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**SURVIVAL OF GENETICALLY ENGINEERED MICROORGANISMS IN THE
ENVIRONMENT AND A TEST OF THE ABILITY TO PREDICT SURVIVAL OF
Pseudomonas chlororaphis 3732RN-L11 IN SOILS ACROSS CANADA**

by

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Dissertation submitted to Faculty of Graduate Studies, Carleton University
in partial fulfillment of the requirements for the degree of Doctor of Philosophy
2001

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Survival of Genetically Engineered Microorganisms in the Environment
and a Test of the Ability to Predict Survival of
Pseudomonas chlororaphis 3732RN-L11 in Soils Across Canada

Submitted by

Thomas A. Edge, B.Sc. (Hons.), M.Sc.

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ABSTRACT

The survival of a genetically engineered microorganism (GEM) is an important consideration in assessing potential risks posed by an environmental release. A scientific literature review identified 102 records that contained the results of 719 experiments comparing the survival of genetically engineered bacteria relative to their parental microorganisms. Experiments were predominantly short-term (e.g. <100 days) laboratory experiments simulating terrestrial agro-ecosystems in northern temperate climates. The survival of the GEMs was the same or lower than parents in 92 % of experiments. GEMs showed a lower survival relative to parents in 36 % of experiments, and an enhanced survival in 8 % of experiments. The results reject categorical hypotheses that GEMs are less fit or more fit than their parental microorganisms.

Linear multiple regression models were developed to examine the ability to predict survival rates of *Pseudomonas chlororaphis* 3732RN-L11 in bulk soil and wheat rhizosphere. Models were developed from GEM survival data generated in the laboratory using intact soil core microcosms and physical, chemical, and biological data on twenty-three soils measured at the time of GEM inoculation. The models were tested by comparing predicted GEM survival rates with observed GEM survival rates from environmental releases at five field sites across Canada over two years. No single soil parameter like % clay proved very useful for predicting GEM survival in soil or wheat rhizosphere. The best multiple regression model for soil selected fungi colony forming

units (CFUs), % soil titanium (positive correlations) and % soil magnesium (negative correlation) for predicting GEM survival slope. The best model for wheat rhizosphere selected soil bacteria CFUs, soil nitrate, % soil potassium (positive correlations) and soil magnesium (negative correlation) for predicting GEM survival slope. While the models were statistically significant, they were not able to reliably predict the survival rate of the GEM in field trial releases. The results demonstrate there can be considerable uncertainty associated with predicting GEM survival for widespread release into soils.

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1. INTRODUCTION

The development of genetic engineering techniques that allow scientists to recombine pieces of DNA from widely different organisms has been described as a major turning point in human history - a scientific revolution. Others describe the advent of genetic engineering techniques simply as an extension of advances in the development of traditional techniques in biotechnology like fermentation or breeding - a scientific evolution. These different perspectives have underlain many debates about the safety of genetic engineering since the early 1970s when the techniques were first developed.

Genetic engineering (or recombinant DNA (rDNA) technology) can be defined as the use of molecular techniques to produce recombinant molecules of DNA containing new genes or novel combinations of genes, usually for insertion into a host cell for cloning. The first genetic engineering experiments appear to have been conceived from an interest of Stanford biochemist Paul Berg to see whether genes from lower organisms could function in the cells of higher organisms (Krimsky 1982). In 1971, Paul Berg sought to construct a hybrid DNA molecule comprised of a segment of a bacterial virus, some additional bacterial genes (galactose metabolizing enzymes), and monkey tumor virus (SV40) to serve as the vector to transfer the DNA into animal cell chromosomes. The hybrid DNA molecule was then going to be inserted into bacterial and animal cells to determine whether the genes were expressed.

Upon hearing about the proposed experiment, Robert Pollack, Cold Spring Harbour Laboratory, N.Y., contacted Paul Berg to express his concern about the safety of inserting tumor virus SV40 DNA into *Escherichia coli*, a normal inhabitant of the human gut (Cavalieri 1981, 160; Pollack, 1994, 10)¹. After some discussions, the Berg experiment was postponed and this led to a program of tests carried out for the U.S. National Institutes of Health at Fort Detrick in Maryland. These tests indicated that the recombinant *E. coli* strains were not more infectious, and that intestines of volunteers who ingested the strains did not become overgrown with the recombinant bacteria - the normal bacteria of the gut prevailed (Pollack, 1994, 11). However, these events had initiated an increasingly public debate about the safety of genetic engineering.

The rDNA safety debate

Krimsky (1982) considered the start of the public debate on the safety of rDNA techniques to be the Gordon Research Conference on Nucleic Acids (June 11-15, 1973, New Hampton, New Hampshire). At this conference Herbert Boyer from the University of California, San Francisco, and others presented results from using restriction enzymes as a way to recombine DNA into a plasmid, thereby creating a hybrid molecule that could be implanted into bacteria. Scientists at this meeting discussed some of the potential

¹ Citations for books to include specific page numbers as suggested by Wise (2000).

biohazard implications of these experiments. They voted to send a letter to the National Academy of Sciences and the National Institute of Medicine expressing their deep concerns about potential hazards to laboratory workers and the public posed by new kinds of hybrid plasmids or viruses, with biological activity of unpredictable nature. They also published the letter in the journal *Science* to more widely publicize their concerns (Singer and Soll 1973).

When rDNA techniques were first discovered, they were accompanied by fear that genetically engineered microorganisms might pose harm to human health. Scientists recommended a voluntary moratorium around the world in 1974 on certain rDNA experiments until their risks could be more properly ascertained (Berg et al. 1974a; 1974b). These experiments included: 1) introducing genetic determinants for antibiotic resistance or bacterial toxin formation into bacterial strains that did not carry such determinants; and 2) recombining DNA from oncogenic or other animal viruses. The concerns about rDNA techniques reached a wider audience with the 1975 Asilomar conference that debated the risks of the new technology and helped establish the basis for rDNA laboratory research guidelines. This conference recognized that little was known about the survival of laboratory strains of bacteria in ecological niches outside the lab, or whether rDNA molecules would enhance or depress their survival (Berg et al. 1975). It was indicated that research needs in this area were a priority and that collaboration was needed with groups skilled in studying bacterial infection and ecological microbiology.

In the 1980s, debate about the risks of genetically engineered organisms shifted from the laboratory to the environment when researchers first proposed to introduce genetically

engineered microorganisms (GEMs) into the environment in small-scale experimental field studies (Brill 1985a,b; Colwell et al. 1985; Davis 1987; Sharples 1987). While biotechnology advocates drew attention to the safe history of use of GEMs in the laboratory (Davis 1987), others cautioned that the absence of human health effects of biotechnology workers in contained environments, though admirable, was not particularly relevant to the question of whether uncontained uses of GEMs would be equally harmless (Sharples 1987). With environmental applications, a myriad of species in ecological communities could be exposed to an introduced microorganism. Campbell (1991) cautioned that, since no activity is totally risk free, appreciable risks can be expected to surface when the scale of the activity is sufficiently large. Sharples (1987) also expressed concern about the transition to larger scales of application - "It is one thing for trained experimenters to apply novel organisms to a 0.2 acre field under close supervision. It will be quite another matter to market commercial products for widespread use by applicators whose major qualification for using them is possessing the cash to acquire them." Similarly, Levin (1991) warned that insufficient attention had been given to the potential for use of a new microbial product to spread widely once it proves its value on a limited scale. He drew attention to the fact that nobody initially had any conception of the scale on which DDT would eventually be used.

GEMs: benefits and risks

The economic, social and environmental benefits from using rDNA techniques to produce genetically engineered microorganisms are potentially enormous. For example, genetically engineered microorganisms can be used to produce pharmaceuticals to alleviate human suffering. Genetically engineered microorganisms have already been used to produce insulin and human growth hormone. These pharmaceuticals were previously only available from biological tissues that could pose a risk of contamination with pathogens (Devillemeur et al. 1992). rDNA technology can also be used to produce vaccines to protect animals and humans from infectious diseases. In addition, genetically engineered microorganisms could be used for environmental purposes such as to treat contaminated sites where naturally occurring microbial communities cannot readily degrade recalcitrant toxic chemicals (Lindow et al. 1989). van Montagu et al. (1995) indicated that genetic engineering of organisms could be used in a variety of ways to enhance human well-being in areas such as controlling disease, eradicating pests, improving agricultural product quality, and rehabilitating polluted ecosystems.

At the same time, however, there are also potential environmental and human health risks associated with genetically engineered organisms developed using rDNA techniques. For example, Holmes et al. (1998) found that a strain of *Klebsiella planticola* genetically engineered for ethanol production adversely affected plant growth in laboratory microcosms unlike its parental microorganism. Another study determined that a strain of *Achromobacter sp* genetically engineered for constitutive production of alkaline phosphatase significantly changed phosphorus cycling in seawater microcosms relative to

its parental microorganism (Sobecky et al. 1996). Doyle et al. (1991) observed that *Pseudomonas putida* genetically engineered to degrade 2,4-dichlorophenoxyacetate significantly affected microbial populations and processes in soil microcosms compared with its parental microorganism. Whether the ecological effects attributed to these GEMs in the laboratory would be transient or likely to pose significant risks upon environmental release is uncertain.

Tiedje et al. (1989) suggested that while most genetically engineered organisms will probably pose minimal ecological risk, some important exceptions may arise. Among their examples of undesirable outcomes to be avoided were genetically engineered microorganisms with a broadened host range that could affect beneficial insects as well as targeted pests, or genetically engineered microorganisms that provided only incomplete degradation of hazardous chemicals leading to the production of even more toxic by-products. The introduction of novel traits could also enable GEMs to become more invasive of natural habitats with the potential for adverse impacts on biological diversity (Edge 1994; Tzotzos et al. 1995).

Most scientists probably do not consider genetic engineering to be inherently hazardous. The National Academy of Sciences (1987) in the United States concluded that “there is no evidence that unique hazards exist either in the use of rDNA techniques or in the movement of genes between unrelated organisms.” A number of scientific reviews have concluded that genetically engineered organisms are generally expected to pose minimal ecological risk (Tiedje et al. 1989; Royal Commission 1989; Mooney and Bernardi 1990; Shorrocks and Coates 1993). Giddings (1998) suggested that GEMs are probably more likely to be beneficial than harmful. Regal (1988) suggested that most genetically

engineered organisms are unlikely to pose any threat to the environment; the challenge was to attain the capacity to identify the small proportion of genetically engineered organisms that might pose a threat.

GEMs: regulations and risk assessment

Microorganisms derived using rDNA methods have been subject to considerably more public scrutiny and government regulatory oversight than microorganisms derived through traditional biotechnology methods. The environmental release of a genetically engineered strain of *Pseudomonas syringae* in a small-scale experimental field study in California in 1987 was greeted by enormous public concern about possible adverse ecological and human health consequences. Associated with this release were activities ranging from a legal challenge by a public interest group to prevent the release, to the use of protective 'moon suits' by workers applying the organism at the release site. Shaw et al. (1992) indicated that outright public hostility associated with early releases of GEMs, and attendant unfavourable publicity, discouraged many scientists from this research.

Many government regulatory programs around the world have also focused requirements for risk assessment more on microorganisms developed through rDNA technology than naturally occurring microorganisms or microorganisms developed through more traditional techniques like chemical mutagenesis. For example, countries in the European Union (EEC Directive 90/220), the United States (Toxic Substances Control Act), Australia (Genetic Manipulation Advisory Committee Guidelines 1998) and Japan (Guidelines for Recombinant DNA Organisms 1995) have requirements for regulatory oversight of microorganisms based largely upon whether or not they have been developed

using recombinant DNA methods. Canada is somewhat unique in requiring notification of microorganisms that are “new” to Canada (Canadian Environmental Protection Act) irrespective of whether they were developed by rDNA techniques. The United Nations Biosafety Protocol has recently introduced a new international regulatory regime that only applies to ‘living modified organisms’ that are developed by modern biotechnology techniques like rDNA technology.

A natural assumption is that the additional public attention and regulatory scrutiny is warranted because genetically engineered microorganisms pose inherently greater risks than non-engineered microorganisms. Some scientists have supported this view and Ho (1997 and 1998, 1) considered genetic engineering biotechnology to be inherently hazardous. Wills (1998) urgently recommended an immediate ban on all environmental releases of genetically engineered organisms that should remain in place indefinitely until all questions concerning evolutionary implications are understood. On the other hand, other scientists have argued for less intrusive regulation of genetically engineered organisms. Szybalski (1985) argued that all regulation of recombinant DNA techniques was against the best interests of society and should be abolished. Miller (1994) argued that there was no longer a need for additional experiments to assess the risks of performing ‘genetic engineering’.

Since the early 1970s, the techniques of genetic engineering have become commonplace in many laboratories around the world. It is now possible, in principle, to recombine pieces of DNA between any two or more organisms. While the pace may have slowed in developing GEMs for environmental release (Wrubel et al. 1997), GEMs can be increasingly expected to be used for manufacturing a variety of products from foods and

pharmaceuticals to industrial enzymes. With the explosive growth occurring in fields like genomics, there will also be increasing opportunities to expand applications of genetic engineering. As a result, GEMs are likely to enter the environment in the future from a variety of possible sources and knowledge about their survival will be important in understanding potential environmental and human health impacts.

The survival of a GEM is widely recognized to be one of the more important considerations in assessing the potential risks posed by an environmental release (Strauss et al. 1986; Fiksel and Covello 1986; Tiedje et al. 1989; Lenski 1992; Smit et al. 1992; Krinsky et al. 1995; Morgan and Winstanley 1996). Survival after environmental release is usually a critical first step if any environmental or human health impacts (beneficial or adverse) are to occur associated with the release of a GEM.

Chapter 2 of this thesis summarizes the scientific literature on what is known about the environmental survival of genetically engineered bacteria from experiments conducted over the past 28 years. This review focuses on a comparison of the environmental survival of genetically engineered bacteria with their non-engineered parental organisms. The analysis of results from experiments identified in this review provides a test of the hypothesis that genetically engineered bacteria are less fit than their parental microorganisms.

Chapter 3 provides the results from laboratory studies conducted on the survival of a genetically engineered bacterium (*Pseudomonas chlororaphis* 3732RN-L11) in soil and rhizosphere microcosms. These studies tested the ability of statistical models to predict the GEM's survival in field trials at five sites across Canada. The final thesis chapter

provides conclusions about the current state of knowledge about environmental survival of GEMs in comparison with their parental microorganisms and about the ability to predict GEM survival upon release.

2. SURVIVAL OF GENETICALLY ENGINEERED BACTERIA IN THE ENVIRONMENT IN COMPARISON TO THEIR PARENTAL MICROORGANISM

Introduction

It has been over a quarter of a century since the first genetically engineered microorganism (GEM) was developed using rDNA techniques. Since that time, millions of experiments with GEMs have been conducted in laboratories around the world without documented hazard to public health attributed to applications of recombinant DNA technology (Berg and Singer 1995). There has also been a growing body of scientific research published on the behavior of GEMs under simulated environmental conditions in laboratories or in environmental field studies.

One of the more common types of study has been to examine the survival of a GEM under simulated environmental conditions in microcosms. These studies endeavor to predict how a GEM will behave once it is released into the environment. How well a GEM survives after environmental release is widely considered to be one of the more important aspects linked to its potential to pose risks (Tiedje et al. 1989; Lenski 1992; Smit et al. 1992). Barring significant gene transfer events, if the microorganism does not survive, it is less likely to have significant impacts beyond the immediate time and location of its release.

Whether a GEM survives will depend primarily on its fitness in the environment where it is introduced. Lenski (1992) considered fitness to encompass the combined effects of all biotic and abiotic interactions on a GEM's capacity to survive and reproduce in a particular environment. There have been a number of arguments put forward to suggest that GEMs will be poor competitors and less fit than their parental organisms upon environmental release (Brill 1985a; Davis 1987; Davies 1988). Tiedje et al. (1989) suggested that many genetically engineered organisms will probably be less fit than the parent organism, although some important exceptions may arise.

Lenski (1993) identified three different models for describing this reduced fitness of genetically engineered microorganisms. The "excess baggage" model suggests that GEMs will be disadvantaged by the extra energetic cost associated with carrying and expressing additional genetic material. The "untuned engine" model suggests there will be a loss of fitness in GEMs from perturbation of biochemical, physiological or developmental processes due to pleiotropic effects from the addition of new genetic material. The untuned engine model suggests not only that there will be an energetic burden from DNA and protein synthesis like the first model, but that foreign genetic material will not fit well in the balanced co-adapted genome of recipients. Finally, the "domesticated species" model suggests that GEMs will be less fit due to a history of relaxed selection and loss of wild-type traits by random genetic drift or secondary selection for other traits during domestication.

Lenski (1992) indicated that ecological interactions between closely related introduced and indigenous microbial populations are likely to be significant in determining the fate

of an introduced GEM. The fitness of a GEM relative to its parental microorganism has been viewed as an important risk assessment consideration (Tiedje et al. 1989; National Academy of Sciences 1989; Wilson and Lindow 1993). Smit et al. (1992) stressed the importance of comparing the survival of GEMs with their parental microorganisms before an environmental release. They suggested that as a general rule, GEMs should not be fitter than their wild-type since this might result in displacement of the parent. Krimsky et al. (1995) suggested that if a GEM is not expected to outcompete its parental strain, but microcosm tests indicate that it does, then further laboratory study should be required before moving to field trials. The fitness of a genetically engineered microorganism relative to its parental microorganism can be inferred by allowing the two to compete in a defined environment and monitoring the rate of change in their relative abundances over a number of generations (Lenski, 1993). Such experiments conducted across a variety of defined environments provide a way to assess relative fitness.

Lenski (1993) indicated that while there were many studies to support the hypothesis that genetically engineered microorganisms are less fit than their non-engineered parental microorganisms, there were also a few noteworthy counter-examples in which genetic modifications unexpectedly enhanced fitness. In recent years there has been a growing body of scientific literature that has compared the survival of GEMs and their parental organisms. However there has not been a comprehensive analysis of this literature. An analysis of this scientific literature could provide some basis to quantify what Lenski (1993) referred to as “many” or “a few” studies in the early 1990s. It could also provide some basis for testing the hypothesis that genetically engineered microorganisms are less fit than their parental organisms.

Methods

A review of the scientific literature was conducted to obtain records that studied the comparative survival of genetically engineered microorganisms and their parental microorganisms under environmental conditions. This literature review was conducted based upon a search of the following databases: Current Contents -Agri/Bio/Environ Sc. (1993-Mar 2001); Environmental Abstracts (1975-Aug 2000); CAB Commonwealth Agricultural Bureaux (1972-Oct 2000); Derwent Biotechnology Abstracts (1982-Mar 2001); Biosis Previews (1969-Feb 2001); Current BioTech Abstracts (1983-Dec 1999); Medline (1966-Dec 2000); NTIS (1964-Mar 2001); and Agricola (1970-Feb 2001).

The review sought records that met the following three criteria: 1) the study was on the survival of a genetically engineered bacterium (developed through rDNA techniques); 2) the study was on survival under environmental conditions such as field trials or simulated environmental conditions such as in aquatic or soil microcosms; and 3) the study provided data on survival. The specific key word search strategy was adapted to each particular database, but was based on a search for the following terms in record titles, abstracts and descriptors:

1. bacteria or bacterium or microbe* or microorganism* or micro organism or rhizobacteri* or rhizobium or rhizobia; and
2. ((genetically or gene or dna) and (engineer* or bioengineer* or modified or altered or manipulated or recombin* or transform*)) or (genetic-engineering or biotechnology); and

3. surviv* or persist* or fate; and

4. air or water or soil or soils or environment or ecosystem or ecosphere or rhizosphere or aquatic or river or lake or rivers or lakes or estuarine or marine or fluvial or terrestrial or land or ground or microcosm.

This search strategy identified a total of 1126 records (CAB - 415; Current Contents - 220; Derwent Biotechnology Abstracts - 172; Biosis Previews - 110; Agricola - 65; Environmental Abstracts - 45; Medline - 45; NTIS - 37; and Current BioTech Abstracts - 17. The number of records identified from Derwent Biotechnology Abstracts and Current BioTech Abstracts are lower than actual records because the search strategy incorporated means of removing records previously captured from other databases. The titles and abstracts for these records were then reviewed to remove duplicate records and those that did not meet the inclusion criteria such as studies that were not on bacteria, where the bacteria were not genetically engineered through use of recombinant DNA techniques, and studies that lacked survival data (e.g. review papers). In most cases, original records were reviewed particularly where abstract information was not conclusive about the relevance of a record.

To identify other potentially relevant literature records that might contain survival data, a parallel gene transfer literature review using the key words [(plasmid or gene or dna or genetic) and (transfer* or flow or movement or migrat* or move or moved)] instead of (surviv* or persist* or fate) was conducted on the same databases. This literature search focused on the terrestrial environment. The titles, abstracts and descriptors of gene

transfer records were reviewed to identify any potential records likely to contain environmental survival data on genetically engineered bacteria.

All survival records obtained were then reduced down to only those which contained data comparing the survival of a genetically engineered bacterium with its parental microorganism. The references cited in GEM/parental survival records were also reviewed to identify any other relevant records.

An analysis was conducted on the final list of records containing data on the survival of GEMs and parental microorganisms. Individual experiments in these records were categorized by whether the survival of a genetically engineered microorganism was 1) significantly less than parents; 2) significantly greater than parents; 3) the same as parents; 4) likely less than parents; and 5) likely greater than parents. Differences in survival were determined as significant based upon results of statistical analyses presented or, if there was no statistical analyses, based upon observation of clearly obvious differences from the data presented.

Results

A total of 102 literature records were obtained that compared the survival of GEMs with their parental microorganisms (Table 1). These records contained data from 719 experiments. Pseudomonads were the predominant group of GEMs studied, occurring in 70 of 102 records (Figure 1). The most commonly studied GEM species were *Pseudomonas fluorescens* (27 records), *Pseudomonas putida* (20 records), and *Escherichia coli* (13 records).

The most common genetic modification among GEMs in these records was the insertion of transposable elements to develop Tn mutants (26 records) (Figure 2). Other more common genetic modifications included insertion of marker genes such as *lacZY* (14 records), *lux* (10 records), *xyIE* (6 records), and *cryIVB* (6 records).

Studies comparing GEM survival with parents were much more common in terrestrial environments than aquatic environments (Figure 3). There were 87 records which conducted studies of GEMs in terrestrial environments compared to only 17 records conducted in aquatic environments. All records involving the study of plant surfaces or rhizospheres were conducted with agriculturally important crop species like wheat (13), bean (8), or corn (7) rather than plants typically found outside of agro-ecosystems (Figure 4). Eighty-eight percent of records were studies conducted under conditions largely simulating northern temperate ecosystems in the United States, Europe or Canada (Figure 5). There were few studies conducted which represented tropical environmental conditions or time periods longer than about 100 days.

Table 1. List of literature records found to compare the survival of genetically engineered bacteria with their parental bacteria (n=102).

Species	Inserted traits	Environment	Authors
<i>Pseudomonas fluorescens</i>	Tn5 mutant	soil	Marshall et al. 2001
<i>Enterobacter agglomerans</i>	Nah G	soil	Utkhede et al. 2000
<i>Pseudomonas putida</i>	lux AB	soil + rhizosphere	Ramos et al. 2000
<i>Pseudomonas fluorescens</i>	Tn5 mutant	soil + rhizosphere	Mirleau et al. 2000
<i>Pseudomonas fluorescens</i>	Tn5 mutant	rhizosphere	Kozdroj 1999
<i>Pseudomonas putida</i>	lux AB + xy/E	soil	Rodriguez-Herva et al. 1999
<i>Moraxella</i> sp.	gfp	soil	Errampalli et al. 1999
<i>Agrobacterium</i> sp.		rhizosphere	Penyalver and Lopez 1999
<i>Pseudomonas fluorescens</i>	lux CDABE	phylloplane	Cirvilleri and Caldarera 1998
<i>Pseudomonas fluorescens</i>	lux	rhizosphere	Kim et al. 1998
<i>Moraxella</i> sp.	gfp	sterile soil	Tresse et al. 1998
<i>Pseudomonas fluorescens</i>	lacZY, aph-1, xy/E	soil + rhiz + phylloplane	De Leij et al. 1998
<i>Klebsiella planticola</i>	pyruvate-decarboxylase gene	soil + rhizosphere	Holmes et al. 1998
<i>Pseudomonas fluorescens</i>	cryIVB + nptII	soil	Guimaraes et al. 1997

Table 1. Continued.

Species	Inserted traits	Environment	Authors
<i>Corynebacterium glutamicum</i>	aprotinin gene	soil	Vahjen et al. 1997
<i>Pseudomonas aureofaciens</i>	<i>lacZY</i>	soil	England et al. 1997
<i>Pseudomonas fluorescens</i>	<i>lux</i>	rhizosphere	Mahaffee et al. 1997
<i>Erwinia carotovora</i>	Tn mutant	soil + water + sediment	Palmer et al. 1997
<i>Pseudomonas fluorescens</i>	<i>lacZ</i>	rhizosphere	Fedi et al. 1997
<i>Pseudomonas putida</i> <i>Pseudomonas fluorescens</i>	<i>cryIVB</i> <i>cryIVB</i>	soil + rhizosphere	Araujo et al. 1996
<i>Bacillus subtilis</i>	<i>lpa-14</i>	soil	Asaka et al. 1996
<i>Pseudomonas fluorescens</i>	<i>lacZY</i>	rhizosphere	Fedi et al. 1996
<i>Pseudomonas aureofaciens</i>	<i>lacZY</i>	rhizosphere	Lamb et al. 1996
<i>Escherichia coli</i>	<i>ala</i> tRNA gene	river water	Alvarez et al. 1996
<i>Achromobacter</i> sp.	<i>phoA</i> hybrid gene	seawater	Sobecky et al. 1996
<i>Pseudomonas fluorescens</i>	Tn5 mutant	soil + rhizosphere	Kozdroj 1996
<i>Escherichia coli</i>	<i>gfp</i>	stream water	Leff and Leff 1996
<i>Pseudomonas fluorescens</i>	<i>cryIVB</i>	soil + rhizosphere	Araujo et al. 1995
<i>Pseudomonas putida</i>	phenoxyacetate degrade gene	soil	Ingham et al. 1995

Table 1. Continued.

Species	Inserted traits	Environment	Authors
<i>Pseudomonas fluorescens</i>	Tn5 mutant	soil + rhizosphere	da Gloria Britto et al. 1995
<i>Escherichia coli</i>	<i>lux</i> ABE	river water	Oliver et al. 1995
<i>Pseudomonas syringae</i>	<i>lux</i> AB	river water	
<i>Pseudomonas fluorescens</i>	<i>lux</i> AB	river water	
<i>Pseudomonas putida</i>	Tn5 mutant	soil	Tang et al. 1995
<i>Pseudomonas aureofaciens</i>	<i>lacZY</i>	soil	England et al. 1995
<i>Pseudomonas fluorescens</i>	<i>lacZ</i>	soil + rhizosphere	van Overbeek et al. 1995
<i>Aeromonas salmonicida</i>	<i>lux</i> AB	seawater	Ferguson et al. 1995
<i>Rhizobium meliloti</i>	<i>lacZY</i> + <i>thyA</i> gene	rhizosphere	O'Flaherty et al. 1995
<i>Pseudomonas fluorescens</i>	<i>PupA</i>	root + cotyledon	Raaijmakers et al. 1995
<i>Pseudomonas corrugata</i>	<i>lacZY</i>	rhizosphere	Ryder et al. 1994
<i>Xanthomonas campestris</i>	<i>lux</i>	soil + rhiz + phylloplane	Dane and Shaw 1994
<i>Xanthomonas campestris</i>	Tn5 mutant	leaf	Newman et al. 1994
<i>Pseudomonas putida</i>	<i>lacZY</i>	soil	Hartel et al. 1994
<i>Pseudomonas fluorescens</i>	<i>lacZ</i>	soil + rhizosphere	Natsch et al. 1994
<i>Pseudomonas sp</i>	aromatics degradation genes	aquifer	Krumme et al. 1994

Table 1. Continued.

Species	Inserted traits	Environment	Authors
<i>Pseudomonas aeruginosa</i>	<i>lacZY</i> + <i>luxAB</i>	soil	Flemming et al. 1994
<i>Pseudomonas fluorescens</i>	<i>nptII</i> + <i>cryIVB</i> genes	soil + rhizosphere	van Elsas et al. 1994
<i>Pseudomonas putida</i>	Hg resistance	soil	Iwasaki et al. 1994
<i>Pseudomonas putida</i>	<i>xyIE</i>	rhizosphere	Raaijmakers et al. 1994
<i>Pseudomonas syringae</i>	Tn5 mutant	leaf surface	Beattie and Lindow 1994
<i>Pseudomonas fluorescens</i>	<i>cryIV</i> gene	soil + rhizosphere	Araujo et al. 1994
<i>Rhizobium leguminosarum</i>	<i>lacZ</i> + <i>mer</i> genes	peat + rhizosphere	Corich et al. 1994
<i>Pseudomonas syringae</i>	Tn5 mutant	leaf	Lindow et al. 1993
<i>Pseudomonas syringae</i>	Tn5 mutant	leaf	Lindow 1993
<i>Pseudomonas putida</i>	Hg resistance	lake + river water	Iwasaki et al. 1993
<i>Streptomyces lividans</i>	lignin peroxidase gene	soil	Crawford et al. 1993
<i>Agrobacterium radiobacter</i>		rhizosphere	Vicedo et al. 1993
<i>Rhizobium meliloti</i>	<i>lux</i>	soil	Cebolla et al. 1993
<i>Pseudomonas syringae</i>	<i>xyIE</i>	air	Israeli et al. 1993
<i>Escherichia coli</i>	<i>xyIE</i>	air	
<i>Pseudomonas aureofaciens</i>	<i>lacZY</i>	soil + rhizosphere	England et al. 1993

Table 1. Continued.

Species	Inserted traits	Environment	Authors
<i>Escherichia coli</i>	antibiotic resistance gene	soil	Doyle and Stotzky 1993
<i>Escherichia coli</i>	pBGH1	river water	Bogosian et al. 1992
<i>Pseudomonas putida</i>	<i>clc</i> genes	river water	Genthner et al. 1992
<i>Pseudomonas putida</i>	2,4-D degradation genes	soil	Short et al. 1992
<i>Pseudomonas syringae</i>		soil	Sato and Sakai 1992
<i>Pseudomonas fluorescens</i>		rhizosphere	Maurhofer et al. 1992
<i>Pseudomonas fluorescens</i>	Tn5 mutant	soil + rhizosphere	Mazzola et al. 1992
<i>Pseudomonas aureofaciens</i>	<i>lacZ</i>	soil + rhizosphere	
<i>Pseudomonas</i> sp.	<i>lacZY</i>	rhizosphere	Fenton et al. 1992
<i>Escherichia coli</i>		lakewater + wastewater + soil	Dott et al. 1991
<i>Pseudomonas fluorescens</i>	Tn5 mutants + <i>cry</i> IVB gene	soil + rhizosphere	van Elsas et al. 1991
<i>Pseudomonas fluorescens</i>		soil + rhizosphere	
<i>Pseudomonas putida</i>	<i>xyIE</i>	lake water	Winstanley et al. 1991
<i>Pseudomonas putida</i>	3-chlorobenzoate gene	activ sludge	McClure et al. 1991
<i>Rhizobium leguminosarum</i>	Tn5 mutant	soil	Postma et al. 1991
<i>Streptomyces lividans</i>	lignin peroxidase	soil	Wang et al. 1991

Table 1. Continued.

Species	Inserted traits	Environment	Authors
<i>Pseudomonas fluorescens</i>	Tn mutants	rhizosphere	Howie and Suslow 1991
<i>Pseudomonas putida</i>	dichlorophenoxyacetate gene	soil	Doyle et al. 1991
<i>Pseudomonas putida</i>	ethylbenzoate gene	soil	Ramos et al. 1991
<i>Streptomyces lividans</i>	<i>xyIE</i>	soil	Wipat et al. 1991
<i>Pseudomonas solanacearum</i>	Tn5 + <i>npt1</i>	soil + rhizosphere	Williamson and Hartel 1991
<i>Pseudomonas solanacearum</i>	<i>pgl</i> and <i>egl</i> genes	soil	
<i>Pseudomonas aureofaciens</i>	<i>lacZY</i>	rhizosphere	Kluepfel et al. 1991
<i>Rhizobium meliloti</i>	<i>nifA</i>	rhizosphere	Ronson et al. 1990
<i>Pseudomonas fluorescens</i>	<i>lacZY</i>	rhizosphere	Cook et al. 1990
<i>Pseudomonas aureofaciens</i>	<i>lacZY</i>	rhizosphere	Kluepfel et al. 1990
<i>Bacillus subtilis</i>	alpha amylase gene	soil + river water	Smalla et al. 1990
<i>Rhizobium leguminosarum</i>	Tn5 mutant	soil	Pillai and Pepper 1990
<i>Escherichia coli</i>	dichlorophenoxyacetate gene	lake water	Awong et al. 1990
<i>Pseudomonas putida</i>		lake water	
<i>Erwinia carotovora</i>	Tn mutant	soil	Orvos et al. 1990
<i>Pseudomonas putida</i>	2,4 TFD degradation gene	soil	Short et al. 1990

Table 1. Continued.

Species	Inserted traits	Environment	Authors
<i>Pseudomonas aeruginosa</i>	Mu dII1681 + lacZ	soil + rhizosphere	Hofte et al. 1990
<i>Erwinia carotovora</i>	Tn mutant	lake water + sediment	Scanferlato et al. 1989
<i>Streptomyces lividans</i>	peroxidase/endoglucanase gene	soil	Wang et al. 1989
<i>Pseudomonas putida</i>	pglA gene	soil + rhizosphere	Yeung et al. 1989
<i>Pseudomonas aeruginosa</i>	pglA gene	soil + rhizosphere	
<i>Bradyrhizobium japonicum</i>	Tn7 mutant	rhizosphere	Liu et al. 1989
<i>Escherichia coli</i>	2,4-D degradation genes	rhizosphere	Morel et al. 1989
<i>Pseudomonas putida</i>		rhizosphere	
<i>Pseudomonas fluorescens</i>	Tn5 mutant	rhizosphere	Lifshitz et al. 1988
<i>Pseudomonas fluorescens</i>	Tn5 mutant	seeds	Loper, 1988
<i>Pseudomonas syringae</i>	Tn5 mutant	leaf	Cooksey 1988
<i>Escherichia coli</i>	Tn mutant		Marshall et al. 1988
<i>Pseudomonas fluorescens</i>	Tn5 mutant	rhizosphere	Thomashow and Weller 1988
<i>Pseudomonas syringae</i>		leaf	Lindemann and Suslow 1987
<i>Pseudomonas fluorescens</i>		leaf	
<i>Pseudomonas cepacia</i>	Tn mutant	soil extract	Walter et al. 1987
<i>Escherichia coli</i>		soil extract	

Table 1. Continued.

Species	Inserted traits	Environment	Authors
<i>Escherichia coli</i>	Drosophila gene	soil	Devanas and Stotzky 1986
<i>Escherichia coli</i>		soil	Devanas et al. 1986
<i>Escherichia coli</i>		soil	
<i>Pseudomonas syringae</i>	Tn5 mutants	leaf	Lindgren et al. 1986

Figure 1. Frequency of bacteria genera studied in literature records comparing survival of genetically engineered bacteria with parental bacteria (n = 102 records).

Figure 2. Frequency of most common traits inserted into genetically engineered bacteria in literature records comparing survival of genetically engineered bacteria with parental bacteria (n = 102 records).

Figure 3. Frequency of environments studied in literature records comparing survival of genetically engineered bacteria with parental bacteria (n = 102 records).

Figure 4. Frequency of plant species studied in literature records comparing survival of genetically engineered bacteria with parental bacteria (n = 49 records).

Figure 5. Frequency of study locations in literature records comparing survival of genetically engineered bacteria with parental bacteria (n = 102 records).

The results from classifying GEM vs parent microorganism survival experiments are presented in Figure 6. Individual experiments were classified as to whether GEM survival was higher, significantly higher, lower, significantly lower, or the same as their parental microorganism. Twelve experiments from van Elsas et al. (1994) were not included because the inoculations of GEMs and parents were not simultaneous. Ninety-two percent of the 707 experiments showed that the survival of GEMs was the same or lower than their parental microorganisms. Forty-four percent of experiments showed a change in survival relative to parental microorganisms with most of these showing a lowering of survival for GEMs. Thirty-six percent of experiments indicated that the relative fitness of GEMs was lower than parental microorganisms compared to only 8 % of experiments indicating GEMs had a relatively enhanced fitness.

Differences in survival results were noted depending upon whether GEMs were studied together with parental microorganisms (co-inoculated) or studied separately (Figure 7). When GEMs were inoculated separately from parents (e.g. different replicate microcosms), their survival was found to be the same in 61 % of experiments. However, when GEMs were co-inoculated with parents (e.g. in the same microcosm), their survival was only found to be the same in 35 % of experiments. When inoculated together, GEMs more commonly showed a lower fitness than parents (51 %) than when they were inoculated separately (32 %). When inoculated together, an increased fitness of GEMs relative to parents was also more common (14 %) than when GEMs were inoculated separately from parents (7 %).

Figure 6. Classification of the survival of genetically engineered bacteria in comparison to their parental bacteria in experiments identified from 102 literature records (n = 719 experiments). Figure includes survival results from all experiments (e.g. where GEM and parent were inoculated together as well as those where inoculations were separate).

Figure 7. Classification of the survival of genetically engineered bacteria in comparison to their parental bacteria in experiments where GEM and parent were inoculated: A) together in mixtures (n = 133 experiments); and B) separately (n = 574 experiments).

A comparison of GEM and parental survival did not identify significant differences between results obtained from laboratory experiments and those from outdoor field trials (Figure 8). Results from laboratory studies indicated that GEMs had the same or lower survival relative to parents in 92% of experiments. Field trials indicated that GEMs had the same or lower survival relative to parents in 97 % of experiments, although the number of field trial experiments (37) was much lower than the number of laboratory experiments (670).

The comparative survival of GEMs was also examined across different environmental conditions (Figure 9). GEMs were found to have the same or lower survival relative to their parents in the vast majority of experiments in soil (92 %), rhizosphere (95 %), lake water (88 %) and river water (100 %). GEMs seemed to have an enhanced fitness relative to parents slightly more often in lake water than other environments, but this was found only in 7 of 59 lake water experiments (12 %) and there was no apparent basis for considering this small difference to be biologically significant.

Most of the common genetic modifications made to GEMs resulted in little change in survival relative to parental organisms except for Tn mutants, *xylE*, and *lacZ* modifications (Figure 10). Tn mutants were commonly found to have a reduced fitness relative to parents (55 % of experiments). GEMs with *xylE* modifications were also commonly found to have a reduced fitness relative to parents (50 % of experiments). Tn mutants appeared to be the modification most commonly resulting in an enhanced fitness of GEMs relative to parents, but this was only in 9 % of experiments. A few other types of genetic modifications were noted in literature records, but there were few experiments with these GEMs to provide for summary analyses.

Figure 8. Classification of the survival of genetically engineered bacteria in comparison to their parental bacteria in experiments conducted in: A) laboratories (n = 670 experiments); and B) field trials (n = 37 experiments).

A

B

Figure 9. Classification of the survival of genetically engineered bacteria in comparison to their parental bacteria in experiments conducted in: A) soil (n = 284 experiments); B) rhizosphere (n = 163 experiments); C) lake water (n = 59 experiments); and D) river water (n = 26 experiments).

A

B

C

D

Figure 10. Classification of the survival of genetically engineered bacteria in comparison to their parental bacteria in experiments where genetic modifications were: A) Tn mutants (n = 207 experiments); B) *lacZY* (n = 44 experiments); C) *xylE* (n = 58 experiments); D) *lux* (n = 61 experiments); and E) *cryIVB* (n = 103 experiments).

A

B

C

D

E

Discussion

This study of the scientific literature identified 102 records and 719 experiments that compared the survival of GEMs and their parental microorganisms under environmental conditions. The results from these experiments provide a growing basis for evaluating the relative competitiveness of GEMs in a variety of environments including terrestrial and aquatic ecosystems. However, the results are also limited by their predominant derivation from short-term laboratory experiments simulating terrestrial agro-ecosystems in northern temperate climates. They are also limited by the fact that most of the GEMs have been modified by transposon mutagenesis or insertion of marker genes designed to have relatively neutral impacts on the fitness. In their review of environmental releases of recombinant microorganisms, Wilson and Lindow (1993) also reported that most of the GEMs they identified had been developed by simple inactivation of genes by transposon insertion or genetic tagging of bacteria with selectable or identifiable markers such as *lacZY*. Additional research will be required to broaden this comparative GEM/parent knowledge base in the future with survival studies conducted on GEMs: 1) with a wider range of potentially more adaptive traits; 2) in climatic zones outside temperate regions; and 3) under conditions more representative of the diversity of ecosystems found outside of agricultural areas.

Despite these limitations, the results of the experiments provide considerable data with which to evaluate the fitness of GEMs relative to their parental microorganisms. GEMs appeared to have enhanced fitness relative to their parental microorganisms in 8 % of 707 experiments examined. These results make it possible to reject the categorical hypothesis

that GEMs are less fit than their parental microorganisms. This hypothesis has been used as a basis for arguments on the safety of environmental releases of genetically engineered microorganisms (Brill 1985a; Davis 1987; Davies 1988). Such generic safety arguments for genetically engineered organisms have been considered to be based upon outdated scientific thinking (Regal 1994). The results also indicate that an additional model is needed to describe the enhanced fitness of GEMs beyond the “excess baggage”, “untuned engine” and “domesticated” models proposed by Lenski (1993).

While the categorical hypothesis can be rejected, nonetheless many experiments examined in this literature review found GEMs to be less fit than their parental microorganisms. GEMs appeared to have reduced fitness relative to their parental microorganisms in 36 % of the 707 experiments examined. However, this is likely an under-estimate of the actual frequency of reduced GEM fitness since over 80% of experiments involved GEMs studied separately from their parental microorganisms. It has been suggested that simultaneous inoculations of mixtures of a GEM and its parent may be more likely to detect differences in survival than separate inoculations (Smit et al. 1992). For example, van Elsas et al. (1994) found separately introduced parent and genetically engineered strains of *Pseudomonas fluorescens* showed a statistically similar decline in soil. However in soil, wheat rhizospheres and wheat rhizoplanes co-inoculated with GEM-parent mixtures, the GEMs showed impaired survival compared to that of the parent. Similarly, Vahjen et al. (1997) found the fitness of a genetically engineered *Corynebacterium glutamicum* to be reduced compared to its parental microorganism, but only when inoculated together in a GEM-parent mixture. They suggested direct competition experiments between GEM and parental strains were more sensitive than just comparing survival rates in separate microcosms.

Lenski (1991) has suggested that it would be difficult or even impossible to estimate fitness of a GEM and parent studied in isolation. This approach ignores the effects each population would have on the other, as mediated by their effects on the environment. Differences in fitness inferred for studies of GEMs in isolation from parents may also require a high degree of replication, particularly if sampling error or variation is large (Lenski 1991). The separate measurements of survival for GEM and parent would each have its own independent error. However, direct studies of fitness of GEM and parent mixtures have an internal control, such that if a particular microcosm replicate was unusual, it would be the same both for the GEM and parent.

The results from this literature review also make it possible to reject the hypothesis that GEMs are more fit than their parental microorganisms. GEMs appeared to have a relatively reduced fitness in 36 % of the 707 experiments examined. GEMs were found to have enhanced fitness relative to their parental microorganisms in only 8 % of the 707 experiments examined. There were only nine literature records that found significantly enhanced GEM fitness relative to parental microorganisms.

Most of the studies (six out of nine) that found significantly enhanced GEM fitness also had inherent selective advantages for the GEM relative to the parental organisms built into the experimental design. This included selective advantages for GEMs based upon experimental amendments of antibiotics (van Elsas et al. 1994; da Gloria Britto de Oliveira et al. 1995), thymidine (O'Flaherty et al. 1995), salicylic acid (Utkhede et al. 2000), ethylbenzoate (Ramos et al. 1991), and phenoxyacetic acid (Short et al. 1992). Awong et al. (1990) noted that the lake water source for their experiments contained

pollutants that could have partly accounted for enhanced survival of two GEMs relative to their parental microorganisms. Dott et al. (1991) and Doyle and Stotsky (1993) did not provide a basis for explaining the enhanced GEM survival observed in soil experiments.

The GEMs that were found to have enhanced survival relative to parental organisms exhibited a variety of inserted traits ranging from antibiotic resistance (Doyle and Stotsky 1993) to degradation of 2,4-D (Short et al. 1992) and degradation of *p*-ethylbenzoate (Ramos et al. 1991). These GEMs were also modified by a variety of different genetic techniques from use of a Tn5 vector (da Gloria Britto de Oliveira et al. 1995) to electroporation with a recombinant plasmid (Utkhede et al. 2000). The range of inserted traits and genetic modification techniques gleaned from these studies did not indicate any clear basis for determining that a particular trait or technique would lead to enhanced GEM survival.

While few experiments were identified in this literature review where GEM fitness was unexpectedly enhanced relative to parental microorganisms, studies conducted in chemostats have found that genetic modifications with the transposon Tn5 enhanced the fitness of certain genotypes of *E. coli* (Biel et al. 1983; Hartl et al. 1983). Lenski (1993) indicated that, although such studies may be over-represented in the scientific literature because their results were so unexpected (relative to studies more commonly finding GEMs with decreased fitness and which perhaps are less likely to be published), they clearly demonstrated there can be unexpected competitive advantages associated with the possession of seemingly superfluous genetic material. More recently, Giddings et al. (1997) found that when a Bt toxin gene (*cryIIIA*) was inserted into *Rhizobium*

leguminosarum, the GEM showed consistently greater than expected nodulation occupancy in competition experiments with the non-engineered parental strain over two years of experiments. This pleiotropic effect was suggested to have resulted from either the inserted transgene itself, or from the positional effect of the Tn5 transposon that carries the gene.

The results of this literature review indicate that the survival of GEMs was the same or lower than their parental microorganisms in over 92% of the 707 experiments examined. When the survival experiments were broken down into classes (e.g. lab versus field, soil versus river water, *lacZY* versus *xyIE* genetic modifications), the survival of GEMs was consistently found to be the same or less than their parents in around 90% or more for each class of experiments examined. These results, though limited to survival which is only one aspect of potential environmental and human health risk, lend support to a number of previous scientific reviews which have suggested that most genetically modified organisms are generally expected to pose minimal ecological risk (National Academy of Sciences 1987; Tiedje et al. 1989; Royal Commission 1989; Mooney and Bernardi 1990; and Shorrocks and Coates 1993). Wrubel et al (1997) indicated that many R&D programs for GEMs have slowed down largely due to the technological difficulty of developing microbial products that can survive and outperform naturally occurring microbes under field conditions. They suggested that much early optimism was based upon results from laboratory experiments which overestimated field performance, and that molecular biologists failed to appreciate the difficulty of establishing introduced microorganisms in the field.

Since the results of the literature review indicate that categorical statements about the fitness of GEMs relative to parental microorganisms are not possible, it is suggested that the risks posed by GEMs should continue to be reviewed on a case by case basis. Case by case reviews would also benefit from better knowledge about those GEMs or traits that are commonly associated with enhanced or lower fitness. While GEMs have frequently been less fit than their parental microorganisms in studies to date, the importance of case-by-case review will likely remain important in the future. Tremendous scientific advances in fields like genomics could lead to many innovative GEMs with potentially more adaptive genetic modifications.

The advances in genomics are part of a profound paradigm shift that is taking place in the way search and discovery research is conducted in biotechnology (Bull et al. 2000). Bull et al. identified 34 bacterial species whose genomes had been sequenced with another 75 in progress. High throughput screening or “biopanning” of DNA sequences and use of advanced bioinformatics tools are providing increasingly rapid search and discovery capacities for identifying genes of interest for biotechnology and recombinant DNA applications. It is also becoming increasingly possible to use genetic engineering to modify biosynthetic pathways such that genetic engineering is no longer limited to the manipulation of single-gene traits (van Montagu et al. 1995, 690).

In addition, Cho et al. (1999) indicated that advances in the ability to create new organisms with minimal genomes could accelerate the pace at which genetically engineered microorganisms are developed. Cho et al. (1999) defined minimal genomes to be the smallest set of genes that allows for replication of an organism in a particular environment. They indicated minimal genomes could be developed by a “top-down”

approach to remove or inactivate the entire set of genes in an organism thought to be unnecessary for replication, or by a “bottom-up” approach to synthesize and assemble the genome and then insert it into a cell environment that allows metabolic activity and replication. The first practical benefits were suggested to be in microbial engineering where minimal organisms might have advantages in requiring less energy or producing fewer waste products that could contaminate biotechnology products. It was suggested that minimal organisms could be used as the basis for novel “designer” bacteria that are created to perform specific tasks and that they could be an important step forward in genetic engineering as it would permit the creation of organisms simply from knowing the sequence of their genomes.

The converging advances occurring in minimal genomes research, genomics, proteomics, robotics, miniaturization, massively parallel and automated sample analysis, and bioinformatics are providing enormous opportunities to better understand and exploit biodiversity and to develop innovative new genetically engineered microorganisms. These developments may lead to environmental releases of increasingly diverse and very complex GEMs whose survival may be difficult to predict.

The accumulated scientific knowledge from 719 survival experiments with GEMs also provides a new perspective for examining the scientific basis of many regulatory regimes which focus notification and risk assessment requirements on genetically engineered microorganisms, but not non-engineered microorganisms. Evidence indicates that only 8% of the experiments to date have shown GEMs to have an enhanced fitness relative to their non-engineered parental microorganisms. However, these same data indicate that 92% of experiments to date have shown non-engineered microorganisms to have the same

or enhanced fitness relative to GEMs that are widely deemed to require rigorous notification and risk assessment. Non-engineered microorganisms actually showed an enhanced fitness relative to GEMs in 36 % of 707 experiments. The figure of 36 % is also probably too low since most experiments with GEMs and parental non-engineered microorganisms were conducted separately as discussed previously.

That non-engineered microorganisms are not more rigorously reviewed is perhaps surprising considering that GEMs represent only a very small fraction of all microorganisms that have been released into the environment to date. For example, Wilson and Lindow (1993) identified 27 field studies of GEMs that had taken place around the world. However, they pointed to the fact that environmental releases of non-engineered microorganisms had long been an integral part of research activities in biological disciplines like plant pathology and entomology. They suggested that hundreds of releases of plant pathogens occur yearly and that there have probably been thousands of releases of plant pathogens since the turn of the century. Similarly, thousands of releases of non-engineered *Rhizobium* species occur yearly around the world. In 1992 alone, Wilson and Lindow (1993) identified at least 138 studies in which plant pathogens were released into field sites and 40 similar studies involving *Rhizobium* species. These authors indicated the actual number of releases was probably higher by a factor of 2 or more. Wilson and Lindow (1993) also indicated that most of these studies had not sought to study the fate of the non-engineered microorganisms nor whether they resulted in any significant environmental effects.

Environmental and human health concerns have already been associated with non-engineered microorganisms proposed for applications in the environment. While non-

engineered strains of *Burkholderia cepacia* may show much promise as agricultural inoculant products for suppressing soilborne plant pathogens, Holmes et al. (1998) drew attention to this species as an emerging pathogen among cystic fibrosis patients and called for a moratorium on its widespread use in agriculture. LiPuma and Mahenthiralingam (1999) also drew attention to potential human health concerns from the use of *Burkholderia cepacia* in environmental bioremediation applications. The species was reported to have been extensively used to degrade trichloroethylene in the groundwater of at least one large U.S. city, although it was unknown how frequent the species was used in commercially available bioremediation inoculant products. Govan et al. (2000) considered *B. cepacia* to be a microbial Jekyll and Hyde with one form presenting agricultural benefits and the other a threat of fatal infections. Liao (1991) also cautioned about using some naturally occurring pseudomonad strains like *Pseudomonas fluorescens* as biocontrol agents since they could cause soft rot in plants.

Defined and undefined microbial consortia products composed of two or more non-engineered strains are also commercially available and likely to expand in use in the future (Royal Society of Canada 2001). Microbial consortia products that are composed of undefined mixtures of microorganisms can also pose significant challenges for environmental and human health risk assessment. Edge (1996) identified 338 commercially available microbial inoculant products for bioremediation-related applications in Canada, a number of which were composed of undefined microbial consortia isolated from sources such as wastewater or livestock manure. Godfrey et al. (1996) screened two of these bioremediation-related products and found low levels of *Pseudomonas aeruginosa* and *E. coli* in one product. It should also be recognized that

quality control associated with manufacturing microbial inoculants for environmental applications is rarely sufficient to exclude the possibility of products containing microbial contaminants posing additional risks. Fincham and Ravetz (1990, 139) cautioned that realities of practice and maintaining standards of quality needed to be considered when assessing hazards of biotechnology applications.

The results from this literature review raise an element of caution in the use of the concept of substantial equivalence for evaluating the environmental safety of GEMs. The concept of substantial equivalence was developed as a means to assist risk assessors in the evaluation of the safety of foods derived through biotechnology. It has been described as embodying the idea that existing organisms used as food, or as a source of food, can be used as the basis for comparison when assessing the safety of human consumption of a food or food component that has been modified or is new (OECD 1993). When developed, the OECD stated the concept was not intended to be applicable to other aspects of biotechnology safety, including environmental safety. More recently, there has been growing interest in examining its application for environmental safety assessments of genetically modified organisms.

Regulatory agencies continue to find substantial equivalence to be a practical tool for guiding food safety assessments (Kearns and Mayer 1999; OECD 2000), although there has been recent debate about its appropriate use (e.g. Millstone et al. 1999; Royal Society of Canada 2000). The application of substantial equivalence to environmental assessments of genetically modified organisms remains to be well examined, although one application might seem to be comparison of survival trends between a GEM and its parent. The results of the present literature review indicate that, while many studies have

shown GEMs to be less fit than their parental organisms, most of these studies have compared the survival of GEMs and parents in separate inoculations. Studies that inoculated a GEM together with its parental microorganism showed a higher incidence of enhanced GEM fitness (14 %) than those studies that inoculated a GEM and its parent separately (7 %). Unless a rigorous experimental approach is taken to evaluate comparative GEM fitness, there remains the possibility that a determination of substantial equivalence in survival could mask an enhanced GEM fitness due to insufficient statistical resolution or a poor experimental design.

The results of this literature review also present a case more for enhanced environmental and human health assessment of releases of non-engineered microorganisms than for reduced oversight of GEMs. Ninety-two percent of experiments demonstrated that non-engineered microorganisms had the same or enhanced survival compared to their GEM derivatives that are subject to more regulatory oversight. Miller (1999) indicated that, by focusing on genetically engineered organisms, the scope of the recently negotiated United Nations Cartagena Protocol on Biosafety would be unscientific and would ignore non-engineered organisms known to present a threat to biodiversity. The results of the literature review are also consistent with less recognized views that a product-based regulatory approach may require more rigorous assessment of non-engineered organisms than in the past (National Academy of Sciences 1989; Campbell 1991; and Levin 1991).

3. PREDICTING SURVIVAL OF *Pseudomonas chlororaphis* 3732RN-L11 IN SOIL AND RHIZOSPHERES: TESTING STATISTICAL MODELS

Introduction

Along with the growing establishment of national and international biotechnology regulatory regimes around the world are growing expectations for accurate assessments of the potential risks posed by environmental releases of genetically engineered microorganisms (GEMs). For example, the recently negotiated United Nations Cartagena Protocol on Biosafety raises expectations for the ability to predict the potential adverse impacts on biodiversity from transboundary movements of GEMs. Enhancing the ability to accurately assess the risk posed by GEMs is an important scientific challenge and an important part of building public confidence in the ability of regulatory regimes to safely manage the tremendous advances occurring in biotechnology and genomics.

A science-based approach for assessing the impacts of environmental releases of GEMs is emerging from the discipline of risk assessment. While the general paradigm of risk assessment has been considered appropriate for assessing the potential environmental impacts of GEMs (Fiksel and Covello 1986; Suter 1993), concepts and methods have only recently been developed for environmental risk assessment and they have largely focused on stressors like chemicals. The extension of such concepts and methods to GEMs has not been well investigated (Levin and Strauss 1991; Sharples 1991; Suter 1993).

A number of preliminary conceptual frameworks have been proposed to guide environmental risk assessment for GEMs and these were reviewed by Strauss (1991). One framework was a six step process proposed by Alexander (1985):

$$\text{Risk} = P_1 * P_2 * P_3 * P_4 * P_5 * P_6$$

where P_1 is the probability of release into the environment, P_2 is the probability of survival, P_3 is the probability of multiplication, P_4 is the probability of dispersal, P_5 is the probability of an adverse ecological effect, and P_6 is the probability of gene transfer to other organisms. The potential for ecological risk was deemed to be dependent upon a sequence of events and was a function of the individual probabilities of the steps in the sequence. From a general conceptual perspective, if risk assessments are to provide scientifically defensible predictions for regulatory decisions, it would be desirable to provide quantitative estimates of the probabilities of the events in this risk sequence.

Smit et al. (1992) considered the ultimate goal of the risk assessment endeavour would be to provide reliable quantitative predictions of the risks associated with the use of microorganisms in the environment. Leiss and Chociolko (1994) indicated that the value of quantitative risk estimates, despite their limitations, has to be judged in relation to the alternatives available as aids in decision-making - hunches, intuitions, and sheer guesswork, all of which are prone to error. Lave (1982) argued that a careful review of scientific evidence and a quantitative risk assessment should be the basis of regulatory decisions, and that decisions without these elements would be uninformed, arbitrary, and unlikely to withstand litigation or induce cooperation from those being regulated. As an example, Colwell et al. (1985) took exception to Brill (1985a) using phrases such as

"seems very small" and "is extremely unlikely" when considering the likelihood of negative effects resulting from environmental introductions of genetically engineered organisms. Colwell et al. (1985) asserted that "the point of risk assessment is to provide sufficient quantitative information about the potential for a negative effect so that the kinds of qualitative and subjective judgements implied in phrases like 'seems very small' are no longer necessary."

Suter (1993) indicated that formal risk assessment methods for genetically engineered organisms have not been well developed or applied and regulatory assessments are performed on an ad hoc basis relying more on expert judgement than on analysis. He warned that as the biotechnology industry develops, the current ad hoc approach to risk assessment will become unfeasible and that standard guidelines, models, and inference procedures should be developed before the need becomes desperate. Therefore, it will be increasingly important for regulatory agencies to know whether accurate quantitative predictions of risk are possible for regulatory decisions regarding potential stressors like GEMs. For example, Kareiva et al. (1996) raised concerns about the ability to assess the invasiveness of genetically engineered organisms and the limitations of making "invasion ecology" a predictive (as opposed to after the fact) science. It should be recognized that quantitative risk assessment is not a panacea, and that it is limited to what can be measured and quantified (Lave 1982).

One of the more important risk assessment considerations surrounding the environmental release of a GEM is whether it will survive (Strauss et al. 1986; Fiksel and Covello 1986; Acea et al. 1988; Alexander 1985, 1988; National Academy of Sciences 1989; Tiedje et al. 1989; Lenski 1992; van Elsas and Trevors 1991; Smit et al. 1992; Krinsky et al. 1995;

Thompson et al. 1995; Morgan and Winstanley 1996). Non-engineered bacteria have been known to persist in soils for years after an environmental release. For example, Diatloff (1977) found *Rhizobium* strains to persist in soils for at least 12 years after a single introduction. Similarly, Brunel et al. (1988) reported *Bradyrhizobium* strains to survive in soils for at least 13 years. Manchee et al. (1994) monitored persistence of *Bacillus anthracis* applied to soil in World War II biological weapons experiments in Scotland and suggested that measurable contamination was likely well into the 21st century.

The likelihood of survival is determined by the fitness of the organism in a new environment that encompasses the combined effects of all biotic and abiotic interactions on the organism's capacity to survive and reproduce (Lenski 1992). If a GEM does not survive, it is generally less likely to pose environmental or human health risks (it is also less likely to perform the function for which it was deliberately released). However, if the GEM survives at high population levels, assessments of risk can become more complicated needing careful consideration of the implications of long-term persistence, dispersal, gene transfer, and potential environmental and human health effects. The Royal Society (2001) recommended that where environmental risks for a biotechnology product are a concern, especially with respect to aspects like the persistence of the organism, exhaustive and long-term testing for these environment risks should be carried out.

This thesis chapter examines the ability to accurately predict the survival of a GEM released into soil and rhizosphere environments. Uncertainty surrounding the prediction of microorganism survival can hamper efforts to provide accurate predictions of the

potential risk or efficacy posed by environmental releases of microorganisms (Alexander 1985; Levidow 1992; Bashan et al. 1995; Vandepitte et al. 1995; van Veen et al. 1997; Giddings 1998). There are several means of predicting GEM survival prior to an environmental release, including the use of laboratory microcosms and statistical modeling. Member countries of the Organisation for Economic Cooperation and Development (OECD) have recognized the value of assessing the survival of GEMs in simulated natural environments such as microcosms for pre-release risk assessment purposes (OECD 1992). Statistical models based upon empirical data, though less recognized and used, may also be useful for predicting survival of GEMs prior to their environmental releases.

Microcosms

A microcosm has been described as an intact, minimally disturbed piece of an ecosystem brought into the laboratory for study and that behaves ecologically like its counterpart in the field (National Academy of Sciences 1989, 117). The use of microcosms has been widely proposed as an effective science-based approach to predict behavior of GEMs before an environmental release (Greenberg et al. 1988; Trevors 1988; Harrass and Sayre 1989; Bentjen et al. 1989; Awong et al. 1990; Fredrickson et al. 1990; Frederick and Pilsucki 1991; Pipke et al. 1992; Smit et al. 1992; Gustafsson and Jansson 1993; Krimsky et al. 1995; Morgan and Winstanley 1996). Tiedje et al. (1989) suggested information on the potential for establishment and possible effects of genetically engineered organisms should be obtained from laboratory tests such as microcosms, before proceeding with carefully planned small-scale field trials to evaluate potential environmental effects. Krimsky et al. (1995) argued that data from microcosm studies should be required for any regulatory approval of a field release of a GEM.

Microcosms have been used to study a variety of behavioral characteristics of GEMs in simulated terrestrial environments. For example, microcosms have been used to study survival of GEMs in soils and rhizospheres (Bentjen et al. 1989; Henschke et al. 1991; Angle et al. 1995, 1996; Vahjen et al. 1997; Gagliardi et al. 2001b). They have also been used to study GEM transport in soil (Trevors et al. 1990; Hekman et al. 1995) and to study the fate of GEMs associated with plants and herbivorous insects (Armstrong et al. 1987). Snyder et al. (1999) used soil microcosms to study insect-mediated dissemination of a GEM and found they were useful for predicting interactions in the field between

plants, insects and rhizobacteria. Microcosms have also been used to study GEM gene transfer processes (Knudsen et al. 1988; Trevors 1988; Hill and Top 1998) and to investigate the potential ecological effects of GEMs (Orvos et al. 1990; Doyle et al. 1991; Jones et al. 1991; Sobecky et al. 1996; Palmer et al. 1997; Holmes et al. 1998; Gagliardi et al. 2001a). With pre-release knowledge obtained from laboratory microcosms, experimental field studies can be better designed to test relevant hypotheses, they may be conducted with better and more clearly understood biosafety precautions, or they may even be considered to pose too significant a risk to proceed.

A variety of different soil microcosms have been developed to simulate environmental conditions for studying GEM survival in terrestrial environments (Bentjen et al. 1989; Fredrickson et al. 1989; Fredrickson et al. 1990; Bolton et al. 1991; Angle et al. 1995; Krinsky et al. 1995). These microcosms can range from small test tubes (Naik et al. 1994), to plastic trays of soil (Armstrong et al. 1987), intact soil cores (Angle et al. 1995) and large environmentally controlled chambers (Gillet and Gile 1976). The soil included in microcosms has ranged from an intact natural soil core (Angle et al. 1995), to soil that has been dried, ground with a mortar and pestle, passed through a 0.242 mm sieve, and sterilized (Naik et al. 1994). Some of these soil microcosms have been validated through field studies and have been shown to closely simulate results in the field (Bolton et al. 1991; Angle et al. 1995; Gagliardi et al. 2001b). Snyder et al. (1999) found a laboratory microcosm useful in predicting the interactions in the field between a GEM and plants and insects.

However, microcosms also have limitations in their ability to simulate natural environmental conditions indicating the importance of obtaining field calibrations.

Kinkel et al. (1996) determined that microcosms seemed most effective at assessing possible differences between parental and recombinant strains, and that they were limited in their ability to predict specific population sizes or plant habitat preferences of bacteria on leaves under field conditions.

The wide variety of soil microcosm designs has prompted concern about the need for microcosm standardization (Angle et al. 1995; Krimsky et al. 1995; Morgan and Winstanley 1996). Angle et al. (1995) indicated that because the design of a microcosm affects survival of a GEM in soil, it is nearly impossible to compare survival data between microcosm studies. Krimsky et al. (1995) reviewed microcosm uses for microbial risk assessment purposes and stressed the importance of designs like intact soil cores that attempt to preserve soils in their natural state as much as possible.

Frederickson et al. (1990) cautioned that microcosms containing homogenized soil do not allow for natural heterogeneity and the maintenance of natural microhabitats where different biological and physicochemical processes may influence microorganism survival. Intact soil microcosms were considered to more closely simulate environmental conditions expected in the field that could affect microbial survival. Krimsky et al. (1995) indicated that standardized microcosm test methods are needed so that it will be possible to compare data from diverse studies, to ensure replicability of results, and to build a body of scientifically defensible knowledge useful for risk assessors.

The development of a standardized soil core microcosm was the objective of research conducted by Angle et al. (1995). These researchers validated a simple intact soil core microcosm that proved useful for predicting survival for a GEM (*Pseudomonas chlororaphis* 3732RN-L11) in soil and wheat rhizosphere at a site in Maryland, USA.

This microcosm is inexpensive (about \$ 1.00/microcosm for materials and construction), easily made from readily available materials, and it does not require much expertise to use. A subsequent validation study found this microcosm was useful for assessing field survival for *Pseudomonas chlororaphis* 3732RN-L11 in soil and wheat rhizospheres at five different field trial sites across Canada (Gagliardi 1998; Gagliardi et al. 2001b). While the microcosm predictions of survival slopes were significantly different from field plot measurements of survival slopes at several sites, for all these cases survival was consistently greater in microcosms than in the field. This increased loss of RN-L11 in field soil compared to microcosms was suggested to correspond to high rainfall rates at some sites (Gagliardi et al. 2001b). These validation studies lent support to application of the intact soil core microcosm for the present objective of testing whether statistical models developed from microcosm-derived survival data for a GEM can reliably predict survival of the GEM in field plots.

Statistical models

To date, prediction of GEM survival has relied largely upon empirical methods such as the use of laboratory microcosms. However, environmental introductions of GEMs have progressed beyond site-specific experimental field studies towards larger-scale applications (Cavalieri 1991; Fox 1992). While empirical methods such as microcosms can provide useful data for assessing survival associated with a site-specific environmental introduction of a GEM, additional inference methods will be needed to assess survival associated with widespread use of a GEM in the environment. Fiksel and Covello (1986) indicated that while empirical methods such as microcosm testing are indispensable for purposes of risk assessment, they must be supplemented by predictive modelling methods.

One of the limitations of microcosms is that they can only represent a few of the potential environmental conditions likely to be encountered by a GEM upon its release into the environment. While tests can be performed in a microcosm representing one set of environmental conditions, it may be unreliable and inappropriate to extrapolate from one set of environmental conditions to another (Berry and Hagedorn 1991). It would be desirable to have a sound scientific basis for making predictions about the likely survival of a GEM at many different release sites without the need to gather laboratory microcosm data representing every possible site. At present, such predictions are often made implicitly in risk assessments based on extrapolations from available knowledge about the biology of a GEM.

Fiksel and Covello (1986) indicated that as risk assessment shifts from most likely outcomes to low-probability outcomes, empirically-based or physical simulation methods become less useful. For example, it is unlikely that low-probability outcomes would be observed in microcosm tests or realistic field trials, although they might appear plausible based on predictive modelling studies. To date, however, most studies of the environmental risks of GEMs have been of a qualitative or semi-quantitative nature and little attempt has been made to develop or apply quantitative models to predict or explain the effects of GEMs (Prosser 1993). Barnthouse (1992) has suggested that regulatory programs could benefit from increased use of models for quantitative risk assessment, for refinement of qualitative decision criteria, or simply as supplemental information for risk assessors.

As more GEMs are released into the environment in the future, it will be important to know whether it is feasible to supplement microcosm methods with useful inference methods such as statistical models for predicting survival trends of GEMs. For example, it would be useful if the survival of a GEM in soil could be accurately predicted based upon knowledge of a few key soil parameters influential for a particular GEM. Prosser (1993) suggested that mechanistic modeling of survival was not currently feasible due to a lack of understanding of the physiology of starvation responses, complicating factors related to the heterogeneity of microenvironments and existence of hot spots of microbial activity. However, he suggested empirical models may prove valuable in quantifying survival in a descriptive manner.

Microorganism survival has been simulated by a number of different statistical models. For example, Vandepitte et al. (1995) evaluated a statistical model based on the

Gompertz function for simulating survival of bacteria introduced into soils. The Gompertz model is an asymmetric logistic model that accounts for an exponential decrease, corrected by a term linked to the difference between population size at time t and its asymptotic value. Vandepitte et al. tested the model against 28 microorganism survival data sets and found a good fit in 21 cases ($R\text{-square} > 0.8$). However, the model only passed all four of their evaluation criteria in 10 of 28 cases. These criteria were: $R\text{-square} > 0.8$; meaningful and stable estimates of parameters; and good simulating power.

Other statistical models have also been used to simulate microorganism survival such as simple exponential decay models (Knudsen et al. 1988) and log linear regression models (Crane and Moore 1986; Hurst 1997; Gagliardi 1998). Crane and Moore (1986) considered a linear regression approach to accurately describe die-off of enteric bacteria under a variety of environmental conditions, although caution was needed in extrapolating results to low bacteria densities since residual populations were often observed at low levels longer than predicted. Gagliardi (1998) and Gagliardi et al. (2001b) found linear regression of log transformed counts over time to adequately describe the survival of a GEM (*Pseudomonas chlororaphis* 3732RN-L11) and provide a means to predict GEM persistence in a variety of soils and rhizospheres. However, these survival models are rarely linked to environmental data in order to predict survival based upon a number of environmental parameters.

A linear multiple regression approach can be taken to develop empirical statistical models for predicting the survival slope of microorganisms based on soil characteristics. In this case, survival slope values are regressed against a number of independent variables representing environmental parameters of the soils being studied. This approach has

proven useful for identifying a number of environmental factors affecting persistence of viruses in soil (Hurst et al. 1980; Hurst 1991, 1997).

In this thesis chapter, linear multiple regression (LMR) models were developed as statistical tools to predict the survival of a genetically engineered microorganism, *Pseudomonas chlororaphis* 3732RN-L11 (hereafter designated RN-L11) introduced into soil and rhizosphere field plots. A linear multiple regression approach offers a number of advantages since it is a statistical technique that is relatively widely used, software programs are readily available, and it is familiar to many risk assessors and regulatory agencies. Hurst (1997) indicated that while linear regression imposes constraints on equations, and may not always provide the best fit for survival data, it is a concept that is simple to understand and produces results that are readily visualized and easily compared.

Similar to the empirical modeling approach summarized by Hurst (1997), linear regression analyses were used to calculate survival slopes (die-off rates) for RN-L11 in different soil and rhizosphere microcosms. These survival slopes were then used to develop predictive LMR models as a function of soil parameters at the time of RN-L11 inoculation in microcosms. A simple LMR modeling approach was taken in order to specify *a priori* statistical models and avoid potential problems of overfitting the data. LMRs were sought that were simple, easily understood and communicated, biologically interpretable, and that contained the minimum number of parameters necessary to describe observed results. The LMR models were developed based upon survival data derived from a laboratory soil microcosm that was validated by Gagliardi et al. (2001b). This validation study demonstrated that the laboratory microcosm was useful for predicting GEM survival in outdoor field plots. The microcosm validation study

conducted by Gagliardi et al. (2001b) also provided a unique survival data set from a multi-year survival study of a GEM in widely different soils and climatic regions across Canada. This data set, supplemented by additional laboratory data collected as part of this thesis, provided a unique opportunity to test the reliability of a multiple regression modeling approach for predicting survival of a GEM.

Materials and Methods

The study described in this chapter was conducted in the laboratory at Carleton University in 1996 (Year 1) and 1997 (Year 2) using soils collected from 6 sites at Agriculture and Agri-Food Canada's Central Experimental Farm in Ottawa, Ontario, Canada. These sites have been used for a variety of agricultural research activities over many decades although soil cores were taken from the soil right at the edge of the fields which was probably less subject to tilling and soil amendments.

The study in the laboratory at Carleton University was conducted simultaneously with a larger soil core microcosm validation study carried out at 6 other locations: the University of Maryland, College Park, MD (Dr. Scott Angle); University of British Columbia, Vancouver, B.C. (Dr. Chris Chanway); University of Saskatchewan, Saskatoon, SK (Dr. Jim Germida); Carleton University, Ottawa, Ont. (Dr. Campbell Wyndham); McGill University, Montreal, Que. (Dr. Charles Greer - 1997 only); and at Agriculture and Agri-Food Canada's Central Experimental Farm in Ottawa, Ont. (Dr. Bob Watson). The soil core microcosm validation study is described by Angle (1998) and Gagliardi et al. (2001b). Study sites for the present study are identified in Table 2.

The genetically engineered bacterium and the materials and methods used in this thesis follow the same protocol used for the microcosm validation study as outlined in Angle (1998). Whereas the microcosm validation study was designed to test the ability of the intact soil core microcosm to predict GEM survival in experimental field releases, the following study examined the ability to predict GEM survival in experimental field releases based on physical, chemical, and biological characteristics of soils. This thesis

Table 2. List of study sites used to gather soil and rhizosphere survival data on *Pseudomonas chlororaphis* 3732RNL-11 for linear multiple regression models.

SITE	CODE	LOCATION	IELD TRIA	PRINCIPLE SCIENTIST
YEAR 1				
1	A	Central Experimental Farm - site A, Ottawa, Ont.	No	Tom Edge, Carleton University
2	B	Central Experimental Farm - site B, Ottawa, Ont.	No	Tom Edge, Carleton University
3	C	Central Experimental Farm - site C, Ottawa, Ont.	No	Tom Edge, Carleton University
4	D	Central Experimental Farm - site D, Ottawa, Ont.	No	Tom Edge, Carleton University
5	E	Central Experimental Farm - site E, Ottawa, Ont.	No	Tom Edge, Carleton University
6	F	Central Experimental Farm - site F, Ottawa, Ont.	No	Tom Edge, Carleton University
7	BC	University of British Columbia, Vancouver, B.C.	Yes	Dr. Chris Chanway, U.B.C.
8	SK	University of Saskatchewan, Saskatoon, Sask.	Yes	Dr. Jim Germida, U. Sask.
9	CL	Carleton University, Ottawa, Ont.	Yes	Dr. Campbell Wyndham, Carleton U.
10	AG	Agriculture and Agri-Food Canada, Ottawa, Ont.	Yes	Dr. Robert Watson, AAFC
11	MD	University of Maryland, College Park, Md	No	Dr. Scott Angle, U. Maryland
YEAR 2				
12	A	Central Experimental Farm - site A, Ottawa, Ont.	No	Tom Edge, Carleton University
13	B	Central Experimental Farm - site B, Ottawa, Ont.	No	Tom Edge, Carleton University
14	C	Central Experimental Farm - site C, Ottawa, Ont.	No	Tom Edge, Carleton University
15	D	Central Experimental Farm - site D, Ottawa, Ont.	No	Tom Edge, Carleton University
16	E	Central Experimental Farm - site E, Ottawa, Ont.	No	Tom Edge, Carleton University
17	F	Central Experimental Farm - site F, Ottawa, Ont.	No	Tom Edge, Carleton University
18	BC	University of British Columbia, Vancouver, B.C.	Yes	Dr. Chris Chanway, U.B.C.
19	SK	University of Saskatchewan, Saskatoon, Sask.	Yes	Dr. Jim Germida, U. Sask.
20	CL	Carleton University, Ottawa, Ont.	Yes	Dr. Campbell Wyndham, Carleton U.
21	AG	Agriculture and Agri-Food Canada, Ottawa, Ont.	Yes	Dr. Robert Watson, AAFC
22	NR	McGill University, Montreal, Que.	Yes	Dr. Charles Greer, McGill U.
23	MD	University of Maryland, College Park, Md	No	Dr. Scott Angle, U. Maryland

study did not involve conducting a field release, although the raw GEM survival data from the microcosm validation study, including data from field releases, were kindly shared and included in statistical analyses.

Bacterium strain

The genetically engineered bacterial strain used in this study was *Pseudomonas chlororaphis* 3732RN-L11 (formerly *P. aureofaciens*). It will subsequently be referred to as RN-L11 in this thesis. This strain and its parent (strain 3732RN) are resistant to antibiotics Rifamycin (100 µg/ml) and Nalidixic Acid (100 µg/ml). The parent organism was originally isolated from the rhizosphere of wheat by Monsanto Corporation in the United States. The genetically engineered strain RN-L11 was developed by inserting a *lacZY* gene cassette into the chromosome using a Tn7-*lacZY* transposon (Barry 1986, 1988). A single copy of the two genes that code for *LacZ* (Beta-galactosidase) and *LacY* (lactose permease) was stably inserted into the chromosome using a modified Tn7 insertion element that lacks the ability to excise or re-insert itself. Colonies of RN-L11 appear blue when grown on a substrate containing X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Both RN-L11 and its parent were developed by Monsanto Corporation for the purpose of enhancing plant growth. The strains produce a prodigious amount of siderophores that chelate iron, possibly inhibiting the growth of pathogens in rhizospheres. The optimal growth temperature for RN-L11 is 28° C.

RN-L11 is a useful microorganism for the purposes of the current study because the strain's genetic modifications allow for it to be detected in soil and wheat rhizospheres with relative ease at concentrations as low as 10 CFU/gram of soil. There is also a growing familiarity with this genetically engineered strain from a variety of studies in laboratory and field settings (Hattemer-Frey et al. 1990; England et al. 1993a; Kluepfel et al. 1994b; Angle et al. 1994; 1995; Leung et al. 1995; England et al. 1995; Gillespie et al. 1995; Angle et al. 1996; Lamb et al. 1996; England et al. 1997; Germida et al. 1998; Nairn and Chanway 1999; Snyder et al. 1999; Gagliardi et al. 2001a; Gagliardi et al. 2001b). Kluepfel (1993) indicated RN-L11 appears to be phenotypically identical to its non-engineered parent suggesting ecological fitness of the GEM is not impaired.

RN-L11 was stored at -80° C as a dense cell suspension in Pseudomonas F broth prepared with 50% water and 50% glycerol.

Media and chemicals

Pseudomonas F Agar (DIFCO Labs, Detroit, MI) with glycerol (Fisher Scientific, Pittsburg, PA) as a major carbon source was used as the selective agar medium. Medium additives included Cycloheximide (Actidione) (100 µg/ml) for fungal inhibition as well as Rifamycin SV (100 µg/ml) and Nalidixic acid (100 µg/ml) (Fluka Chemicals, St. Louis MO) to inhibit other bacteria. X-Gal (Diagnostic Chemical Ltd, Oxford, CT) was added at 80 µg/ml as an agent to distinguish RN-L11 from other soil bacteria that generally do not metabolize lactose (a milk sugar). The antibiotics and X-Gal all came from the same

lot to maintain consistency throughout the experiment. In one instance in 1997, an additional antibiotic, Nystatin, was added to the agar media to address some fungal contamination problems encountered.

The agar medium was prepared in 1000 ml Pyrex Erlenmeyer flasks containing: 1) 19 g pre-made *Pseudomonas* F medium; 2) 5 ml glycerol; 3) 500 ml distilled/ deionized water; and a magnetic stir-bar. The flasks were autoclaved at 121° C for 30 minutes and then cooled to 55° C. Supplements were then added in the following order: 0.5 ml X-Gal stock (60 mg in 0.5 ml N,N-Dimethylformamide); 0.5 ml Nalidixic acid stock (100 mg in 0.5 ml of 1N NaOH (40 g/l NaOH)); 0.5 ml Cycloheximide (Actidione) stock (100 mg in 0.5 ml HPLC grade methanol); 0.5 ml Rifamycin SV stock (100 mg in 0.5 ml HPLC grade methanol). Agar plates were then poured and allowed to dry for about 48 hours.

Liquid culture broth used to grow the inoculant was the same formula as the agar medium except the agar, cycloheximide and X-Gal were omitted. The antibiotics Rifamycin and Nalidixic acid were included to maintain selection pressure on the inoculant strain.

The buffered water used for dilution tubes was made with 1000 ml distilled/deionized water and 5 ml of stock solution I and 1.25 ml of stock solution II. Stock solution I was made from 50 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in 1000 ml distilled/deionized water. Stock solution II was made from 34 g KH_2PO_4 dissolved in 1000 ml distilled/deionized water. The dilution tubes were kept to a pH between 6 and 7 and they were sterilized prior to use.

Soil and rhizosphere microcosms

The soil microcosms consisted of 17.5 cm long hollow cores made from 5 cm diameter polyvinyl chloride plumbing pipe (Figure 11). One end of the core was tapered on the outside edge to ease penetration into the ground. Cores were hammered into the ground until about one inch remained above the soil surface. A trench was then dug around the cores and they were individually removed sideways. The bottom of each core was covered with aluminum foil held in place by an elastic band. These cores thus provided an intact soil core with minimal disturbance to the natural soil structure. Both the core and the core with soil were weighed to enable tracking of water loss and maintenance of soil moisture over the course of the experiment.

Rhizosphere microcosms were identical to the soil microcosms except they were planted with Canadian prairie spring wheat seeds (hard red spring wheat, *Triticum aestivum*, cultivar A.C. Karma) just prior to inoculation with the RN-L11 (see Microcosm inoculation section). Seed remaining from year one was refrigerated and re-distributed for use in year 2. Three to five wheat seeds were planted in a shallow hole in the centre of soil microcosms. About seven days after seed germination, the rhizosphere cores were thinned to one plant per core.

Figure 11. Photograph of Year 1 rhizosphere microcosms (intact soil core with one wheat seedling per core) from sites A to F at the Central Experimental Farm, Ottawa, Ont. The photograph was taken at day 43 after microcosms with wheat seeds were inoculated with *Pseudomonas chlororaphis* 3732RN-L11. Soil microcosms were the intact soil core without wheat seeds planted.

Once collected from the field in early May, all the soil microcosms were placed randomly in a growth chamber at Carleton University. The growth chamber was kept at 22° C (approximately the 5 year mean growing season temperature for wheat in Canada) and a relative humidity of 70 % over the duration of the experiment. A photoperiod cycle of 12 hours night and 12 hours daylight was established. Three replicate soil cores from each site were also collected in order to determine the soil moisture content as a percentage of field capacity (also see microcosm critique of field capacity in Discussion section). The weight of the soil was determined in each replicate core at 100% water saturation and then after drying at 72° C for 72 hours in an oven in order to calculate 60% of field capacity. The soil cores were closely monitored over the experiment for both growth of plant weeds and water loss. Any weeds growing in the soil microcosms (and closely associated soil) were carefully removed. Cores were weighed regularly to monitor water loss and to maintain about 60% soil moisture content, particularly for the rhizosphere cores which required more water to support plant growth. Cores were watered with sterile, distilled water.

Soil collection and analysis

Soils were analyzed from 11 sites in Year 1 and 12 sites in Year 2 (Table 2). Six sites at the Central Experimental Farm (Agriculture and Agri-Food Canada) in Ottawa, Ontario, Canada were chosen to represent a variety of different agricultural soil types in the Experimental Farm area. Soil cores at each site were taken at the edge of agricultural fields (just outside the cultivated part of the field) in early May. The areas chosen were

flat and without rocks or stones that would make soil core collection difficult. The soil surface was cleared by hand of any sticks, rocks and plant material without disturbing the soil structure before taking the soil cores.

Thirty soil cores were collected at each site in Year 1 and Year 2. Twenty-four cores were used for the microcosm experiment (12 soil and 12 rhizosphere cores) and three replicates were used to calculate the 60% soil moisture levels as described above. In addition, three replicate soil cores from each site were shipped for analysis of physical, chemical and biological characteristics in the Soil Testing Laboratory at the University of Maryland, College Park, Md. These cores were lightly watered with sterile distilled water, wrapped in a plastic bag for shipping, and then maintained as intact soil cores at 4°C until the soil was extracted for analysis within about 7 days after collection from the ground. The soil was extracted from the cores and sieved through a 4 mm sieve to remove large stones and debris. Samples of this fresh soil were then used in analyses of the biological characteristics of the soils in the microbiology laboratory of Dr. Scott Angle at the University of Maryland, College Park, MD. The remaining fresh soil was air-dried over two days and used for additional analyses of physical and chemical parameters. Air-dried soil samples were passed through a grinder to crush large particles and provided to the University of Maryland Soil Testing Laboratory (Patterson Hall, College Park, MD 20742) for analyses following their standard test methods and described in University of Maryland (1978). Air-dried soil samples were also provided to the lab of Dr. Rufus Chaney, U.S. Department of Agriculture, Beltsville, MD for chemical analyses following their Standard Operating Procedure 4 summarized below and described in McGrath and Cunliffe (1985). Finally, oven-dried soil samples were also analyzed by Scanning Electron Microscope at Carleton University.

A summary of the physical, chemical, and biological soil parameters used in this study are provided in Table 3 and the associated methods are described as follows:

Physical parameters

% sand, % silt, % clay (sand, silt, clay) - Soils were measured at the University of Maryland Soil Testing Laboratory. 50 g of air-dried soil was added to 250 ml of water and 5 ml of 5% sodium pyrophosphate and allowed to stand overnight. Additional water was then added and stirred for 5 minutes. The soil suspension was transferred to a Buoyoucos cylinder and a hydrometer was added along with water to bring the volume to 1130 ml. The hydrometer was removed, and the cylinder was shaken and inverted several times to obtain a uniform suspension. The cylinder was then placed on the table and hydrometer added. After 40 seconds, the hydrometer mark at the surface of the suspension was read and the temperature of the suspension taken. At this point, the sand fraction is settled (> 0.05 mm) and % sand was calculated after considering a temperature correction. After 2 hours, the hydrometer and temperature readings were taken again. At this point, only the clay fraction is still suspended (< 2 microns), and % clay was calculated after considering a temperature correction. % silt was then obtained by subtracting % sand and clay from 100%.

Table 3. List of soil parameters measured at the time of *Pseudomonas chlororaphis* 3732RNL-11 inoculation into microcosms. Parameter abbreviations and units identified in third column.

SOIL PHYSICAL PARAMETERS

1	clay	% clay (% dry soil weight)
2	sand	% sand (% dry soil weight)
3	silt	% silt (% dry soil weight)

SOIL BIOLOGICAL PARAMETERS

4	bact	total soil bacteria (CFU/g. of soil)
5	pseudo	total soil pseudomonads (CFU/g. of soil)
6	fung	total soil fungi (CFU/g. of soil)
7	bio1-bio96	96 well Biolog GN plate readings (optical density) (96 metabolic reactions - bio1 to bio96)
8	bio#	significant Biolog wells (number of wells > 0.49 od out of 96)
9	biopt	cumulative Biolog wells (cumulative optical density)

SOIL CHEMICAL PARAMETERS

10	pH	pH
11	cec	cation exchange capacity (meq/100g)
12	ca	exchangeable calcium (kg/ha)
13	mg	exchangeable magnesium (kg/ha)
14	ko	K ₂ O (kg/ha)
15	po	P ₂ O ₅ (kg/ha)
16	no	NO ₃ (kg/ha)
17	ss	soluble salts (ppm soil/water suspension)
18	org	% organic matter (% dry soil weight)
19	carb	% carbon (% molecular weight)
20	nitr	% nitrogen (% molecular weight)
21	hydr	% hydrogen (% molecular weight)
22	aacd-aana	atomic absorption elemental analyses (% molecular weight) (11 elements - Cd, Zn, Cu, Mn, Ni, Pb, Fe, K, Ca, Mg, Na)
23	semsi-semcu	Scanning electron microscope elemental analyses (% mol. wt) (16 elements + summed total elemental reading (semtot) - Si, Al, Ti, Cr, Fe, Mg, Mn, K, Ca, Na, Cl, Zn, S, Ni, P, Cu)

Chemical parameters

pH (pH) - Soils were analyzed at the University of Maryland Soil Testing Laboratory. 20 g of air-dried soil were added to 20 ml of distilled water and allowed to stand for one hour. pH was then measured using a Fisher Scientific Accumet 915 pH meter with a glass electrode assembly.

Ca (ca) - Soils were analyzed at the University of Maryland Soil Testing Laboratory. Exchangeable calcium (kg/ha) was measured using a flame photometer (Technicon AutoAnalyzer II System) which determines its concentration by the intensity of light emission characteristics at 623 nm when oxidized in a natural gas - compressed air flame. Lithium was used as an internal standard at 671 nm.

Mg (mg) - Soils were analyzed at the University of Maryland Soil Testing Laboratory. Exchangeable magnesium (kg/ha) was measured using the AutoAnalyzer II. A highly coloured indicator dye, magnesium blue, was used which forms a blue complex with magnesium in an alkaline solution. The intensity of the colour developed was determined at 630 nm.

K₂O (ko) - Soils were analyzed at the University of Maryland Soil Testing Laboratory. Exchangeable potassium (kg/ha) was measured using a flame photometer which determines its concentration by the intensity of light emission characteristics at 768 nm when oxidized in a natural gas - compressed air flame on an AutoAnalyzer II. Lithium was used as an internal standard at 671 nm.

P₂O₅ (po) - Soils were analyzed at the University of Maryland Soil Testing Laboratory. The molybdate-vanadate colorimetric method was used to determine phosphorus P₂O₅ (kg/ha). The intensity of the yellow colour developed with this method was measured at 420 nm using the AutoAnalyzer II.

NO₃ (no) - Soils were analyzed at the University of Maryland Soil Testing Laboratory. Nitrate (NO₃) (kg/ha) was analyzed by colorimetric method using the AutoAnalyzer II.

CEC (cec) - Soils were analyzed at the University of Maryland Soil Testing Laboratory. Total cation exchange capacity (meq/100 gm) was calculated as the sum of exchangeable acidity (= 4(6.6 - buffered pH)) and the following cations: Mg, K, and Ca (meq/100 gm). This method is routinely used in this laboratory and is based on these cations being the major ones likely to be present in soils from the northeastern United States.

Soluble salts (ss) - Soils were analyzed at the University of Maryland Soil Testing Laboratory. Soluble salts (ppm) were determined by measuring electrical conductivity of the soil-water suspension and referring to a standard curve based on conductivity and high purity sodium chloride.

% organic matter (org) - Soils were analyzed at the University of Maryland Soil Testing Laboratory. % organic matter was measured by weight loss on ignition at 360° C for 2 hours. The soil samples were first pre-dried at 120° C for 1 hour.

% carbon, % hydrogen, % nitrogen (carb, hydr, nitr) - Soils were analyzed at the University of Maryland Soil Testing Laboratory. Soil samples were analyzed by gas combustion using a LECO CHN600 analyzer.

Scanning electron microscope (SEM) elemental analyses (semsi - semtot) - The element composition of soils from Years 1 and 2 was examined using a JEOL JSM-6400 scanning electron microscope (SEM) at the Carleton University Research Facility for Electron Microscopy (CURFEM). A Link Analytical x-ray elemental analysis unit attached to the scanning electron microscope was used to obtain % elemental composition data for soil samples. Soil samples from 3 soil cores from each site were oven dried and a small soil sample from a core was then spread onto double-sided carbon tape on small aluminum posts. SEM scans of the carbon tape alone did not indicate any elemental contamination likely to adversely affect the elemental analyses. The aluminum posts with soil samples were then coated with a thin layer of carbon (10-20 nm) before being mounted for observation in the scanning electron microscope. The SEM scans the surface of a sample with a finely focused electron beam that stimulates the emission of electrons and x-rays from the sample surface. The x-rays emitted are characteristic of the elements present in the sample and the x-rays are collected and analyzed to identify elements in the sample.

The elemental composition of each soil was measured by performing 3 replicate x-ray analyses for each soil sample from a given soil core. This resulted in 9 x-ray analyses per site (3 x-ray analyses of a soil sample from each of the 3 replicate soil cores from a given site). A cobalt sample was analyzed at regular intervals during the soil analyses for the gain calibration to ensure reliability of SEM analyses. Each x-ray analysis was for 100

seconds at probe current of 0.8 nanoamperes, 20 kV accelerating voltage, a working distance of 39 mm and a magnification of 50x. These x-ray analyses provided a crude composite analysis of many different soil particles characterizing each soil sample rather than an analysis of a specific soil particle (e.g. clay particle) within a sample. While quantitative SEM elemental microanalysis has typically used soil samples impregnated within resin and then either thin sections or polished blocks prepared to satisfy smooth flat surface requirements, it is possible to obtain useful semi-quantitative data from x-ray analyses of rough soil surfaces (Sullivan and Bush 1997; L.A. Sullivan, personal communication 1998). Elemental composition of soil (as a % of total molecular weight) was obtained with a Link ZAF-4 program by analyses of the area under peaks corresponding to elements detected in the energy dispersive x-ray spectras. In order to allow comparison of % elemental values between samples, the total % elemental detection was adjusted to 100% for each sample and percentages for each element were adjusted accordingly. The elements detected by the SEM analyses were: Si, Al, Ti, Cr, Fe, Mg, Mn, K, Ca, Na, Cl, Zn, S, Ni, P, Cu. The total of all elements detected was also measured (semtot).

Atomic absorption elemental analyses (aacd - aana) - Air-dried soil samples from Year 2 only were digested in hot nitric acid and provided to the laboratory of Dr. Rufus Chaney, U.S. Department of Agriculture, Beltsville, MD for ICP atomic absorption analyses following their Standard Operating Procedure 4 and described by McGrath and Cunliffe (1985). Data were obtained on the concentration of the following elements: cadmium (Cd); zinc (Zn); copper (Cu); manganese (Mn); nickel (Ni); lead (Pb); iron (Fe); potassium (K); calcium (Ca); magnesium (Mg) and sodium (Na).

Biological parameters

Total soil bacteria (bact) - Soils were analyzed in the laboratory of Dr. Scott Angle, University of Maryland, when fresh soil was removed from soil cores. Three or four replicate soil cores from each site from each year were analyzed for total bacteria counts as defined by colonies counted on a selective medium described below. The soil from each core was removed and sieved through a 4 mm sieve to remove large stones and debris. Similar to the microcosm sampling protocol, 10.0 grams of the soil were then weighed out and added to 95 ml of sterile distilled water in a Waring blender for extraction of bacteria. Since 10 g of soil takes up about 5 cc of volume, the Protocol considered that the extractant provided approximately a 10^{-1} dilution. The extractant was blended in the Waring blender for one minute, whereupon the suspension was allowed to settle for an additional minute before sampling. The basic method for obtaining plate counts involved estimating the likely count of total bacteria, devising an appropriate dilution scheme, and using a spiral-plating device (Spiral Systems Inc., Cincinnati, OH, Model D) for plating onto a selective medium. The selective medium used for obtaining counts of total bacteria was described by Buyer (1995) as a medium that inhibits the growth of *Bacillus mycoides* and is relatively nonselective for other bacteria thus providing an advantage for enumerating soil bacteria. After inoculation, all plates were inverted and incubated in the dark at 22° C for about 48 hours before counting colonies. The total bacteria counts were expressed in terms of colony forming units per gram of fresh soil (CFU/g), recognizing that this would not account for the bacteria that were not culturable with this medium.

Total soil pseudomonads (pseudo) - Soils were analyzed in the laboratory of Dr. Scott Angle, University of Maryland, when fresh soil was removed from soil cores. Three or four replicate soil cores from each site from each year were analyzed for total pseudomonad counts as defined by colonies counted on a selective media described below. The soil from each core was extracted and sieved through a 4 mm sieve to remove large stones and debris. Similar to the microcosm sampling protocol, 10.0 grams of the soil were then weighed out and added to 95 ml of sterile distilled water in a Waring blender for extraction of bacteria. Since 10 g of soil takes up about 5 cc of volume, the Protocol considered that the extractant provided approximately a 10^{-1} dilution. The extractant was blended in the Waring blender for one minute, whereupon the suspension was allowed to settle for an additional minute before sampling. The basic method for obtaining plate counts involved estimating the likely count of total pseudomonads, devising an appropriate dilution scheme, and using a spiral-plating device (Spiral Systems Inc., Cincinnati, OH, Model D) for plating onto a selective medium. The selective medium used for obtaining counts of total pseudomonads was described as *Pseudomonas* Agar F by Difco, Detroit, Michigan. This was the same medium used in the microcosm sampling analyses for the GEM except it lacked the antibiotics described previously. This medium was described as providing for the easy and rapid identification of most *Pseudomonas* organisms. After inoculation, all plates were inverted and incubated in the dark at 22° C for about 24 hours before counting colonies. The total pseudomonad counts were expressed in terms of colony forming units per gram of fresh soil (CFU/g) recognizing that this would not account for the pseudomonads that were not culturable with this medium.

Total soil fungi (fung) - Soils were analyzed in the laboratory of Dr. Scott Angle, University of Maryland, when fresh soil was removed from soil cores. Three or four replicate soil cores from each site from each year were analyzed for total fungi counts as defined by colonies counted on a selective medium described below. The soil from each core was removed and sieved through a 4 mm sieve to remove large stones and debris. Similar to the microcosm sampling protocol, 10.0 grams of the soil was then weighed out and added to 95 ml of sterile distilled water in a Waring blender for extraction of bacteria. Since 10 g of soil takes up about 5 cc of volume, the Protocol considered that the extractant provided approximately a 10^{-1} dilution. The extractant was blended in the Waring blender for one minute, whereupon the suspension was allowed to settle for an additional minute before sampling. The basic method for obtaining plate counts involved estimating the likely count of total fungi, devising an appropriate dilution scheme, and using a spiral-plating device (Spiral Systems Inc., Cincinnati, OH, Model D) for plating onto a selective medium. The selective medium used for obtaining counts of total fungi was described by Buyer et al. (1989). These authors indicated a wide variety of fungi were found to grow on this medium. After inoculation, all plates were inverted and incubated in the dark at 22° C for about 48 hours before counting colonies. The total fungi counts were expressed in terms of colony forming units per gram of fresh soil (CFU/g), recognizing that this would not account for the fungi that were not culturable with this medium.

Biolog data analyses (bio1 - bio96, bio#, biopt) - Soils from Year 2 only were analyzed using BIOLOG GN microplates in the laboratory of Dr. Scott Angle, University of

Maryland. Fresh soil was removed from three or four replicate soil cores from each site and was then analyzed for total microbial community function as defined by carbon source utilization on BIOLOG microplates. Utilization of a carbon source is detected as an increase in the respiration of cells in microplate wells, leading to irreversible reduction of a tetrazolium dye. The soil from each core was removed and sieved through a 4 mm sieve to remove stones and organic debris. Similar to the microcosm sampling protocol, 10.0 grams of the soil was then weighed out and added to 95 ml of sterile distilled water in a Waring blender for extraction of bacteria. Since 10 g of soil takes up about 5 cc of volume, the Protocol considered that the extractant provided approximately a 10^{-1} dilution. The extractant was blended in the Waring blender for one minute, whereupon the suspension was allowed to settle for an additional minute before sampling. After extraction of samples for total bacteria, pseudomonads and fungi, BIOLOG samples were extracted from the blender and further diluted 1:1000 in 0.85% NaCl. Then 150 μ l was inoculated into each of the 96 wells of a BIOLOG GN microplate with a multichannel pipettor. BIOLOG microplates were then covered and incubated at 22° C in the dark for 72 hours. After incubation, all plates were read in a microplate reader equipped with a 590 nm barrier filter (Molecular Devices, E-MAX with Softmax). The 96 optical density readings from each microplate were stored as an ASCII file for subsequent statistical analysis. Parameters used in statistical analyses included the optical density reading from the blank well (**bio1**) and each of the other wells (**bio2-bio96**), the summed optical density readings for total well colour development of the tetrazolium dye over 95 wells **biopt** (a measure of total metabolism of the microbial community), and **bio#** which was the number of wells (out of 95) that produced visible reactions corresponding to optical density readings >0.49 at 590 nm.

Microcosm inoculation

The RN-L11 inoculant was prepared by plunging a sterilized loop into the frozen broth, extracting and streaking a loopful onto the selective agar medium used throughout this study. A single colony was used to start a 10 ml overnight broth culture. Then 0.2 ml from this broth was added to a flask with 200 ml of liquid medium. The liquid medium was cultured about 12 hours at 28° C in a shaker set at a constant 175 rpm to reach an Optical Density (OD-660) of 0.800 (approximately 5×10^9 CFU/ml) as measured using a spectrophotometer. At this point the cells were in mid to late log phase and the entire flask was cooled in an ice bath to stop cell growth.

The cells were pelleted by filling two sterile 250 ml polypropylene bottles with the liquid medium and centrifuging them for 20 minutes at 4500 rpm. The supernatant was removed and the cells were resuspended in chilled sterile distilled water with vigorous shaking. The RN-L11 inoculant was prepared in these centrifuge bottles by adding chilled sterile distilled water to reach an Optical Density (OD-660) of 0.400 (approximately 5×10^8 CFU/ml). The inoculant was kept in an ice bath until inoculation within about 2 hours. A serial dilution of the inoculant was also made in order to obtain plate counts of the inoculant concentration.

All microcosms were inoculated about one week after collection from the field. Each microcosm was inoculated with 2.5 ml of RN-L11 inoculant using a sterile micropipette.

The inoculum was applied evenly over the soil surface of the soil microcosms and over the planted seeds in the rhizosphere microcosms. The inoculant was allowed to soak into the soil for at least 3 to 4 hours before sampling for Day 0 GEM counts in soil.

Microcosm sampling

Soil microcosms were sampled at Day 0, Day 14, and at a third time depending upon the observed die-off rate that was generally either Day 42 or Day 56. For rhizosphere microcosms, sampling had to await germination and development of the rhizosphere, and so Day 0 sampling took place at 14 days after inoculation of the seeds. Subsequent rhizosphere samplings were then at Day 28 and Day 84. At each sampling time, 4 replicate soil cores and 4 replicate rhizosphere cores were sampled from each site. Photographs were taken to record plant growth in the rhizosphere cores at each sampling time. Microcosm sampling entailed tapping on the sides of the cores with a hammer until the soil or soil and associated rhizosphere fell out onto a 4 mm stainless steel sieve.

For each soil microcosm, the soil was sieved through the 4 mm screen into a catch pan to remove larger organic and inorganic debris like sticks and stones, and thoroughly mixed in the catch pan. Then 10.0 grams of the soil was weighed out and added to 95 ml of sterile distilled water in a Waring blender for extraction of RN-L11. The blender had a glass container with stainless steel blades and operated at high speed (22, 000 rpm). Since 10 g of soil takes up about 5 cc of volume, the Protocol considered that the extractant provided approximately a 10^{-1} dilution. The extractant was blended in the Waring blender for one minute, whereupon the suspension was allowed to settle for an additional minute before sampling. Previous experiments showed that blending for one

minute followed by one minute of settling yields the highest viable count of RN-L11 from soil and rhizospheres (Angle et al. 1994).

For each rhizosphere microcosm, instead of sieving the soil, the soil was removed from around the rhizosphere and loosely adhering clumps of soil were gently cleaned from the roots. The plant portion was then cut off at the root crown and the entire rhizosphere (roots including any soil that remained tightly adhered to the roots) was weighed and recorded as rhizosphere fresh weight. This entire fresh rhizosphere was then added to sterile distilled water in the Waring blender for extraction of RN-L11. The amount of distilled water added was 100 ml minus the water volume equivalent to the weight of the fresh rhizosphere in grams (e.g. 4.5 g rhizosphere = 4.5 ml distilled water). The Protocol considered that extractant provided about a 10^{-2} dilution. The extractant containing the fresh rhizosphere was then blended in the Waring blender for one minute, whereupon the suspension was allowed to settle for an additional minute before sampling.

Selective plating techniques

The basic method for obtaining plate counts involved predicting the survival trend of RN-L11 based on previous knowledge and devising an appropriate dilution scheme. The plating method used three plates at three different dilutions to cover the expected RN-L11 concentration from a soil or rhizosphere microcosm. Three replicate one ml extractant samples were removed from the blender by sterile micropipette and added to three test tubes each containing 9 ml of buffered diluent. Each of these test tubes served as the first 10^{-1} dilution in three replicate series of dilution test tubes. After adding the 1 ml sample containing RN-L11 to a dilution tube, the dilution tube was thoroughly shaken before

micropipetting 1 ml for the next dilution tube. Then 100 μ l was micropipetted onto the surface of the selective agar plates for all dilution test tubes from a 10^{-2} dilution or higher dilution. For counts below a 10^{-2} dilution (i.e. for very low population densities), 1 ml of undiluted sample was plated to give a 10 CFU/gm limit of detection. Rhizosphere samples were plated in the same way except that the 1 ml of undiluted sample was plated below the 10^{-3} dilution for a CFU detection limit of 10^2 CFU/gm.

The inoculum was spread on the agar plates until they were dry by a glass “hockey stick” sterilized in ethanol and flamed dry. The plating method used three replicate dilution series (nine plates) for each microcosm core. All plates were inverted and incubated in the dark at 28° C for 48 hours before counting colonies. Generally, the dilution series allowed one plate with between 20-300 blue colonies per plate to be used from each dilution, and the mean of three plates was then used as the final count for each microcosm. The soil microcosm counts were expressed in terms of colony forming units per gram of fresh soil (CFU/g). The rhizosphere plate counts were expressed as colony forming units per gram of fresh rhizosphere. Since the detection limit for RN-L11 in soil was 10 CFU/g of soil and 100 CFU/g of rhizosphere, a count of zero was not valid. If all plates for a replicate were zero but there were mean counts for other replicates at that sampling time and treatment, then a value halfway between the limit of detection and zero was used for that replicate. For situations within replicates, where a single plate was zero with counts on other plates, a ‘one’ was inserted if the mean count was less than 10 or it was counted as missing data if the mean count was greater than 10 CFU.

In Year 2, contamination of some plates was found to preclude obtaining reliable counts of RN-L11. Contamination, possibly by a fungal species, appeared to crowd out and

prevent growth of RN-L11 colonies. The source of this contamination was not determined, but it could have resulted from the antibiotic Actidione losing its activity. In order to reduce contamination, 100 µg/ml of another antibiotic, Nystatin, was added to the agar to provide a more selective medium for RN-L11. Additional data were collected which indicated that addition of the Nystatin helped to control the contamination but did not adversely affect the growth of RN-L11 (in a test, soil samples showed significantly decreased contamination and RN-L11 plate counts of 77, 71 and 75 CFUs for agar plates with 25, 50 and 100 µg/ml of Nystatin).

Experimental field releases of GEM

Experimental field releases were conducted as part of the larger microcosm validation study and raw plate count data were kindly shared for inclusion in current analyses. A detailed description of the methods for the field releases of RN-L11 is provided by Angle (1998) and Gagliardi et al. (2001b). At each site across Canada, eight 1 m² field plots were cleared of surface plant and organic material and separated by 1 m between each plot. Four plots were planted with wheat for the rhizosphere treatment and four plots were left fallow. Corrugated plastic edging 15 cm high was buried 7.5 cm deep around each plot to prevent RN-L11 runoff and root interactions between plots as in the previous study by Angle et al. (1995). The field plots were watered when necessary and weeded like the laboratory soil microcosms that were obtained from an area adjacent to the field plots so that soil conditions would be very similar. Field plots were inoculated at the same time as laboratory microcosms and sampling of soil and wheat rhizospheres was also timed to coincide with sampling of the laboratory cores. The plots were inoculated using a polyethylene gravity-fed watering bottle to slowly distribute the inoculum as

droplets over the entire soil surface in each plot. Field soil cores were obtained with 15 cm deep stainless steel T-handle soil corers, while field rhizospheres were randomly selected and obtained by clipping the plant at the soil surface and using a sterilized trowel to dig out the entire rhizosphere around an individual plant.

Statistical Analysis of Data

Statistical analyses were conducted using SAS statistical programs (version 6.08) and Microsoft Excel. Linear regression equations were first calculated to estimate RN-L11 survival slope values from laboratory soil and rhizosphere microcosm data representing each site. These survival slopes were then used in multivariate data sets with physical, chemical, and biological soil parameters in order to calculate linear multiple regression (LMR) models for predicting RN-L11 survival slope based on soil characteristics. Since sample sizes were small (e.g. $n = 11$ sites in Year 1 and $n = 12$ sites in Year 2), data were pooled for Year 1 and Year 2 to calculate soil and rhizosphere Year 1&2 LMRs based on 23 sites. Exploratory analyses were also conducted on LMRs calculated from only Year 1 or Year 2 data. A validation of the Year 1&2 LMR models was performed by comparing predicted GEM survival slopes from LMR models created using laboratory microcosm data with observed RN-L11 survival slopes from environmental releases in field plots.

Calculation of Survival Slopes

Linear regression equations were calculated for RN-L11 survival in laboratory soil and rhizosphere microcosms representing the six Experimental Farm sites in Ottawa. Equations were also calculated for RN-L11 survival in laboratory soil and rhizosphere

microcosms representing five other sites (six in Year 2) used in the microcosm validation study (Angle 1998; Gagliardi et al 2001b). The scientists in this study kindly shared raw data (CFU) that was used for calculations in the present study.

The Experimental Farm RN-L11 survival data was first visually examined for outlier data points such as those CFU counts in Year 2 that were influenced by a contamination problem found on some selective agar plates. RN-L11 CFU data points were deleted for several cores in Year 2 (primarily soil cores at time 28 days and above) because they were clearly observed as outliers (unusually low counts) and they were obtained from agar plates that exhibited high contamination. After removal of these few data points, CFU count data was transformed ($\log_{10}$) to enable calculation of survival slopes over time for soil and rhizosphere by least squares linear regression analysis (PROC REG).

Linear regression equations were characterized by survival slope values, slope standard errors, R-square values, adjusted R-square values, and Prob>F values. In addition, data scatter diagrams were examined, and analyses were performed on residual values to evaluate statistical assumptions and the fit of linear regression models. Residuals were analyzed to determine if they were normally distributed (Wilks-Shapiro test) and studentized residuals and Cook's distance values were examined to detect outlier and influential observations.

Linear regression equations for the six Experimental Farm sites were calculated based on soil or rhizosphere sampling at three times (e.g. generally day 0, 28 and 56 for soil; and day 14, 42 and 98 for rhizospheres). However, survival data from the microcosm validation study sites (Angle 1998; Gagliardi et al 2001b) were collected on more than

three sampling times and over a longer sampling period. It was decided that all data from the microcosm validation study would be used in regression analyses (e.g. 5-7 sampling times) but only within the sampling periods used in the present study (e.g. about 60 days for soil and about 100 days for rhizospheres).

Univariate analyses

Univariate analyses of survival slopes and the soil physical, chemical and biological parameters at each site were conducted using the SAS PROC UNIVARIATE procedure. Parameters were first examined to see if they were normally distributed by examining frequency histograms for each parameter and results from the Wilks-Shapiro test. Soil parameters were also examined after log10, square root, inverse ($1/X$), exponential (X^2), and trigonometric sine function transformations to determine if their distribution appeared more normal. If there was a significant improvement detected from examining frequency histograms and results from Wilks-Shapiro tests, the transformed parameter was used in subsequent analyses. These univariate analyses were also conducted separately for Year 1 and Year 2 data although since sample sizes were small (e.g. $n = 11$ sites in Year 1), it was more difficult to test for normality because the power of the test is low (e.g. a parameter must be highly non-normal in distribution for a test to detect non-normality).

The soil parameters used for correlation and multivariate analyses were the mean value for three or four replicate cores from each site. Correlations between soil parameters, and between soil parameters and survival slopes, were examined with the Pearson correlation coefficient and the non-parametric Spearman rank-order correlation test and using SAS PROC CORR.

Development of linear multiple regression models

Linear multiple regression (LMR) models were developed for predicting RN-L11 survival slopes from data sets comprised of laboratory microcosm-derived survival slope values and the measures of physical, chemical, and biological soil characteristics at each site at the time of inoculation. LMR predicted survival slopes were then compared with the observed survival slopes obtained from the laboratory microcosms and from the experimental field releases. These comparisons allowed for a validation of the accuracy of LMR predictions of RN-L11 survival in the experimental field releases, and a comparison of the accuracy of LMR-derived and microcosm-derived survival slope predictions of RN-L11 survival in field plots.

In order to address concerns about a small sample size (e.g. $n = 11$ sites in Year 1 and $n = 12$ sites in Year 2), data were pooled ($n=23$ sites) to calculate Year 1&2 LMR models for soil and rhizosphere. A weighted multiple regression approach was taken to calculate the LMRs in order to give less weight in calculations to those sites that were known to have survival slope values with higher variance. A weighting approach takes into account the unequal slope variances that are a violation of an assumption of linear regression models (homoscedasticity) and can introduce bias into estimates of regression coefficients and variances of estimated mean values (Freund and Wilson 1998). For each LMR calculation, sites were weighted by the reciprocal of the microcosm survival slope standard error ($1/\text{slope standard error}$). Typically the LMR coefficients and R-square values from weighted regression regressions are quite close to those of unweighted regressions although the weighting more clearly represents the nature of the underlying

distribution of the dependent variable (Freund and Wilson 1998).

In order to calculate each weighted LMR model, a four-step approach was taken based upon maximizing adjusted R-square values using the fewest number of soil parameters, and minimizing statistical concerns for aspects like multicollinearity. Multicollinearity results from correlations between independent variables and can lead to imprecise estimates of regression coefficients. Freund and Wilson (1998) indicate that multicollinearity is not a violation of the assumptions underlying a regression analysis, although it can impair the usefulness of a regression analysis, particularly with respect to interpretation of regression coefficients. While the regression may do a good job of estimating the dependent variable, it can be difficult to tell which of the independent variables are responsible.

In the first step, several multiple regression procedures were performed on the multivariate data set to identify a reduced number of soil parameters to include in the final LMRs. The first procedures performed were SAS PROC REG-MAXR and -STEPWISE (SLE=0.30 and SLS=0.25). The MAXR procedure seeks to find the best one-parameter model, the best two-parameter model and so on. It first finds the one-parameter model producing the highest R-square. Another parameter is added that yields the highest increase in R-square and each of the parameters is compared to each parameter not in the model. The MAXR procedure determines if removing one parameter and replacing it with another increases the R-square. It compares all possible parameter switches and makes the switch that produces the largest increase in R-square. The process continues until there is no switch that increases the R-square and the resulting model (e.g. two-parameter, three-parameter etc.) is considered the best the

procedure could find. The STEPWISE procedure identifies the best soil parameters based on maximizing the F statistic for a parameter's contribution to the model. This procedure adds the soil parameter with the highest computed F-to-enter value for the model. At each step, the procedure also evaluates all the parameters in the model and deletes ones that do not have a significant F statistic. The procedure ends when none of the parameters outside the model has an F statistic significant enough to meet the predefined level for entry, and every parameter in the model is significant such as to not warrant removal.

MAXR and STEPWISE procedures can be a useful approach to screening many parameters but they do not examine all possible sets of soil parameter combinations. As a result, useful soil parameters for predicting RN-L11 survival that were identified from MAXR and STEPWISE procedures were selected for more thorough analysis by the ADJRSQ procedure. The ADJRSQ procedure examines all possible combinations of parameters and identifies sets of parameters with the highest adjusted R-square values. Since the ADJRSQ requires extensive computation time, only 10 parameters could be practically analyzed by this procedure. These 10 parameters were selected to be the best 8 eight parameters as identified by the MAXR and STEPWISE procedures along with the parameters % clay and pH. The latter two parameters were included because of their relative ease of measurement and a theoretical basis for assuming their importance in influencing survival (Alexander 1986; England et al. 1993b; Trevors et al. 1994; Gillespie et al. 1995; Stotzky 1986, 1997; van Veen et al. 1997). The results of the first step were best LMR models based on between one and six soil parameters as measured by adjusted R-square values.

The second step involved examining correlations between soil parameters in order to remove highly correlated parameters from models that could result in concerns about multicollinearity. Hurst (1991, 1997) suggested that if two parameters were highly correlated, it would probably be better to allow only one to be included in a LMR model. As indicated previously, when multicollinearity is present, estimates of the regression coefficients are unstable and their interpretation becomes more difficult. As a result, the best LMR models based on between one and six soil parameters were those with the highest adjusted R-square and where parameters were not highly correlated (e.g. Pearson/Spearman coefficient about < 0.7).

The third step involved plotting adjusted R-square and R-square values against the number of soil parameters used in developing LMR models. The best LMR model was judged to be the one to maximize the adjusted R-square with the fewest number of soil parameters. It has been suggested that the adjusted R-square may be a more useful statistic than the R-square in situations when the sample size is small compared to the number of independent variables (Freund and Wilson 1998). In many cases, the plotted R-square values reached a plateau (and adjusted r-square values peaked) around 3-4 soil parameters. To reduce concern about statistical overfitting, final LMRs were sought with four or fewer soil parameters.

The fourth and final step involved analysis of LMR diagnostics tests. Similar to previous diagnostics for simple linear regression models, scatter diagrams of residual values were examined and analyses were performed on residual values to evaluate the fit of calculated LMR models. Residuals were plotted to determine whether they met assumptions of normality and homoscedasticity and whether there was any evidence for the presence of

curvilinear relationships. Residuals were also tested for normality (Wilks-Shapiro test) and studentized residuals and Cook's distance values were examined to detect outlier and influential observations. Freund and Wilson (1998) indicated studentized residual values exceeding 2.5 have been used to detect outlier observations markedly different from the rest of a dataset. Cook's distance is an overall measure of the influence of an observation on the estimated regression coefficients, and Ryan (1997) indicated a frequently used cutoff value has been 1.0.

Variance Inflation Factors (VIF) were examined in the output of regression analyses to assess concerns for multicollinearity. A VIF value of 10, although without theoretical basis, is often used as a cutoff value to detect multicollinearity and indicate a serious affect on estimation of a regression coefficient (Freund and Wilson 1998). However, Freund and Wilson (1998) also cautioned that VIF values needed to be evaluated relative to the overall fit of the regression model. They suggested that VIF values $> 1/(1-R\text{-square})$ imply stronger relationships among independent variables than their relationship to the dependent variable. Variance proportions were also examined to detect parameter contributions to multicollinearity. The parameters involved in multicollinearities can be identified by their relatively large variance proportions in principal components with small eigenvalues (variances) (Freund and Wilson 1998).

Soil and rhizosphere LMRs were developed for Year 1&2 pooled data (n=23).

Exploratory soil and rhizosphere LMRs were also calculated for Year 1 data (n=11) and Year 2 data (n=11). A Year 2a LMR (n=12) was calculated from Year 2 data plus another site and additional soil parameters that were added to the study in Year 2. All LMRs were characterized by regression coefficient values, R-square values, adjusted R-

square values, and Prob>F.

Verification of LMRs

A jackknife analysis was performed to examine the stability of soil parameters included in Year 1&2 LMR models. Based upon the pooled data set (n=23), twenty-three LMR models were calculated, leaving one site out each time in calculations. Each jackknife LMR model was developed to consist of whichever best four soil parameters were selected by the previously described LMR building steps. The frequency of soil parameters appearing in these twenty-three different LMR models was then used to assess Year 1&2 LMR parameter stability.

Exploratory principal component analyses were also conducted on the Year 1&2 data. The SAS PROC PRINCOMP procedure (correlation matrix) was performed to develop principal components that were new variables representing all the soil parameters. These principal components were then used as composite soil parameters in the steps outlined previously to develop LMRs. The first four principal components accounting for most of the variation in the data were analyzed by the SAS PROC REG procedure to examine whether the principal components could be useful for predicting survival slopes. The importance of soil parameter weights for each principal component could also be used to assess LMR stability by comparing those parameters most highly weighted to those soil parameters selected in the Year 1&2 LMRs.

A verification step for each Year 1&2 LMR model was performed by comparing predicted survival slopes with those observed from laboratory microcosm measurements.

This comparison was done on a quantitative basis by plotting LMR predicted slope values vs observed microcosm slope values. This allowed a simple visual test for assessing the accuracy of LMR predictions as was used by Hurst et al. (1992). The comparison was also done on a qualitative basis by dividing observed microcosm survival slope values into high and low survival categories based on the median slope value. The percentage error rate for classification of slopes using the LMR models was then determined. However, this verification is biased and could be expected to show reasonably accurate predictions since the observed slope values were also used in the calculations to develop the LMRs. A less biased verification step was also performed using a jackknife 'leave one out' test (SAS-REG - macro jackknife). This jackknife procedure re-calculated the Year 1&2 soil and rhizosphere LMRs leaving one site out each time, and applied each LMR to predicting the slope of the site left out. Predicted and observed survival slopes were then compared.

Additional cross-validation studies were also conducted as verification of the LMRs for predicting RN-L11 survival in soil and rhizospheres. The data were split into two groups in order to calculate different LMRs for predicting RN-L11 survival in soil and in rhizospheres from data collected in Year 1 (n=11), and from the equivalent data collected in Year 2 (n=11). This permitted a cross-validation test to determine whether the LMR calculated for one year could reliably predict survival slopes for the other year. In Year 2, another site was added to the study, and additional biologic and soil chemical data were collected. To check whether the additional site and soil parameters influenced calculation of Year 2 soil and rhizosphere models, Year 2a models (n=12) were calculated for soil and rhizosphere to include the additional site and soil parameter data.

Validation of LMRs

A final validation step for Year 1&2 LMR models compared predicted survival slopes with observed survival slopes from environmental releases in experimental field plots. This comparison enabled a quantitative and qualitative evaluation of how well the Year 1&2 LMRs predicted survival of RN-L11 in the outdoor field plots. A similar test of microcosm-derived survival slopes was also conducted which enabled a comparative evaluation of the accuracy of the LMR method and the microcosm method for predicting survival of RN-L11 in the experimental field releases.

Results

Soil characteristics

The results of soil analyses are provided in Tables 4 to 7. They show a considerable range for key soil parameters like % clay (2% to 37%), pH (5.8 to 7.9) and % organic matter (2.4% to 6.2%). Figure 12 summarizes the range of soil texture among study sites. The soil core microcosm protocol performed well for all sites and this range of soil types served as a useful test for the effectiveness of the microcosm protocol. Worms and soil invertebrates were found alive in the cores at sampling stages as late as 98 days after inoculation suggesting the microcosms could adequately maintain some soil mesofauna over the course of months. No adverse ecological effects from RN-L11 inoculation were noticed in the microcosms during the experiment based on cursory visual observations.

The data also show considerable soil variability between years at some sites despite the close proximity of core sampling locations. Parameters such as soluble salts were clearly not normally distributed and were not used in subsequent LMR analyses. The SEM analyses provided consistent soil measurements, although these analyses should not be regarded as a rigorously quantitative. More accurate SEM elemental analyses of soil typically require embedding the soil in a matrix and polishing the matrix until a very flat surface is obtained for X-ray analyses purposes. The rough surface of the samples used in this study resulted in total X-ray readings from 37 % to 51 % that are below readings obtained from more rigorous sample treatment methods. However, SEM data from analyses of rough surfaces can still provide useful semi-quantitative data for comparing soil samples (Sullivan and Bush 1997; Sullivan, personal communication 1998).

Table 4. Soil physical and biological parameter measurements at time of *Pseudomonas chlororaphis* 3732RN-L11 inoculation into microcosms. Site and soil parameter abbreviations and units are shown in Tables 2 and 3 respectively. Values are the mean of three or four replicates soil cores.

SITE-YR 1	sand	silt	clay	pseudo	fung	bact		
A	49.33	17.33	33.33	5.84	4.79	7.06		
B	62.67	25.33	12.00	5.62	5.22	7.38		
C	36.00	26.67	37.33	5.81	5.57	7.40		
D	89.67	7.33	3.00	6.09	5.27	7.24		
E	46.67	22.00	31.33	6.21	5.78	7.57		
F	55.00	26.33	18.67	6.07	5.69	7.32		
BC	86.33	9.00	4.67	5.98	4.35	6.71		
SK	34.33	44.00	21.67	6.26	5.81	7.62		
CL	59.33	28.67	12.00	6.00	5.15	7.18		
AG	81.33	16.00	2.67	6.21	5.56	7.50		
MD	43.33	42.00	14.67	6.09	5.16	7.23		
SITE-YR 2	sand	silt	clay	pseudo	fung	bact	bio#	biopt
A	51.00	17.67	31.33	6.64	5.98	7.28	56.3	95.3
B	66.00	22.33	11.67	6.52	5.99	7.35	59.0	94.0
C	48.00	16.00	36.00	6.60	6.06	7.17	55.0	83.5
D	92.67	4.33	3.00	6.39	5.44	6.92	13.3	27.8
E	44.00	26.33	29.67	6.60	5.75	7.09	58.3	93.4
F	61.00	24.33	14.67	6.66	6.05	7.23	58.3	91.1
BC	88.67	8.00	3.33	6.28	5.31	6.92	38.3	56.9
SK	35.00	45.33	19.67	6.59	6.26	7.37	47.5	70.9
CL	78.67	14.33	7.00	6.42	6.07	7.09	56.3	82.1
AG	84.33	13.67	2.00	6.58	6.25	7.12	56.0	78.2
MD	46.00	40.00	14.00	6.65	5.88	7.19	57.3	82.8
NR	60.00	21.67	19.00	6.51	5.38	7.08	23.5	40.7

Table 5. Soil chemical parameter measurements at the time of *Pseudomonas chlororaphis* 3732RN-L11 inoculation into microcosms. Site and soil parameter abbreviations and units are identified in Tables 2 and 3 respectively. Values are the mean of three or four replicate soil cores.

SITE- YR1	pH	cec	org	ss	ca	mg	no	po	ko	carb	hydr	nitr
A	7.07	18.47	2.70	103.0	5580.6	778.0	5.23	655.9	265.8	1.107	0.567	0.087
B	7.47	49.00	4.13	147.7	8062.5	410.3	38.38	824.7	610.8	2.067	0.487	0.153
C	6.60	19.77	5.43	179.7	5764.3	787.0	54.95	453.6	417.4	2.547	0.857	0.180
D	5.80	6.87	2.80	103.0	1549.0	196.0	42.86	176.6	360.3	1.480	0.357	0.113
E	6.63	17.57	4.60	116.3	4747.3	872.5	26.13	547.7	498.0	2.240	0.670	0.150
F	7.70	56.57	3.93	420.0	10892.4	655.6	17.02	78.8	160.2	2.207	0.567	0.153
BC	6.13	5.77	5.57	102.0	1008.7	183.3	19.41	160.2	218.4	2.877	0.630	0.143
SK	7.53	29.97	5.93	143.3	6188.4	1189.8	23.15	211.7	573.4	3.333	0.940	0.260
CL	7.53	46.90	3.57	152.3	6918.6	734.7	41.66	210.2	106.4	1.767	0.497	0.140
AG	6.23	5.57	3.57	102.0	993.4	237.1	21.80	484.2	324.8	1.560	0.370	0.087
MD	5.80	5.33	2.63	102.0	1067.7	247.1	16.43	109.0	136.3	1.213	0.373	0.093

SITE- YR2	pH	cec	org	ss	ca	mg	no	po	ko	carb	hydr	nitr
A	7.47	27.10	3.48	103.0	8882.3	1042.0	13.29	593.6	391.3	1.963	0.483	0.143
B	7.60	27.00	3.20	103.0	9387.1	567.1	24.34	1226.4	487.6	1.830	0.340	0.157
C	7.27	26.73	4.07	103.0	8718.1	741.8	16.58	588.0	328.5	1.920	0.527	0.143
D	5.87	6.49	2.53	103.0	1432.1	140.4	14.63	140.7	246.4	1.613	0.300	0.120
E	6.77	24.60	4.20	103.0	6981.3	1224.5	21.35	621.6	468.5	2.057	0.577	0.130
F	7.93	33.92	3.21	197.7	11818.2	985.6	20.01	94.1	219.9	2.023	0.377	0.127
BC	5.83	5.06	4.39	103.0	737.3	79.1	24.49	221.0	142.2	2.303	0.380	0.130
SK	7.37	26.31	6.24	175.3	7829.5	1378.0	32.11	246.8	782.1	3.640	0.757	0.297
CL	7.40	26.37	3.41	152.3	8949.5	893.4	89.60	181.4	241.2	1.880	0.327	0.157
AG	6.20	5.47	2.87	103.0	944.2	193.8	20.31	569.7	256.5	1.640	0.273	0.103
MD	5.90	6.74	2.41	103.0	1397.8	313.6	14.04	185.2	170.6	1.220	0.323	0.120
NR	5.87	10.29	3.69	103.0	2543.1	298.7	22.55	310.6	190.4	1.630	0.373	0.133

Table 6. Soil chemical parameter measurements on microcosm soils obtained by scanning electron microscope (sem) analyses. Site and soil parameter abbreviations are shown in Tables 2 and 3 respectively. Values are the mean of n=9 measurements on three replicate soil cores.

SITE-YR1	sems1	sema1	semd1	semcr	semfe	emm	emm	semk	semca	sema	semcl	sems	semin1	semp	semcu	semtot
A	55.85	17.90	1.31	0	11.22	3.64	0.07	3.51	3.37	2.50	0	0.07	0	0.35	0.19	48.60
B	61.85	15.58	1.00	0	9.44	2.30	0.11	3.22	3.73	1.99	0.01	0.14	0	0.52	0.12	46.86
C	56.20	18.11	1.36	0	11.09	3.50	0.09	3.57	3.26	2.18	0	0.02	0	0.34	0.26	49.83
D	60.26	18.36	1.08	0	9.88	2.24	0.00	2.68	2.58	2.20	0	0.28	0	0.45	0.00	41.99
E	56.69	17.16	1.54	0	11.56	3.53	0.11	3.71	3.04	2.03	0.01	0.02	0	0.37	0.22	47.80
F	58.02	17.25	1.07	0	10.39	2.91	0.03	3.82	4.21	1.48	0.05	0.11	0	0.45	0.19	46.37
BC	58.21	21.50	1.33	0	8.99	1.51	0.03	1.25	3.24	2.14	0	0.48	0	1.04	0.28	40.19
SK	68.71	14.89	0.79	0	7.56	1.76	0.16	2.77	2.28	0.66	0	0.12	0	0.09	0.21	45.46
CL	63.78	15.33	0.89	0	8.04	2.38	0.02	3.26	3.56	2.06	0	0.15	0	0.37	0.15	47.28
AG	59.93	18.05	1.11	0	9.63	1.88	0.04	2.61	2.90	1.77	0.01	0.29	0	1.59	0.20	47.34
MD	75.61	13.02	1.36	0	6.02	0.80	0.14	2.11	0.38	0.09	0	0.07	0	0.15	0.26	48.95

SITE-YR2	sems1	sema1	semd1	semcr	semfe	emm	emm	semk	semca	sema	semcl	sems	semin1	semp	semcu	semtot
A	56.88	17.37	1.27	0.03	10.95	3.56	0.05	3.63	3.42	2.49	0	0.03	0	0.28	0.04	46.72
B	59.59	17.56	1.02	0	10.17	2.21	0.07	3.34	3.43	1.79	0	0.06	0	0.58	0.16	50.40
C	56.97	17.89	1.20	0	10.73	3.39	0.07	3.33	3.57	2.48	0	0.05	0	0.20	0.12	48.85
D	57.40	20.43	1.22	0	11.10	2.44	0.00	2.65	2.77	0.99	0	0.34	0	0.55	0.12	37.07
E	55.46	19.49	1.43	0	11.09	3.44	0.11	3.45	2.98	2.02	0.02	0.06	0	0.27	0.19	51.17
F	56.64	19.21	1.02	0.02	10.22	2.95	0.20	3.90	4.04	1.16	0.02	0.09	0	0.31	0.22	49.12
BC	56.73	23.86	1.33	0	8.24	1.46	0.00	1.17	3.12	2.45	0	0.38	0	0.98	0.29	38.41
SK	70.32	14.57	0.75	0	7.13	1.81	0.15	2.77	1.78	0.52	0	0.12	0	0.03	0.05	39.77
CL	62.62	16.43	0.91	0	7.81	2.54	0.08	2.90	3.70	2.33	0	0.13	0	0.51	0.05	39.35
AG	60.59	17.19	1.08	0.02	9.23	2.23	0.08	2.10	3.26	2.24	0	0.30	0	1.55	0.14	42.22
MD	75.14	13.71	1.38	0	5.82	0.75	0.11	2.06	0.40	0.12	0	0.02	0	0.20	0.28	47.12
NR	62.85	16.23	1.03	0	9.37	2.58	0.11	3.40	2.29	1.56	0	0.07	0.04	0.37	0.09	46.63

Table 7. Soil chemical parameter measurements on microcosm soils obtained by atomic absorption elemental analyses. Site and soil parameter abbreviations and units are shown in Tables 2 and 3 respectively. Values are the mean of three replicate soil cores.

SITE-YR 2	aacd	aaZn	aacu	aamn	aanl	aspb	asfe	ask	asca	asmg	asna
A	0	92.55	25.24	418.1	29.08	19.04	24513	5357	12891	11674	409.5
B	0	43.53	11.72	443.9	11.16	12.25	15478	1699	8605	3487	164.0
C	0	90.76	26.06	460.3	32.03	12.25	28497	4237	6679	9621	389.8
D	0	29.53	17.43	119.5	6.01	7.13	11596	773	1463	2091	118.0
E	0	98.86	27.14	470.8	36.62	13.29	32872	6798	4010	11771	443.1
F	0.037	45.40	19.90	754.1	15.64	14.97	19342	2509	14541	7306	352.1
BC	0	29.01	8.70	140.0	6.76	5.00	12843	299	1788	2139	154.6
SK	0.448	86.80	16.13	647.4	16.05	7.88	18103	2460	4163	4663	102.9
CL	0	43.68	11.27	353.5	10.08	9.74	11323	2374	11767	5469	183.3
AG	0	44.31	52.98	162.8	8.06	137.27	9617	627	1553	2214	150.9
MD	0	34.16	6.46	327.4	8.47	15.23	9718	586	575	1337	59.6
NR	0.075	62.16	11.57	395.0	20.46	9.93	19081	1497	2554	6615	160.8

Figure 12. Range of soil textures represented by the 23 study sites as plotted on a soil texture diagram - source: Research Branch, Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, http://sis.agr.gc.ca/cansis/glossary/texture._soil.html.

Linear regression survival slopes

The results of linear regression analyses for survival in soil and rhizosphere microcosms and field plots from each site are provided in Tables 8 and 9. Figures 13 and 14 show the range between highest and lowest survival slopes for soil and rhizosphere microcosms over 23 sites. All regressions were significant at the $p < 0.05$ level although in some instances, the regression models accounted for relatively low amounts of variation in the data (e.g. Year 1 Saskatchewan soil R-square = 0.32). The analysis of regression residuals indicated linear regressions generally provided good models for RN-L11 survival. An attempt to account for the lower confidence in some estimates of survival slopes was made by using lower weights ($1/\text{slope standard error}$) for these slopes in subsequent weighted LMR model calculations.

Population levels of RN-L11 declined over all observation periods after every inoculation. This was observed in soils and rhizospheres from each site. The range of microcosm survival slopes was greater for soils (-0.0102 to -0.1561 CFU/g. of soil/day) than for rhizospheres (-0.0120 to -0.0608 CFU/g. of root/day). RN-L11 generally persisted longer in the rhizosphere than in bulk soil at each site. The mean survival slope in soil microcosms was -0.0657 CFU/g. of soil/day ($n=23$) compared to a slower die-off rate in rhizosphere microcosms of -0.0314 CFU/g. of root/day. RN-L11 also generally had a faster die-off in field plots than in microcosms. The mean field plot survival slope was -0.0807 CFU/g. of soil/day in soil ($n=9$) and -0.0383 CFU/g. of root/day for rhizospheres.

Table 8. Results from linear regression analyses of *Pseudomonas chlororaphis* 3732RN-L11 survival in bulk soil in microcosms and field plots. Slope values are log CFU/g. of soil/day. Site abbreviations are in Table 2.

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SITE	SLOPE	STD ERROR	R-SQUARE	F-VALUE	PROB>F
YEAR 1 SOIL MICROCOSMS					
A	-0.0102	0.00198	0.7257	26.45	0.0004
C	-0.0345	0.00691	0.7134	24.893	0.0005
CL	-0.0352	0.00387	0.79	82.772	0.0001
SK	-0.0372	0.01156	0.3201	10.357	0.004
BC	-0.0409	0.00158	0.9574	673.528	0.0001
B	-0.0429	0.00343	0.9398	156.143	0.0001
AG	-0.049	0.0028	0.9214	304.927	0.0001
E	-0.0679	0.00862	0.8613	62.102	0.0001
D	-0.0873	0.01242	0.8319	49.472	0.0001
F	-0.0972	0.01445	0.8189	45.206	0.0001
MD	-0.0991	0.01073	0.795	85.305	0.0001
YEAR 1 SOIL FIELD PLOTS					
SK	-0.0481	0.01239	0.406	15.04	0.0008
CL	-0.0624	0.00345	0.9371	327.606	0.0001
AG	-0.0725	0.00655	0.8035	122.666	0.0001
BC	-0.0798	0.00566	0.869	199.067	0.0001
YEAR 2 SOIL MICROCOSMS					
CL	-0.0424	0.00465	0.8221	83.204	0.0001
B	-0.0504	0.00525	0.9023	92.324	0.0001
F	-0.0522	0.00621	0.91	70.774	0.0001
C	-0.0551	0.00698	0.874	62.437	0.0001
NR	-0.0602	0.01024	0.5704	34.527	0.0001
AG	-0.0642	0.00589	0.844	119.066	0.0001
A	-0.0762	0.01326	0.7855	32.965	0.0003
BC	-0.0828	0.00568	0.8909	212.336	0.0001
SK	-0.0886	0.01203	0.7948	54.242	0.0001
D	-0.089	0.01238	0.8378	51.653	0.0001
E	-0.0931	0.00896	0.9391	107.995	0.0001
MD	-0.1561	0.01303	0.8885	143.47	0.0001
YEAR 2 SOIL FIELD PLOTS					
AG	-0.0774	0.01478	0.5551	27.452	0.0001
BC	-0.0816	0.00739	0.8245	122.117	0.0001
CL	-0.0854	0.00768	0.873	123.698	0.0001
SK	-0.0907	0.01022	0.849	78.727	0.0001
NR	-0.128	0.01475	0.8069	75.212	0.0001

Table 9. Results from linear regression analyses of *Pseudomonas chlororaphis* 3732RN-L11 survival in wheat rhizospheres in microcosms and field plots. Slope values are log CFU/g. of root/day. Site abbreviations are in Table 2.

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SITE	SLOPE	STD ERROR	R-SQUARE	F-VALUE	PROB>F
YEAR 1 RHIZOSPHERE MICROCOSMS					
A	-0.012	0.00405	0.468	8.796	0.0141
CL	-0.02	0.00294	0.6392	46.06	0.0001
SK	-0.0205	0.00333	0.6108	37.657	0.0001
BC	-0.0247	0.0035	0.6569	49.783	0.0001
AG	-0.0303	0.00583	0.509	26.955	0.0001
E	-0.0387	0.00452	0.8801	73.422	0.0001
MD	-0.042	0.00447	0.7728	88.415	0.0001
B	-0.0447	0.00237	0.9727	356.047	0.0001
C	-0.0453	0.00307	0.9561	217.558	0.0001
F	-0.0525	0.00704	0.8474	55.539	0.0001
D	-0.0608	0.00386	0.9613	248.341	0.0001
YEAR 1 RHIZOSPHERE FIELD PLOTS					
SK	-0.0167	0.00432	0.4036	14.89	0.0009
CL	-0.0258	0.00371	0.6871	48.307	0.0001
AG	-0.0381	0.00756	0.5363	25.448	0.0001
BC	-0.0389	0.00596	0.6593	42.579	0.0001
YEAR 2 RHIZOSPHERE MICROCOSMS					
E	-0.0179	0.00284	0.7986	39.645	0.0001
CL	-0.0209	0.00365	0.5997	32.957	0.0001
SK	-0.0223	0.00509	0.5164	19.222	0.0004
BC	-0.0237	0.00653	0.3365	13.189	0.0012
C	-0.0243	0.00353	0.8403	47.344	0.0001
D	-0.0253	0.00466	0.7468	29.493	0.0003
A	-0.0266	0.00304	0.8844	76.541	0.0001
B	-0.0308	0.00469	0.8117	43.108	0.0001
AG	-0.032	0.00618	0.5086	26.915	0.0001
F	-0.0348	0.00366	0.9001	90.079	0.0001
NR	-0.0352	0.0045	0.7018	61.196	0.0001
MD	-0.0358	0.00382	0.7999	87.939	0.0001
YEAR 2 RHIZOSPHERE FIELD PLOTS					
CL	-0.0259	0.00459	0.6389	31.845	0.0001
SK	-0.0265	0.0085	0.3633	9.699	0.0063
BC	-0.0527	0.00747	0.6932	49.714	0.0001
AG	-0.0577	0.00925	0.6834	38.853	0.0001
NR	-0.0621	0.00838	0.7138	54.864	0.0001

Figure 13. The range of *Pseudomonas chlororaphis* 3732RN-L11 soil survival slopes showing the largest and smallest slopes measured in soil microcosms from 23 sites over two years.

Figure 14. The range of *Pseudomonas chlororaphis* 3732RN-L11 rhizosphere survival slopes showing the largest and smallest slopes measured in rhizosphere microcosms from 23 sites over two years.

Linear multiple regression (LMR) models

No single soil parameter proved very useful for predicting RN-L11 survival suggesting the need for a multivariate approach. The only statistically significant ($p < 0.05$; $n = 23$) Spearman correlation coefficients were between soil microcosm survival slope and total pseudomonads (pseudo $r = 0.45$), and % soil sodium (semna $r = -0.42$) (Table 10). There were no significant correlations ($p < 0.5$; $n = 23$) between rhizosphere microcosm survival slopes and soil parameters. Table 10 also shows the considerable variation in soil parameter correlations between years. For example, % soil calcium (semca) was highly correlated with soil survival slope in Year 2 but not Year 1. Many soil parameters were highly correlated with survival slope in one year but not in the other.

Exploratory principal component analyses of the Year 1&2 soil parameter data found that the first five principal components accounted for 74% of the variation in the soil data (PC 1 = 29%; PC 2 = 16%; PC 3 = 13%; PC 4 = 9%; and PC 5 = 7%). However, none of these principal components were significantly correlated with soil survival slope and an LMR composed of the first four components was not a statistically significant predictor of soil survival ($p = 0.051$; $R\text{-square} = 0.39$). Similarly for the rhizosphere, an LMR comprised of the first four components was not a statistically significant predictor of rhizosphere survival ($p = 0.98$; $R\text{-square} = 0.02$). The principal components were found to be a relatively poor means to predict RN-L11 survival slopes. Interpretation of 'composite' principal components can also be difficult, particularly for those principal components accounting for a very small amount of variation in the original data set. In addition, many soil parameters would have to be measured to use these principal component LMRs as predictive tools, making them less practical to apply to new sites.

Table 10. Spearman correlation coefficients between soil parameters and *Pseudomonas chlororaphis* 3732RN-L11 survival slopes in soil and rhizosphere microcosms over two years of lab experiments. The ten highest parameter correlations and % clay are shown. Statistical significance ($p < 0.05$) is marked by *. Soil parameter abbreviations are shown in Table 3.

SOIL SURVIVAL SLOPE CORRELATIONS

YEAR 1 (n=11)		YEAR 2 (n=11)		YEAR 1&2 (n=23)	
parameter	Coeff.	parameter	Coeff.	parameter	Coeff.
semna	-0.55	semca	-0.91 *	pseudo	0.45 *
po	-0.51	semti	0.68 *	semna	-0.42 *
hydr	-0.49	ca	-0.67 *	semca	-0.41
pseudo	0.43	pH	-0.66 *	pH	-0.36
mg	-0.43	cec	-0.55	no	-0.34
semmg	-0.4	fung	-0.5	semti	0.32
clay	-0.4	semna	-0.44	hydr	-0.31
semsi	-0.35	nitr	-0.4	po	-0.3
pH	-0.31	semk	-0.39	cec	-0.28
semca	-0.3	no	-0.36	org	-0.27
org	-0.3	clay	0.06	clay	-0.12

RHIZOSPHERE SURVIVAL SLOPE CORRELATIONS

YEAR 1 (n=11)		YEAR 2 (n=11)		YEAR 1&2 (n=23)	
parameter	Coeff.	parameter	Coeff.	parameter	Coeff.
no	0.37	org	-0.74 *	bact	0.4
fung	0.36	no	-0.6	mg	-0.32
po	-0.3	carb	-0.57	semna	-0.26
hydr	-0.29	hydr	-0.56	po	-0.23
mg	-0.28	nitr	-0.54	pH	-0.22
nitr	0.27	pseudo	0.43	hydr	-0.21
pH	-0.25	mg	-0.35	sems	-0.2
semal	0.25	bact	0.33	pseudo	-0.2
bact	0.23	semcu	-0.33	org	-0.18
semfe	0.21	ko	-0.32	semti	0.16
clay	-0.12	clay	-0.19	clay	-0.11

The results of multiple regression analyses to determine the number of soil parameters to include in LMRs are shown in Figures 15 and 16. The resulting soil and rhizosphere Year 1&2 LMRs are shown in Table 11. Also shown in Table 11 are the results of other exploratory LMR analyses that calculated LMRs based only on Year 1 or Year 2 data. No power, exponential or polynomial regressions for individual soil parameters against survival slopes could account for as much variation in the data as the Year 1&2 LMRs.

The Year 1&2 LMR models for soil and rhizosphere were statistically significant ($p < 0.05$). The partial regression coefficients for all soil parameters were significant ($p < 0.05$) except for $\log_{10} \text{no} (\text{NO}_3^-)$ in the Year 1&2 rhizosphere LMR. This parameter was maintained in the model because it significantly improved the model adjusted R-square, its importance was observed in subsequent jackknife analyses of LMR stability, and its partial regression coefficient or p-value was 0.08. Consideration was given to including a fourth parameter semna^2 (% soil sodium) in the Year 1&2 LMR for soil. However, it was not included since it was judged not to significantly improve the model adjusted R-square, its importance was not observed in subsequent jackknife analyses of LMR stability, and its partial regression coefficient was not significant ($p = 0.12$).

Analysis of Year 1&2 LMR residuals and studentized residuals did not indicate a need to consider curvilinear relationships or the need for any further data transformations for the LMRs beyond those original transformations to improve normal distributions.

Scatterplots of each soil parameter in LMRs against survival slopes did not indicate non-linear relationships. Variance inflation factors were close to one suggesting little concern about multicollinearity in either Year 1&2 soil or rhizosphere LMRs. There were no observations with undue influence on LMR models as judged by Cook's D values.

Figure 15. R-square improvements for Year 1&2 Soil LMRs ($n = 23$) based upon the number of soil parameters included in the LMR model.

Figure 16. R-square improvements for Year 1&2 Rhizosphere LMRs ($n = 23$) based upon the number of soil parameters included in the LMR model.

Table 11. Linear Multiple Regression models calculated to predict survival slopes for *Pseudomonas chlororaphis* 3732RN-L11 in soil and wheat rhizospheres. Soil parameter abbreviations are in Table 3. AR-squ = adjusted R-square. bio59 = Biolog sebacic acid oxidation; bio5 = tween 40 oxidation; bio72 = glycyl-L-glutamic acid oxidation; bio18 = D-glucose oxidation.

LMR	LMR model	Fvalue	Prob>F	R-squ	AR-squ
Soil LMR models					
Year 1&2 (n=23)	$Y = 0.02108(\text{semmg}) - 0.03634(\text{funga}) - 0.07347(\text{semti}) + 0.17801$	11.54	0.0002	0.65	0.59
Year 1 (n=11)	$Y = 0.01779(\text{semna}^2) - 0.03443(\text{semmg}) + 0.00008(\text{mg}) + 0.00181(\sqrt{\text{po}}) - 0.10862$	7.71	0.0151	0.84	0.73
Year 2 (n=11)	$Y = 0.01697(\text{sin[ca]}) - 0.09092(\text{semti}) - 0.02854(\text{sin[semca]}) + 0.01008(\text{semfe}) - 0.07022$	30.72	0.0004	0.95	0.92
Year 2a (n=12)	$Y = 0.11814(\text{sin[semna]}) - 0.01015(1/\text{bio59}) + 0.08458(\text{bio72}) - 0.1422$	12.86	0.002	0.83	0.76
Rhizosphere LMR models					
Year 1&2 (n=23)	$Y = 0.00136(\sqrt{\text{mg}}) - 0.02658(\text{bact}) - 0.01302(\log_{10}\text{no}) - 0.00149(\text{semk}^2) + 0.15976$	7.44	0.001	0.62	0.54
Year 1 (n=11)	$Y = 0.07294(\text{semti}) + 0.00007(\text{mg}) + 0.08520(\log_{10}\text{sems}) + 0.00006(\text{semtot}^2) - 0.21088$	9.05	0.0103	0.86	0.76
Year 2 (n=11)	$Y = 0.00689(\log_{10}\text{clay}) - 0.72357(\log_{10}\text{bact}) + 0.01059(\log_{10}\text{no}) + 0.02270(\log_{10}\text{ko}) + 0.51392$	21.74	0.001	0.94	0.89
Year 2a (n=12)	$Y = 0.01078(\text{bio18}) - 0.03179(1/\text{org}) - 0.03872(\text{bio5}) + 0.00057$	42.76	0.0001	0.94	0.92

The Year 1&2 soil LMR indicated that higher fung (total soil fungi - CFU/g of soil) and semti (soil % titanium as measured by scanning electron microscopy) were associated with faster GEM die-off in soil (Table 11). Higher values of semmg (soil % magnesium as measured by scanning electron microscopy) were associated with slower RN-L11 die-off in soil. There were also significant correlations (pearson coefficient <0.05) between these parameters and other soil parameters not included in the Year 1&2 soil LMR. For example, fung (total soil fungi) was significantly correlated with total soil pseudomonads ($r = 0.75$). Semmg (soil % magnesium) was significantly correlated with % clay ($r = 0.60$).

The Year 1&2 rhizosphere LMR suggested that higher values of bact (total soil bacteria - CFU/g. of soil), no (nitrate NO_3 (kg/ha)), and semk (soil % potassium as measured by scanning electron microscopy) were associated with faster RN-L11 die-off in rhizospheres. Higher values of mg (exchangeable magnesium (kg/ha)) were associated with slower RN-L11 die-off in rhizospheres. Similarly, there were also significant correlations (pearson coefficient <0.05) between these parameters and other soil parameters not included in the Year 1&2 rhizosphere LMR. Total bacteria was significantly correlated with K_2O ($r = 0.57$), % sand ($r = -0.46$). Soil % potassium was significantly correlated with soil % magnesium ($r = 0.85$), cation exchange capacity ($r = 0.68$), and % clay ($r = 0.67$). Magnesium was significantly correlated with pH ($r = 0.66$), % clay ($r = 0.72$), % soil magnesium ($r = 0.49$). Despite their anticipated importance, the soil parameters % clay and pH were not selected for the Year 1&2 LMRs even though attempts were taken to retain them through preliminary steps to reduce soil parameter numbers.

While some soil parameters from the Year 1&2 LMRs were also included in Year 1 or Year 2 LMRs, there were no parameters found in common between all soil LMRs or all rhizosphere LMRs. There was no stability of soil parameter selection between the exploratory LMR models calculated for Year 1 and Year 2. In this cross-validation, none of the soil parameters included in the Year 1 soil LMR or the Year 1 rhizosphere LMR were also included in their corresponding Year 2 LMRs.

The addition of another site and more soil parameter data for calculating the Year 2a LMRs also resulted in instability in soil predictors compared to the original Year 2 LMRs. The biolog parameters included in the Year 2a soil LMR suggested higher soil oxidation capability for sebacic acid (bio59) was associated with faster RN-L11 die-off, while higher soil oxidation capability for glycyl-L-glutamic acid (bio72) was associated with slower RN-L11 die-off in soils. The biolog parameters selected in the Year 2a rhizosphere LMR suggested higher soil oxidation capability for tween 40 (bio5) was associated with faster RN-L11 die-off while higher soil oxidation capability for α -D-glucose (bio18) was associated with slower RN-L11 die-off in rhizospheres. However, caution is needed in interpreting the Year 2a LMRs since about 140 soil parameters were considered in the development of these LMRs (e.g. including >96 biolog parameters), and spurious correlations with survival slope would be expected for several soil parameters.

Verification of LMR models

The results of the jackknife LMR analysis to examine parameter stability of the Year 1&2 LMRs are provided in Table 12. These data confirmed the importance of the original soil parameters as they were consistently selected as useful in predicting survival slope. The most frequently selected soil parameter for predicting RN-L11 survival in soil was semti (soil % titanium as measured by scanning electron microscopy). It was selected in 19 of 23 LMRs. The most frequently selected soil parameter for predicting RN-L11 survival in rhizospheres was mg (exchangeable magnesium (kg/ha)). It was selected in 22 of 23 LMRs. While multicollinearity can cause the signs (e.g. '-' or '+') of regression coefficients to be incorrect, the results from this jackknife LMR indicated there were no incorrect regression coefficient signs to suggest multicollinearity influences.

A test to verify the accuracy of Year 1&2 LMR model predictions for microcosm survival slopes suggested the LMRs provided reasonably accurate survival predictions for soil microcosms (Figure 17) and rhizosphere microcosms (Figure 18). In qualitative terms, the Year 1&2 LMRs were also able to provide reasonably correct predictions of whether a microcosm survival slope should be classified as a high or low survival slope (Table 13). For example, the Year 1&2 soil LMR incorrectly classified seven of twenty three soil microcosm survival slopes (30% error rate), and the Year 1&2 rhizosphere incorrectly classified five of twenty three rhizosphere microcosm survival slopes (22% error rate). Both Year 1&2 LMRs were more accurate at classifying the twenty-three survival slopes than the LMRs developed only from either Year 1 or Year 2 data.

Table 12. Parameter stability of Year 1&2 linear multiple regression models (LMRs) as measured by frequency of soil parameter selection in 23 LMRs calculated using a jackknife LMR procedure. Soil parameter abbreviations are in Table 3).

Soil parameter	Year 1&2 Soil LMR	Year 1&2 Rhizosphere LMR
$\sqrt{\text{mg}}$	1	22
semk ²	1	19
semti	19	0
bact	0	18
semmg	17	0
log ₁₀ no	0	16
fung	16	0
semna ²	7	0
semcu	7	0
log ₁₀ po	3	5
pseudo	5	1
log ₁₀ semp	3	2
$\sqrt{\text{clay}}$	2	1
log ₁₀ sems	2	1
ph	2	0
semfe	2	0
$\sqrt{\text{silt}}$	0	2
log ₁₀ ko	0	2
1/hydr	1	0
sin(ca)	1	1
1/nitr	1	0
semmn	1	0
$\sqrt{\text{cec}}$	1	0
log ₁₀ carb	0	1
1/org	0	1

However, a limitation of the preceding verification tests is their bias from testing the LMRs with the same survival slopes that were used to develop the LMRs. The results of less biased jackknife LMR analyses to test Year 1&2 LMR survival predictions are presented in Figure 19 for soil and Figure 20 for rhizospheres. These “leave-one-out” verification tests showed the Year 1&2 LMRs were capable of providing reasonably accurate predictions for new survival slopes not used to develop the LMRs in the first place.

The cross-validation studies for Year 1 and Year 2 LMRs indicated that the LMRs developed from data in one year could not be used to accurately predict survival slopes in the other year. Table 13 shows that while Year 1 and Year 2 LMRs could correctly classify the microcosm survival slopes from their perspective years reasonably well, they usually had error rates of more than 50% for classifying microcosm survival slopes from the other year.

Figure 17. Comparison of Year 1&2 LMR predictions for *Pseudomonas chlororaphis* 3732RN-L11 soil survival slopes with observed survival slopes in soil microcosms (n = 23 microcosm sites). Slope values were negative and were converted into positive values to ease comparison.

Figure 18. Comparison of Year 1&2 LMR predictions for *Pseudomonas chlororaphis* 3732RN-L11 rhizosphere survival slopes with observed survival slopes in rhizosphere microcosms (n = 23 microcosm sites). Slope values were negative and were converted into positive values to ease comparison.

Table 13.

Error rates (%) for linear multiple regression models (LMRs) in classifying survival slopes for *Pseudomonas chlororaphis* 3732RN-L11 into high or low slope classes. High or low slope classes were distinguished by median slope values for 23 microcosms (soil = -0.0602; rhizosphere = -0.0303) or 9 field plots (soil = -0.0798; rhizosphere = -0.0381).

Observed survival slopes	SOIL LMRs				RHIZOSPHERE LMRs			
	Year 1&2	Year 1	Year 2	Year 1&2	Year 1	Year 2	Year 1	Year 2
Year 1&2 field plots (n=9)	56	44	22	33	44	56	44	56
Year 1&2 microcosms (n=23)	30	35	35	22	43	30	43	30
Year 1 microcosms (n=11)	27	18	55	9	27	45	27	45
Year 2 microcosms (n=11)	27	55	18	36	64	9	64	9

Figure 19. Comparison of jackknife Year 1&2 LMR predictions for *Pseudomonas chlororaphis* 3732RN-L11 soil survival slopes with observed survival slopes in soil microcosms (n = 23 microcosm sites). Slope values were negative and were converted into positive values to ease comparison.

Figure 20. Comparison of jackknife Year 1&2 LMR predictions for *Pseudomonas chlororaphis* 3732RN-L11 rhizosphere survival slopes with observed survival slopes in rhizosphere microcosms (n = 23 microcosm sites). Slope values were negative and were converted into positive values to ease comparison.

LMR model validation

Validation of the Year 1&2 LMR models involved comparing their predicted survival slopes with observed survival slopes in field plots in the environment (Figures 21 and 22). The Year 1&2 LMRs were not very accurate for predicting survival slopes in either soil or rhizosphere field plots. Year 1&2 soil LMR predictions were calibrated to observed soil field plot survival slopes by the regression equation $Y = -0.0623$ (predicted slope) $+ 0.0842$; $R^2 = 0.003$. Year 1&2 rhizosphere LMR predictions were calibrated to observed rhizosphere field plot survival slopes by the regression equation $Y = 0.8937$ (predicted slope) $+ 0.0115$; $R^2 = 0.2758$. However, sample sizes for validation were small ($n=9$) and one of the soil field plot sites had an unexpectedly rapid die-off (possibly due to rainfall) that was influential for the poor soil regression equation above.

Year 1&2 LMRs were also not very accurate from a qualitative perspective for predicting whether soil or rhizosphere field plot survival slopes should be classified as either high or low (Table 13). The Year 1&2 rhizosphere LMR incorrectly classified three of nine field plot survival slopes (error rate = 33%), while the Year 1&2 soil LMR incorrectly classified five of nine field plot survival slopes (error rate = 56%). While the classification error rate of the Year 1&2 rhizosphere LMR was marginally lower than the Year 1 and Year 2 rhizosphere LMRs, the classification error rate of the Year 1&2 soil LMR was higher than both the Year 1 and Year 2 soil LMRs.

Figure 21. Comparison of Year 1&2 LMR predictions for *Pseudomonas chlororaphis* 3732RN-L11 soil survival slopes with observed survival slopes in soil field plots (n = 9 field trial sites). Slope values were negative and were converted into positive values to ease comparison.

Figure 22. Comparison of Year 1&2 LMR predictions for *Pseudomonas chlororaphis* 3732RN-L11 rhizosphere survival slopes with observed survival slopes in rhizosphere field plots (n = 9 field trial sites). Slope values were negative and were converted into positive values to ease comparison.

Figures 23 and 24 compare the accuracy of LMR and microcosm approaches for predicting survival slopes in soil and rhizosphere field plots respectively. In many cases both the Year 1&2 LMR and microcosm derived slope predictions under-estimated the survival slope observed in field plots. The microcosm approach appeared to be the more conservative approach since it only over-estimated RN-L11 die-off in the field for one of nine soil sites and one of nine rhizosphere sites. In addition, these over-estimations were small. The Year 1&2 LMR over-estimated RN-L11 die-off in the field for three of nine soil sites and four of nine rhizosphere sites. These over-estimations were larger than those obtained from microcosms. Year 1&2 LMRs predicted higher survival slopes than microcosms did for five of nine soil sites and seven of nine rhizosphere sites.

Year 1&2 LMR predicted survival slopes were closer than microcosm predicted slopes to observed field plot survival slopes at six of nine soil sites and five of nine rhizosphere sites. However, both Year 1&2 LMRs and microcosms had relatively poor ability to correctly classify field plot survival slopes into high and low classes. The soil microcosms were determined to have a classification error rate of 44 % and predicted survival slopes could be related to observed soil field plot survival slopes by the regression equation: $Y = 0.489 (\text{predicted slope}) + 0.0535$; $R^2 = 0.194$. The rhizosphere microcosms were determined to have a classification error rate of 56 % and predicted survival slopes could be related to observed rhizosphere field plot survival slopes by the regression equation $Y = 2.4055 (\text{predicted slope}) - 0.0231$; $R^2 = 0.6998$.

Figure 23. Comparison of Year 1&2 LMR and microcosm predictions for *Pseudomonas chlororaphis* 3732RN-L11 soil survival slopes with observed survival slopes in soil field plots (n = 9 field trial sites). Slope values were negative and were converted into positive values to ease comparison. Site abbreviations are in Table 2.

Figure 24. Comparison of Year 1&2 LMR and microcosm predictions for *Pseudomonas chlororaphis* 3732RN-L11 rhizosphere survival slopes with observed survival slopes in rhizosphere field plots (n = 9 field trial sites). Slope values were negative and were converted into positive values to ease comparison. Site abbreviations are in Table 2.

Discussion

The current study was unique in being able to provide a multi-site field test for linear multiple regression (LMR) models developed to predict survival of a GEM in soils and rhizospheres. While there have been studies to validate soil microcosm results for predicting GEM survival in field plots (Bolton et al. 1991; Angle et al. 1995; Gagliardi et al. 2001b), the author is unaware of studies testing statistical models to predict GEM survival across as wide a range of soils and climatic conditions as used in this thesis. Although Bashan et al. (1995) examined soil parameter correlation with survival of *Azospirillum brasilense* in bulk soil and rhizospheres of 23 soil types, there was no specification of multivariate predictive models nor was there a field validation aspect to test the importance of the soil parameters for predicting survival in their study .

Soil microcosm protocol evaluation

The soil microcosm protocol as outlined by Angle (1998) was found to be a relatively easy and inexpensive approach to simulate a variety of agricultural soils and wheat rhizosphere habitats. However, it was noted that the protocol may be somewhat limited in its range of applicability and that there are aspects that should be revised or used with caution to more reliably predict survival of genetically engineered microorganisms (GEMs).

A limitation of the protocol could be its applicability for handling a wide range of soils and rhizosphere conditions. For example, plants species with extensive and rapidly

growing root systems might be less amenable to study in this relatively small soil core. In addition, there could be limitations for studying GEM survival in soils with very high clay content, or soils with a lot of material that would not pass through the 4mm sieve. While soil samples composed of 37 % clay could be processed through the 4mm sieve in a reasonably short time, soils with very high clay content would be more difficult and time consuming to process. In addition, soils with a lot of stones or organic matter that would not pass through the 4mm sieve could result in loss of associated GEM cells from subsequent protocol steps. These limitations are more likely to be relevant for studying GEM survival in soils outside of agricultural ecosystems.

The protocol approach to standardization of soil water should be revised to enable more reliable means to predict GEM survival across soil types. The current approach to standardization is based on maintaining soil water at 60 % of maximum retentive capacity (saturation). However, different soils maintained at the same % saturation can vary in their amount of biologically available water depending upon the texture of the soil (Topp 1993; Brady and Weil 1999). For this reason, the current microcosm protocol may not effectively control available soil water across a wide variety of soil types and this could influence GEM survival.

The implications of standardizing soil water based on % saturation may be most apparent when using the protocol to compare GEM survival across very different soil types. Brady and Weil (1999, 205) outline the general relationship between soil water content and soil texture. As texture becomes finer from sands to silt loams, there is a general increase in soil field capacity, soil wilting coefficient and potentially available water. As a result, at the same soil water content level, a sand soil may be well above field capacity with

excess water for plants and sub-optimal soil aeration, while a clay soil may be well below field capacity (and wilting coefficient) such that water is not available for plant growth. Standardization based on % saturation can thus mask biologically important differences in soil water and plant growth conditions that could influence GEM survival.

A more suitable approach may be to standardize soil water content based upon field capacity for a given soil. At field capacity, air will have replaced water in larger soil pores and gravitational draining of water will have become negligible. Soils at field capacity will hold the most amount of water useful to plants and there will be sufficient pore space filled with air to allow optimal aeration for most aerobic microbial activity and for growth of most plants (Brady and Weil 1999). While the soil water content would vary between soils of different texture, there would be a more constant amount of biologically relevant water and aeration across soil types.

Calculations in Appendix 1 show the importance of this approach to standardizing soil water across soil types in the current study. Soil water content varied between sites despite the Protocol's attempt to standardize at 60% retentive capacity. In the case of *Pseudomonas chlororaphis* 3732RN-L11 used in this thesis, Rivera (1996) found that variation in soil water content had no significant effect on the survival of this strain in soil and wheat rhizospheres. However, the approach to standardize soil water based upon field capacity would be more important when studying the survival of those microorganisms more sensitive to soil moisture and anaerobic conditions.

There are several other aspects of the Protocol that will require careful attention in order to derive reliable GEM survival predictions including:

1) initial GEM dilution - The current Protocol assumes that adding 10 grams of fresh soil containing the GEM to 95 ml of water will lead to an initial 10^{-1} dilution. However, this is only a rough approximation, and the initial dilution is actually based upon the number of GEMs relative to the dry weight of the 10 grams of fresh soil rather than relative to the water portion of the soil that is being diluted. While subsequent steps in the dilution series are each 10^{-1} dilutions by design, the initial dilution will actually be greater than 10^{-1} for most soils (in some cases possibly approaching 10^{-2}). As a result, colony forming units (CFUs) for the GEM obtained using this Protocol will often be an underestimate of the actual GEM CFUs/g of soil. For those applications of the Protocol seeking only the GEM survival slope in a given soil, the rough approximation of the initial dilution would be less important than applications seeking an accurate estimate of the exact number of GEM CFUs present at a certain time. For these latter applications, the amount of fresh soil to be added to the 95 ml of water could be adjusted such that a 10^{-1} dilution of the water content of the soil was obtained.

2) soils high in organic matter – In addition to the previously mentioned problem of the possible loss of GEMs associated with organic matter not passing through sieves, attention should also be paid to the presence of organic matter for soil water measurements. The current Protocol measures soil water content by weighing soils before and after drying in an oven at 72 C for 72 hours. It is assumed that weight loss is only due to removal of water. However, if very accurate soil water content measurements are needed, consideration should to be given to the presence of large amounts of organic matter in some soils. Volatilization and oxidation of organic materials may lead to additional weight losses from soil components other than water (Topp 1993). While this

weight loss would not likely be a major consideration for many mineral soils, and drying at 72 C rather than more typically at 105 C may reduce volatilization of organic materials, additional weight loss may still be an issue for soils very high in organic matter. Topp (1993) also drew attention to the need to check the accuracy of maintaining temperature control in ovens.

3) soils with stones - In addition to the previously mentioned problem of the possible loss of GEMs associated with stones not passing through sieves, attention should also be paid to the presence of stones for soil water measurements. Gardner et al. (1991) indicated that the water content of the stone fraction of a soil sample is often considered to be negligible except where stones are porous.

4) compacted soils – the intact soil core microcosm was designed to simulate the natural soil structure as closely as possible in the laboratory. However, the procedure to obtain cores requires the use of a rubber mallet to hammer the cores into the soil. In some instances, this hammering may result in some compacting of soil in the cores. This compacting could crush soil macropores and large micropores into smaller pores resulting in more limited plant root penetration and decreased water retention and pore space aeration at field capacity (Brady and Weil 1999). Caution should thus be taken when interpreting GEM survival results that may have been obtained from intact soil cores influenced by compaction.

Finally, the protocol approach to measuring water content was a gravimetric approach. This approach is a destructive technique requiring removal of a soil sample for obtaining measurements. For situations where close monitoring of soil water in microcosms may

be required over the course of experiments, another approach to measuring water content would be needed such as using Time Domain Reflectometry as outlined by Topp et al. (1980). O'Brien and Oberbauer (2001) describe a portable, inexpensive (\$350 US) reflectometer that could be adapted by inserting probes into soil microcosms to measure changes in a soil's dielectric constant and thus monitor volumetric water content.

GEM survival

Population levels of RN-L11 declined over time after introduction into all microcosms and field plots in this study. The survival rates for RN-L11 observed in this study were similar to results for RN-L11 observed in other survival studies by Angle et al. (1995) and Gagliardi et al. (2001b). The survival rates were also generally comparable to *Pseudomonas* survival rates reported in the scientific literature by van Elsas et al. (1991a). These authors reported *Pseudomonas* survival rates in soil ranged from about 0.02 to 0.1 log CFU/g. of soil /day.

The decline of RN-L11 in a variety of soils was consistent with many other studies that have shown that population levels of microorganisms introduced into non-sterile soils decline over time (Liang et al. 1982; Acea et al. 1988; Alexander 1986, 1988; van Elsas and Trevors 1991; van Veen et al. 1997). This decline has also been observed in many studies of genetically engineered microorganisms (Bolton et al. 1991; Smit et al. 1992; Angle et al. 1995; Vahjen et al. 1997; Gagliardi et al. 2001b). Van Veen et al. (1997) considered that growth of introduced microorganism populations in microbiologically undisturbed soils was a rare phenomenon. Introduced microorganisms generally do not compete and survive well with microorganisms already established in naturally occurring

microbial communities (Alexander 1988; van Veen et al. 1997; Hurst 1997).

The inability of microorganisms to increase in numbers when added to natural soil was reported as early as 1909 by Russell and Hutchinson (1909). This general phenomenon has been called soil bacteriostasis for bacteria (Brown 1973) and soil microbiostasis for microorganisms in general (Ko and Chow 1976). The phenomenon of soil microbiostasis is widespread in soils (Brown 1973; Ko and Chow 1977; Ho and Ko 1985; van Veen et al. 1997). van Veen et al. (1997) attributed the phenomenon to the scarcity of nutrient resources and the hostility of the soil environment to introduced microorganisms due to a variety of adverse abiotic and biotic factors. A mathematical model developed by van der Hoeven et al. (1996) predicted that even if a GEM was assumed to be 'fitter' than indigenous microorganisms, the advantage of being already present and occupying the most favourable sites in soil was often sufficient to counterbalance small differences in fitness.

Population levels of RN-L11 almost always declined more slowly in rhizospheres than bulk soil in microcosms and field plots in this study. The slower decline of RN-L11 in rhizospheres likely reflects the original source of isolation of the GEM's parent and its natural adaptation more to wheat rhizosphere rather than bulk soil. Rhizospheres are known to differ from bulk soil by exhibiting enhanced microbial numbers and activity and more nutrient-rich conditions from the presence of root exudates such as sugars, amino acids and proteins (Kluepfel 1993; Angle et al. 1996; Sorensen 1997; Lugtenberg and Dekkers 1999). Since RN-L11 is a rhizosphere colonizer (Hattemer-Frey et al. 1990; Kluepfel et al. 1994b; Angle et al. 1996; Gagliardi et al. 2001b) and naturally adapted to wheat rhizospheres, it would generally be expected to survive better in wheat

rhizospheres than in bulk soil.

LMR evaluation

From a very coarse qualitative perspective, the Year 1&2 LMR predictions were consistent with the principle of microbiostasis, and correctly predicted that RN-L11 populations trends would decline in all soil and rhizosphere field plots. The LMR predictions were also consistent with the known origin and biology of the GEM and correctly predicted that RN-L11 populations would generally decline faster in soil field plots than in rhizosphere field plots. In this qualitative sense, the Year 1&2 LMRs were reliable for predicting general RN-L11 population trends in field plots. However, beyond these very general qualitative predictions, LMRs could not provide accurate quantitative predictions of RN-L11 survival rates in environmental field plots.

There are many factors that can influence the survival rate of a GEM introduced into the environment. The particular survival slope or die-off rate exhibited by a GEM will be influenced by the characteristics of the microorganism strain as well as the environment into which it will be introduced. For example, microorganisms are known to adopt a variety of survival strategies such as the formation of dormant spores, cysts, viable but non-culturable states, vegetative cells, including ultramicrobacteria in which metabolic activity is reduced and cells have increased resistance to stress and adverse environmental conditions (Roszak and Colwell 1987). As reported by Gagliardi et al. (2001b), there was rarely a case where viable RN-L11 cells were not recovered in the present study, and there was no evidence of RN-L11 entering a viable but non-culturable state.

A wide variety of environmental factors are also known to influence the survival rate of introduced microorganisms. In soil environments, parameters like temperature, moisture, pH, nutrient concentrations, soil type, and presence of competitors, predators, and plants are among the more important determinants of survival (Alexander 1986; van Elsas and Trevors 1991; van Veen et al. 1997). Alexander (1986) indicated that bacteria populations introduced into soils may be markedly reduced by the grazing activities of indigenous protozoa. These protozoa may reduce bacteria population levels by 1, 2 or 3 logarithmic orders of magnitude if the initial bacterial density is high. Van der Hoeven et al. (1996) suggested that pore structure of soil is a key factor controlling the fate of bacteria since protozoan predation of bacteria would be dominant in pores with large neck sizes, while virtually absent in smaller pores. Prosser (1993) also indicated that predation by protozoa and soil animals were likely to be major factors influencing the survival and establishment of GEM microbial inocula. Recorbet et al. (1992) demonstrated the role of protozoa-free soil microflora competitors in reducing populations of introduced microorganisms. This diverse range of potentially influential soil characteristics makes it very difficult to provide accurate predictions of GEM survival in soils.

In this study, none of the soil parameters measured were sufficiently correlated with RN-L11 survival rate in soil or rhizospheres to be reliable predictors on their own. While Gagliardi et al. (2001b) reported significant correlations between RN-L11 survival rate and pH, carbon-to-nitrogen ratios and nickel levels, these correlations were not significant in the larger data set used in the present study. It is possible that none of the soil parameters were of critical importance to the survival of the RN-L11. It is also possible the range of values for the soil parameters encountered in the relatively typical agricultural soils used in this study did not present a significant enough environmental

challenge to be clearly influential in affecting RN-L11's survival. With the soil environment being so complex, perhaps no single parameter would likely have a predominant influence on survival within the range of agricultural soils used in this study. These results are consistent with views of Sayler and Ripp (2000) that the vast array of environmental factors influencing GEMs will make the derivation of a reliable modeling approach for GEM survival a daunting task.

Another study that tried to predict bacterial survival based upon soil factors was conducted by Bashan et al. (1995) across 23 different soils. Bashan et al. found percentages of clay, nitrogen, organic matter and water-holding capacity in soils were positively associated with survival of *Azospirillum brasilense*, while percentages of CaCO_3 , fine sand and rough sand were negatively associated with survival. However, they found only one of 15 soil parameters (CaCO_3) to be significantly correlated (negatively) with survival slope of *Azospirillum brasilense*.

While no single soil parameter proved very valuable for predicting RN-L11 survival rates, it was possible to develop linear multiple regression models that explained a significant amount of the soil and rhizosphere survival slope variation in microcosms. In addition, some of the soil parameters selected for the Year 1&2 LMRs appeared to have some biological relevance for understanding survival of RN-L11 in soil, although no evidence is provided of causal relationships.

The Year 1&2 soil LMR indicated that fung (total soil fungi - CFU/g. of soil), semmg (soil % magnesium) and semti (soil % soil titanium) were influential for predicting RN-L11 survival in soil. Fungi are often the dominant component of soil biomass and they

are known to produce bacteriolytic enzymes and antibacterial antibiotics, and to attack living bacteria (Thorn 1997). Since the total number of fungi was also significantly correlated with the total number of pseudomonads ($r = 0.75$), it could represent a measure of the population size of the naturally occurring microbial flora, including pseudomonads closely related to RN-L11, that would be competing with RN-L11 for space and nutrients in bulk soils. Indigenous microorganisms competing for available resources in soil have been considered a biotic factor that can adversely affect the survival of introduced microorganisms (Recorbet et al. 1992; van Veen et al. 1997). Acea et al. (1988) suggested populations of bacteria introduced into soil may have been reduced because of their susceptibility to starvation, protozoa predation, and possibly antibiotic-producing or lytic microorganisms.

It is uncertain how semti (soil % titanium) was related to a higher RN-L11 soil die-off rate. Higher values of titanium are known to be associated with more weathered soils (Foth 1990, 154 and 160) but this is of uncertain significance to RN-L11. On the other hand, there may be some biological significance to the relationship between higher semmg (soil % magnesium as measured by scanning electron microscopy) and enhanced RN-L11 survival in bulk soils. It is possible to speculate there may be a link between higher soil magnesium and the presence of certain clay minerals influential to the survival of an introduced GEM. The clay mineralogy of soils is known to have a significant effect on bacterial survival (Marshall 1975).

Finer soil particles like clays generally contain more magnesium than coarser soil particles like silt and sand (Troeh and Thompson 1993, 257). Magnesium is also more commonly associated with certain clay minerals like vermiculite (Bohn et al. 1985, 82;

Troeh and Thompson 1993, 258) and montmorillonite (Bohn et al. 1985, 76, 89 and 296; Foth 1990, 158). Montmorillonite and vermiculite clays are 2:1 lattice clays that are typically part of the smaller particle size fraction of clay minerals ($<0.0002\text{mm}$ diameter) and which have a relatively high cation exchange capacity (Foth 1990, 160). Mokuwunye and Melsted (1972) and Camberato and Pan (2000) suggested that soils with the highest 2:1 lattice clay content (e.g. montmorillonite) generally have the highest magnesium content. Preliminary X-ray diffraction analyses of a few of the same soils used in the present study indicated the presence of clay minerals like vermiculites and chlorites at SK and CL sites which showed enhanced RN-L11 survival (Gagliardi et al. 2001b). These clay minerals were not detected at BC and AG sites where RN-L11 survival was relatively poor (Gagliardi et al. 2001b).

The 2:1 clay minerals like montmorillonite have been associated with enhanced survival of bacteria species in soil (Marshall and Roberts 1963; Marshall 1964; Marshall 1975; Bushby and Marshall 1977; Osa-Afiana and Alexander 1982; and Stutz et al. 1989). Dessication resistance studies have also shown enhanced survival of *Klebsiella aerogenes* in soil (Bitton et al. 1976) and *Pseudomonas* species in mineral powder (Dandurand et al. 1994) associated with montmorillonite compared to other clay minerals. Dandurand et al. (1994) summarized several possible reasons for increased dessication resistance and bacterial survival in the presence of montmorillonite, including 1) the small particle size (and high soil surface area) associated with this clay mineral could slow soil desiccation resulting in enhanced bacterial survival; and 2) that these clays increase bacterial desiccation resistance by removing more water from bacterial cells leading to reduced metabolic activity and a resting stage capable of better desiccation tolerance.

The presence of montmorillonite clays in soil is known to be influential for many microbial activities. Montmorillonite clays have been shown to maintain pH stability, enhance nitrification, enhance bacterial growth and retard the growth of fungi more than other clay minerals (Stotzky 1986). Marshall (1975) indicated montmorillonite clays have been associated with enhanced bacterial survival through protection from fungi, X-rays, bacteriophage attack, and the maintenance of suitable pH and water in soils. In addition, the small particle size of montmorillonite clays may also provide for smaller pore spaces and protection of introduced microorganisms from predators like protozoa. A number of studies have shown the importance of smaller soil pore spaces for providing bacteria protection from predatory protozoa (Vargus and Hattori 1986; Postma and van Veen 1990; Postma et al. 1990; Heijnen and van Veen 1991; Heijnen et al. 1993; Wright et al. 1993; Wright et al. 1995; van der Hoeven et al. 1996; van Veen et al. 1997). The smaller pore spaces in montmorillonitic clay soils may also provide protection from competition and more uniform and stable microhabitats for microorganisms (Postma et al. 1990).

The Year 1&2 LMR model for rhizosphere indicated that bact (total soil bacteria - CFU/g. of soil), mg (exchangeable magnesium (kg/ha)), no (nitrate) and semk (soil % potassium) were influential for predicting rhizosphere survival slope. Total soil bacteria could represent a measure of the population size of the naturally occurring microbial flora that would be competing with RN-L11 to colonize the rhizosphere. Studies have shown that inhibiting the indigenous microflora using antibiotics has resulted in the establishment of inoculant microorganisms in rhizospheres at higher levels than those obtained in controls without antibiotics added (Bashan 1986). It is uncertain how higher values of nitrate and % soil potassium were associated with faster RN-L11 die-off. Both

are essential plant nutrients.

Mg (exchangeable magnesium) was significantly correlated with % clay ($r = 0.72$) and pH ($r = 0.66$). This measure of soil magnesium was more representative of the extractable, or biologically available magnesium in soils, as opposed to % soil magnesium measured by scanning electron microscopy. It is possible that higher exchangeable soil magnesium was related to enhanced RN-L11 survival associated with certain clay minerals in the rhizosphere or that it represented factors associated with a more fertile environment for plant growth and rhizosphere development. Interestingly, Evans et al. (1993) found survival of a *Rhizobium leguminosarum* strain in soil was most strongly correlated with exchangeable magnesium (e.g. more than other parameters like % clay). These authors could not elucidate a clear role for Mg in influencing survival, although they suggested that it might have been related to clay mineralogy and higher magnesium concentrations associated with those clay minerals favourable to protecting the bacteria from soil desiccation and high temperature.

Year 1&2 LMRs for soil and rhizospheres were both consistent in indicating that soil magnesium was positively associated with RN-L11 survival, while indigenous microflora abundance (either total fungi or total bacteria) was negatively associated with RN-L11 survival. It had been anticipated that % clay would have been an important soil characteristic for predicting RN-L11 survival rates. Many studies have found that increased clay content in soil (e.g. soil texture or % clay) was associated with enhanced bacteria survival (Marshall and Roberts 1963; Al-Rashidi et al. 1982; van Elsas et al. 1986; van Elsas et al. 1989; Heijnen and van Veen 1991; van Elsas et al. 1991b; Heijnen et al. 1993b; Trevors et al. 1994; Bashan et al. 1995; Howell et al. 1996). However, there

have also been studies finding that clay content did not significantly influence bacteria survival (Mahler and Wollum 1981; Mubiru et al. 2000). While Bashan et al. (1995) found % clay to be positively associated with survival of *Azospirillum brasilense* in 23 soil types, the relationship was not statistically significant.

There has been a general recognition of the importance of clay in influencing bacteria survival in soil (Alexander 1986; England et al. 1993b; Stotzky 1997; van Veen et al. 1997; van Overbeek and van Elsas 1997). Clays can provide a readily available source of exchangeable inorganic nutrients, they can scavenge soluble toxic chemicals, and their ability to retain water is critical for many microbial activities (Stotzky 1986). In the current study, however, % clay did not prove useful for predicting RN-L11 survival rates. It is possible that % clay was too coarse a measurement for predicting survival rates since it does not reflect the presence/absence of different types of clay minerals which may be of more importance to the survival of introduced microorganisms. For example, the quantity of montmorillonite clay minerals rather than the entire clay fraction (e.g. % clay) may be a more useful predictor of RN-L11 survival rate. However, time and costs associated with sample preparations for clay mineral analysis would need additional study prior to its consideration in routine regulatory or monitoring work.

Despite jackknife and other statistical analyses that provided a reasonable verification of Year 1&2 LMRs, and the apparent biological relevance of some of the LMR parameters, the Year 1&2 LMRs did not provide accurate predictions of RN-L11 survival slopes in field plots. There are a variety of sources of variation that could have contributed to this lack of quantitative predictive ability such as weather influences on GEM survival slopes in field plots but not in laboratory microcosms. Gagliardi et al. (2001b) suggested that

cooler mean monthly temperature and 30-40 mm rainfall events were correlated with enhanced and lower GEM persistence in fallow soil plots respectively. They noted sudden drops in GEM population levels at CL in 1996 and NR in 1997 after heavy rainfall events. In these instances, the microcosm may not have provided a sufficiently accurate simulation of environmental conditions in the field

Additional reasons for the lack of LMR predictive ability could have included that: 1) the soil parameters chosen in this study were not critical to the survival of RN-L11; 2) the different soil types used in this study may not have provided a sufficient environmental challenge to establish a significant range of survival slopes; 3) the variation associated with soil parameter and survival slope measurements may have masked small but significant relationships; and 4) while data scatterplots and LMR diagnostic analyses did not indicate any obvious nonlinear relationships between soil parameters and survival slopes, it is possible some relationships were not well represented by linear equations (while linear relationships are easier to understand and communicate, they may not accurately simulate complicated interactions between microorganisms and diverse characteristics of the soil environment).

Prosser (1993) indicated caution was required in interpreting empirical models for predicting survival because of factors like unaccounted variation in the shape of survival curves or the inaccuracy of the experimental data used to develop the models that can frequently prevent precise characterization of survival. Particular caution must be observed in developing LMRs based upon small sample sizes. For example, this study found there was no parameter stability between years for LMRs, and the LMRs developed with data from one year ($n=11$) could not accurately predict survival in the other year.

Small sample sizes are a common practical problem for many science-based regulatory decision-making situations and they may preclude rigorous statistical interpretation of LMRs for predicting survival.

Uncertainty challenges

This study demonstrated that considerable uncertainty was associated with accurately predicting the survival of RN-L11. It should also be recognized that these survival predictions were for soil, which is only one medium or compartment of the environment. While rhizosphere survival was also investigated, survival predictions were only directly applicable to wheat plants. Widespread commercial applications of a GEM like RN-L11 would also require consideration of survival in other environmental compartments ranging from aerosols to surface waters. In developing mathematical models for predicting the potential spread of a GEM in an agricultural setting, Lewis et al. (1996) considered that a GEM might end up in at least eight distinct compartments, each with different transport attributes (aerosols, dust, plant surfaces, surface soil, subsurface soil, surface water, groundwater and rhizosphere). Modeling the potential spread suggested that even an infrequent mobilization of a GEM from a sedentary compartment to another more mobile environment compartment, can substantially increase the invasive speed of a GEM which they would not have predicted without their model.

Gillespie et al. (1995) showed surface runoff of RN-L11 could be an important mechanism for movement to nontarget environments. It has also been shown that RN-L11 can invade aerial stem and foliage tissues of wheat plants (Kluepfel and Tonkyn 1991; Kluepfel et al. 1994a). Lamb et al. (1996) found that RN-L11 moved naturally

from the roots into aerial tissues of all 16 plants, from six families that were tested.

Snyder et al. (1999) subsequently demonstrated in laboratory and field experiments that the grasshopper, *Melanoplus femurrubrum* could readily acquire and maintain RN-L11 from corn plants and transmit it to new plants. These authors indicated that, while RN-L11 might have been considered only capable of spreading in soil by passive movements through groundwater or colonization through intertwined plant roots, experiments now indicate it routinely moves into plant stems and can come into contact with winged herbivorous insects above the soil line. These insects can acquire and transmit RN-L11 to new plants, which could greatly speed its spread and make containment at the point of release unlikely.

Uncertainty associated with predicting survival of RN-L11 would also have implications for predicting aspects such as gene transfer. England et al. (1997) were able to demonstrate using PCR techniques that a 630-bp DNA fragment of a Tn-*lac* fusion element inserted chromosomally in RN-L11 persisted in non-autoclaved soil for at least 4 weeks after inocula of killed RN-L11 cells were introduced. They suggested their results showed the possibility that RN-L11's genetically engineered DNA could be transferred to the indigenous microbial community by transformation.

This thesis has highlighted the difficult scientific challenges for accurately predicting the fate of GEMs in the environment. Kareiva et al. (1996) indicated they had such little faith in models and short-term experiments for predicting invasions of genetically engineered organisms, that they advocated their use more for setting up appropriate monitoring programs to detect a problem before it became too late. These concerns suggest a need to place more emphasis on obtaining accurate characterizations of the

hazard posed by a GEM rather than attempting to accurately characterize potential exposure. Lenski (1993) indicated that evaluation of the likelihood that a GEM will persist following release into the environment is complex and costly, and should be required only when the intended application is not similar to previous applications with a safe record of use and there exist plausible scenarios for significant adverse environmental effects. Giddings (1998) suggested it would generally be better to accept the likely persistence of GEMs or their transgenes after environmental release, and minimize associated risks accordingly. The level of potential hazard may then be the best guide to determining the level of effort and accuracy required for predicting GEM survival.

4. CONCLUSIONS

One of the first comprehensive scientific reviews of the safety of genetic engineering concluded that “there is no evidence that unique hazards exist either in the use of rDNA techniques or in the movement of genes between unrelated organisms” (National Academy of Sciences 1987). However, Colwell (1988) criticized this review as being oversimplified and remarkably sanguine. He considered that in the absence of ecological experience with the products of genetic engineering, predictions of possible hazards and assurances of safety are equally conjectural. Similarly, Krimsky (1991, 141) cautioned that the conclusion of the U.S. National Academy of Sciences had the flavour of ideology rather than science, and that it was based upon scant empirical data.

In the intervening years, many studies have sought to obtain empirical data and ecological experience with genetically engineered microorganisms. Probably the most common approach has been to study survival of GEMs in laboratory microcosms that simulate ecological conditions in soil or water. It will be increasingly important to have a thorough compilation of this existing knowledge about GEM survival under simulated and actual ecological conditions in order to evaluate environmental releases of GEMs in the future.

In addition, we will need to understand the limitations in our ability to predict GEM survival in diverse ecosystems. The accuracy of survival predictions for GEMs could be of importance for many situations ranging from evaluating the efficacy of a GEM in commercial applications, to evaluating disinfection technologies for reducing

environmental releases of GEMs from contained facilities, to assessing the potential scale of environmental contamination from use of genetically engineered biological weapons. While the use of a GEM as biological weapon may be an unlikely event, concerns have been raised about the potential use of GEMs as biological weapons (Novick and Shulman 1990; Edge 1999). The former Soviet Union had an active genetic engineering research program that successfully transferred toxin genes from *Bacillus cereus* into *B. anthracis* (Pomerantsev et al. 1997), inserted the gene for myelin toxin into the plague bacterium *Yersinia pestis*, and inserted genes from the viruses Venezuelan equine encephalitis and Ebola into the genome of Vaccinia virus, a close relative of smallpox (Alibek 1999, 166 and 261). Lederberg (1998) considered the *B. anthracis* strain outlined above to be the first example of an “artificially contrived” new human pathogen.

Studies comparing the survival of GEMs with their non-engineered parental organisms can be particularly useful for evaluating the competitiveness of GEMs. The literature review conducted for this thesis identified 102 literature records containing the results of 719 experiments that compared the fitness of GEMs relative to their parental microorganisms. The survival of GEMs was found to be the same or lower than their parental microorganisms in 92 % of the experiments examined. GEMs showed a relatively enhanced fitness in only 8 % of survival experiments. The results from this literature review, though limited to survival which is only one aspect of potential environmental and human health risk, lend support to previous scientific reviews which have suggested that most genetically modified organisms are expected to pose minimal ecological risk. However, the results also reject the categorical hypothesis that GEMs are less fit than their parental organisms.

Perhaps more importantly, the literature review results also identified the converse of the above. Ninety-two percent of experiments indicated that the survival of non-engineered microorganisms was the same or enhanced relative to their engineered counterparts. The results of this literature review present a case more for enhanced environmental and human health assessment of environmental releases of non-engineered microorganisms than for reduced oversight of genetically engineered microorganisms. It is possible that with public and regulatory attention directed more towards GEMs, the potential environmental and human health risks associated with an extensive and growing use of non-engineered microorganisms in the environment have been overlooked. This is in spite of the widely claimed need for science-based regulations that focus on products of biotechnology rather than the specific techniques used to produce them.

While the results of the literature review suggest that environmental survival of most GEMs can be predicted to be the same or lower than their parental organisms, the results from laboratory and modeling studies indicate that it will be very difficult to reliably predict specific survival rates for GEMs in the environment. While microcosms can provide useful predictions of GEM survival for site-specific environmental introductions (Bolton et al. 1991; Angle et al. 1995; Gagliardi et al. 2001b), additional inference methods are needed to predict survival associated with more widespread use of a GEM. The soil microcosm validation study by Gagliardi et al. (2001b) provided the first known opportunity to validate a statistical model for making quantitative predictions for the survival of a GEM in soil and rhizospheres across a wide range of different climatic conditions and different soils.

Linear multiple regression models were developed to predict survival rates for the GEM *Pseudomonas chlororaphis* 3732RN-L11 in soil and wheat rhizospheres based upon physical, chemical, and biological soil characteristics of the soil at the time of GEM inoculation. The models were developed from survival data generated in laboratory microcosms and were tested by comparing survival predictions with the survival of the GEM measured in outdoor field plots across Canada. While these statistical models appeared statistically significant they were not able to reliably predict, quantitatively or qualitatively, the survival rate of the GEM introduced into field plots across Canada. These microcosm/modeling studies demonstrated there was considerable uncertainty associated with predicting the survival of a GEM in different soils. Statistical models like those developed in this study might be better used as exploratory tools for studying the influence of specific soil parameters on GEM survival or for designing better field studies and monitoring programs, rather than as rigorous predictive tools.

The microcosm/modeling studies also identified the significant challenges involved in accurately predicting the environmental fate of a GEM. Despite considerable cost and effort involved in developing predictive statistical models, it was not possible to reliably predict survival in bulk soil or wheat rhizospheres for *Pseudomonas chlororaphis* 3732RN-L11 in this study. However, it should be considered that these survival predictions were for only one compartment of the environment (soil) and were directly applicable to the rhizosphere of only one plant species (wheat). For widespread use of a GEM, predictions of survival in other environmental compartments such as water, plant surfaces etc would also be needed. In addition, survival is only the first step in determining the fate of a GEM. Prediction of other aspects such as GEM dispersal and gene transfer events would also be required. The large cumulative uncertainty involved

in predicting the environmental fate of a GEM suggests emphasis could be better placed on a sound characterization of the potential hazards these organisms might pose. The level of hazard and related uncertainty associated with a GEM would then determine the level of effort to be devoted to predicting the survival and fate of the GEM in the environment.

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Appendix 1. Soil water content calculations for soils obtained from the Central Experimental Farm study sites in Year 1 and 2.
Study site abbreviations are in Table 2. Data obtained from intact soil cores.

YEAR 1	SITE A			SITE B			SITE C			SITE D			SITE E			SITE F		
Soil parameter	core1	core2	core3	core1	core2	core3	core1	core2	core3	core1	core2	core3	core1	core2	core3	core1	core2	core3
soil wt (100% sat) ¹	594	606	506	562	597	559	529	430	527	544	512	521	517	489	551	603	636	568
soil wt (oven dried) ¹	467	489	404	448	469	439	393	313	395	406	378	385	377	353	402	477	497	443
soil vol. ^{2,3}	176	185	152	169	177	166	148	118	149	153	143	145	142	133	152	180	188	167
soil bulk density ⁴	1.54	1.54	1.54	1.58	1.54	1.53	1.38	1.33	1.41	1.4	1.36	1.37	1.34	1.31	1.34	1.56	1.52	1.52
water wt (100% sat) ¹	127	117	102	114	128	120	136	117	132	138	134	136	140	136	149	126	139	125
water wt (60% sat) ^{1,5}	76	70	61	68	77	72	82	70	79	83	80	82	84	82	89	76	83	75
water vol. (60%) ^{2,5}	76	70	61	68	77	72	82	70	79	83	80	82	84	82	89	76	83	75
%water (wt-60% sat) ⁶	16	14	15	15	16	16	21	22	20	20	21	21	22	23	22	16	17	17
%water (vol-60% sat) ⁷	25	23	24	24	25	25	29	30	28	29	29	29	30	30	30	25	25	26
% sand ⁸			49			63			36			90			47			55
% silt ⁸			17			25			27			7			22			26
% clay ⁸			33			12			37			3			31			19
total bacteria (log CFU/g soil) ⁹			7.1			7.4			7.4			7.2			7.6			7.3
total fungi (log CFU/g soil) ⁹			4.8			5.2			5.6			5.3			5.8			5.7
total pseudomonads (log CFU/g soil) ⁹			5.8			5.6			5.8			6.1			6.2			6.1
soil survival slope ⁹			-0.01			-0.04			-0.03			-0.09			-0.07			-0.1
rhizosphere survival slope ⁹			-0.01			-0.05			-0.05			-0.06			-0.04			-0.05

¹ all weights in grams

² all volumes in cubic centimeters

³ soil volume (cc) = soil dry weight (g) / 2.65

⁴ soil bulk density (g/cc) = soil dry weight (g) / soil and water total volume (cc)

⁵ water wt or vol. (60%) = water weight or volume (100 % saturation) x 0.6

⁶ % water (wt-60% sat) = weight of water at 60 % saturation (g) / dry weight of soil (g) x 100%

⁷ % water (vol-60% sat) = volume of water at 60 % saturation (cc) / volume of water at 100 % saturation (cc) + volume of dry soil (cc) x 100%

⁸ mean of three cores but using different cores than for water characterization

⁹ regression slope for GEM in soil (log CFU/g of soil/day) or rhizosphere (log CFU/g of root/day)

Appendix 1 con't. Soil water content calculations for soils obtained from the Central Experimental Farm study sites in Year 1 and 2. Study site abbreviations are in Table 2. Data obtained from intact soil cores.

YEAR 2	SITE A			SITE B			SITE C			SITE D			SITE E			SITE F		
	core1	core2	core3	core1	core2	core3	core1	core2	core3	core1	core2	core3	core1	core2	core3	core1	core2	core3
soil wt (100% sat) ¹	589	533	552	558	555	640	535	548	559	551	584	596	549	569	522	489	563	530
soil wt (oven dried) ¹	463	425	429	448	445	523	412	420	426	424	455	476	415	419	399	382	448	414
soil vol. ^{2,3}	175	160	162	169	168	197	155	158	161	160	172	180	157	158	151	144	169	156
soil bulk density ⁴	1.54	1.59	1.51	1.61	1.6	1.67	1.48	1.47	1.45	1.48	1.51	1.59	1.43	1.36	1.46	1.52	1.58	1.52
water wt (100% sat) ¹	126	108	123	110	110	117	123	128	133	127	129	120	134	150	123	107	115	116
water wt (60% sat) ^{1,5}	76	65	74	66	66	70	74	77	80	76	77	72	80	90	74	64	69	70
water vol. (60%) ^{2,5}	76	65	74	66	66	70	74	77	80	76	77	72	80	90	74	64	69	70
%water (wt-60% sat) ⁶	16	15	17	15	15	13	18	18	19	18	17	15	19	21	19	17	15	17
%water (vol-60% sat) ⁷	25	24	26	24	24	22	27	27	27	26	26	24	27	29	27	25	24	26
% sand ⁸			51			66			48			93			44			61
% silt ⁸			18			22			16			4			26			24
% clay ⁸			31			12			36			3			30			15
total bacteria (CFU/g soil) ⁹			7.3			7.4			7.2			6.9			7.1			7.2
total fungi (CFU/g soil) ⁹			6			6			6.1			5.4			5.8			6.1
total pseudomonads (CFU/g soil) ⁹			6.6			6.5			6.6			6.4			6.6			6.7
soil survival slope ⁹			-0.08			-0.05			-0.06			-0.09			-0.09			-0.05
rhizosphere survival slope ⁹			-0.03			-0.03			-0.02			-0.03			-0.02			-0.04

¹ all weights in grams

² all volumes in cubic centimeters

³ soil volume (cc) = soil dry weight (g) / 2.65

⁴ soil bulk density (g/cc) = soil dry weight (g) / soil and water total volume (cc)

⁵ water wt or vol. (60%) = water weight or volume (100 % saturation) x 0.6

⁶ % water (wt-60% sat) = weight of water at 60 % saturation (g) / dry weight of soil (g) x 100%

⁷ % water (vol-60% sat) = volume of water at 60 % saturation (cc) / volume of water at 100 % saturation (cc) + volume of dry soil (cc) x 100%

⁸ mean of three cores but using different cores than for water characterization

⁹ regression slope for GEM in soil (log CFU/g of soil/day) or rhizosphere (log CFU/g of root/day)