in commercial inoculants and sterile compost granules on Day 0 of the survival monitoring using standard plating experiment[†].

Inoculant[‡]	Rhizobia CFU g^{-1}
EA	4.88×10^6 (1.60×10^6)[§]
PA	3.63×10^7 (4.44×10^6)
SZ	9.28×10^7 (4.66×10^6)
BU	2.53×10^7 (5.68×10^6)
LT	1.90×10^4 (7.08×10^3)

[†] Enumeration was performed immediately following granule inoculation.
[‡] The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; Peat granules prepared from peat obtained from Becker Underwood, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech; Milwaukee, WI.
[§] Number in brackets is the standard deviation of the mean of 4 replicates.

The properties of the non-sterile and sterile raw compost materials are presented in Table A2 (Appendix A).

The gamma-irradiated granules were enumerated prior to use to ensure sterility. The *Rhizobium* culture preparation and carrier inoculation were carried out in the same fashion as previously described. An enumeration of the *Rhizobium* cells present in the commercial inoculants and those applied to the compost granules on Day 0 of the experiment was completed and is presented in Table 3.2.4. All samples were replicated four times.

Survival monitoring via standard plating

Survival monitoring was carried out as previously described for the non-sterile composts, for a period of 224 days. The lower limit of detection used for the non-sterile compost enumerations was applied to the sterile, inoculated composts as well.

Survival monitoring via sterile Leonard jars

Upon the termination of the sterile compost survival experiment, the 224-day-old inoculated composts and commercial inoculants were tested for their ability to nodulate field pea (*Pisum sativum*), cv. Delta.

Leonard jars were assembled as described by Somasegaran and Hoben (1994). Briefly, pre-washed, sterile vermiculite was placed into each inverted bottle and lamp wick was run through the center of the bottle down into the nutrient reservoir filled with nitrogen-free liquid Fähraeus medium (Somasegaran and Hoben, 1994).

The apparatus was wrapped in Kraft paper and autoclaved (60 minutes, 121°C at 15 psi). Three holes (2.5 cm depth) were made in the sterile vermiculite in each Leonard jar apparatus; 1.0 g of inoculant was placed in each hole and covered with sterile vermiculite to a depth of 1 cm. One surface sterilized seed (Vincent, 1970) was placed in each hole and covered with sterile vermiculite. A summary of the enumeration of the inoculants on Day 0 of this study is presented in Table 3.2.5.

Shortly after germination, autoclaved polypropylene beads were placed on the surface of the vermiculite in each Leonard jar apparatus to reduce moisture loss. Plants were also staked soon after germination and thinning (thinning from 5 to 2 plants per pot was performed when plants were well established, approximately 2.5 inches high). Plants were grown and randomized weekly in the growth chamber with a cycle of 16 hour day/25°C and 8 hour night/20°C (temperature ramped). All treatments were examined for number of nodules, shoot and root biomass, and total N in the shoot material. In order to obtain the plant shoot and root material for parameter evaluation, the plant shoot material was removed at the soil surface using pruning shears. The root

Table 3.2.5. Enumeration of rhizobia from 224-day-old inoculated composts, commercial inoculants, and in control vermiculite on Day 0 of the survival monitoring using sterile Leonard jars experiment.

Inoculant[†]	Rhizobia CFU g^{-1}
EA	5.85×10^4 (1.43×10^4)[‡]
PA	9.88×10^3 (6.74×10^3)
SZ	5.36×10^5 (1.13×10^5)
BU	3.43×10^7 (1.49×10^6)
LT	2.76×10^4 (6.73×10^4)
Control Vermiculite[§]	$< 1 \times 10^3$[¶]

[†] The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech, Milwaukee, WI.
[‡] Number in brackets is the standard deviation of the mean of 4 replicates.
[§] Number of rhizobia present in the sterile control vermiculite used in the Leonard jars.
[¶] $< 1 \times 10^3$ is the lowest limit of detection used in this experiment.

systems were then gently separated from the remaining soil, rinsed clean with tap water, padded dry and evaluated.

Evaluation of rhizobia on pea

The wet and dry weights of the shoot and seed materials were determined. Dry weights of the samples were taken after drying the plant material in paper bags at 40°C for three days. Upon drying, the shoot and seed material (when applicable) was ground using a cyclone grinder (Cyclotec ™) in preparation for analysis of total shoot nitrogen (N) content. Shoot total N content determinations were completed using a LECO CNS-2000

Analyzer.

3.2.4 Ability of *Rhizobium*-inoculated compost granules to nodulate pea plants (*Pisum sativum*) under sterile conditions

Experimental parameters

The treatments used in this study were sterile EA, PA and SZ compost granules. The granules were either left un-inoculated or freshly inoculated with a commercial strain of *R. leguminosarum* bv. *viciae* (M1). Two commercial granular inoculants, BU and LT were included as positive controls. A negative control that contained no inoculant also was included in the study. Plants were sampled six weeks after planting. Each treatment was replicated seven times.

Leonard jar experiment

Leonard jars were assembled as described section 3.2.3. The treatments were enumerated on Day 0 and the data summarized in Table 3.2.6. Shortly after germination, autoclaved polypropylene beads were placed on the surface of the vermiculite in each Leonard jar apparatus to reduce moisture loss. Plants were also staked soon after germination and thinning (as described in section 3.2.3). Plants were grown and randomized weekly in a growth chamber with a cycle of 16 hour day/25°C and 8 hour night/20°C (temperature ramped).

All treatments were examined for the number of nodules, shoot and root biomass and total N of the shoot material. Plant material was sampled as described in section 3.2.3.

Evaluation of rhizobia on pea

The evaluation of nodules, and shoot, root and seed material was completed as outlined in section 3.2.3.

Table 3.2.6. Enumeration of rhizobia in inoculant and control vermiculite on Day 0 of the experiment to determine if *Rhizobium*-inoculated sterile compost granules promote the nodulation of pea plants (*Pisum sativum*) under sterile conditions[†].

Treatment[‡]	Rhizobia CFU g^{-1} of inoculant
EA inoculated	1.32×10^7 (6.45×10^5)[§]
EA uninoculated	$< 1 \times 10^3$[¶]
PA inoculated	1.27×10^8 (7.73×10^6)
PA uninoculated	$< 1 \times 10^3$
SZ inoculated	3.60×10^8 (1.47×10^7)
SZ uninoculated	$< 1 \times 10^3$
BU	2.90×10^7 (4.53×10^6)
LT	9.75×10^4 (1.26×10^3)
Control Vermiculite	$< 1 \times 10^3$

[†] Enumeration was performed immediately following granule inoculation.
[‡] The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech, Milwaukee, WI.
[§] Number in brackets is the standard deviation of the mean of 4 replicates.
[¶] $< 1 \times 10^3$ is the lowest limit of detection used in this experiment.

3.2.5 Ability of *Rhizobium*-inoculated compost granules to promote the nodulation of pea plants (*Pisum sativum*) in field soil: pot studies.

Field soil pot study using *Rhizobium*-inoculated non-sterile composts

Experimental parameters

Following the results of the survival screenings, the EA and PA composts were selected for further use. The treatments included in this study were non-sterile EA and PA compost granules. The granules were either un-inoculated or freshly inoculated with a

commercial strain of *R. leguminosarum* bv. *viciae* (M1). Two commercial granular inoculants, BU and LT were included as positive controls. A negative control that contained no inoculant also was included in the study. Two sampling times were selected: 43 days after planting (DAP) and 67 DAP. These sampling times represented the flowering and maturity physiological stages (respectively) of the plants grown in growth chamber conditions in this experiment. Four pots were sampled per sampling day for each treatment. The cultivar of field pea used was Delta.

The clay loam bulk soil used in this study was collected from Meadow Lake, SK. The soil was air-dried (3 days, 25°C) and screened to pass a 2 mm sieve. The macronutrient content of the soil was determined by Enviro-Test Laboratories, Saskatoon, SK. ($\mu g/g^{-1}$): N, 20.2; P, 5.9; K, 125; S, 15.6. The pH of the soil was 6.9 and the electrical conductivity was 0.3 mS/cm.

Soil (2 kg) was weighed into 8-inch plastic pots (n=8 per treatment). The pots were watered with distilled water to 60% field capacity, covered with plastic bags and allowed to equilibrate at room temperature for one week prior to seeding. Germination tests were performed on the pea seeds. Germination tests were performed by placement of seeds on moistened filter paper in glass petri plates. The filter paper was maintained in a moist condition throughout the three days of storage at room temperature (the petri plate was wrapped in tinfoil to reduce exposure to light).

Five holes, 2.5 cm deep, were made in each pot. Thirty milligrams of inoculant were divided (6 mg per hole) into each of five holes (per pot). The holes were then covered with soil to a depth of one centimeter and one surface sterilized seed placed in each hole. The seeds were covered with remaining soil and placed in the growth chamber. An enumeration of all of the treatments was performed on YEM and Congo

Table 3.2.7. Enumeration of rhizobia in inoculants and control soil on Day 0 of the field soil pot study using non-sterile granular compost[†].

Treatment[‡]	Rhizobia CFU g^{-1} of inoculant
EA Inoculated	6.89×10^7 (1.41×10^6)[§]
EA Uninoculated	$< 1 \times 10^3$[¶]
PA Inoculated	1.07×10^7 (8.96×10^5)
PA Uninoculated	2.75×10^4 (3.40×10^4)
BU	2.18×10^7 (3.40×10^6)
LT	9.75×10^4 (3.86×10^4)
Control Soil[#]	1.17×10^5 (2.75×10^4)

[†] Enumeration was performed immediately following granule inoculation.
[‡] The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech, Milwaukee, WI.
[§] Number in brackets is the standard deviation of the mean of 4 replicates.
[¶] $< 1 \times 10^3$ is the lowest limit of detection used in this experiment.
[#] Count of native rhizobia present per gram of control field soil.

red agar plates on the day of seeding (Table 3.2.7). Upon germination, pots were thinned to 2 plants per pot and watered with tap water to 60% water holding capacity throughout the study. After thinning, autoclaved polypropylene beads were placed on the surface of the soil to reduce moisture loss. The plants were staked soon after thinning, grown, and randomized weekly in a growth chamber with a cycle of 16 hour day/25°C and 8 hour night/20°C (temperature ramped).

Four pots per treatment were harvested at flowering, approximately 43 days after planting (DAP). The remaining pots were harvested 67 DAP. All treatments, for both sampling dates, were examined for the number of nodules, shoot and root biomass and total N of the shoot material.

Evaluation of rhizobia on pea

The evaluation of nodules, and the shoot and root material was completed as outlined in section 3.2.3.

Field soil pot study using *Rhizobium*-inoculated sterile composts

Experimental parameters

A second field soil pot study in the growth chamber was also conducted. The treatments included sterile EA, PA and SZ composts freshly inoculated with a commercial strain of *R. leguminosarum* bv. *viciae* (M1). Two commercial granular inoculants, BU and LT were included as positive controls.

A negative control that contained no inoculant was also included in the study. Plants were sampled 43 and 67 DAP. Three pots were sampled per sampling day for each treatment. The cultivar of pea was Delta.

The clay loam bulk soil used in this study was collected from Carlyle, SK. The soil was air-dried (3 days, 25°C) and screened to pass a 2 mm sieve. The macronutrient content of the soil was determined by Enviro-Test Laboratories, Saskatoon, SK. ($\mu g/g^{-1}$): N, 5.8; P, 4.0; K, 196; S, 2000. The pH of the soil was 7.5 and the electrical conductivity was 2.9 mS/cm.

Soil (1.5 kg) was weighed into 6-inch plastic pots (n=6 per treatment). The pots were watered with distilled water to 60% water holding capacity, covered with plastic bags and allowed to equilibrate at room temperature for one week prior to seeding. Germination tests were performed (22°C) on the pea seeds, as previously described. Five holes (2.5 cm deep) were made in each pot. One gram of inoculant was put into each of five holes (per pot). The holes were then covered with soil to a depth of one

Table 3.2.8. Enumeration of rhizobia in inoculants and control soil on Day 0 of the field soil pot study using sterile granular compost[†].

Treatment[‡]	Rhizobia CFU g^{-1} of inoculant
EA Inoculated	1.32×10^7 (6.45×10^5)[§]
EA Uninoculated	$< 1 \times 10^3$[¶]
PA Inoculated	1.27×10^8 (7.73×10^6)
PA Uninoculated	$< 1 \times 10^3$
SZ Inoculated	3.60×10^8 (1.47×10^7)
SZ Uninoculated	$< 1 \times 10^3$
BU	2.90×10^7 (4.53×10^6)
LT	9.75×10^4 (1.26×10^3)
Control Soil[#]	1.88×10^4 (2.63×10^3)

[†] Titre was performed immediately following granule inoculation.
[‡] The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech, Milwaukee, WI.
[§] Number in brackets is the standard deviation of the mean of 4 replicates.
[¶] $< 1 \times 10^3$ is the lowest limit of detection used in this experiment.
[#] Count of native rhizobia present per gram of control field soil.

centimeter and one surface sterilized seed was placed in each hole. The seeds were covered with remaining soil and placed in the growth chamber. An enumeration of all of the treatments was performed on YEM and Congo Red agar plates on the day of seeding and the data summarized in Table 3.2.8.

Upon germination, pots were thinned to 2 plants per pot and watered with tap water to 60% field capacity. This water content was maintained throughout the study.

After thinning, autoclaved polypropylene beads were placed on the surface of the soil to reduce moisture loss. Plants were staked soon after thinning, grown, and

randomized weekly in the growth chamber with a cycle of 16 hour day/25°C and 8 hour night/20°C (temperature ramped).

Three pots per treatment were harvested at flowering, approximately 43 DAP. The remaining pots were harvested 67 DAP. All treatments, for both sampling dates, were examined for the number of nodules, shoot and root biomass and total N of the shoot material.

Evaluation of rhizobia on pea

The evaluation of nodules, and shoot and root material was completed as outlined in section 3.2.3. Means comparison was completed using least significant difference (LSD).

3.2.6 Statistics

All data were analyzed using the analysis of variance (ANOVA) procedure in Costat statistical software program Ver. 6 (Cohort statistical software). Analysis of means was performed using the least significant difference (LSD) test.

3.3 RESULTS

Survival of *Rhizobium leguminosarum* bv. *viciae* on non-sterile EA, PA and SZ compost granules

Survival monitoring via standard plating

The *Rhizobium*-inoculated compost granules varied in their capacity to support the survival of rhizobia. For example, the non-sterile SZ compost granules failed to support acceptable populations of culturable rhizobia after approximately 27 days of incubation at 4°C (Figure 3.3.1). Conversely, the non-sterile EA and PA compost granules supported culturable populations of rhizobia for approximately 70 days, after which time the limit of detection was reached and enumerations ceased (Figure 3.3.1). Of the two non-sterile EA and PA composts, survival after 70 days was greatest for the EA compost The BU and LT commercial inoculants that were used as positive controls for survival varied greatly in their culturable *Rhizobium* populations (Figure 3.3.1). The BU inoculant consistently supported high populations of rhizobia.

The peat granules prepared and inoculated in the same fashion as the compost granules demonstrated the capacity to support a large culturable population of rhizobia, comparable to that of the commercial inoculants (Figure 3.3.1). Mean values and standard deviations for the enumerations on each sampling day are presented in Tables B1 and B2 (Appendix B).

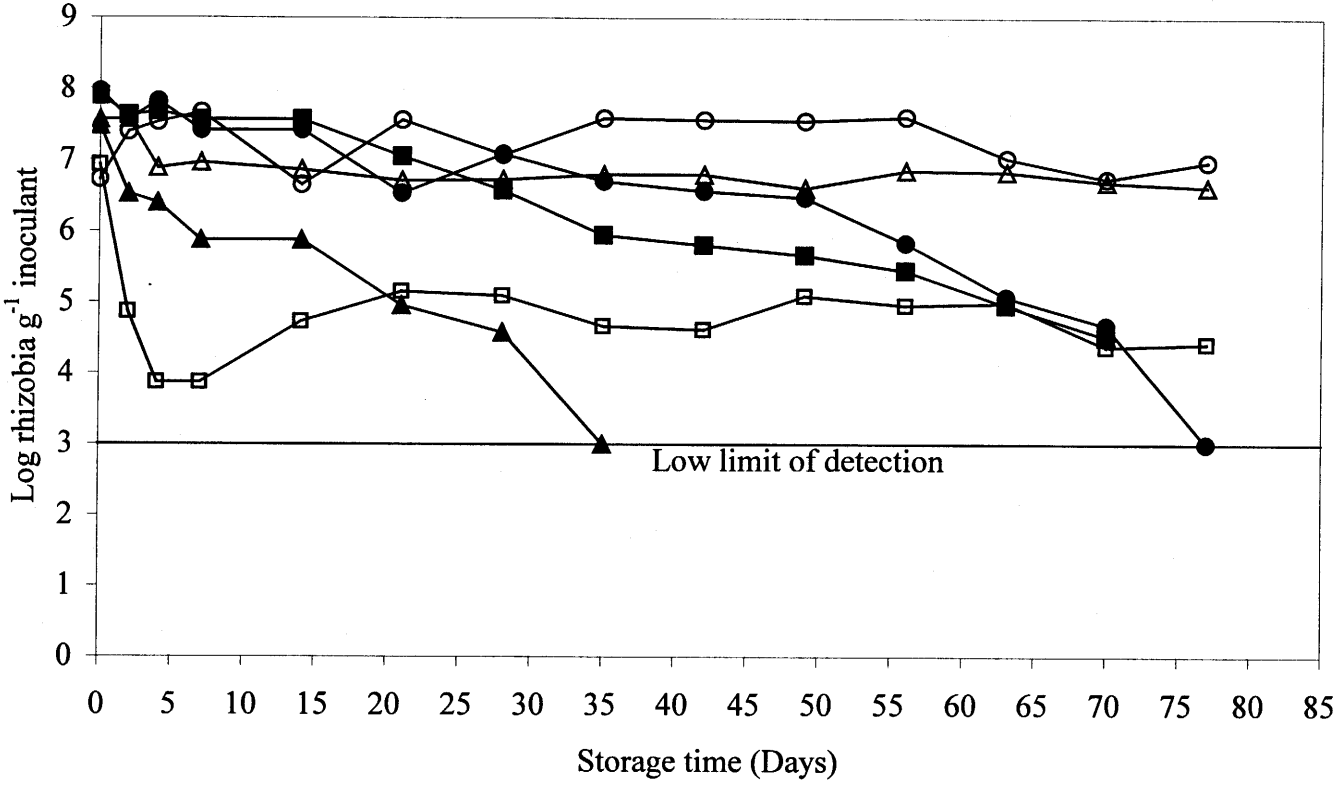

Figure 3.3.1. Survival of *R. leguminosarum* bv. *viciae* on non-sterile EA compost granules (●), PA compost granules (■), SZ compost granules (▲), peat granules (Δ), and commercial inoculants [BU (○) and LT(□)] stored at 4°C. The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; powdered peat and BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech, Milwaukee, WI..

Survival of *Rhizobium leguminosarum* bv. *viciae* on sterile EA, PA and SZ compost granules

Survival monitoring via standard plating

Sterile compost was very similar in its chemical characteristics to the non-sterile compost (Table A2, Appendix A). All three sterile composts were able to support the survival of rhizobia well past the 70 days seen with the non-sterile composts (Figure 3.3.2). All of the sterile composts inoculated with rhizobia performed similarly to the commercial inoculants to a certain extent. However, after 224 days of storage, the EA compost had the highest percentage (1.2%) of the original *Rhizobium* populations still culturable on the granules as compared to the PA and SZ compost granules (0.03 and 0.58 % of original, respectively). These results were similar to the trends seen when the non-sterile composts were enumerated. The mean values and standard deviations for the enumerations on each sampling day are listed in Table B3 (Appendix B).

Survival monitoring via sterile Leonard jars

Survival of rhizobia was monitored in a sterile Leonard jar system to determine whether the culturable populations on the sterile EA, PA and SZ compost granules contained rhizobia capable of nodulating pea. The 224-day-old inoculants still contained rhizobia capable of nodulating pea (Table 3.3.1). The number of nodules found on pea roots varied significantly from 0 nodules for the control treatment to a high of 149 nodules for the SZ treatment. The SZ compost resulted in a significantly higher number of nodules as compared to all of the treatments, except the PA compost granules. The PA and SZ compost granules produced the highest shoot total nitrogen values (80.54 and 85.34 mg nitrogen) of the three inoculated composts, but these values

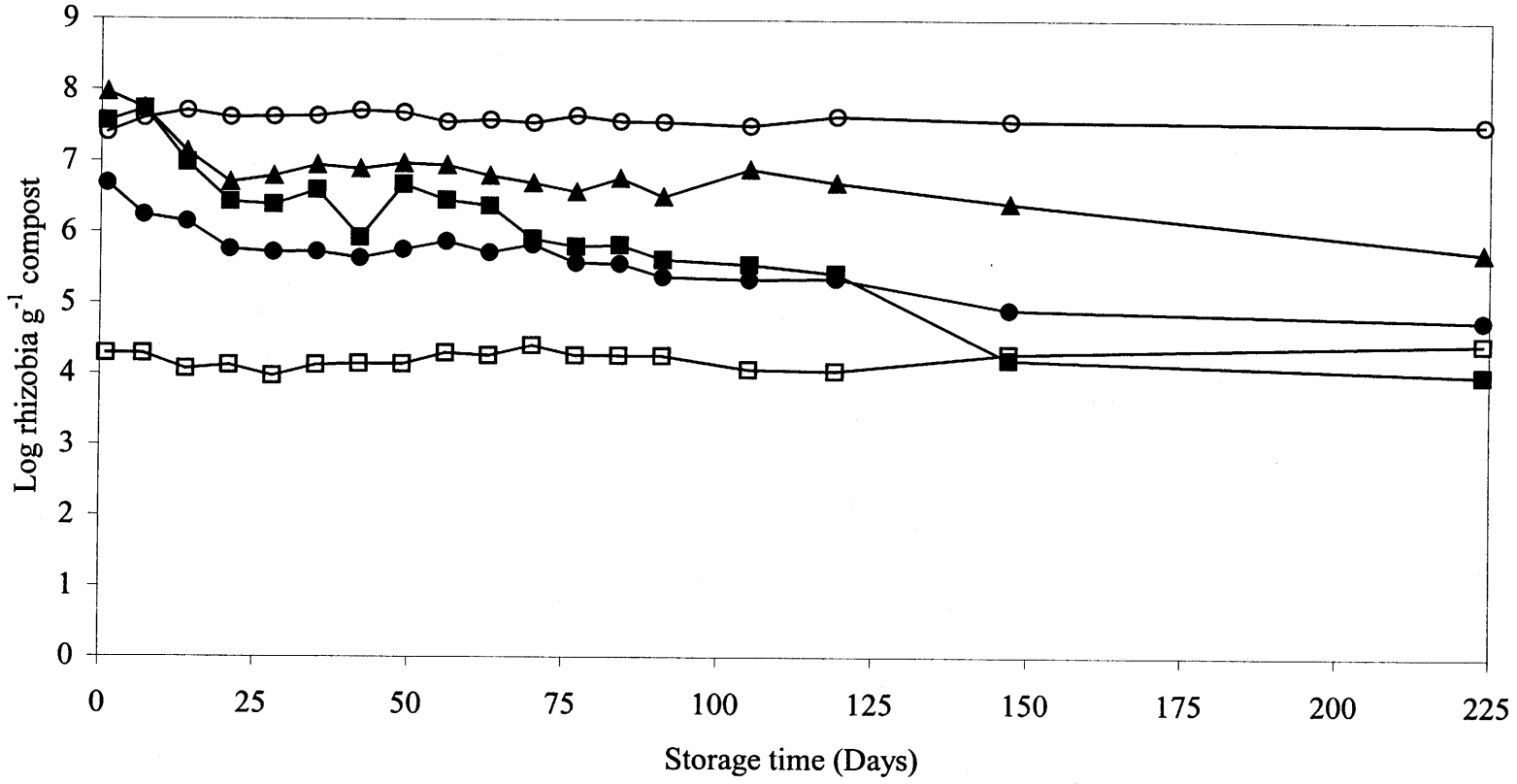

Figure 3.3.2. Survival of *R. leguminosarum* bv. *viciae* on sterile EA compost granules (●), PA compost granules (■), SZ compost granules (▲) and commercial inoculants [BU(○) and LT(□)] stored at 4°C. The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech, Milwaukee, WI.

Table 3.3.1. Ability of 224-day-old *Rhizobium*-inoculated granular compost and commercial *Rhizobium* inoculants to promote the nodulation of pea plants (*Pisum sativum*) in sterile Leonard jars.

Treatment[†]	Number of nodules (per replicate)	Nodule mass dry weight (per replicate) (mg)	Shoot biomass (per replicate) (g)	Total shoot nitrogen[‡] (per replicate) (mg/g)
EA	26 c,d[§]	0.04 b	1.50 c	52.23 c
PA	112 a,b	0.33 a	2.40 c	80.54 c
SZ	149 a	0.33 a	2.52 c	85.34 c
BU	80 b,c	0.22 a	5.40 b	177.66 b
LT	28 c,d	0.07 b	7.81 a	235.41 a
Control[¶]	0 d	0.00 b	0.34 d	7.40 d

[†] The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech; Milwaukee, WI.

[‡] Total nitrogen = Shoot nitrogen content x Shoot biomass.

[§] Means with the same letter in columns were not significantly different at $P<0.05$ (LSD).

[¶] No inoculant added.

were still lower than those of the BU and LT commercial inoculants (177.66 and 235.41 mg nitrogen respectively).

Despite having more nodules, plants treated with the compost-based inoculants yielded lower shoot biomass and shoot total N (Table 3.3.1).

Ability of *Rhizobium*-inoculated sterile EA, PA and SZ compost granules to promote the nodulation of field pea (*Pisum sativum*) under sterile conditions

The ability of BU and LT commercial inoculants and sterile EA, PA and SZ compost granules (freshly inoculated with *Rhizobium*-M1) to deliver viable, nodulating rhizobia in a sterile Leonard jar environment was assessed. All of the inoculated compost treatments consistently yielded high numbers of nodules after 6 weeks of growth, all of which were greater than the nodule counts for the BU commercial

inoculant (Table 3.3.2). The dry weight of the nodules also followed this trend (Table 3.3.2). However, after six weeks of growth the inoculated compost treatments resulted in shoot total N contents less than the BU and LT commercial inoculants, but still greater than the un-inoculated controls.

Ability of *Rhizobium*-inoculated EA, PA and SZ compost granules to promote the nodulation of field pea (*Pisum sativum*) in field soil: pot study

Agricultural soil pot study using non-sterile EA, PA and SZ compost granules inoculated with *Rhizobium leguminosarum* bv. *viciae* (M1)

The ability of inoculated non-sterile EA, PA and SZ compost granules and two commercial *Rhizobium* inoculants (BU and LT) to deliver viable, nodulating rhizobia in a field soil pot study environment was assessed. No differences were seen between the composts, commercial inoculants and control treatments at 43 DAP for the number of nodules, shoot and root biomass and nitrogen content of the shoot material (data not shown). However, at 67 DAP the root biomass for the inoculated PA compost granules and un-inoculated PA compost granules was greater than those of all other treatments (Table 3.3.3). In addition, the nitrogen content for the un-inoculated PA treatment was significantly higher than those of all other treatments (Table 3.3.3).

Agricultural soil pot study using sterile EA, PA and SZ compost granules inoculated with *Rhizobium leguminosarum* bv. *viciae* (M1)

The ability of two commercial *Rhizobium* inoculants (BU and LT) and sterile EA, PA and SZ compost granules inoculated with rhizobia to deliver viable, nodulating rhizobia in a field soil pot study environment was assessed. No significant differences were seen between the compost treatments and the commercial inoculants. However,

Table 3.3.2. Ability of sterile compost granules inoculated with *Rhizobium leguminosarum* bv. *viciae* (M1) to promote the nodulation of field pea (*Pisum sativum*) under sterile conditions.

Treatment[†]	Number of nodules (per replicate)	Nodule dry weight (per replicate) (mg)	Shoot biomass (per replicate) (g)	Root biomass (per replicate) (g)	Total shoot nitrogen[‡] (per replicate) (mg/g)
EA	333 a[§]	0.74 b	0.789 bcd	0.93 d	30.59 d
EA un[¶]	0 c	0.00 c	0.474 e	1.58 b,c	6.07 e
PA	312 a	1.18 a	0.847 bc	0.94 d	35.86 cd
PA un	0 c	0.00 c	0.593 de	1.95 ab	7.35 e
SZ	310 a	1.01 a	0.979 b	1.26 cd	40.14 c
SZ un	0 c	0.06 c	0.629 cde	2.21 a	9.32 e
BU	85 b	0.11 c	1.620 a	1.73 b	67.96 b
LT	301 a	1.20 a	1.799 a	0.80 d	78.50 a
Control[#]	0 c	0.00 c	0.604 de	1.20 cd	7.11 e

[†] The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech, Milwaukee, WI.

[‡] Total nitrogen = Shoot nitrogen content x Shoot biomass.

[§] Means with the same letter in columns were not significantly different at $P<0.05$ (LSD).

[¶] un = un-inoculated.

[#] No inoculant added.

Table 3.3.3. Ability of non-sterile compost granules inoculated with *Rhizobium leguminosarum* bv. *viciae* (M1) to promote the nodulation of field pea (*Pisum sativum*) in field soil 67 days after planting: pot study.

Treatment[†]	Number of nodules (per replicate)	Shoot biomass (per replicate) (g)	Root biomass (per replicate) (g)	Total shoot nitrogen[‡] (per replicate) (mg/g)
EA	41 a[§]	5.25 a	0.52 b	40.04 b
EA un[¶]	39 a	5.52 a	0.60 b	42.19 b
PA	70 a	4.64 a	0.87 a	35.65 b
PA un	88 a	5.46 a	0.91 a	60.04 a
BU	39a	4.95 a	0.55 b	43.55 b
LT	40a	4.61 a	0.63 b	33.01 b
Control[#]	37 a	4.95 a	0.52 b	35.94 b

[†] The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech, Milwaukee, WI.

[‡] Total nitrogen = Shoot nitrogen content x Shoot biomass.

[§] Means with the same letter in columns were not significantly different at $P<0.05$ (LSD).

[¶] un = un-inoculated.

[#] No inoculant added.

the inoculated composts were similar to the commercial inoculants in the amount of shoot total nitrogen 43 DAP (Table 3.3.4). The un-inoculated composts were consistently lower than their inoculated forms but not significantly different from them (Table 3.3.4). No significant differences between treatments for the shoot and root biomass, and shoot total N contents were detected at 67 DAP (data not shown).

Table 3.3.4. Ability of sterile compost granules inoculated with *Rhizobium leguminosarum* bv. *viciae* (M1) to promote the nodulation of field pea (*Pisum sativum*) in field soil 43 days after planting: pot study.

Treatment[†]	Number of nodules (per replicate)	Shoot biomass (per replicate) (g)	Root biomass (per replicate) (g)	Total shoot nitrogen[‡] (per replicate) (mg/g)
EA	167 a[§]	0.98 a	0.34 a	21.83 a
EA un[¶]	168 a	1.10 a	0.24 a	19.84 a
PA	213 a	1.45 a	0.26 a	31.94 a
PA un	193 a	1.45 a	0.33 a	26.20 a
SZ	112 a	1.25 a	0.30 a	29.05 a
SZ un	140 a	1.23 a	0.38 a	24.73 a
BU	175 a	1.11 a	0.24 a	26.29 a
LT	133 a	1.32 a	0.23 a	38.66 a
Control[#]	87 a	0.91 a	0.22 a	21.07 a

[†] The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech, Milwaukee, WI.

[‡] Total nitrogen = Shoot nitrogen content x Shoot biomass.

[§] Means with the same letter in columns were not significantly different at $P<0.05$ (LSD).

[¶] un = un-inoculated.

[#] No inoculant added.

3.4 DISCUSSION

The lack of the long-term survival of *Rhizobium* on the non-sterile compost granules was expected as studies have shown increased survival in sterile carriers versus non-sterile carriers (Date and Roughley, 1977; Roughley and Vincent, 1967). Moreover, it is generally thought that rhizobia are not good competitors (Olsen *et al.*, 1994; Paau, 1991). Although the granular composts used in this study contained sufficient nutrients and an appropriate level of moisture, the rhizobia were unable to compete with the indigenous bacteria present in the compost. This was hypothesized to be the case because when the compost granules were sterilized by gamma irradiation the survival of rhizobia was prolonged well past the 30 days seen with the non-sterile compost granules.

Similarly, it was observed that the number of non-rhizobia colonies observed on the non-sterile enumeration plates increased with time while the culturable *Rhizobium* colonies decreased (data not presented). Aside from the sterilization, all other factors pertaining to the composts remained the same. For example, analyses indicated very little difference between the non-sterile and sterile compost granules both chemically and physically. A study done by Graham-Weiss and colleagues (1987) suggested that sterilization via irradiation produces chemicals that are toxic to rhizobia. This was not likely a limiting factor in this study, as rhizobia survived well on the sterile compost granules. Gamma irradiation should be considered the prime choice for the sterilization of these composts due to the non-detrimental effects of the sterilization treatment. The sterile SZ compost granules supported numbers of *Rhizobium* comparable to the BU

commercial inoculant for the first 100 days of storage at 4°C. After that time, the *Rhizobium* populations decreased, but potential still remained for long-term survival.

The moisture content of the granules was initially adjusted to and remained at approximately 20% (Abd-Alla and Wahab, 1995). Several studies indicated that moisture contents of 35-50% should be the target range for *Rhizobium* inoculants (Denardin and Freire, 2000; Rodriguez-Navarro *et al.*, 1991). A higher moisture content could have positively influenced the survival of *Rhizobium* on the compost granules, and thus should be further examined.

The shoot total nitrogen measured after 6 weeks of growth in sterile conditions (Table 3.3.1) indicated lower shoot total nitrogen values in comparison to the commercial inoculants, when 224-day-old inoculated compost granules were used. However, in this case the total nitrogen values obtained with the inoculant were higher than those of the un-inoculated control. The calculated amount of nitrogen in the plant shoot biomass indicated that N-content was not directly related to the number of nodules on the roots.

In a second sterile-environment study, freshly-inoculated compost granules resulted in plant total nitrogen concentrations comparable to those of the commercial inoculants, while the un-inoculated granules produced plant total nitrogen values lower than those of the commercial inoculants but comparable to that of the control treatment.

The use of inoculated non-sterile granular composts in field soil pot studies produced variable nodulation and levels of shoot total nitrogen. For example, the un-inoculated PA compost granule treatment produced the highest shoot total nitrogen levels of all of the treatments. The inoculated treatments, including the commercial

inoculants did not seem to influence nodulation or shoot total nitrogen levels. The lack of consistent nodulation may have been a result of two factors.

Firstly, the soil nitrogen levels were relatively high, in the range of 20.2 $\mu g\ g^{-1}$ of soil. It has been found that high soil nitrogen levels reduce the effectiveness of legume inoculants because the plant preferentially uses the available soil nitrogen (Nutman, 1976). In addition, high levels of nitrate in the soil also have been shown to inhibit attachment of rhizobia to the root, root hair curling and a number of other important steps in the formation of the symbiosis (Carroll and Mathews, 1990). Another factor that may have contributed to the lack of consistent nodulation was the number of rhizobia applied to each seed. For example 6 mg of inoculant were added to each seed in the non-sterile compost experiment. This application rate roughly translated to approximately 10^5 rhizobia per seed. Studies have shown that higher numbers of rhizobia (in the range of 10^6 rhizobia g^{-1} inoculant) are necessary to produce effective and consistent nodulation (Burton, 1976). Similarly, a study done by Burton and Curley (1965) indicated that a minimum of 2×10^5 rhizobia $seed^{-1}$ was required for successful nodulation. The moisture content may have also been a limiting factor in the failure of the inoculated compost granules to promote survival and consistent nodulation when added at time of pea seeding in field soil pot studies. Because rhizobia do not form endospores, they are somewhat susceptible to desiccation in both soil and carrier materials (Bushby and Marshall, 1977b). Studies have indicated that carriers with higher moisture contents or high clay contents tended to promote better survival in the soil environment, resulting in more consistent nodulation (Biederbeck and Geissler, 1993; Bushby and Marshall, 1977a). Increased survival and nodulation consistency in these studies was thought to be due to the added protection against desiccation.

The second field soil pot study using sterile-inoculated granular compost resulted in results similar to the first pot study. In this case, the soil nitrogen levels were lower than those of the first study (5.8 versus 20.2 $\mu g\ g^{-1}$ soil). In addition, one gram of inoculated granules was added to each seed to obtain a concentration of approximately 10^7 or more rhizobia per seed. The combination of low soil nitrogen and higher rhizobia per seed resulted in consistent nodulation.

The ability of the selected sterile composts to support large populations of rhizobia could play an important role in the production of alternative carrier materials for *Rhizobium*. Future work should investigate whether manipulation of water content in the composts would prolong the survival of rhizobia on the composts as well as aid in the survival of rhizobia in the soil environment. It would also be interesting to test the potential of inoculated compost to be used as a seed coat treatment for legumes to provide producers with another form of inoculant application.

4.0 PRODUCTION OF A GRANULAR COMPOST CARRIER FOR PHENANTHRENE DEGRADING BACTERIA

4.1 INTRODUCTION

Petroleum oil is a very necessary and important commodity in today's world. However, when it is spilled in terrestrial or aquatic environments it becomes a serious environmental contaminant that may adversely affect ecosystem health. Polycyclic aromatic hydrocarbons (PAHs) present in petroleum oil are considered harmful to animals and humans (Edwards, 1983; Juhasz *et al.*, 1996; Pothuluri and Cerniglia, 1994). Due to these health concerns, technologies have been developed to remove PAHs from the environment as efficiently, effectively and as timely as possible. Some technologies developed for the removal of petroleum from contaminated soil are bioremediation (the use of naturally-occurring bacteria for the remediation of contaminated soil) and phytoremediation (the use of plants and their associated root bacteria for the remediation of contaminated soil) (Altas, 1995a; Bollag *et al.*, 1994; Flathman and Lanza, 1998; Nyer and Gatliff, 1996).

Bioremediation technology has evolved greatly since some of the first successful documented cases of its application to terrestrial oil spills (Atlas, 1995a). The addition of nutrients, purified bacterial isolates, consortia of bacterial isolates or different combinations of these factors has greatly assisted in the success of this technology. Studies conducted by Atlas (1995a) and Steffensen and Alexander (1995) indicated great remediation success when nutrients such as nitrogen and phosphorus were added to the contaminated environment. Similarly, the remediation of PAHs suspended in solution

has been greatly enhanced by the addition of single bacterial isolates and consortia capable of PAH degradation (Boyle *et al.*, 1998; Juhasz *et al.*, 1996; Madsen and Kristensen, 1997).

Most research has focused on the bioremediation of PAHs suspended in aqueous-based solutions. However, when applying this technology to the terrestrial environment complications arise because the soil is a very heterogeneous environment. One obstacle is the successful application of viable PAH-degrading bacteria to the contaminated soil.

Some studies have suggested that the number of bacteria applied to the soil greatly influences bioremediation rates (Ramadan *et al.*, 1990). Inherent in this suggestion is the importance of successful application of sufficient viable, degradative bacteria to the contaminated environment. The addition of PAH-degrading bacteria or fungi to contaminated soil in liquid culture form has had varied success (Boyle *et al.*, 1998; Madsen and Kristensen, 1997; Moller and Ingvorsen, 1993). However, newly improved or novel methods of inoculation need to be developed to obtain consistent degradation results in the soil environment. Little work has been done on examining the use of carrier materials to distribute PAH-degrading bacteria to the contaminated soil environment. The focus of this series of studies was to identify a carrier (granular compost) and to examine the role that granulated compost could play in the successful application of PAH-degrading bacteria to PAH-contaminated soil. The overall objectives were:

1. To assess the survival of PAH-degrading bacteria on the granular carrier material; and,
2. To assess the ability of PAH-degrading bacteria, inoculated onto compost granules, to degrade phenanthrene in the soil.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial isolations and screening for phenanthrene degradation

Contaminated soil samples (0–25, 25–50 cm sampling depths) were collected from Hendon, SK and frozen (–80°C) until processed. The total detectable hydrocarbon (TDH) concentration of the soil samples (determined in the field using a Gas Tech modular analyzer) varied from 0–170 parts per million (ppm) TDH. Randomly selected bacterial isolates isolated from the previously mentioned contaminated soils and select, purified bacterial cultures were screened for their ability to degrade phenanthrene using a sublimation procedure (Alley and Brown, 2000). Phenanthrene was the hydrocarbon of choice because it is fairly easy to degrade, being a three-ringed structure. It is also one of the less toxic PAHs present in petroleum oil. Finally, it is generally thought of as the model hydrocarbon in most PAH degradation studies.

Isolation of bacteria from contaminated soil involved standard plating where a 5 g soil sample was aseptically transferred into a 95 mL bottle of sterile PBS. The sample was shaken for 20 minutes at 22°C on a rotary shaker (400 rpm). Serial dilutions (1:10) were made immediately after shaking, and 0.1 mL of the 10^3, 10^4, and 10^5 dilutions were plated out on mineral salts agar (MSA) plates and cooled to a temperature of ~4°C in preparation for sublimation. Mineral salts agar plates (100 by 15 mm diameter) consisting of 1.0 g KNO_3, 0.38 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.05 g $FeCl_3.6H_2O$, and 15 g Difco granulated agar per litre of distilled water were prepared. The solution was adjusted to a pH of 7.0 with 10% HCl (vol/vol).

Bacterial isolates from a purified culture collection were maintained in glycerol at –80°C. Isolates of interest were removed from –80°C storage and quickly thawed in 37°C water. A loop-full of culture was aseptically streaked onto a 1/10-strength Trypticase Soy Agar (TSA) plate to check for purity. Stock cultures were immediately returned to –80°C storage. Pure cultures were re-streaked onto MSA plates (4 isolates per plate) and cooled to a temperature of ~4°C in preparation for phenanthrene sublimation.

The sublimation procedure outlined by Alley and Brown (2000) was used to screen the various bacteria for their ability to degrade phenanthrene. A 140 mm diameter glass Pyrex® petri plate bottom was filled with sand, leveled and placed on a thermostatically controlled aluminum hot plate (Fisher Thermix®). The hot plate was heated to 95°C, the temperature at which phenanthrene sublimes. An aluminum dish containing (~2 g) phenanthrene was placed on the heated sand bath immediately prior to sublimation. The surface temperature of the sand bath was monitored by a thermometer that was inserted just below the surface of the sand.

To carry out the sublimation, the lid of a cooled MSA plate containing a spread dilution or streaked-out purified isolate was removed and the MSA plate was inverted over the heated aluminum dish containing the phenanthrene. A second aluminum dish filled with crushed ice was then placed on top of the inverted MSA plate to control the temperature of the MSA during the 5-minute sublimation process. The 5 minute period was sufficient to deposit a visible, hazy white, layer of phenanthrene over the surface of the MSA spread plate. The sublimed plates were inverted, bagged in a Petri plate sleeve, and stored in a chemical flow hood (6 days, 22–24°C). The plates were examined for colony growth and zones of clearing. The zones of clearing were indicated

by a clearing of the white phenanthrene layer directly surrounding the bacterial colonies. The phenanthrene-degrading bacteria isolated from the contaminated soil were purified and re-tested (using the previously described sublimation technique) for their ability to degrade phenanthrene prior to identification based on their fatty acid methyl ester (FAME) profiles as determined by gas chromatography. A select number of these isolates were chosen for further study and -80°C storage.

Identification of phenanthrene-degrading bacteria

All successful phenanthrene-degrading bacteria were identified based on their fatty acid methyl ester (FAME) profiles obtained via gas chromatograpic analysis using MIDI Corporation's (Newark, DE. USA) software and extraction procedures (Siciliano and Germida, 1997). A similarity index (SI) was assigned to each isolate that indicated the degree of homology between the fatty acid profiles of the unknown isolate and known isolates in the system software. A SI of 1.0 was considered a perfect match while anything less than 0.3 was considered an invalid match.

For the long-term storage of the selected isolates an 8-mL test tube blank of sterile 1/10 Tryptic Soy Broth (TSB) was inoculated with a loop-full of a purified isolate and grown on a rotary shaker (160 rpm for 48 h at 27°C) (Siciliano *et al.*, 1997). Duplicate -80°C glycerol stock cultures were prepared for each isolate (Gerhardt *et al.*, 1994).

4.2.2 Carrier selection and inoculation

The carriers selected for use in the survival and phenanthrene degradation studies were non-sterile EA, PA and SZ granular composts. The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg

Table 4.2.1. Enumeration of phenanthrene-degrading *Rahnella aquatilis* (B1) and *Arthrobacter globiformis* (B2) present on compost granules stored at 4°C and 22°C on Day 0 of the survival study[†].

Inoculant[‡]	B1	B2
	CFU g^{-1} compost	
EA	1.0×10^7	1.0×10^7
PA	1.0×10^8	1.0×10^8
SZ	1.0×10^8	1.0×10^8

[†]Titre was performed immediately following granule inoculation.
[‡]The granules were prepared from powdered raw product supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK.

Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK. The granules were aseptically inoculated with one of two selected phenanthrene-degrading bacteria (B1= *Rahnella aquatilis*; B2=*Arthrobacter globiformis*) via a sterile spray bottle with adjustable misting head. Compost granules were spread onto a piece of sterile Kraft paper inside of the flow hood during the inoculation to ensure sterile and consistent inoculation was obtained.

4.2.3 Survival of phenanthrene-degrading bacteria on granular compost

Experimental parameters

The treatments included in this study were non-sterile granular EA, PA and SZ composts (Table 4.2.1) inoculated with two different purified bacterial isolates: B1 (*Rahnella aquatilis)* and B2 (*Arthrobacter globiformis)* and stored at two different temperatures (4 and 22°C). The titre of phenanthrene-degrading bacteria on Day 0 of the experiment is presented in Table 4.2.1. All of the samples were replicated four times.

Sample Preparation

Samples (4.5 g) of the inoculated compost were weighed out and placed into sterile polyethylene Whirlpac® bags and sealed. All samples were stored in a larger polyethylene bag and kept in a cardboard box (to remove the samples from exposure to light) at 4 and 22°C for 240 days.

Survival monitoring via standard plating and phenanthrene overlay

On each sampling day, one 4.5-g package of granules from each treatment was removed from storage. Each inoculant (1 g) was then diluted in 99 mL of sterile PBS (n=4) and vigorously hand shaken for 30 seconds, allowed to rest for 30 seconds, and then shaken for an additional 30 seconds. Serial dilutions (1:10) in the range of 10^4-10^7 were made into blanks of sterile PBS. Aliquots (0.1 mL) of these dilutions were plated on MSA plates. Plates were cooled to a temperature of ~4°C in preparation for phenanthrene sublimation as described in section 4.2.1. The plates were sublimed with phenanthrene, inverted, bagged and stored in the chemical flow hood (6 days, 22-24°C) prior to enumeration.

4.2.4 Efficiency of inoculated granular compost in phenanthrene-spiked soil

Continuous, non-destructive sampling experiment

Experimental parameters

The compost of choice was 135-day-old SZB2 (Saskatoon Zoo compost inoculated with a phenanthrene-degrading strain of *A. globiformis* (B2), previously stored at 4°C). The compost contained approximately 5 x 10^7 *A. globiformis* g^{-1} compost granules at time of use in the present study.

The clay loam soil used in this study was collected from Meadow Lake, SK. The soil was air-dried (3 days, 25°C) and screened to pass a 2-mm sieve. The macronutrient content of the soil was determined by Enviro-Test Laboratories, Saskatoon, SK ($\mu g/g^{-1}$): N, 20.2; P, 5.9; K, 125; S, 15.6. The pH of the soil was 6.9 and the electrical conductivity was 0.3 mS/cm.

The treatments included soil without phenanthrene, soil plus phenanthrene, soil plus phenanthrene and SZB2. Each treatment was replicated three times. Samples were stored at 22°C and sampled weekly for a period of ~ 13 weeks. Destructive sampling was not used in this experiment (i.e. reaction vessels containing the various treatments were reused for subsequent sampling days without opening the reaction vessel lid). A standard curve was created to determine the concentration of volatile phenanthrene in the reaction vessel head-space, Figure 4.2.1.

Reaction vessel preparation

The reaction vessels were 75-mL Erlenmeyer flasks with 20 grams of soil containing the various treatments. The flasks were sealed with rubber stoppers throughout the experiment to facilitate the capture and subsequent measurement of volatile phenanthrene in the head-space of the reaction vessel (area between the soil and the stopper).

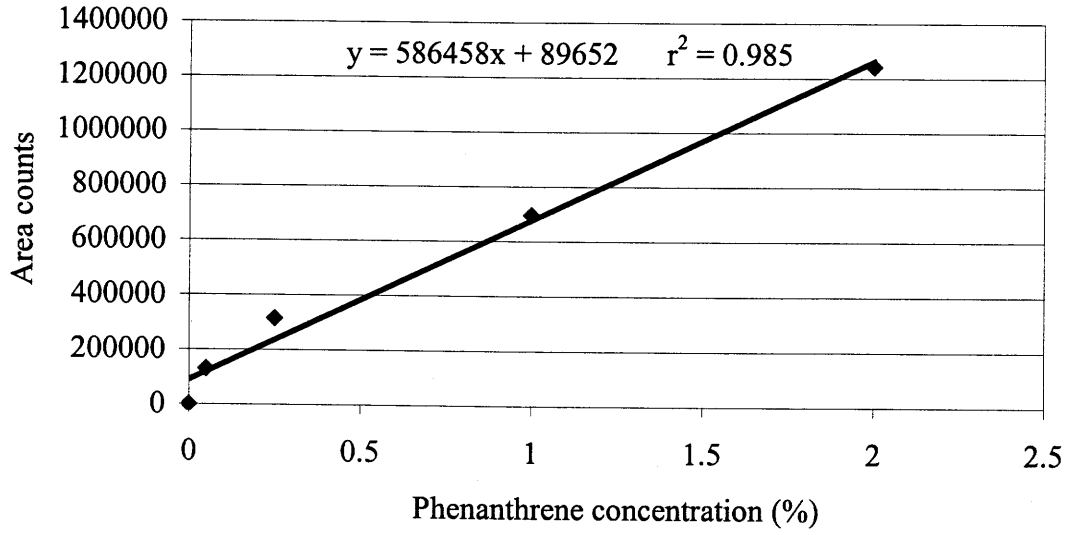

Figure 4.2.1. Standard curve of the volatile phenanthrene in the head-space of reaction vessels, measured by solid phase micro-extraction.

With treatments containing no phenanthrene or inoculant the soil moisture was adjusted to 20% of water holding capacity, homogenized and then 20 g of soil were transferred to each 75-mL Erlenmeyer flask. The relatively low moisture content ensured the integrity of the granules.

For treatments containing phenanthrene, the phenanthrene was dissolved in n-pentane and a sterile spray bottle with an adjustable misting head was used to apply the phenanthrene to the soil to achieve a concentration of 1% phenanthrene g^{-1} soil. With treatments requiring just phenanthrene, the phenanthrene was added, soil moisture was adjusted to 20%, homogenized, and transferred to 75-mL Erlenmeyer flasks.

The treatments requiring phenanthrene and granular compost contained 2.74 g of granular compost per 20 g of 1% phenanthrene-spiked soil (soil moisture adjusted to 20%). The mixture was homogenized and then transferred to 75-mL Erlenmeyer flasks.

The inoculation rate was chosen to achieved a concentration of approximately 6.85 x 10^6 *A. globiformis* g^{-1} soil.

All flasks were sealed with stoppers and stored (22°C). No venting of the flasks was performed (i.e. exchange of head-space air with atmospheric air via removal of the stoppers). Volatile phenanthrene present in the head-space of the reaction vessels was measured using SPME.

Solid phase micro-extraction sampling procedure

The volatile phenanthrene in the head-space of the reaction vessels was measured using Solid Phase Micro-Extraction (SPME). Briefly, a micro-filament (100 μm non-bonded, polydimethylsiloxane-coated, obtained from Supelco–Sigma-Aldrich Co.) was inserted into the reaction vessel septum and incubated (45 min at 30°C). During the incubation period the volatile phenanthrene in the head-space of the reaction vessel adsorbed to the filament. The amount of volatile phenanthrene adsorbed on the filament was then measured using a HP5890 Series II gas chromatography system equipped with a HP-S (Crosslinked 5% PH ME Siloxane 30 m x 0.32 mm x 0.25μm film thickness) capillary column, flame ionization detector (FID), and Hewlett-Packard ChemStation software. The carrier gas was helium (70 psi), the FID was operated using hydrogen gas (20 psi) and ultra pure oxygen (40 psi). The analysis temperatures for the various components of the system were as follows: detector 225°C and injection port 250°C. The sample run time was approximately 30.7 minutes. Further details of the chromatography settings are presented in Appendix D.

Distribution of phenanthrene-degrading bacteria

Experimental parameters

A sample containing the phenanthrene-spiked soil with inoculated granules was taken from the previous study. The treatment selected was the SZ compost that was inoculated with B2 (*Arthrobacter globiformis*) and previously stored at 4°C.

Sample preparation

The intact granules were manually separated and removed from the phenanthrene-spiked soil. One-gram portions were then serially diluted (1:10) in sterile PBS ($10^3 - 10^7$) and plated on MSA plates. The spread plates were then sublimed with phenanthrene and enumerated after 6 days of incubation at 22°C under a chemical flow hood.

Batch, destructive sampling experiment

Experimental Parameters

The compost of choice was 174-day-old SZB2 (Saskatoon Zoo compost inoculated with a phenanthrene-degrading strain of *A. globiformis*). The compost contained approximately 1 x 10^8 *A. globiformis* g^{-1} compost granules. This inoculated compost had been previously stored at 4°C.

The clay loam soil used in this study was collected from Meadow Lake, SK. The soil was air-dried (3 days, 25°C) and screened to pass a 2-mm sieve. The macronutrient content of the soil was determined by Enviro-Test Laboratories, Saskatoon, SK ($\mu g/g^{-1}$): N, 20.2; P, 5.9; K, 125; S, 15.6. The pH of the soil was 6.9 and the electrical conductivity was 0.3 mS/cm.

The treatments included: soil without phenanthrene, soil plus phenanthrene (P), soil plus phenanthrene and SZB2 intact granules (PB2G), soil plus phenanthrene and SZB2 crushed granules (PB2C), and lastly soil plus phenanthrene and fresh, viable B2 cells suspended in sterile water (PL). Each treatment was replicated three times.

During the experiment the samples were stored at 22°C for a period of 150 days. This experiment involved destructive sampling, whereby samples were not used at later processing dates. A standard curve was created to determine the concentration of volatile phenanthrene in the reaction vessel head-space (Figure 4.2.1).

Reaction vessel preparation

With treatments requiring no phenanthrene the soil moisture was adjusted to 20%. The soil was homogenized and then transferred (20 g) to each 75-mL Erlenmeyer flask.

Phenanthrene was added to the soil to achieve a concentration of 1% phenanthrene g^{-1} soil by misting procedure (use of sterile spray bottle with adjustable misting head, phenanthrene was dissolved in n–pentane). With treatments requiring just phenanthrene the spiked soil moisture was adjusted to 20%, mixed well, and transferred to 75-mL Erlenmeyer flasks.

With treatments requiring phenanthrene and inoculated compost, 5 g of either intact or crushed inoculated compost granules were added to 20 g of phenanthrene-spiked soil, soil moisture was adjusted to 20%, homogenized, and then transferred to 75-mL Erlenmeyer flasks. This achieved a concentration of approximately 2.5×10^7 *A. globiformis* g^{-1} soil. Freshly prepared liquid inoculant was added to phenanthrene-spiked soil to obtain a concentration of 2.5×10^7 *A. globiformis* g^{-1} soil. All bacterial inoculants used were prepared immediately prior to use.

All flasks were sealed with stoppers and stored (22°C). No venting of the flasks was performed. Volatile phenanthrene present in the head-space of the reaction vessels was measured using SPME.

4.3 RESULTS

Bacterial isolations and screening for phenanthrene degradation

Soil samples (n=27) collected (0-25 and 25-50 cm depths) from Hendon, SK contained varying amounts of total detectable hydrocarbons (TDH) (Table 4.3.1). The average number of phenanthrene-degrading bacteria present in the soil samples at the two depths was 2.68×10^3 and 7.59×10^3 CFU g^{-1} soil for the 0-25 and 25-50-cm depths, respectively (Table 4.3.1). The difference in the culturable phenanthrene-degrading bacterial populations between the two sampling depths did not vary greatly. However, when comparing the two depths for an individual soil sample (e.g. samples 4.1 and 4.2 0-25 and 25-50 cm) the phenanthrene-degrading bacterial populations were generally one log unit higher at the 25-50 cm depth (Table 4.3.1). This trend corresponded to higher TDH levels at the 25-50 cm depth as well.

One hundred and sixty-eight bacterial isolates capable of degrading phenanthrene were isolated from the Hendon soil samples. The ability of these isolates to degrade phenanthrene was determined by providing phenanthrene as the sole source of carbon for bacterial growth using the sublimation technique. Phenanthrene-degrading isolates were identified via their fatty acid methyl ester (FAME) profiles. The phenanthrene-degrading bacteria identified represented 25 different genera based on SIs of 0.3 or greater (Table 4.3.2).

Table 4.3.1. Total detectable hydrocarbon concentration and total phenanthrene-degrading bacterial enumerations of soil samples collected from Hendon, SK.

Soil sample identification number	Sampling depth (cm)	Total detectable hydrocarbon concentration[†] (%)[§]	Total phenanthrene-degrading bacteria (CFU g^{-1} soil)[‡]
1.1	0 – 25	55	1.0×10^3
1.2	25 – 50	0	2.0×10^3
2.1	0 – 25	45	0.0×10^0
2.2	25 – 50	25	8.0×10^3
3.1	0 – 25	25	3.0×10^3
3.2	25 – 50	90	2.2×10^3
4.1	0 – 25	50	4.0×10^3
4.2	25 – 50	65	1.9×10^4
5.1	0 – 25	25	5.0×10^2
5.2	25 – 50	75	7.0×10^3
6.1	0 – 25	49	1.8×10^4
6.2	25 – 50	48	2.0×10^3
7.1	0 – 25	75	2.0×10^3
7.2	25 – 50	0	1.5×10^3
8.1	0 – 25	20	4.0×10^3
8.2	25 – 50	50	4.2×10^4
9.1	0 – 25	50	2.5×10^3
9.2	25 – 50	49	5.0×10^3
10.1	0 – 25	40	0.0×10^0
10.2	25 – 50	49	5.0×10^2
11.1	0 – 25	60	0.0×10^0
11.2	25 – 50	100	1.2×10^4
12.1	0 – 25	20	0.0×10^0
12.2	25 – 50	170[¶]	5.0×10^2
13.1	0 – 25	45	0.0×10^0
13.2	25 – 50	47	2.0×10^3
14.1	0 - 25	0	2.5×10^3

[†] Total detectable hydrocarbon concentration (%) determined in the field using a Gas Tech modular analyzer.

[‡] Phenanthrene-degrading bacteria isolated via phenanthrene overlay technique (Alley and Brown, 2000).

[§] Value is a percentage of detectable hydrocarbons in a soil sample, machine generated (Gas Tech modular analyzer).

[¶] A value of 170 was achieved based on a calculated level of TDH (modulator was calibrated to 100% per unit of soil and therefore calculated the value based on its calibration).

Table 4.3.2. Diversity of phenanthrene-degrading bacteria isolated from petroleum-contaminated soil.

Identification	Number of isolates[†]	Percentage of total
No growth on BBL[‡]	54	32
No match [¶]	15	9
Arthrobacter oxydans	35	21
Arthrobacter globiformis	15	9
Microbacterium sp.	11	7
Deinococcus sp.	6	4
Pseudomonas sp.	4	2.4
Stenotrophomonas sp.	4	2.4
Chryseobacterium sp.	2	1.2
Pseudomonas sp.	2	1.2
Acinetobacter sp.	1	0.6
Alcaligenes piechaudii	1	0.6
Arthrobacter ilicis	1	0.6
Bacillus coagulans	1	0.6
Brevibacterium sp.	1	0.6
Brevundimonas diminuta	1	0.6
Cellulomonas turbata	1	0.6
Chryseobacterium sp.	1	0.6
Clavibacter sp.	1	0.6
Kocuria rosea	1	0.6
Microbacterium barkeri	1	0.6
Micrococcus lylae	1	0.6
Nocardia restricta	1	0.6
Paenibacillus polymyxa	1	0.6
Pseudomonas balearica	1	0.6
Pseudomonas syringae	1	0.6
Rahnella aquatilis	1	0.6
Shingomonas sp.	1	0.6
Variovorax sp.	1	0.6
Xanthobacter agilis	1	0.6

[†] Total number of bacterial isolates (n=168).
[‡] No growth on media required for FAME analysis.
[¶] No match with the bacteria in the FAME library.

The most frequently encountered genera of phenanthrene-degrading bacteria included *Arthrobacter* sp. (n=51), *Microbacterium* sp. (n=12) and *Pseudomonas* sp. (n=8). Only isolates with a similarity index of 0.6 or greater were considered for further investigation.

Thirty-two percent of the bacteria isolated from the contaminated soil would not grow on the growth medium required to prepare the isolates for FAME identification and as a result could not be identified. Similarly, *ca.* 9% of the isolates grew on the growth medium but could not be matched to the database (i.e. bacterial identification library) and as a result were not identified. Two isolates selected for further study were *Rahnella aquatilis* (SIN = 0.824) and *Arthrobacter globiformis* (SIN = 0.712). These bacteria will be referred to as B1 and B2 respectively throughout the remainder of this chapter. Both isolates demonstrated rapid and efficient phenanthrene degradation in relation to the other isolates that were screened.

In addition to the isolates from the Hendon soil samples, a number of purified bacteria (n=80) isolated from canola and wheat rhizosphere soil and *Rhizobium* isolates also were tested for their ability to degrade phenanthrene. These isolates included *Clavibacter* sp., *Pseudomonas* sp., *Bacillus* sp., *Arthrobacter* sp., *Brevibacillus* sp., *Flavobacterium* sp., *Paenibacillus* sp., and *Rhizobium* sp. All of these isolates tested negative for phenanthrene degradation.

Survival of phenanthrene-degrading bacteria on granular carrier

Survival of *Rahnella aquatilis* and *Arthrobacter globiformis* was monitored on non-sterile EA, PA and SZ granular compost, stored at 4 and 22°C. There was no difference in survival of *R. aquatilis* or *A. globiformis* in any of the composts stored at 4 or 22°C after 114 days (Figures 4.3.1-4.3.4). However, after 240 days of storage, the

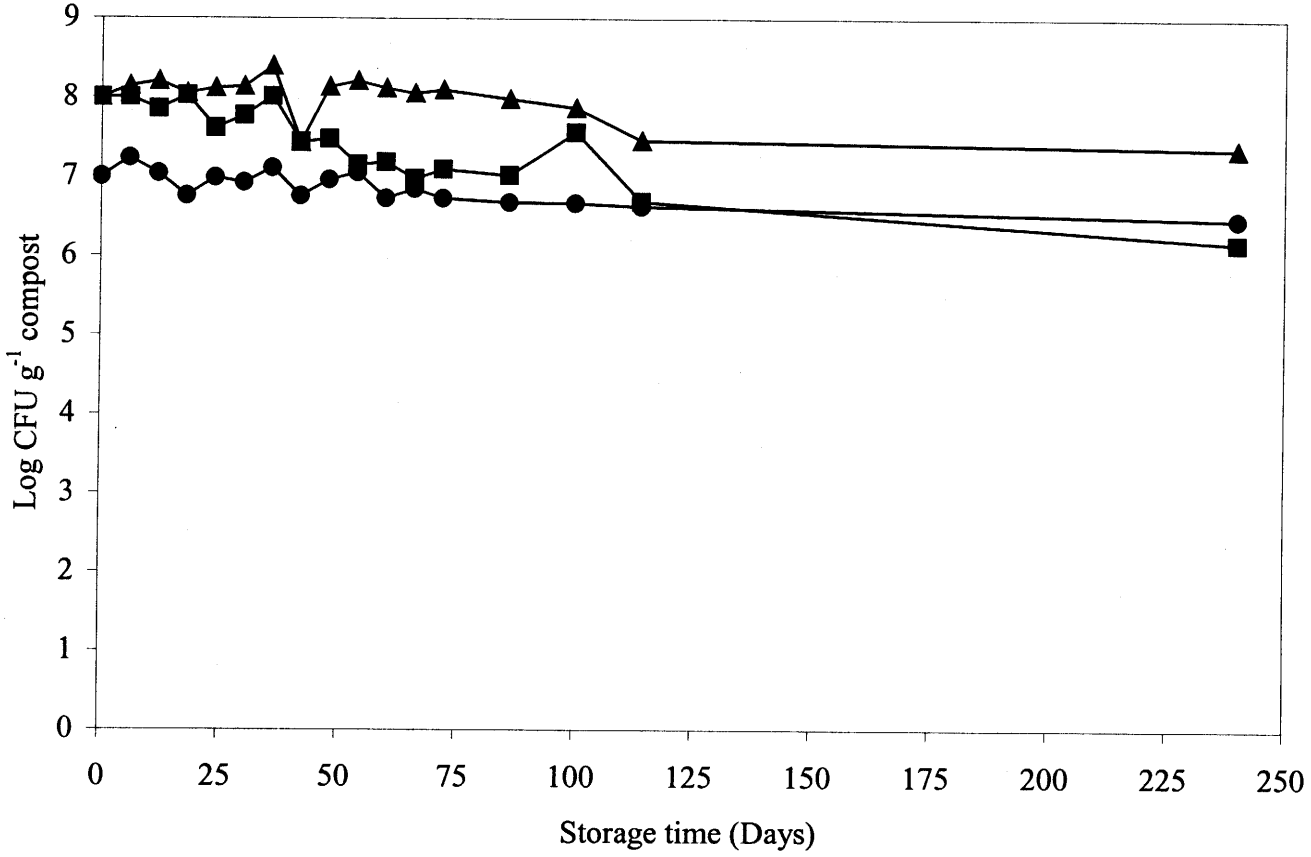

Figure 4.3.1. Survival of *Rahnella aquatilis* (B1) on EA compost granules (●), PA compost granules (■), SZ compost granules (▲), stored at 4°C. The raw products used to produce the compost granules were supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK.

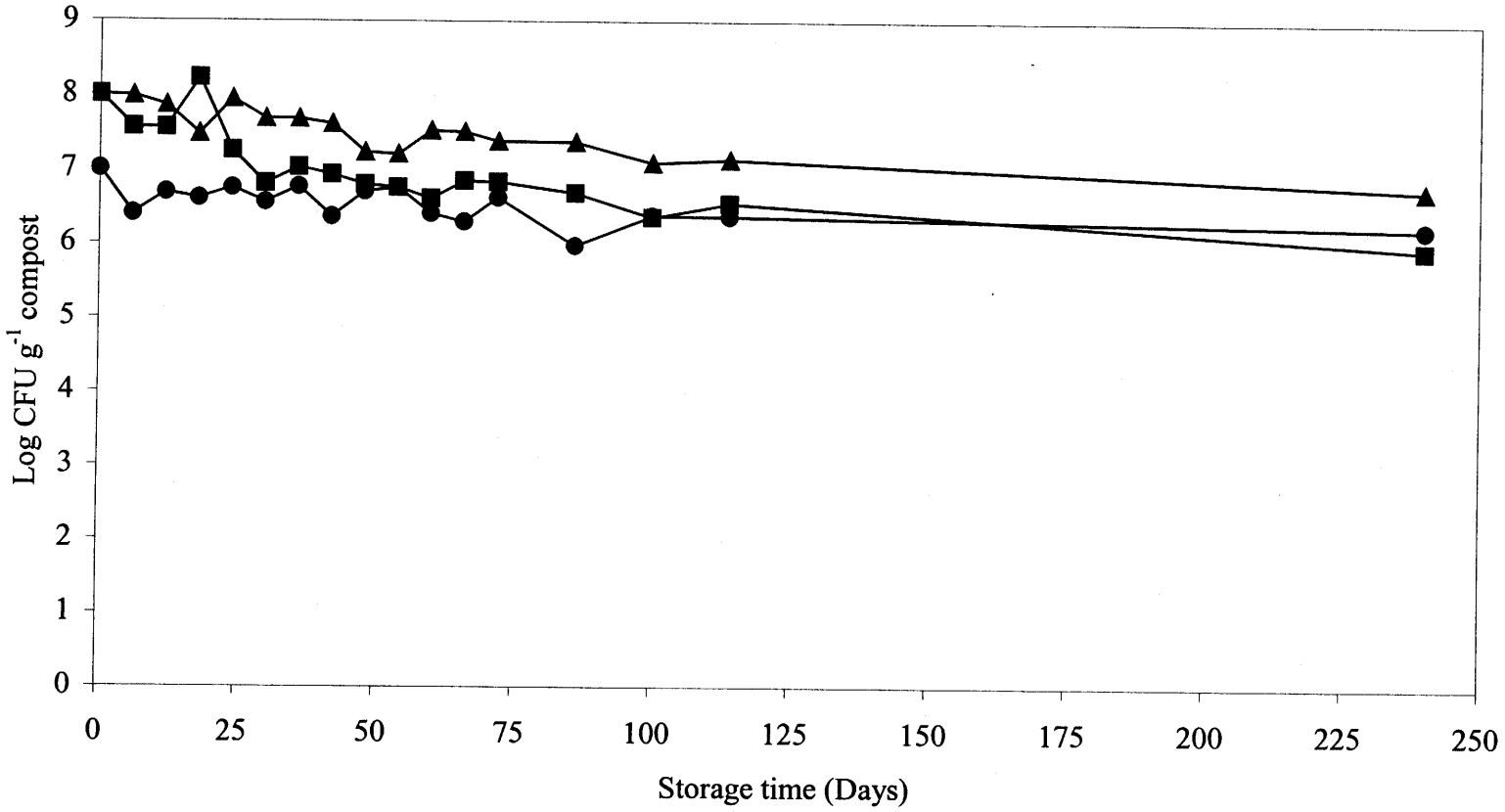

Figure 4.3.2. Survival of *Rahnella aquatilis* (B1) on EA compost granules (●), PA compost granules (■), SZ compost granules (▲), stored at 22°C. The raw products used to produce the compost granules were supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK.

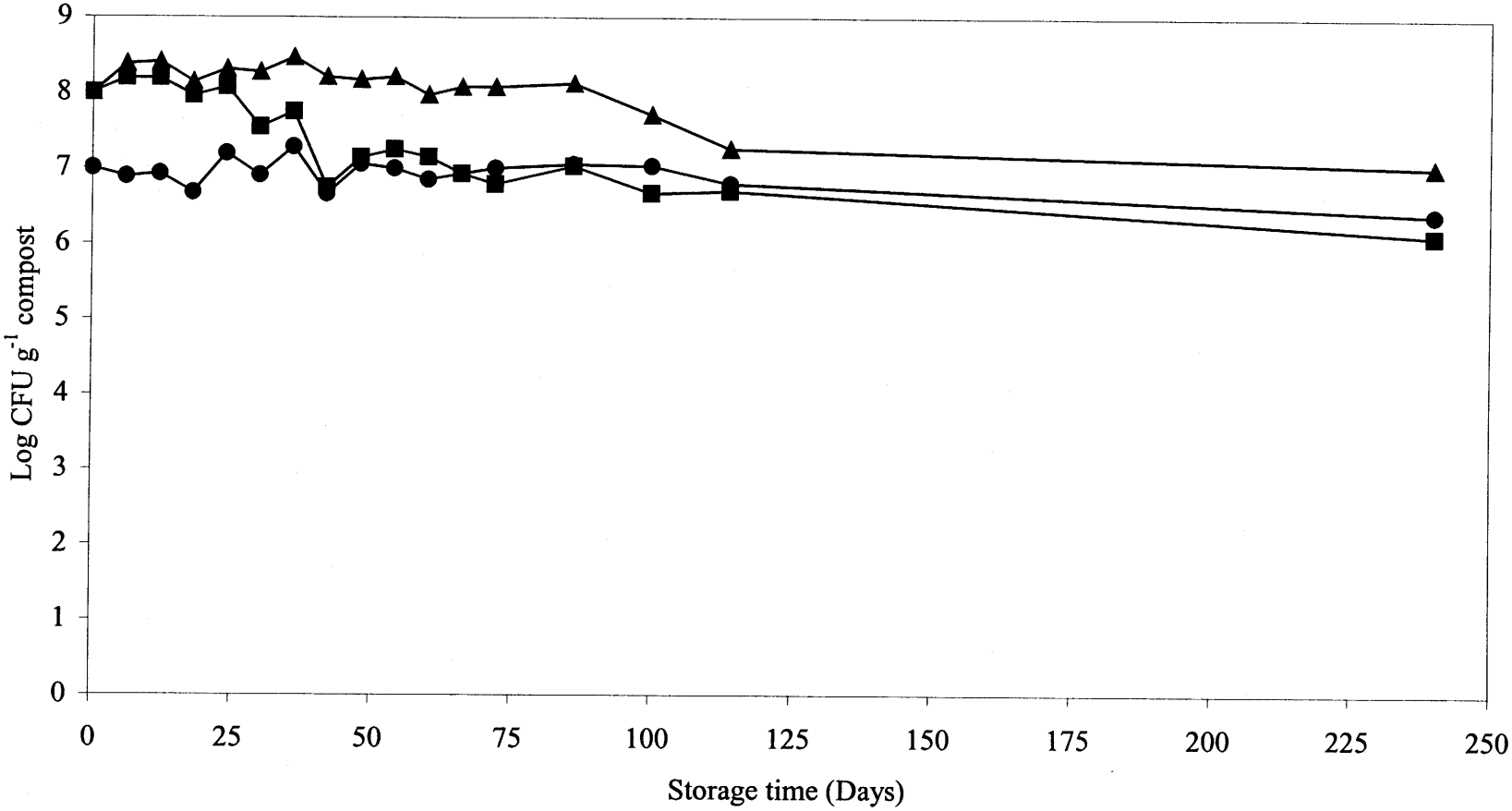

Figure 4.3.3. Survival of *Arthrobacter globiformis* (B2) on EA compost granules (●), PA compost granules (■), SZ compost granules (▲), stored at 4°C. The raw products used to produce the compost granules were supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK.

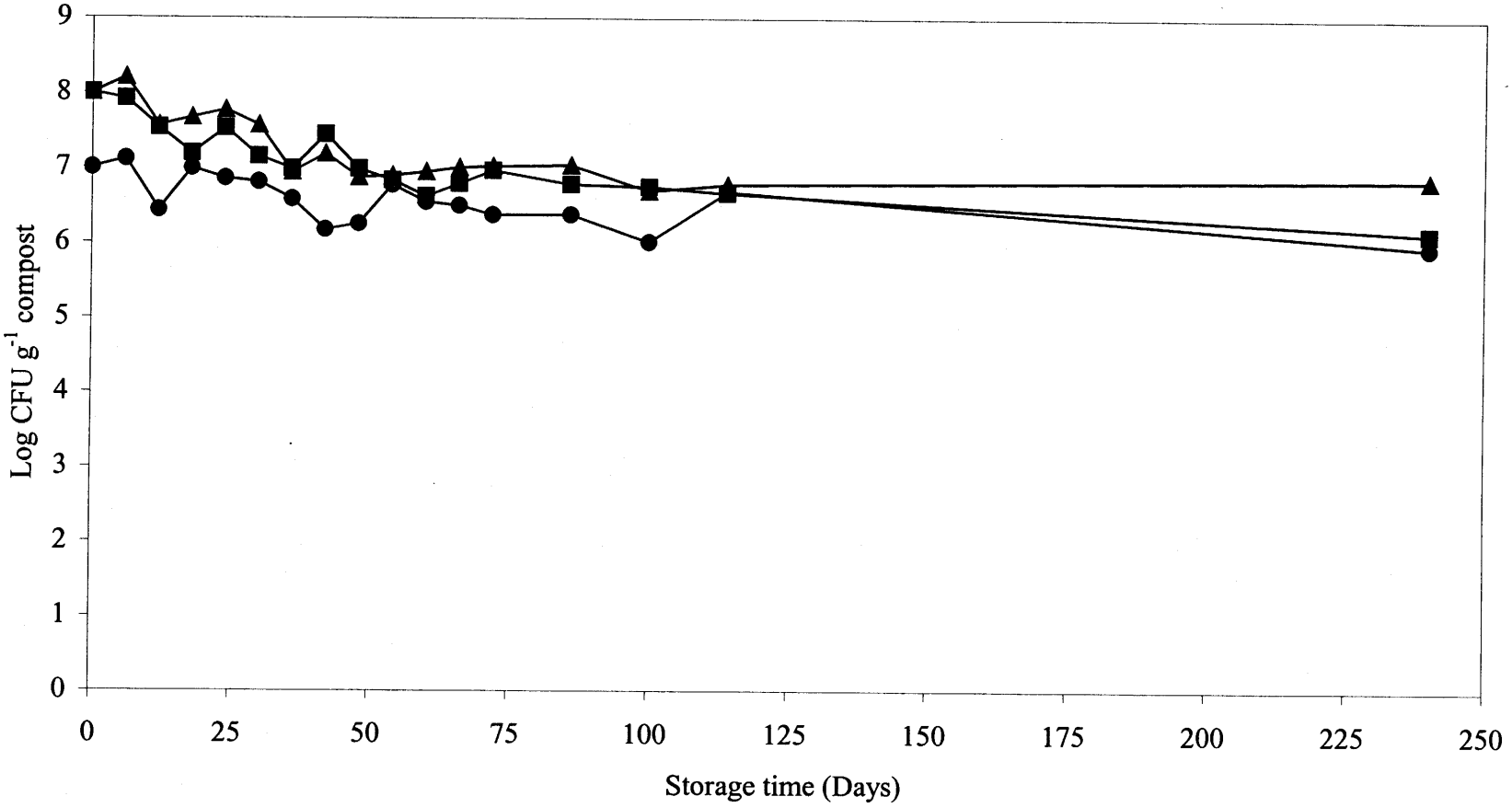

Figure 4.3.4. Survival of *Arthrobacter globiformis* (B2) on EA compost granules (●), PA compost granules (■), SZ compost granules (▲), stored at 22°C. The raw products used to produce the compost granules were supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK.

R. aquatilis phenanthrene-degrading populations inoculated onto the three composts (stored at 4°C) and the *A. globiformis* phenanthrene-degrading populations inoculated onto the EA and SZ compost granules (stored at 4°C) were one log unit higher than when stored at 22°C (Figures 4.3.1 - 4.3.4). The phenanthrene-degrading *R. aquatilis* and *A. globiformis* survived best on the EA and SZ composts. For example, the percentage of original *R. aquatilis* on the composts after 240 days of storage at 4°C was 29.8 and 23.0% for the EA and SZ composts respectively. The PA compost only resulted in 1.5% of the original *R. aquatilis* after 240 days of storage at 4°C. A similar trend was seen with the *A. globiformis* (i.e. the percentage of original enumeration after 240 days of incubation at 4°C was 25, 10.6, and 1.3% for the EA, SZ and PA composts respectively. The means and standard deviations of the populations enumerated on each sampling day are presented in Tables C1-C4 (Appendix C).

Efficiency of inoculated granular compost in phenanthrene-spiked soil

Continuous, non-destructive sampling experiment

The ability of *A. globiformis* inoculated onto compost granules to degrade phenanthrene in soil was examined (Figure 4.3.5). Addition of the inoculant did not enhance the degradation of phenanthrene in the spiked soil. That is, there was no significant decrease in the measurable concentration of volatile phenanthrene in the head-space of the reaction vessels after 139 days of incubation.

The distribution of the phenanthrene-degrading bacteria in the soil and on the compost granules was determined following completion of the degradation experiment. The titre of phenanthrene-degrading bacteria present in the soil and SZ compost granules (of the inoculated treatment) following the incubation period indicated that the bacteria did not completely re-locate off of the granules and into the soil environment. The titre

of phenanthrene-degrading bacteria on the granules was 4.87×10^5 CFU g^{-1} (originally 5×10^7 CFU g^{-1}) and in the reaction-vessel soil was 6.69×10^4 CFU g^{-1}. These results suggested that the delivery of the inoculant may have been inefficient. Therefore, a second study examining mode of inoculant application was completed to address this question.

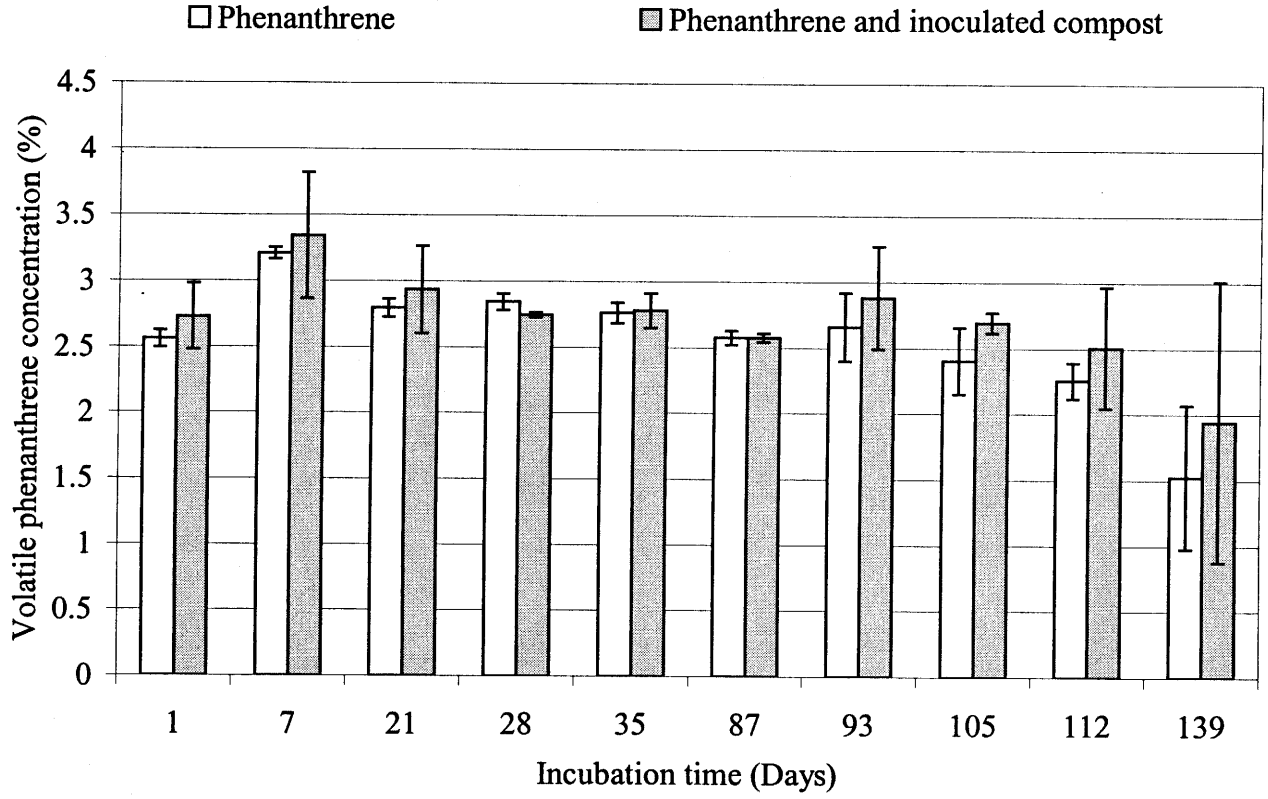

Figure 4.3.5. Concentration of volatile phenanthrene following the addition of SZ compost granules inoculated with *Arthrobacter globiformis* (135-day-old inoculant). A control treatment measured 0% phenanthrene throughout the sampling period (data not shown). Error bars indicate standard deviations for the individual sampling days (n=3 replications).

Batch, destructive sampling experiment

The phenanthrene degradation capabilities of bacteria inoculated into phenanthrene-spiked soil using various inoculation methods was examined. The reaction vessels in this experiment were single use only.

During the first 60 days of this incubation, phenanthrene measured in the head-space of the reaction vessels decreased slightly (from ~3.5% at Day 0 to ~1.75% by Day 60) (Figure 4.3.6).

After 150 days of incubation, the crushed granule and liquid bacterial formulations promoted the highest rate of degradation as seen by a decrease in the amount of measurable volatile phenanthrene (Figure 4.3.6).

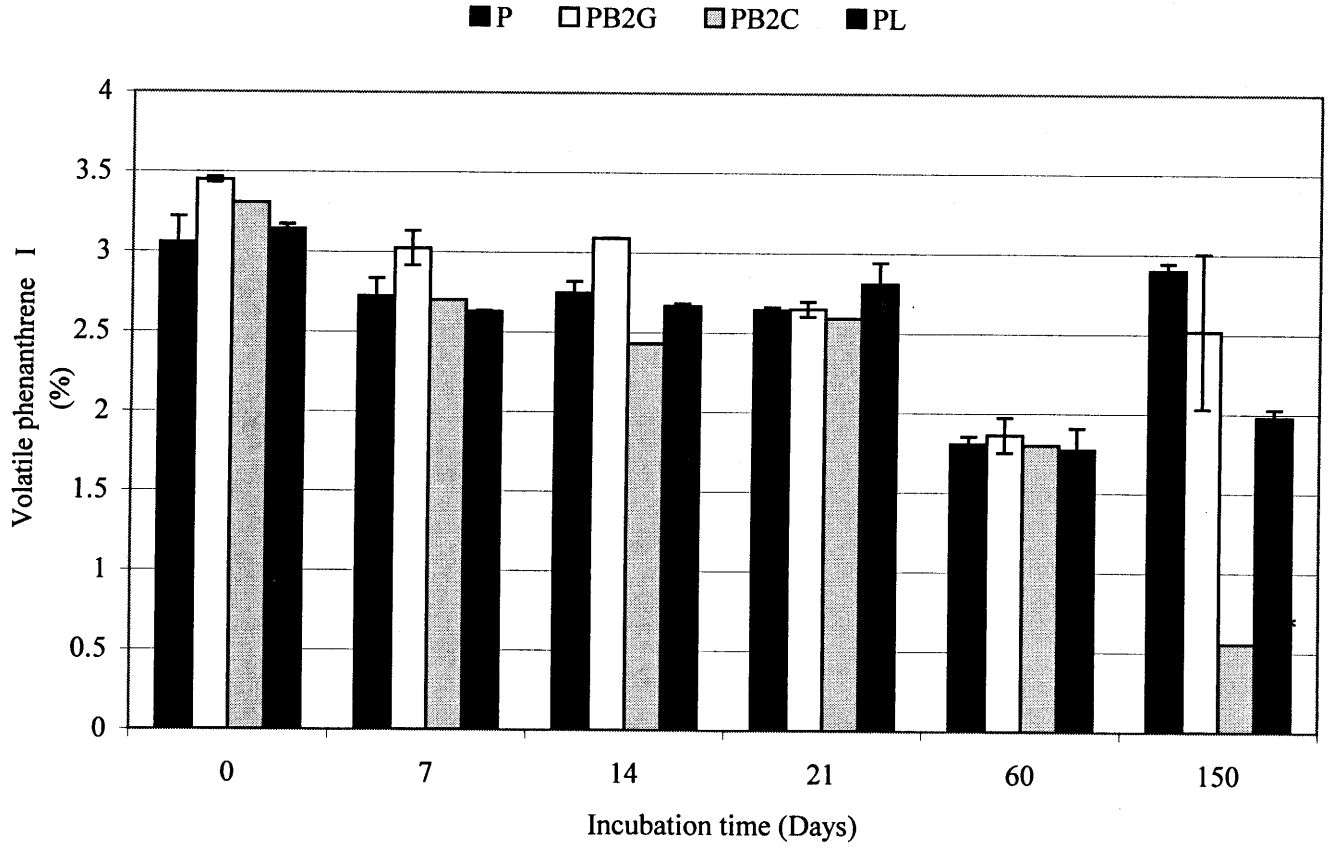

Figure 4.3.6. Concentration of volatile phenanthrene following the addition of *Arthrobacter globiformis*-inoculated whole granules (PB2G), crushed granules (PB2C) and liquid *A. globiformis* cells (PL) into phenanthrene-spiked soil and soil containing no inoculant (P). A control treatment measured 0% phenanthrene throughout the sampling period (data not shown). Error bars indicate standard deviations for the individual sampling days (n=3 replications). * Only two samples were available at the 150 day mark so no standard deviation was determined for this sample time.

4.4 DISCUSSION

Generally, an increase in soil depth results in a decrease in bacterial populations and diversity due to a decrease in oxygen and available nutrients (Ellis *et al.*, 1990; Riser-Roberts, 1998; Steffensen and Alexander, 1995). Higher phenanthrene-degrading bacterial populations were enumerated at the 25-50 cm depth for most of the soil samples from Hendon as opposed to the 0-25 cm depth. Higher TPH levels were measured at the 25-50 cm depth as well, which may explain the high populations of phenanthrene-degrading bacteria present at this depth. That is, higher concentrations of the growth substrate (contaminant) would support higher bacterial populations.

The diversity of phenanthrene-degrading bacteria isolated from the Hendon soil samples was similar to the diversity of bacteria in PAH-contaminated soils reported in other studies (Chaîneau *et al.*, 1999; Reilley *et al.*, 1996; Riser-Roberts, 1998; Whyte *et al.*, 1998).

Compost is rich in nutrients and is very similar to soil. Due to these properties it generally contains many different types of bacteria that are present in high numbers.

Some of the genera isolated from the composts used in this thesis included *Arthrobacter* spp., *Paenibacillus* spp., *Clavibacter* spp., and *Bacillus* spp. Thus, it is not unreasonable to predict that survival of the *Arthrobacter globiformis* and *Rahnella aquatilis* (phenanthrene-degrading strains) on the compost granules would be achievable. Indeed, when stored at 4°C the composts supported very high populations of these two phenanthrene-degrading bacteria for a period of 240 days, after which time,

enumerations were no longer performed. Due to the similarity of these degrading bacteria to those indigenous to the compost, competition between the two groups of bacteria was not expected, especially when the composts were stored at 4°C and bacterial growth was subdued. However, storage of the inoculated compost granules at 22°C resulted in decreased populations of these phenanthrene-degrading bacteria on the compost granules. The increased temperature may have increased the growth of the indigenous bacteria and as a result their numbers increased at a rate faster than the introduced bacteria thus resulting in competition for available resources or production of antibacterial compounds specific for the PAH-degrading bacteria. Similar results have been reported in studies where native rhizobia and other microbial contaminants present in carrier materials were responsible for reduced survival of *Rhizobium* strains that were inoculated onto the carriers, resulting in subsequent reduced nodulation rates (Olsen *et al.*, 1994). Similarly, an increased temperature may have not been conducive to the growth requirements for the degrading bacteria.

Addition of inoculated granular compost to phenanthrene-spiked soil did not result in an increase in the rate of phenanthrene degradation as compared to uninoculated compost added to the phenanthrene-spiked soil. One explanation may be that the phenanthrene-degrading bacteria did not migrate off of the granules and into the spiked soil in a concentration high enough to produce a significant level of degradation. This was indeed shown to be one possible explanation when the soil and manually-removed compost granules from this experiment were enumerated for the number of phenanthrene-degrading bacteria present in each entity. Another possibility may have been that the bacteria/phenanthrene contact in the soil was poor due to the surface area of the granules relative to the surface area of the soil. In addition, in either case soil

moisture content could have played a role, perhaps being too low (20%) to facilitate movement of bacteria to sites of phenanthrene contamination.

To address the question of bacterial migration the experiment pertaining to different inoculant application techniques reinforced the importance of inoculant/soil contact for phenanthrene degradation. Inoculation of phenanthrene-spiked soil with inoculated crushed granules resulted in the highest level of phenanthrene degradation of all of the application techniques after 150 days of incubation. Based on these preliminary findings, there is potential for the addition of inoculated crushed granules to have a two-fold benefit for remediation programs. Firstly, the addition of the inoculant would add a high number of PAH-degrading bacteria (could be a consortium or a few isolated strains) that could assist in the degradation of the PAHs in the soil. Due to the fine nature of the ground compost granules, incorporation into the contaminated soil could contribute to successful microbe/contaminant contact. Secondly, the compost itself may have been a very important source of nutrition for the PAH-degrading bacteria. For example, the addition of fine, powdered compost enriched the contaminated soil environment, making the environment more conducive for bacterial growth and proliferation. The addition of the inoculant in this fashion could help to reduce two of the major obstacles, population dynamics and nutrient depletion.

A second method of application would be the application of the crushed granules to seeds that would be used in phytoremediation programs. Some research has investigated the possibility of coating seeds capable of germination and growth in PAH-contaminated soil with PAH-degrading bacteria. The central idea was that the PAH-degrading bacteria would be introduced at an early stage into the rhizosphere of the plant and as a result enhance root growth and development. At the same time the developing

PAH-degrading bacterial populations would function to reduce the contaminant surrounding the root. Some groups have had good success with this type of technology.

The ability of the selected non-sterile composts to support large populations of phenanthrene-degrading bacteria could play an important role in remediation technologies. Further studies may include: inoculating the crushed compost granules with different strains of PAH-degrading bacteria. For instance strains that have been proven to be successful PAH degraders in a soil environment with the subsequent application of this inoculant to contaminated soil; secondly, investigation into the coating of seeds, used in phytoremediation projects, with the inoculated crushed compost granules could be performed and compared to uninoculated plants.

5.0 SUMMARY AND CONCLUSIONS

Different microbial inoculants such as those used in legume production and soil remediation programs are widely used and are an important part of their respective industries. However successful, these inoculants are currently being improved and optimised. There are a number of different basic requirements inoculants must have in order to be of value to consumers. Firstly, a successful inoculant will contain the most effective strains of microorganisms in relation to their given tasks. A successful carrier will also have a long storage potential, be free from harmful contaminants, have the ability to support large populations of the intended microorganisms and be relatively inexpensive and easy to apply. On top of these requirements, it is also beneficial for the inoculant to have characteristics that make it applicable to a range of different environmental situations such as vastly different climatic zones and different soil zones.

Rhizobium inoculants have long been implemented in legume production with a good record of success. Modes of application of *Rhizobium* in legume production have, in the past, included the dipping of seeds in liquid culture, the coating of seeds with a carrier material containing the rhizobia, and the application of a separate granular carrier material containing the rhizobia, during the seeding process.

A number of carrier materials have been tried, but the most successful *Rhizobium* carrier to date is peat. Peat has good moisture holding capacity, buffering capacity, and high nutritional value that all positively influence the growth and

survival of rhizobia. However, in some cases inoculant-quality peat is difficult and expensive to obtain. In addition, the variability in quality between different batches of peat and the potential reduction in sufficient quantities to meet industrial demands are some concerns currently being discussed. A number of different carriers have been proposed to act as alternatives to peat. One that has received little attention is compost. The first chapter of this thesis investigated the possibility of using compost as an alternative carrier material for *Rhizobium.* Three composts varying in their constituents were chosen and a granular form of each was prepared. A series of studies examined the ability of non-sterile and sterile granular compost to support large populations of rhizobia. In addition growth chamber studies examined the ability of the granular carriers to deliver nodulating rhizobia to pea plants in both sterile (Leonard jar) and non-sterile (soil pot studies) environments.

Survival on all three composts was extended past 30 days when the compost granules were gamma irradiated prior to inoculation and storage. As a result of the irradiation, the elimination of competitive bacteria in the composts increased the probability of survival for the introduced rhizobia. In addition, the irradiation of the compost granules did not result in any detrimental effects or result in the production of compounds toxic to rhizobia as seen in other studies.

Several studies discuss the concerns surrounding the use of standard plate counts for the enumeration of rhizobia in inoculants. For example, culturable rhizobia may not necessarily retain the ability to nodulate legumes despite their culturability, therefore resulting in an incorrect assessment of the nodulation capabilities of a given inoculant. For this reason, after the completion of the survival

study the inoculant was then applied to pea seeds in a sterile Leonard jar environment. The rhizobia still present in the composts successfully nodulated the peas. The application of the inoculant resulted in a significant increase in the total nitrogen found in the plant tissue as compared to the control but this increase was not significantly greater than the total nitrogen content obtained with the commercial inoculants.

The use of sterile and non-sterile compost granules, inoculated with *Rhizobium,* to nodulate field pea in field soil (pot study) was examined and indicated variable nodulation. In the case of the non-sterile compost experiment, the soil nitrogen levels were too high for successful symbiosis, resulting in poor nodulation. However, the application of *Rhizobium*-inoculated sterile compost granules to peas in soil with a lower nitrogen level resulted in a different outcome. Lower plant total nitrogen values were produced in the inoculated compost treatments as compared to commercial inoculants but they were significantly higher than the control treatment total nitrogen values.

The need for a successful inoculant formulation for bioremediation projects is at the forefront of remediation technology development. The lack of consistent degradation results seen with the addition of hydrocarbon-degrading microorganisms to contaminated soil has prompted research in this area. To examine this problem a series of experiments were done to firstly, develop a granular carrier material for phenanthrene-degrading bacteria that would assist in the application of the bacteria to contaminated soil, and secondly to examine the ability of the granular carrier to deliver the bacteria in a contaminated soil environment.

The same three composts used in the *Rhizobium*-carrier production experiments were also tested for their ability to promote the growth and survival of two strains of phenanthrene-degrading bacteria, *Rahnella aquatilis* and *Arthrobacter globiformis*. After 240 days of storage, the counts of these two bacteria were generally one log unit higher when the composts were stored at 4°C verses 22°C. The composts were able to support high numbers of these different bacteria, despite not being sterilized prior to inoculation. The SZ compost (135 days old), inoculated with *A. globiformis*, was added to phenanthrene-spiked soil. This addition did not result in a significant decrease in the amount of measurable volatile phenanthrene. However, when different formulations of the SZ-*A. globiformis* inoculant were added to the phenanthrene-spiked soil, degradation was seen. After 150 days of incubation, the crushed granule formulation resulted in the most substantial reduction in measurable volatile phenanthrene. This reduction was thought to be due to the improved inoculant/contaminant contact obtained by adding crushed granules versus whole granules.

Preliminary results presented in this thesis indicated that compost has the ability to promote extended survival of both *Rhizobium leguminosarum* bv. *viciae* and also phenanthrene-degrading bacteria. Preliminary results obtained in this thesis regarding the use of granular compost in delivering these organisms to the soil environment indicated potential for success. Further examination into the mode of application, i.e. granular or seed coat in the case of *Rhizobium* application as well as phenanthrene-degrading bacteria application could determine the suitability and potential of this product for industrial use.

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APPENDIX A: Chemical and microbiological data of raw compost materials.

Table A1. Supplier and general description of the compost and commercial peat materials screened for use in developing a granular carrier for rhizobia.

Supplier	ID Code	Description
Becker Underwood; Saskatoon, SK	Peat	Inoculant quality peat.
LiphaTech; Milwaukee, WI	Peat 1	Inoculant quality peat.
Corcan Agribusiness; Prince Albert, SK	PA	Composted cattle manure mixed with straw.
EcoAg Initiatives; Calgary, AB	EA	Horticultural grade compost from cattle manure and animal bedding.
Forestry Farm Park Saskatoon Zoo; Saskatoon, SK	SZ	Composted animal waste and bedding material mixed with horticultural waste, grass clippings, and leaves.

Table A.2. Chemical analysis of non-sterile (NS) and sterile (S) raw compost[†].

Analyte	EA-NS	EA-S	PA-NS	PA-S	SZ-NS	SZ-S
			$\mu g\ g^{-1}$			
Aluminum	16600	16600	20400	23800	31300	36200
Arsenic	3.5	7	4.4	4.7	6.6	7.1
Barium	180	160	180	200	220	240
Berylium	<0.5	<0.5	0.5	0.6	0.8	1
Boron	<1	<1	<1	<1	<1	<1
Cadmium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Calcium	44200	29800	18800	28500	25900	23800
Chloride[‡]	8100	300	2900	3400	700	700
Chromium	20	20	24	26	27	9.6
Cobalt	4.5	4.7	5.8	6.3	7.4	7.4
Copper	42	10	29	48	20	19
Iron	11000	15100	15200	17300	21700	22800
Lead	5	9	7	7	14	14
Magnesium	10000	3900	8400	10100	12500	12300
Manganese	310	340	350	370	430	390
Molybdenum	2	2.5	1.3	2.1	0.9	2.3
Nickel	12	14	15	17	21	21
Nitrogen[¶]	1.26	1.33	0.81	1.01	0.37	0.36
Phosphorus	9700	840	4200	7200	1200	1200
Potassium	22900	2200	15200	17700	9300	10400
Selenium	1.1	1.2	<0.5	0.6	<0.5	0.6
Silver	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Sodium	3900	230	2100	2500	360	420
Strontium	54	39	47	67	58	59
Sulfur	3400	3600	1900	3000	860	820
Titanium	180	400	420	460	490	720
Vandium	32	31	42	49	57	60
Zinc	220	53	130	210	89	91
Zirconium	3.9	10	9.1	11	15	6.6

[†] The raw products were supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK.
[‡] Water soluble chloride.
[¶] Total Kjeldahl nitrogen.

APPENDIX B: Enumeration of *Rhizobium leguminosarum* bv. *viciae* inoculated onto compost granules.

Table B1. Mean values and standard deviations (SD) of enumerations of *Rhizobium* present on non-sterile EA compost (EA), PA compost (PA) and SZ compost (SZ).[†]

Storage time (Days)	EA	SD	PA	SD	SZ	SD
	Rhizobia g^{-1} compost					
0	9.25×10^7	1.71×10^7	7.88×10^7	1.75×10^7	3.00×10^7	1.47×10^7
2	3.75×10^7	8.66×10^6	4.25×10^7	9.57×10^6	3.38×10^6	1.11×10^6
4	6.63×10^7	8.54×10^6	4.75×10^7	9.57×10^6	2.50×10^6	2.89×10^6
7	2.63×10^7	5.19×10^6	3.69×10^7	9.56×10^6	7.50×10^5	6.45×10^5
14	2.64×10^7	5.76×10^6	3.70×10^7	9.42×10^6	7.50×10^5	6.45×10^5
21	3.45×10^6	7.23×10^5	1.13×10^7	2.27×10^6	9.05×10^4	2.41×10^4
28	1.22×10^7	2.14×10^6	3.80×10^6	1.15×10^6	3.80×10^4	1.37×10^4
35	5.06×10^6	1.34×10^6	8.88×10^5	3.97×10^5	1.00×10^3	ND
42	3.70×10^6	7.53×10^5	6.40×10^5	1.26×10^5	1.00×10^3	ND
49	3.00×10^6	7.07×10^5	4.65×10^5	1.19×10^5	1.00×10^3	ND
56	6.83×10^5	1.19×10^5	2.80×10^5	5.29×10^4	1.00×10^3	ND
63	1.20×10^5	4.83×10^4	9.13×10^4	2.93×10^4	1.00×10^3	ND
70	4.75×10^4	1.44×10^4	3.25×10^4	3.18×10^4	1.00×10^3	ND
77	1.00×10^3	ND	1.00×10^3	ND	1.00×10^3	ND

[†] The raw products were supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK. ND = Not Determined.

Table B2. Mean values and standard deviations (SD) of enumerations of *Rhizobium* present on non-sterile peat (Peat) granules and commercial granular inoculant (BU and LT). †

Storage time (Days)	Peat	SD	BU	SD	LT	SD
	Rhizobia g^{-1} compost					
0	3.70×10^7	2.12×10^6	2.49×10^7	2.43×10^6	7.50×10^6	2.93×10^6
2	3.75×10^7	2.38×10^6	2.49×10^7	6.42×10^6	7.39×10^4	1.01×10^5
4	7.75×10^6	2.51×10^6	3.39×10^7	7.55×10^6	7.50×10^3	1.00×10^4
7	9.26×10^6	3.46×10^6	4.64×10^7	4.27×10^6	7.50×10^3	1.00×10^4
14	7.33×10^6	2.57×10^6	4.53×10^6	1.75×10^6	5.38×10^4	1.75×10^4
21	5.20×10^6	2.38×10^6	3.68×10^7	1.23×10^7	1.43×10^5	4.27×10^4
28	5.38×10^6	2.73×10^6	1.19×10^7	2.44×10^6	1.25×10^5	2.38×10^4
35	6.33×10^6	1.40×10^6	3.88×10^7	3.59×10^6	4.63×10^4	2.50×10^4
42	6.30×10^6	1.28×10^6	3.70×10^7	4.69×10^6	4.13×10^4	2.02×10^4
49	4.08×10^6	8.27×10^5	3.59×10^7	3.94×10^6	1.24×10^5	1.11×10^4
56	7.15×10^6	2.13×10^6	4.05×10^7	4.36×10^6	9.13×10^4	6.34×10^4
63	6.90×10^7	1.41×10^6	1.07×10^7	8.96×10^5	9.75×10^4	3.86×10^4
70	4.98×10^6	6.99×10^5	5.53×10^6	7.18×10^5	2.38×10^4	9.46×10^3
77	4.23×10^6	1.22×10^6	9.55×10^6	7.94×10^5	2.63×10^4	1.49×10^4

† The raw products were supplied by the following suppliers: Peat from Becker Underwood, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech, Milwaukee, WI.

Table B3. Mean values and standard deviations (SD) of enumerations of *Rhizobium* present on sterile EA compost (EA), PA compost (PA) and SZ compost (SZ) granules and commercial granular inoculants (BU and LT)[†].

Storage Time (Days)	EA	SD	PA	SD	SZ	SD	BU	SD	LT	SD
					Rhizobia g^{-1} compost					
1	4.88×10^6	1.60×10^6	3.63×10^7	1.60×10^6	9.28×10^7	1.60×10^6	2.53×10^7	5.68×10^6	1.93×10^4	7.08×10^3
7	1.75×10^6	2.08×10^5	5.40×10^7	2.08×10^5	5.51×10^7	2.08×10^5	3.99×10^7	2.78×10^6	1.93×10^4	7.08×10^3
14	1.41×10^6	2.75×10^5	9.60×10^6	2.75×10^5	1.36×10^7	2.75×10^5	5.14×10^7	1.17×10^7	1.16×10^4	3.71×10^3
21	5.79×10^5	1.58×10^5	2.66×10^6	1.58×10^5	5.04×10^6	1.58×10^5	4.13×10^7	4.27×10^6	1.33×10^4	5.56×10^3
28	5.24×10^5	1.29×10^5	2.45×10^6	1.29×10^5	6.15×10^6	1.29×10^5	4.23×10^7	6.30×10^6	9.38×10^3	2.84×10^3
35	5.35×10^5	1.11×10^5	3.93×10^6	1.11×10^5	8.80×10^6	1.11×10^5	4.35×10^7	7.29×10^6	1.34×10^4	3.50×10^3
42	4.33×10^5	7.79×10^4	8.48×10^5	7.79×10^4	7.83×10^6	7.79×10^4	5.18×10^7	7.89×10^6	1.39×10^4	2.81×10^3
49	5.75×10^5	8.84×10^4	4.74×10^6	8.84×10^4	9.35×10^6	8.84×10^4	4.85×10^7	1.91×10^6	1.38×10^4	5.00×10^2
56	7.49×10^5	1.46×10^5	2.84×10^6	1.46×10^5	8.86×10^6	1.46×10^5	3.58×10^7	5.95×10^6	1.99×10^4	1.93×10^3
63	5.20×10^5	7.11×10^4	2.36×10^6	7.11×10^4	6.29×10^6	7.11×10^4	3.84×10^7	4.07×10^6	1.83×10^4	2.75×10^3
70	6.73×10^5	5.39×10^4	8.13×10^5	5.39×10^4	4.96×10^6	5.39×10^4	3.53×10^7	3.01×10^6	2.55×10^4	5.74×10^3
77	3.71×10^5	6.22×10^4	6.31×10^5	6.22×10^4	3.74×10^6	6.22×10^4	4.43×10^7	4.87×10^6	1.84×10^4	9.46×10^2
84	3.66×10^5	6.32×10^4	6.69×10^5	6.32×10^4	5.85×10^6	6.32×10^4	3.70×10^7	1.41×10^6	1.83×10^4	8.66×10^2
91	2.39×10^5	5.45×10^4	4.16×10^5	5.45×10^4	3.23×10^6	5.45×10^4	3.60×10^7	3.03×10^6	1.81×10^4	1.31×10^3
105	2.20×10^5	1.91×10^4	3.58×10^5	1.91×10^4	7.93×10^6	1.91×10^4	3.23×10^7	1.26×10^6	1.16×10^4	1.38×10^3
119	2.25×10^5	2.48×10^4	2.66×10^5	2.48×10^4	5.09×10^6	2.48×10^4	4.39×10^7	5.78×10^6	1.11×10^4	1.03×10^3
147	8.21×10^4	8.27×10^3	1.60×10^4	8.27×10^3	2.59×10^6	8.27×10^3	3.76×10^7	1.80×10^6	1.95×10^4	3.32×10^3
224	5.85×10^4	1.37×10^4	9.88×10^3	1.37×10^4	5.36×10^5	1.37×10^4	3.43×10^7	1.49×10^6	2.76×10^4	6.73×10^3

[†] The raw products were supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK; SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK; BU granular inoculant from Becker Underwood, Saskatoon, SK; and LT granular inoculant from LiphaTech, Milwaukee, WI.

APPENDIX C: Enumeration of phenanthrene-degrading bacteria inoculated onto compost granules.

Table C1. Enumeration of phenanthrene-degrading *Rahnella aquatilis* (B1) inoculated on non-sterile EA compost granules (EAB1) and PA compost granules (PAB1) stored at 4°C and 22°C. SD = standard deviation of replicate plates for each day (n=4)[†].

Incubation Time (Days)	EAB1 4°C	SD	EAB1 22°C	SD	PAB1 4°C	SD	PAB1 22°C	SD
	Colony forming units g^{-1} compost							
0	1.00×10^7	0	1.00×10^7	0	1.00×10^8	0	1.00×10^8	0
6	1.71×10^7	1.12×10^7	2.50×10^6	1.68×10^6	1.00×10^8	1.52×10^7	3.65×10^7	8.10×10^6
12	1.10×10^7	3.94×10^6	4.88×10^6	3.33×10^6	7.11×10^7	1.03×10^7	3.60×10^7	2.84×10^7
18	5.72×10^6	5.54×10^5	4.08×10^6	4.16×10^5	1.06×10^8	7.69×10^6	1.70×10^8	2.21×10^7
24	9.70×10^6	3.98×10^6	5.61×10^6	1.66×10^6	4.08×10^7	1.38×10^6	1.78×10^7	5.10×10^6
30	8.43×10^6	3.46×10^6	3.61×10^6	1.74×10^6	5.96×10^7	1.43×10^7	6.44×10^6	2.17×10^6
36	1.29×10^6	1.65×10^6	5.88×10^6	1.04×10^6	1.03×10^8	1.26×10^7	1.07×10^7	3.06×10^6
42	5.75×10^6	1.13×10^6	2.33×10^6	2.36×10^5	2.76×10^7	9.41×10^6	8.56×10^6	6.57×10^5
48	9.18×10^6	2.85×10^6	5.04×10^6	2.62×10^6	2.98×10^7	1.34×10^7	6.26×10^6	1.40×10^6
54	1.13×10^6	2.29×10^6	5.71×10^6	3.42×10^5	1.43×10^7	3.59×10^6	5.70×10^6	1.90×10^6
60	5.40×10^6	1.21×10^6	2.55×10^6	8.96×10^5	1.53×10^7	6.36×10^6	4.09×10^6	1.50×10^6
66	7.04×10^6	2.31×10^6	1.98×10^6	2.07×10^6	9.48×10^6	7.49×10^5	7.03×10^6	2.95×10^6
72	5.36×10^6	2.16×10^6	4.20×10^6	3.21×10^6	1.24×10^7	5.79×10^6	6.81×10^6	3.72×10^6
86	4.80×10^6	1.30×10^6	9.45×10^5	1.21×10^5	1.05×10^7	2.79×10^6	4.88×10^6	2.11×10^6
100	4.71×10^6	1.98×10^6	2.41×10^6	4.50×10^5	3.69×10^7	2.19×10^7	2.30×10^6	2.34×10^4
114	4.29×10^6	4.77×10^5	2.36×10^6	1.03×10^6	4.94×10^6	1.96×10^6	3.53×10^6	7.42×10^5
240	2.98×10^6	7.70×10^5	1.68×10^6	5.40×10^5	1.47×10^6	3.75×10^5	8.71×10^5	2.60×10^5

[†] The raw products were supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK.

Table C2. Enumeration of phenanthrene-degrading *Rahnella aquatilis* (B1) inoculated on non-sterile SZ compost granules (SZB1) stored at 4°C and 22°C. SD = standard deviation of replicate plates for each day (n=4)[†].

Incubation Time (Days)	SZB1 4°C	SD	SZB1 22°C	SD
	Colony forming units g^{-1} compost			
0	1.00×10^8	0	1.00×10^8	0
6	1.39×10^8	2.72×10^7	9.73×10^7	2.74×10^7
12	1.61×10^8	8.54×10^7	7.25×10^7	3.66×10^7
18	1.13×10^8	2.89×10^7	2.99×10^7	7.55×10^6
24	1.31×10^8	2.37×10^7	8.80×10^7	2.33×10^7
30	1.38×10^8	2.94×10^7	4.79×10^7	1.45×10^7
36	2.49×10^8	1.44×10^7	4.78×10^7	1.82×10^7
42	2.72×10^7	3.88×10^6	4.08×10^7	5.61×10^6
48	1.37×10^8	5.27×10^6	1.70×10^7	1.26×10^6
54	1.62×10^8	2.24×10^7	1.60×10^7	6.13×10^5
60	1.32×10^8	3.57×10^7	3.33×10^7	1.09×10^7
66	1.13×10^8	1.33×10^7	3.20×10^7	4.69×10^6
72	1.26×10^8	3.67×10^7	2.40×10^7	9.20×10^6
86	9.64×10^7	1.55×10^7	2.35×10^7	7.39×10^6
100	7.50×10^7	1.62×10^7	1.24×10^7	3.39×10^6
114	2.90×10^7	2.07×10^7	1.39×10^7	3.09×10^6
240	2.30×10^7	6.92×10^6	5.55×10^6	2.18×10^6

[†] The raw compost products were supplied by: SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK.

Table C3. Enumeration of phenanthrene-degrading *Arthrobacter globiformis* (B2) inoculated on non-sterile EA compost granules (EAB2) and PA compost granules (PAB2) stored at 4°C and 22°C. SD = standard deviation of replicate plates for each day (n=4) [†].

Incubation Time (Days)	EAB2 4°C	SD	EAB2 22°C	SD	PAB2 4°C	SD	PAB2 22°C	SD
	Colony forming units g^{-1} compost							
0	1.00×10^7	0	1.00×10^7	0	1.00×10^8	0	1.00×10^8	0
6	7.75×10^6	1.19×10^6	1.31×10^7	3.42×10^6	1.56×10^8	4.67×10^7	8.33×10^7	9.07×10^6
12	8.50×10^6	7.78×10^6	2.75×10^6	8.66×10^5	1.55×10^8	3.07×10^7	3.43×10^7	1.63×10^7
18	4.75×10^6	9.32×10^6	9.96×10^6	6.84×10^6	9.11×10^7	2.19×10^7	1.56×10^7	8.50×10^6
24	1.56×10^7	4.95×10^6	7.30×10^6	3.16×10^6	1.18×10^8	3.21×10^7	3.39×10^7	1.08×10^7
30	8.14×10^6	4.07×10^6	6.50×10^6	6.69×10^6	3.50×10^7	1.93×10^6	1.42×10^7	6.56×10^6
36	1.92×10^7	3.84×10^6	3.88×10^6	8.15×10^5	5.58×10^7	1.31×10^7	9.80×10^6	1.17×10^6
42	4.61×10^6	3.09×10^6	1.51×10^6	1.43×10^5	5.61×10^6	2.01×10^6	2.80×10^7	2.71×10^6
48	1.15×10^7	4.27×10^6	1.81×10^6	3.53×10^5	1.39×10^7	5.35×10^6	9.76×10^6	2.15×10^6
54	9.98×10^6	4.96×10^6	6.01×10^6	1.81×10^6	1.78×10^7	4.41×10^5	6.87×10^6	4.30×10^6
60	7.13×10^6	1.64×10^6	3.55×10^6	1.09×10^6	1.41×10^7	1.02×10^7	4.23×10^6	1.10×10^6
66	8.59×10^6	2.10×10^6	3.18×10^6	2.19×10^6	8.58×10^6	2.43×10^6	6.20×10^6	1.45×10^6
72	1.01×10^7	2.23×10^6	2.40×10^6	1.12×10^6	6.23×10^6	6.89×10^5	9.33×10^6	2.03×10^6
86	1.13×10^7	2.73×10^6	2.43×10^6	4.02×10^5	1.08×10^7	4.02×10^6	6.11×10^6	1.72×10^6
100	1.09×10^7	1.11×10^6	1.04×10^6	1.73×10^5	4.74×10^6	1.62×10^6	5.69×10^6	2.41×10^6
114	6.31×10^6	9.59×10^5	4.89×10^6	2.54×10^6	5.08×10^6	2.31×10^6	4.69×10^6	1.68×10^6
240	2.50×10^6	1.11×10^6	8.89×10^5	2.75×10^5	1.29×10^6	1.64×10^5	1.36×10^6	2.43×10^5

[†] The raw products were supplied by the following suppliers: EA compost from EcoAg Initiatives, Calgary, AB; PA compost from Corcan Agribusiness, Prince Albert, SK.

Table C4. Enumeration of phenanthrene-degrading *Arthrobacter globiformis* (B2) inoculated on non-sterile SZ compost granules (SZB2) stored at 4°C and 22°C. SD = standard deviation of replicate plates for each day (n=4)[†].

Incubation Time (Days)	SZB2 4°C	SD	SZB2 22°C	SD
	Colony forming units g^{-1} compost			
0	1.00×10^8	0	1.00×10^8	0
6	2.39×10^8	7.07×10^7	1.61×10^8	6.45×10^7
12	2.57×10^8	1.14×10^8	3.66×10^7	2.86×10^7
18	1.38×10^8	3.78×10^7	4.68×10^7	4.12×10^7
24	2.06×10^8	5.58×10^7	5.90×10^7	3.22×10^7
30	1.87×10^8	4.44×10^7	3.71×10^7	3.93×10^7
36	2.94×10^8	2.87×10^8	8.83×10^6	1.78×10^6
42	1.61×10^8	6.55×10^7	1.54×10^7	2.67×10^6
48	1.49×10^8	2.06×10^7	7.56×10^6	9.25×10^5
54	1.63×10^8	5.74×10^7	7.96×10^6	8.02×10^5
60	9.38×10^7	2.03×10^7	8.93×10^6	2.51×10^6
66	1.19×10^8	5.72×10^7	1.02×10^7	1.77×10^6
72	1.19×10^8	2.65×10^7	1.06×10^7	2.93×10^6
86	1.34×10^8	2.82×10^7	1.10×10^7	4.24×10^6
100	5.14×10^7	2.18×10^7	4.99×10^6	9.26×10^6
114	1.83×10^7	1.00×10^7	6.10×10^6	1.97×10^6
240	1.06×10^7	1.14×10^6	7.15×10^6	1.55×10^6

[†] The raw compost products were supplied by: SZ compost from Forestry Farm Park Saskatoon Zoo, Saskatoon, SK.

APPENDIX D: Solid Phase Micro-Extraction gas chromatography parameters.

Solid Phase Micro-Extraction Gas Chromatography Parameters

OVEN\DETECTOR

Runtime (min): 30.7

Zone Temperatures:

	State	Setpoint
Inl. A	ON	225 C.
Inl. B	OFF	50 C.
Det. A	ON	250 C.
Det. B	OFF	50 C.
Aux.	OFF	50 C.

Oven Zone:

Oven max	300 C.
Equib Time	2.00 Min.
Oven State	ON
Cryo State	OFF
Ambient	25 C.
Cryo Blast	OFF

Oven Program:

	Setpoint
Initial Temp.:	40 C.
Initial Time:	5.00 Min.

Level	Rate (C/min.)	Final Temp.(C)	Final Time (min)
1	7.50	225	1.00

Purge Valve Settings:

Purge A/B:

	Init Value	On Time (Min.)	Off Time (Min.)
A (Valve 3)	Off	3.00	30.67
B (Valve 4)	Off	0.00	0.00

A - Splitless Injection: Yes
B - Splitless Injection: No

VALVES/RELAYS

Initial Setpoints:

5890 Valves:

Valve 1:	Off
Valve 2:	Off
Valve 3 (Purge A):	Off
Valve 4 (Purge B):	Off

Detector Information

Detector A:

Type	FID
State	ON

Detector B:

Type	FPD
State	OFF

Signal Information

Save Data: Signal 1

Signal 1:

Signal	Det. A
Data rate	5.000 Hz.
Peakwidth	0.053 min.
Start Time	0.00 min.
Stop Time	650.00 min.

Signal 2:

Signal	Testplot
Data rate	5.000 Hz.
Peakwidth	0.053 min.
Start Time	0.00 min.
Stop Time	650.00 min