**Soil Microcosms as Environmental Research Tools for the Study of
Microorganism Gene Transfer in Soil Environments**

By

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**Dissertation submitted to Faculty of Graduate Studies, Carleton
University in partial fulfillment of the requirements for the degree of
Master of Science**

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Chair, Department of Biology

Thesis Supervisor

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Abstract

The evaluation of risk involved in the release of genetically modified micro-organisms (GMO) to the environment has environmental and economic implications. In this project we released a strain of *Pseudomonas putida*, genetically modified to contain a self-transmissible atrazine-degradation plasmid, in the field and in contained soil microcosms. We also monitored the persistence of atrazine degradation in the soil following release. Atrazine-degrading bacteria thrived during the first 48 hr after release and then rapidly declined in numbers. To follow the horizontal transfer of atrazine degradation genes, transconjugant bacteria were isolated and identified. Of the 150 potential transconjugants isolated 10 were chosen for further characterization. Of these, 8 were *Pseudomonad* spp. isolated directly from experimental trials, and 2 were *Arthrobacter* from subsequent enrichment experiments. This study demonstrated that horizontal gene transfer can occur in the soil and is therefore an important consideration for any environmental release of a GMO.

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1. Introduction

The steady development and application of microbiological and genetic techniques has resulted in numerous organisms designed for intended environmental release. This ability has a large economic potential; organisms have been patented encompassing a wide range of environmental applications including synthesis of agricultural chemicals, biomass conversion, industrial chemical production, biosensor activities, energy, mining/metal recovery, nitrogen fixation, polymer production, oil recovery and pollutant/waste degradation. Bacteria modified to express catabolic functions beneficial to an environment have been developed at a rapid rate. In turn this has spurred scientists to explore the microbiology of the environment and its interactions, because a better understanding is required to help comprehend and assess the impact of introducing genes not normally found in a particular environment.

Bioremediation of landfills, accidental spill sites, improved crop yield, biological pest control, and protection from frost, are major environmental breakthroughs of applied microbiology (Sussman *et al.*, 1988). For the genetically modified organism (GMO) to be of commercial interest it must show a significant impact on its target, be economically feasible, and have little to no risk to the environment.

1.1 Safety concerns surrounding the release of GMOs into the environment

Bacteria, genetically modified for use in the environment, have widespread applications, from use in closed systems such as containers for remediation, to more open systems such as environmental release.

The current safety debate surrounding the production and use of GMOs has pushed for the development of methods for accurately predicting the environmental fate of foreign DNA in the environment. One of the critical issues in this debate is the ability of bacteria to maintain and transfer plasmids, which has a significant impact on the safety of GMOs intended for release into the environment. When planning a study using a bacterium containing genes that can move between bacteria, such as those on a plasmid or transposable element, the risk is amplified since the DNA of the introduced organism can transfer to indigenous organisms by horizontal DNA movement. When the genes of interest are contained on a transmissible plasmid, it is therefore essential to assess the survival of the host as well as the persistence of the genes.

Often it is the horizontal transfer of plasmids containing catabolic genes that allows bacteria to survive under selective pressure in the environment rather than advantageous mutations. This gives rise to many questions surrounding the risk assessment of bacteria engineered to contain conjugative plasmids:

Will the indigenous population acquire the plasmid and maintain active genes? Would transfer of the plasmid occur under nonselective conditions? Will the plasmid be lost from the population once the selective pressure is no longer present?

The long-term stability of the introduced genes needs to be assessed to determine or predict their future impact. An example is the research by Ramos-Gonzalez *et al.* (1991), in which the transfer of a catabolic plasmid was studied. A thorough investigation revealed that the genes responsible for the catabolic function were located on a transposon. This conferred instability in some potential recipients and resulted in rearrangements in the original plasmid as revealed by a decrease in size when recovered from transconjugants. These results emphasize the potential difficulty in following introduced genes in the environment and reveal some of the technical challenges that researchers must face to accurately detect these genes.

1.1.1 GMO risk assessment and standardized validation

The potential undesirable effects of introducing a GMO into the environment must be explored in detail. A major concern of this technology is an irreversible and damaging effect on the natural flora and fauna. To assess the risks associated with the release of a GMO, a standardized method must be validated in a contained environment to accurately predict the behavior of the GMO upon uncontained release. Questions to be addressed include: Can the

introduced host strain survive and find a niche in the proposed environment? Will the genes survive in the indigenous population if the host dies out? Will the current ecosystem balance of indigenous organisms be altered undesirably, for example will the introduction of the GMO cause the loss of another organism from the environment?

There have been studies that have sought to validate laboratory-based methods for accurately predicting the fate of GMOs in the environment. Since the environmental fate of introduced genes is difficult to predict, it is important to establish and standardize in-house methods to accurately detect the behavior of the GMO and to have these methods validated with field trials.

Once a genetically modified bacterium has been prepared for release into the environment, there are a number of considerations. The survival rate must be carefully monitored: Will the introduced bacteria be fit enough to compete with indigenous bacteria? How suited to the intended release site is the host? Will the phenotype be affected by the environmental conditions? And in the case of horizontal gene movement, will the genes be easily identified if transferred to other indigenous bacteria?

The focus in many studies has been to explore the potential for gene transfer in a soil environment (Richaume *et al.*, 1992; Smit *et al.*, 1992; Smit *et al.*, 1998; Dronen *et al.*, 1998). Horizontal gene transfer has been shown to occur

in situations where there is a selective pressure, such as conferring antibiotic resistance or the ability to degrade heavy metals (Davis, 1996; Levy, 1994; Salyers and Shoemaker, 1996; Van der Meer *et al.*, 1992).

1.1.2 Canadian policy

Canada has a notification system, which is applied to all organisms that are new to the Canadian environment. This initiative is the Canadian Environmental Protection Act (CEPA). The notification procedure was completed for this study and the GMO approved for field trial release (Germida, unpublished).

1.1.3 Microcosms as risk assessment tools

Soil microcosms have been valuable experimental systems for a range of studies, such as monitoring the breakdown of xenobiotic compounds, nitrogen transformation and survival studies. Soil microcosms are generally set up as an intact core or a mixed soil sample and can be used for any soil system although the type of microcosm chosen may vary. Since there are often strict regulations surrounding the environmental release of GMOs, microcosms have been extensively used in these kinds of studies. The microcosm is used to assess the risk of a GMO prior to a field trial. Its value therefore depends on its ability to provide results that are consistent with field release experiments. Many early studies exploring gene transfer were completed in

microcosms (Graham and Istock, 1978; Graham and Istock, 1979; Iwasaki *et al.*, 1994).

Various types of microcosms have been designed in an attempt to mimic environmental conditions (Daehler and Strong, 1996). These range from very simple to very complex systems, but a relatively simple, inexpensive system with the ability to accurately reflect field behavior is the most desirable. For soil systems, microcosms have been used that can range in size from a few grams of soil in a bottle to a complex system containing a large section of undisturbed soil.

The effect of microcosm size and sterility was investigated by Henschke *et al.* (1989) to monitor the survival of a *Pseudomonas fluorescens* containing a plasmid. A study by Brokamp and Schmidt (1991) tracked gene transfer to another inoculated group into the sterile microcosm. Bolton *et al.* (1991a) designed an intact core microcosm and compared the Pseudomonad activity to a field site. The general conclusion from these studies is that soil microcosms were good indicators of bacterial activity in the field including the potential for survival and distribution.

Richaume *et al.* (1992) explored another aspect of microcosm design; whether there are appreciable differences in using an intact soil core versus a homogenized soil sample. An intact core of the field site more closely mimics

the field by maintaining the soil structure. In this study, survival of a *Pseudomonas* strain was compared between microcosms, a climate controlled chamber and a field trial. The results of the field and growth chamber were in agreement and thought to be due to the similarity in temperature in these systems (Bolton *et al.*, 1991). Angle *et al.* (1995) carried out one of the first comparative studies using a simple microcosm consisting of a 15 cm X 5 cm tube of polyvinyl chloride (PVC) containing a core of soil. They found that experiments performed in this simple, inexpensive microcosm were able to provide accurate predictions of field behavior of *Pseudomonas aureofaciens* chromosomally modified to carry the *lacZY* genes. In comparison to soil samples that had been homogenized and sieved to create a uniform soil sample, the intact microcosm proved to be more accurate.

Studies designed to track the movement of foreign genes, are often confined to microcosms, not to necessarily mimic a natural environment but rather to create a controllable one. Some studies have concentrated on specific potential recipients by sterilizing the soil in the microcosm and then introducing the potential recipients. An example is the interspecific transfer of large plasmids within *Streptomyces* (Ravel *et al.*, 2000). Other studies have isolated transconjugants from the soil but have used a system that is not comparable to many other studies. For example, a study by Top *et al.* (1995) used soil that was collected from several years earlier that lacked indigenous

bacteria . Because there were no indigenous bacteria, this cannot be used to predict the behavior or impact of the introduced GMO in a natural environment.

Although many studies have explored the survival of GMOs in field-trials and microcosm experiments, the majority of studies have focused on organisms which have had genetic changes made to their chromosomal DNA. It is far more complicated to follow the DNA of GMOs containing plasmids harboring foreign DNA. In this case it is desirable to monitor not only the host bacterium but also any possible recipients. However, detection of all possible recipients is difficult because a large percentage of soil bacteria are difficult to culture. The growth conditions and media constituents must support as broad a range of microorganisms as possible.

Validating the use of a contained microcosm for predicting the environmental fate of a GMO necessitates a field trial for comparison. The research in this thesis is a continuation of an effort started in 1990 with Environment Canada to evaluate the appropriateness of soil microcosms as risk assessment tools for GMOs planned for release into the terrestrial environment. The first set of experiments indicated that the microcosm experiments provided a good indication of GMO behavior in the rhizosphere and soil environment (Gagliardi *et al.*, 2001). In this study, the GMO contained markers on its chromosome

and survival of its host, *Pseudomonas chlororaphis*, was monitored in a wheat rhizosphere and in soil.

1.2 Description of the host bacterium- *Pseudomonas putida*

Pseudomonas putida is a member of a common, heterogeneous group of microorganisms referred to as Pseudomonads. Pseudomonads are gram-negative bacteria that are metabolically versatile, bioactive, and prolific colonizers of surfaces, including soil matter and the rhizosphere (OECD, 1997). These chemo-organotrophs have a respiratory, non-fermentative type of metabolism and are usually catalase and oxidase-positive (OECD, 1997). *P. putida* reproduces through binary fission and is asporogenous (OECD, 1997).

Pseudomonads have the ability to live in a diverse range of habitats and maintain catabolic pathways on their chromosomes or on a plasmid, making them an attractive host for insertion of engineered genes. Many plasmids found in Pseudomonads are important for biodegradation applications. As a result, numerous studies have used *P. putida* as the host organism in GMO studies (Araujo *et al.*, 1996; Awong *et al.*, 1990; Doyle *et al.*, 1991; Hartel *et al.*, 1994; Inghem *et al.*, 1995; Iwasaki *et al.*, 1994; Lifshitz *et al.*, 1988; Raaijmaker *et al.*, 1994; Ramos *et al.*, 2000; Short *et al.*, 1990; Tang *et al.*, 1995; Winstanley *et al.*, 1991; Yeung *et al.*, 1989). Because Pseudomonads are bioactive, fast-growing, and prolific colonizers of plant surfaces, they are

able to suppress or out-compete pathogenic and other deleterious microorganisms (OECD, 1997) The ability to colonize plant surfaces is a result of pili on the bacteria (De Groot *et al.*, 1994; Vesper, 1987) and the production of agglutinin on the root surface (Anderson *et al.*, 1983; Glandorf *et al.*, 1994).

Pseudomonas strains have been used in studies concerning viability of GMOs containing plasmids in the environment. Field trials have been carried out using genetically modified strains of *Pseudomonas* including *P. aureofaciens* (lacZY), *P. fluorescens* (lacZY), *P. putida* (siderophore deletion) and *P. syringae* (Ice⁻). Conjugation to indigenous species has been demonstrated for both soil and rhizosphere experiments (Van Elsas *et al.*, 1988, Van Elsas *et al.*, 1990). Transformation of Pseudomonads can also occur if extracellular DNA is attached to a solid particle protecting it from exonucleases (Lorenz and Wackernagel, 1990; Stewart and Sinigalliano, 1990).

The *P. putida* used in this study was previously isolated from the experimental field site. This is an important feature because it increases the possibility that the GMO could occupy the niche currently inhabited by its wild type. McClure *et al.* (1991) noted the importance of using GMOs suited for growth in the chosen environment. In their experiments, the transconjugant that acquired the genes was better suited to growth in the environment and outlived the

inoculating host (McClure *et al.*, 1991). A number of other studies have found similar results (Brookamp and Schmidt, 1991; Chatterjee *et al.*, 1982; Golovleva *et al.*, 1988; Kilbane *et al.*, 1983; Ramos *et al.*, 1991). In contrast, a study with *P. putida* engineered to degrade 2, 4-DCP and a comparable non-engineered strain were found to have no differences in their ability to survive (Short *et al.*, 1990). Drahos *et al.* (1988) reported similar findings with an engineered strain of *Pseudomonas*. These findings highlight the importance of pre-release testing to determine the ability of the inoculated bacteria and the potential transconjugants to survive in the environment, especially in competition with the wild-type bacterial strains.

1.3 Biodegradation

Biodegradation is the breakdown of organic matter by organisms. The ability of naturally occurring bacteria which have the ability to degrade contaminants has been studied since the mid-twentieth century. Bioremediation exploits the ability of organisms to help alleviate an environmental problem; for example cleaning up oil spills by degrading the oil *in situ*. Bioremediation is generally carried out in one of two ways: fertilizers or other growth promoting agents are added to the site to aid the growth of the naturally occurring bacteria which can degrade the target, or a bacteria from elsewhere with the ability to degrade the contaminant is added. This clean-up strategy is often more attractive than other methods, which either contain the contamination or incinerate it, as it avoids the need to remove large amounts of soil or water.

Often the strains used are naturally occurring; however, with the current knowledge of genetic engineering it is possible to modify a bacterium from a contaminated site to contain genes that will enable it to use the target as a substrate. Bioaugmentation is the addition of a strain with the ability to degrade a contaminant to the site.

1.3.1 Plasmid ADP, atrazine degrader

The *Pseudomonas* sp. ADP was identified by Mandelbaum *et al.* (1995) and has been described in detail (Boundy-Mills *et al.*, 1997; De Souza *et al.*, 1996; De Souza *et al.*, 1998a; De Souza *et al.*, 1998b; De Souza *et al.*, 1998c). The *Pseudomonas* contains a self-transmissible plasmid that harbors four genes able to degrade the herbicide atrazine (figure 1). The 108Kb plasmid was recently sequenced and is 9Kb larger than initially reported (Boundy-Mills *et al.*, 1997). Figure 2 is a diagram of the ADP-1 plasmid indicating locations of the atrazine degrading (*atz*) genes and IS1071 transposable elements (genbank accession #U66917). The *atzA* gene encodes an atrazine chlorohydrolase which is the first step of the metabolic pathway involving the dechlorination of atrazine to hydroxyatrazine (De Souza *et al.*, 1995), the *atzB* gene encodes a hydroxyatrazine ethylamidohydrolase responsible for the deamidation of hydroxyatrazine to N-isopropylammelide (Boundy-Mills *et al.*, 1997) and the *atzC* gene encodes N-isopropylamide isopropylamidohydrolase that converts N-isopropylammelide to cyanuric acid and isopropylamine (Sadowsky *et al.*, 1998), see figure 1.

It has been demonstrated that the genes *atzA*, *atzB* and *atzC* are homologous to genes from diverse bacteria (De Souza *et al.*, 1998a). De Souza *et al.* (1998a) found the plasmid was transmissible to other bacteria using experiments with *E. coli*. It was also shown that the ADP plasmid does not carry the genes required to degrade cyanuric acid (De Souza *et al.*, 1998b).

There are two intact copies and one partial copy of the class II transposable element IS1071 on the pADP-1 plasmid which boarder the *atzA* and *atzB* genes (figure 2). IS1071 has been found in a wide range of catabolic sequences (Di Gioia *et al.*, 1998; Wyndham *et al.*, 1994). It is thought that IS1071 plays an important role in the mobilization of genes onto plasmids and has been found on many catabolic plasmids which confer the ability to degrade pollutants (Di Gioia *et al.*, 1998; Wyndham *et al.*, 1994). Class II transposons have been shown to be involved in gene mobilization and recombination of catabolic genes (Wyndham *et al.*, 1994). The IS1071 insertion elements, flanking the *atzA* and *atzB* genes, may have resulted from these genes moving onto the ADP plasmid from a smaller plasmid.

Figure 1: The atrazine degradation pathway by the *atzA*, *atzB* and *atzC* genes on the catabolic plasmid ADP-1 from *P. ADP*

Figure 2: The atrazine catabolic plasmid from *P. ADP*. Adapted from sequence provided by Genbank (accession # U66917)

It was hypothesized these elements played a role in localizing all the catabolic atrazine genes on the same plasmid for effective survival in the presence of atrazine. This is a likely hypothesis as the bacteria containing the plasmid were isolated from a heavily atrazine contaminated site (Mandelbaum *et al.*, 1995).

Pseudomonas ADP has been used in studies attempting to bioremediate atrazine-contaminated sites. Mandelbaum *et al.* (1995) found a 17% reduction in the level of atrazine from a site containing 1,500 µg of atrazine per g of soil. The plasmid ADP was chosen for this project as it did not contain genes which are undesirable for release into the environment, such as antibiotic resistance. Also there was no evidence of bacteria with the ability to degrade atrazine in the site chosen for release.

1.4 Introducing a plasmid capable of horizontal transfer into a terrestrial environment

Two main points to consider when introducing genes into the environment are the survival of the host cell and the survival of the foreign DNA in the indigenous population. Generally, the introduction of a host organism that commonly inhabits the intended environment survives better than foreign organisms. For example, Compeau *et al.* (1988) studied survival of *P. fluorescens* and *P. putida* strains in sterile and non-sterile soil and found that if one strain had colonized the soil, the other strain was prevented from

finding a niche. Gene transfer is known to occur in the soil and rhizosphere environment, though it is difficult to assess the rate of transfer. The frequency may be highly affected by surrounding conditions and microcosms provide a method to predict their behavior. When exploring the movement of foreign genes it is important to be able to identify the organism that has acquired the genes. An understanding of the potential recipients of the foreign genes should be carried out prior to release. This can be completed in a contained system, giving insight into potential recipients but is not intended as a prediction of field behavior.

The three mechanisms of gene transfer between bacteria living in the environment are; transduction, transformation and conjugation. Transduction is the movement of genetic material by bacteriophages, the host range is often narrow for bacteriophages.. Transformation involves the uptake of naked DNA from the environment by a recipient organism, this method requires the DNA to be stably maintained in the environment (i.e. not degraded). The third mechanism is conjugation, this is the transfer of genetic material directly between cells via the pili. This study is employing a conjugative plasmid and it is expected that conjugation will be the main method of horizontal transfer.

1.4.1 Conjugation

Conjugation is generally accepted as the most frequent mode of horizontal transfer in the environment and has been detected in both the rhizosphere and in soil (Lilley *et al.*, 1994; Smit *et al.*, 1998; Sorensen 1997; van Elsas *et al.*, 1988) and aquatic environments (Bale *et al.*, 1987; Muela *et al.*, 1994; Sandaa and Enger, 1994). Davidson (1999) has written a comprehensive review entitled 'Genetic Exchange in the Environment' which highlights current knowledge of bacterial gene transfer and the topic has also been covered by others (Wellington and van Elsas, 1992; Veal *et al.*, 1992).

The transfer of conjugative plasmids has been demonstrated to occur between Pseudomonads in a number of non-rhizosphere and rhizosphere soil environments both in microcosms and *in situ* (Lilley *et al.*, 1994; Trevors and Berg, 1989; van Elsas *et al.*, 1988).

For conjugation to occur, cell-to-cell contact is required. It is therefore more frequent in areas which are highly populated with bacteria such as the rhizosphere (Lilley *et al.*, 1994), or when bacteria are attached to a soil component such as clay or organic matter (Trevors and Berg, 1989; Stotzky *et al.*, 1991). In wheat plant root (van Elsas *et al.*, 1988) and sugarbeet (Lilley *et al.*, 1994) conjugation studies, survival of the donor and recipient, as well as frequency of plasmid transfer, decreased with increasing distance from the plant root.

If the genes confer a selective advantage to the host in the intended environment, studies have shown that the frequency of surviving bacteria is increased. Beneficial plasmids often transfer under selective pressure in soil microcosms (Brokamp and Schmidt, 1991; Ramos-Gonzalez *et al.*, 1991). The fitness of the host bacterium decreases when a large plasmid has been inserted unless it is beneficial to the organism. This is because the presence of plasmid DNA increases the metabolic load carried by the organism (Grabherr, 2002; Paulsson, 2002; Tiedje *et al.*, 1989). Conversely, the presence of transposons may aid in the survival of the introduced genes, as was the case in studies on *E. coli* growth containing a Tn5 transposon, which showed a higher survival than the parental strain (Biel and Hartl, 1983). A study by Chao *et al.* (1983) found that strains containing a Tn10 transposon were more competitive than the strains lacking the Tn10, suggesting that the presence of transposable elements aids in the survival of genes by allowing movement, for example onto the chromosome. The studies to date indicate that the persistence of genes introduced in the environment are for the most part unpredictable, so standardized microcosm experiments are essential before any field trial.

1.5 Studies with *P. putida* and *P. ADP*

In microcosms, plasmid transfer has been reported from *P. putida* to indigenous soil bacteria (Dejonghe *et al.*, 2000) and from *Pseudomonas sp.* B13 to *P. putida* as well as to indigenous bacteria in activated sludge (Ravatt *et al.*, 1998). Other examples of *P. putida* strains being used in

bioremediation include degradation of naphthalene, sulphonic acid, dimethylphenol, and polyaromatic hydrocarbons (OECD 1997). For many chemicals such as camphor, xylene, toluene, salicylate, naphthalene, isopropyl-benzene, styrene, cinnamic acid, and 3-chloro-benzoic acid, the metabolic genes are plasmid encoded (OECD 1997). Using pLV1013 as a marker plasmid in *P. putida* PaW8, Macnaughton *et al.* (1992) investigated the effect of soil texture on survival and found that introduced bacteria survived better in soils with higher clay content. Compeau *et al.* (1988) studied survival of *P. fluorescens* and *P. putida* strains in sterile and non-sterile soil and found if one strain had colonized the soil, the other was prevented from finding a niche. The durability of *P. putida* WCS 358, was demonstrated by survival in distilled water for over a year (Lynch, 1990), thus indicating the viable cells can survive on very low energy under starvation conditions.

Madsen and Alexander (1982) studied the movement of *P. putida* in soil and found the cells remained near the surface unless a rhizosphere system was present. Stressed *P. putida* communities can be used as an indicator of environmental organic pollution levels (Heipieper *et al.*, 1995). It has been reported that the addition of *P. putida* to soil or water did not affect other microorganisms or enzyme activity (OECD 1997).

Studies in both microcosms and field plots have been carried out using modified strains of *P. putida* marked with an antibiotic resistance marker (Compeau *et al.*, 1988; Dupler and Baker, 1984).

P. ADP has been used in a study by Shapir *et al.* (1998) to assess its bioremediation potential of waste from an atrazine manufacturing plant. The study was carried out in a wastewater system and results indicated the strain was useful for biodegradation (Shapir *et al.* 1998). *P. ADP* was also successfully used in a bioreactor for remediation of atrazine-contaminated soil (Newcombe and Crowley, 1999).

1.6 Release of a GMO into the soil and rhizosphere - characteristics and considerations

GMO release into soil environments provides a challenge for the detection and monitoring of the introduced genes. For a review of microorganisms introduced into soil and their survival see van Veen *et al.* (1997). There are two main methods for detecting the genes in the environment: phenotypically and genotypically. However, determining that an organism is present can be particularly difficult in a soil environment (Ford and Olsen, 1988). The phenotypic approach depends on the ability to culture the bacteria and on the ability to easily detect the products of the genes of interest. This method is relatively simple and inexpensive, although is restricted to quantify only the organisms that can grow on the chosen media and express the genes of

interest. The genotypic approach, is a characterization of the organism's genetic composition such as the sequence of a specific gene. The genotypic approach is more expensive than the phenotypic approach but this method gives a quantification of the genes in the environment, however, it does not indicate how frequently the genes are expressed. One of the most accurate methods of identifying a recipient organism is from analysis of the sequence of the 16S rRNA gene. Large databases of 16S rRNA sequences exist so it is possible to identify the organism.

Soil characteristics can have a significant effect on the survival of introduced bacteria (Godbout *et al.*, 1995; Ramos-Gonzalez *et al.*, 1991). In these studies they concluded that soils with a higher than normal organic content and slightly lower water content provided the highest survival rate for the GMO. Soil systems are naturally heterogenic and combinations of biotic and abiotic factors affect the survival of introduced organisms. Soils with a higher ratio of clay and silt are more favorable for survival (van Veen *et al.*, 1997). Other factors that can affect the survival of bacteria include pH, temperature, surface sorption (O'Donnell and Hopkins, 1993) and controllable factors such as the concentration of the introduced GMO and the dispersal method (Ford and Olsen, 1988).

The environmental factors discussed above can also impact the rate of conjugation, for example, the transfer rate is higher with a high level of carbon

in the soil (1,139 mg per liter) (Fernandez-Astorga *et al.*, 1992) or in the presence of clay, silt and roots, as they all provide a surface for attachment of the GMO (OECD, 1997). Temperatures below optimum for growth of the host *P. putida* can significantly slow the rate of gene transfer (OECD, 1997). However pH did not show a significant impact on gene transfer over a range of normal soil pH levels (Fernandez-Astorga *et al.*, 1992).

The method of sampling organisms is also an important consideration. The method must take into consideration the soil matrix. A well-mixed sample partially negates the errors of soil sampling (Atlas and Bartha, 1981). The most common method is a sub-sample of the soil shaken with a buffer to create a suspended dilution. This sample is then processed for phenotypic detection, generally by plating on selective media or genotypic detection involving isolation of nucleic acids. The type of soil can affect the ability to produce a sub-sample of soil, since bacteria are more easily extracted from soil with a high sand content compared to soils with a high clay content. This is because microorganisms often form strong bonds to clay particles in the soil (OECD, 1997) and remain in soil aggregates (Hopkins *et al.*, 1991). Several methods, such as homogenization, chemical dispersants, cation exchange resins, and differential centrifugation, have been developed to disrupt these soil-microbe associations so that bacteria can be extracted (Bakken, 1985; Faegri *et al.*, 1977; Herron and Wellington, 1990; Hopkins *et al.*, 1991; MacDonald, 1986).

Many studies have shown that, in general, the population levels of microorganisms introduced into soils decline over time (van Veen *et al.*, 1997; Bolton *et al.*, 1991; Macnaughton *et al.*, 1992; Ramos *et al.*, 1991; Recorbet *et al.*, 1993; Gagliardi *et al.*, 2001; Filonov *et al.*, 1999), although at least one study found an increase in the population levels (Dupler and Baker, 1984). Introduced microorganisms generally do not compete well with microorganisms already established in naturally occurring microbial communities (van Veen *et al.*, 1997).

Compared to pure soil, the rhizosphere can provide an improved environment for survival and conjugation (Gamliel and Katan, 1992) due to the acidity of the soil and the enriched amounts of carbohydrates, amino acids, organic acids and proteins from plant secretions and bacterial metabolism (Rouatt and Katznelson, 1961). Pseudomonads are root-colonizing bacteria and generally develop niches in the rhizosphere system of many plants since the rhizosphere provides a surface for bacterial attachment. Costa *et al.* (2000) found the numbers of *P. ADP* increased around the rhizosphere and the degree of atrazine degradation was higher carried out in containers as rhizosphere studies compared to soil studies.

1.7 The herbicide atrazine as a selective pressure agent

Atrazine is a herbicide commonly used for the control of broad leaf weed control in corn crops. Atrazine is a member of the s-triazine herbicides (for structure see figure 1) and is degraded by bacteria in some soils by the sequential removal of the functional group. It is the most commonly used s-triazine herbicide, up to 73×10^6 lb is released per year in the USA (Mandelbaum *et al.*, 1993; Short and Colborn, 1999). Atrazine is applied before or after plant growth begins. Atrazine is absorbed mostly by roots and also by leaves of plants. It moves up through the plant, and builds up in the margins of the leaves. Atrazine acts by inhibiting photosynthesis in plants and is therefore highly effective in killing these plants. Plants, which are sensitive to atrazine, do not metabolize (or break down) atrazine. Tolerant plants metabolize atrazine to hydroxyatrazine and amino acid conjugates. Hydroxyatrazine is then broken down further by de-alkylation and hydrolysis (USA Department of Agriculture. <http://infoventures.com/e-hlth/>).

The end metabolite of the bacterial degradation of atrazine is cyanuric acid, which can be degraded by enzymatic ring cleavage to CO_2 and NH_3 (Radosevich *et al.*, 1995). Atrazine, as well as its metabolites such as hydroxyatrazine, have been shown to persist in the environment for extended periods of time. The half-life of atrazine at 25 C° at a pH of 4 was 244 days although this is reduced in the presence of humic material (Goodrich *et al.*, 1991; Jones *et al.*, 1991; Lerch *et al.*, 1999; Solomon *et al.*, 1996; Spectrum

Laboratories. <http://www.speclab.com/compound/c1912249.htm>). The persistence of this chemical is an important environmental concern. As a result, the degradation of atrazine in the environment has also been the focus of many studies attempting to bioremediate field sites (Struthers *et al.*, 1998).

The plasmid ADP-1 has genes that code for enzymes to degrade atrazine to cyanuric acid (figure 1), and the host organism was selected with the ability to mineralize the cyanuric acid (Cook *et al.*, 1985). Organisms with the ability to degrade atrazine are able to use it as their sole nitrogen source (Ostrofsky *et al.*, 1997; Pussemier *et al.*, 1997).

1.8 Objectives

In this project we set out to explore if the simple PVC soil microcosms validated in the previous study (Gagliardi *et al.*, 2001) can be used to predict field behavior of a GMO engineered to contain the conjugative plasmid pADP-

1. We are seeking to answer four main questions:

- 1) Will the GMO find a niche in the parental strain environment?
- 2) Can transconjugants be cultured using a selective plating method?
- 3) Will the plasmid be stably maintained in transconjugants?
- 4) Do trends from the microcosm trials reflect the results from the field studies?

This is the first time that a soil microcosm experiment and field trial will be compared for their ability to assess horizontal gene transfer from a GMO containing a conjugative plasmid. A conjugative plasmid was chosen since this was the most likely mechanism of horizontal transfer and this would test the limits of the microcosm for predicting field behavior. A pictorial description of experimental design is shown in figure 3.

Figure 3: Schematic experimental design for the standardized validation of a *P. putida* modified to carry the ADP-1 plasmid from *P. ADP*

2.0 Methods

2.1 Selection media

Selective plating was used for isolating the inoculated bacteria and soil and rhizosphere bacteria with the ability to degrade atrazine was selective plating. The plates were used within a week of being poured and were monitored for fourteen days after use. *Pseudomonas* F-agar was used to distinguish the host bacteria, which was resistant to rifampicin and naladixic acid. GATZ media was used to confirm the bacteria were able to degrade atrazine (for minimal media A base see appendix 1)

2.1.1 Pseudomonas F-agar

The *Pseudomonas* F agar was prepared in 100 ml Pyrex Erlenmyer flasks containing 19 g of the pre-made *Pseudomonas* F medium, 5 ml of glycerol, 500 ml ddH₂O and a magnetic stir bar. The media was autoclaved at 121°C for 20 minutes. After autoclaving, the flasks were put into a water bath pre-warmed to 60°C, with a water level slightly higher than the media level in the flask. Once the media had cooled to 60°C the following filter sterilized solutions were added: D-glucose (2 ml of a 1.6 M stock solution to a final concentration of 8 mM), rifampicin (1 ml of a 20 mg/ml stock solution to a final concentration of 50 µg/ml), potassium tellurite (500 µl of a 20 mg/ml stock solution to a final concentration of 25 µg/ml), cyclohexamide (2 ml of a 20 mg/ml stock solution to a final concentration of 100 µg/ml) and nalidixic acid (200 µl of a 20 mg/ml stock solution to a final concentration of 10 µg/ml). The

plates were poured, allowed to set and dried inverted on the bench top over night.

2.1.2 Selective media containing atrazine

The GATZ media was prepared in a 1000 ml Pyrex Erlenmeyer flask containing 360 ml of Medium A with no nitrogen source (-N) base (appendix 1) was autoclaved at 121°C for 20 minutes. After autoclaving, the flasks were put into a water bath pre-warmed to 60°C, with a water level slightly higher than the media level in the flask. Once the media had cooled to 60°C the 40 ml of 10 X P was mixed into the Medium A-N base. Then the following filter sterilized solutions were added; D-Glucose (2 ml of a 1.6 M stock solution to a final concentration of 8 mM), rifampicin (1 ml of a 20 mg/ml stock solution to a final concentration of 8 mM), rifampicin (1 ml of a 20 mg/ml stock solution to a final concentration of 50 µg/ml), potassium tellurite (500 µl of a 20 mg/ml stock solution to a final concentration of 25 µg/ml), Cyclohexamide (2 ml of a 20 mg/ml stock solution to a final concentration of 100 µg/ml) and naladixic acid (200 µl of a 20 mg/ml stock solution to a final concentration of 10 µg/ml).

The plates prepared before the field trial were made in two layers with the top layer containing the atrazine. Once the bottom layer was poured (10 ml) and had dried the top layer containing the atrazine was prepared. A sterile 50 ml Falcon tube was placed in a beaker of hot water (to prevent rapid cooling of the agar), 40 ml of the above agar was poured into the Falcon tube and 100 µl of concentrated 0.4M atrazine (1 g atrazine/11.6 ml of N,N-

dimethylformamide) was added to give a final concentration of 1mM. The tube was immediately inverted to evenly disperse the atrazine precipitate. Approximately 8 ml was then poured to create the top layer on the plate.

Plates made during the field and microcosm trials were made in one layer using the above agar with 100 µl of commercial atrazine (50% active ingredient) per 100 ml of media. This was added directly to the medium in the 1000 ml Pyrex flasks, mixed, and the molten agar was poured into the plates.

After the agar hardened, the plates were inverted and allowed to dry for 48 hours on the bench top. Freshly prepared plates were used for each sampling to avoid increased background numbers due to inactivation of the antibiotics.

2.2 Creation of CR30RNSpADPTel

The strain CR30RNSpADPTel was created for use in an ongoing microcosm validation project for Environment Canada (Gagliardi *et al.*, 2001). The original host is *Pseudomonas putida* (denoted as CR30). This strain was isolated from the rhizosphere soil of canola (*Brassica napus*) plants that were grown in soil microcosms from the Carleton University field plot. Identification was confirmed by FAME (fatty acid methyl ester) analysis, BIOLOG assays (BIOLOG Inc., Hayward, California, USA) and 16S rDNA gene sequencing (MIDI Laboratories, Newark, NJ).

The strain was selected based on its ability to metabolize cyanuric acid the end metabolite from the degradation of atrazine by pADP-1, (see figure 1). A series of selective pressure plating experiments containing increasing levels of antibiotics were completed to obtain a strain with chromosomally encoded resistance to the antibiotics: rifampicin, nalidixic acid and streptomycin all at concentrations of 100 µg/ml, this strain was denoted CR30RNS. The strain *P. putida* CR30RNS was engineered to contain the plasmid ADP-1 from *P. ADP* to which the genes *kilAtelAB* from *Klebsiella aerogenes*, which encoded the ability to reduce potassium tellurite to tellurium metal, were added (see appendix 2).

2.3 Probes

Probes for each of the atrazine genes were initially made from vectors supplied by Topp *et al.* (2000). All restriction enzymes and buffers were from New England Biolabs (NEB, Beverly, Mass., USA). *AtzA* was cut from pMD4 with *Apal* and *PstI*, *atzB* from pATZ-2 with *BglII* and *EcoRI*, and *atzC* from pTD-2 with *Clal* and *HincII*. The probe for the *kilAtelAB* genes was created from the *NotI* fragment of vector pUCNotI.Tel. Restriction digestion was carried out according to the manufacturers' instructions. DNA, 0.4-2.0 µg, was placed in a 1.0 ml Eppendorf tube, 2 µl of appropriate buffer was added [pMD4 - Buffer 4 (20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM diothreitol, pH 7.5), pATZ-2 – unique buffer (50 mM

NaCl, 100 mM Tris-HCl, 10 mM MgCl₂, 0.025% Triton X-100, pH 7.5) and bovine serum albumin, BSA (100 µg/ml final concentration), pTD-2 – buffer 4 and BSA (100 µg/ml final concentration), pUCNotI.Tel- Buffer 3 (50 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl, 1mM dithreitol, pH 7.9) and BSA (100µg/ml final concentration)]. The reaction volume was brought to 36 µl with sterile ddH₂O and 2 units of appropriate restriction enzyme added. The reactions were incubated at 37°C, except pMD4 which was incubated at 25°C, for 3-6 hr The reactions were stopped with the addition of ethylenedinitriolo tetra-acetic acid (EDTA) to a final concentration of 10 mM.

The probes created from the vectors did not give a strong signal during detection; this was most likely due to the poor compatibility between some of the restriction enzymes and buffers, and therefore a low concentration of DNA for labeling. Following the field trial, the probes were made using PCR generated fragments the labeling follows the procedure outlined below.

Once the digestions were completed, the fragments (*atzA* - 0.6 Kb, *atzB* -1.2 Kb, *atzC* - 0.75 Kb, *kilAtelAtelB* - 1.2 Kb) were ran on a low melt agarose gel (NuSieve, GCG, Mandel Scientific, Guelph, Ontario, Canada), 1.5%, 55 V, 1.5 hr, and excised out. The excised bands were gene cleaned [6X volume 6 M NaCl set at 55°C for 15 minutes, 20 µl glass milk 37°C for 5 min, the glass milk with bound DNA was then pelleted and washed 3 times with cold New solution, the DNA was eluted in distilled water or TE buffer (Tris – EDTA)] and

stored at -20°C. The probes were Dig-Labeled according to manufacturers' instructions [10 ng - 3.0 µg template DNA, 5 µl hexanucleotide mixture (1.56 mg/ml random hexanucleotides, 100 mM MgCl₂, 500 mM Tris—HCl, 1 mM dithioerythritol, 2 mg/ml BSA, pH 7.2), 5 µl dNTP labeling mix (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile DIG-dUTP, pH 6.5), sterile de-ionized/distilled water (to a total volume of 47.5 µl), Klenow enzyme (1 µl)].

2.4 Genomic and plasmid preparations

The sequence of the plasmid pADP-1 was completed just prior to the field trial (Acc# U66917, Martinez *et al.*, 2001). The large size, 108 Kb, created difficulties in separation of the plasmid intact. Initially, genomic DNA was isolated for a check of foreign gene presence following the protocol outlined in Maniatis *et al.* (1989). The cells were grown overnight at 200 rpm with shaking (New Brunswick Scientific Inc., G24 Environmental incubator shaker, model 34309263002985, NJ, USA) in 10 ml GATZ medium with 1 mM atrazine (Chem Service, PA, USA) and 8 mM D-glucose. Once growth was evident the cells were collected by centrifugation (4000 X g, 20 min, 4°C), OD readings were not used as the presence of atrazine as a fine precipitate gave inaccurate readings. The cells were washed three times with sterile ddH₂O and once in 1 X SSC [8.75 g NaCl, 4.11 g tri-sodium acetate (BDH, Inc., Dorset, UK) in 800 ml ddH₂O, pH 8.0). The cells were then re-suspended in 950 µl lysis solution [10 µl lysozyme (400 mg/ml) 10 µl RNase (10 mg/ml;

Pharmacia, NJ, USA), 930 µl sterile ddH₂O] and incubated at 37°C for 20 min. Sodium dodecyl sulphate (SDS)(50µl of a 10% solution, BDH Inc., Dorset, UK) was added and the tubes placed in a water bath at 60°C for 15 minutes. Proteinase K, 10µl of a 20mg/ml stock (Roche, QC, Canada) was added and incubated at 60°C for a further 60 min. One ml of buffered phenol (pH 8.0) was added to the solution, mixed and centrifuged for 20 min, 4°C, and 12,000 rpm. The aqueous phase was removed and to the remaining solution 1 ml of phenol and 1 ml of CHCl₃ added vortexed and centrifuged as above. The aqueous phase was again removed and 1 ml of CHCl₃ was added, vortexed, and centrifuged at 12, 000 rpm for 20 min at 4°C. To the recovered aqueous phase 1/10 of the volume of the solution of sodium acetate (3 M, pH 4.8) was added and mixed well. A 2.5 volume of 100% ice cold ethanol was then added to the solution and left overnight at -20°C. The solution was centrifuged at 12,000 rpm for 10 min at 4°C to pellet the DNA, the pellet was washed with ice cold 70% ethanol and centrifuged. The ethanol was removed by aspiration and the DNA re-suspended in sterile ddH₂O or 0.5 mM TE (Tris – EDTA) buffer.

An integral part of the study involved tracking the movement of the pADPTel plasmid, requiring a reliable separation method that could be used to accurately determine the presence/absence of a large plasmid. The method, which initially gave optimum results was described by Speijer *et al.* (1999). The cells were grown overnight in 5ml tryptone yeast extract (TYE) (1%

tryptone, 1% yeast extract, 0.5% NaCl, 1.6% agar) with antibiotic, rifampicin and naladixic acid, selection or GATZ liquid media. The cells were collected by centrifugation and washed 2 times with PettIV (10 mM Tris-HCL, pH 7.6, 1 M NaCl), and re-suspended in 1 ml of the PettIV buffer. Cells were embedded in 1.6% low melt agarose, using an equal volume of cell suspension and agarose, in the molds for pulse field gel electrophoresis (PFGE) (Bio-Rad, ON, Canada). The plugs were incubated for 30 hrs at 37°C in EC lysis solution (6 mM Tris-HCL [pH7.6], 1M NaCl, 100 mM EDTA [pH7.5], 0.5% wt/vol Brij 58, 0.2% wt/vol deoxycholate, 0.5% wt/vol Sarcosyl, 1 mg/ml lysozyme, 20ug/ml RNase). The EC solution was then removed and ESP solution added (0.5 M EDTA [pH 9.5], 1% wt/vol lauryl sarcosine, 1 mg/ml Proteinase K) and incubated for 48 hrs at 50°C. The plugs were washed and stored (4°C) in TE buffer.

2.5 Southern blot/probe hybridization

The chromosomal DNA from the CR30RNSpADPTel strain was separated by gel electrophoresis [0.8% agarose, 15 V, 24 hours]. The plugs of agarose containing samples for separation of the pADPTel plasmid were run with ¼ of the plug embedded in a 0.8% agarose gel, 0.5X TE buffer, for 24 hrs at 200 V, 10-20 s switch times in the pulse field gel electrophoresis (PFGE) chamber (CHEF-DR II Pulsed Field Electrophoresis system, Bio-Rad Laboratories, ON, Canada). The agarose gel was blotted on a nylon membrane using a vacuum transfer apparatus (Vacugene XL, Pharmacia, NJ, USA) with a pressure of 55

mbar. The gel was flooded with 15-25 ml of denaturation solution (0.5 M NaCl, 0.5 M NaOH) for 15 min, removed and replaced with neutralization solution (1.5 M NaCl, 1 M TrisHCL, pH 7.5) for 15 min, the chamber was then flooded with 20 X SSC (1000 ml ddH₂O, 175 g NaCl, 88.2 g tri-NaCitrate pH 7.0, 1000 ml ddH₂O water) for one hr. The wells were marked with a pencil and the membrane separated from the agarose gel and washed 2 times in 20 X SSC for 10 minutes, the membrane was then UV cross linked for 5 min and allowed to dry on the bench top.

Probe hybridization was completed as outlined in Maniatis (1989) the membrane was blocked in hybridization buffer (5X SSC, 0.02% SDS, 0.1% Na-lauroyl sarcosine, 1% skim milk powder (Carnation) for 1 hour at room temperature. Depending on the probe strength, 1-10 µl was diluted in the hybridization buffer (atrazine probes made from the vectors required a minimum of 10 µl of labeled probe whereas probes made from PCR product had a higher concentration of labeled DNA and generally required only 1-3µl of labeled probe). The probes were denatured by being placed in a boiling water bath for 15 min and rapid cooling in an ice bath. 10-15 µl of this hybridization solution was added to the hybridization tube containing the membrane and put in a rotating oven (Robbins Scientific, Model 400) overnight at 68°C. The membrane then underwent a series of washes to remove un-hybridized probe. The first wash was at room temperature, twice

for 5 min with 2X SSC and 0.1% SDS, the second at 68°C twice for 15 min in 0.1X SSC and 0.1% SDS.

The following steps were all carried out at room temperature. The membrane was equilibrated with Buffer 1 (100 mM Tris pH7.5, 150 mM NaCl and 0.3% Tween 20) for 5 minutes. Buffer 1, 10ml with 2% blocking reagent, was added and put in the rotating oven for 30-60 min. This solution was then replaced with Buffer 1 with 2% blocking reagent and 1µl anti-Dig-AP, and the membrane was placed back in the rotating oven for 30-60 min. The membrane was washed in Buffer 1 twice for 15 min at room temperature and equilibrated in Buffer 3 for 5-10 min (100 mM Tris pH 9.5, 100 mM NaCl).

Detection of probe hybridization was carried out using either a chemiluminescent or colourimetric method. For the chemiluminescent method the membrane in a heat sealable bag was covered with buffer 3 and sprinkled with CSPD, approximately 2 drops per square inch. The bag was sealed after removing as many air bubbles as possible. The membrane and cassette were incubated at 37° for 15 min and then exposed to X-OMAT film (Kodak) between intensifying screens to increase signal detection. Development times varied from 20 min to 2 hr depending on probe strength. For the colourimetric method the membrane was also placed in a heat sealable bag to which a solution of 15 ml Buffer 3 and 45 µl NBT and 35 µl 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) was added. The bag was sealed, removing as many

air bubbles as possible, and left in a dark place overnight. Washing in Buffer 1 for 5 minutes stopped the reaction, the membrane was air-dried.

2.6 Field and microcosm experimental trials

The field plots and microcosms were inoculated on the same day, the schedule of sampling was based on preliminary pre-trial microcosm studies (data not shown).

2.6.1 Site Preparation

The site was cleared a week prior to the inoculation using a rotary soil tiller to create a uniform soil surface free of organic debris. The four treatments in the field for the study were field soil with atrazine (FSA), field soil without atrazine (FS), field rhizosphere with atrazine (FRA), and field rhizosphere without atrazine (FR) with each completed in triplicate. Twelve plots, each one meter square with one meter separating each plot, were marked and a plastic edging was placed around each (see figure 4 and appendix 2). The edging was set into the ground so that 6 cm remained above ground. The area was watered to set the edging in place.

2.6.2 Microcosm preparation

The core soil samples were removed from within the field site (figure 4, figure 5, appendix 3). The evening before the core removal the site was watered manually. The cores were made from PVC plumbing pipe, measuring seven

inches long with a diameter of two inches. One end of the pipe was filed to facilitate ground penetration. Each core was weighed empty and the weight recorded on the core with indelible marker.

The cores were driven into the ground with a mallet in rows with 2 cm left about ground surface. A trench was then dug around them and they were removed sideways (see figure 4). The bottom of the cores were covered with aluminum foil and secured with an elastic band.

The cores were immediately transported to the laboratory and weighed; the total weight was recorded on the outside of the core with indelible marker. They were then placed in a growth chamber, which was set to provide a cycle of twelve hours full spectrum lighting and twelve hours darkness.

2.6.3 Planting

The canola strain used was an atrazine resistant variety. The planting occurred immediately before inoculation. The canola in the field plots was planted with 5 seeds in 25 evenly spaced areas of each of the six rhizosphere plots. The place in which each set of five seeds was planted was marked with a small plastic stake. Each rhizosphere core was planted with five seeds near the center of the core. Once germination occurred, the cores were thinned to one plant per core; the rhizosphere plots were thinned to 25 evenly spaced plants.

2.6.4 Inoculation

All growth stages of the inoculum were completed at 28°C with shaking (200 rpm). A single colony of CR30RNSpADPTel from a GATZ+ plate was grown overnight in 10 ml Medium A-N (no agar) containing 0.5 mM atrazine, 8 mM D-glucose, rifampicin (50 µg/ml), and tellurite (25 µg/ml). The 10 ml was then transferred to a 500 ml flask containing 250 ml of the above medium and grown over night. The following day (early log phase) 250 µL of this culture was used to inoculate a second flask containing 250 ml of Medium A-N containing glucose and atrazine in the above concentrations. Once the media began to turn turbid, TYE was added to the inoculum. The cells were grown to an OD₆₀₀ of 0.8 (late log phase), this occurred at 24 hr after the addition of TYE. Placing the flask in an ice bath while swirling stopped the growth.

The cells were pelleted in sterile 500 ml polypropylene tubes at 5000 g for 20 min. The centrifuge and rotor were chilled to 4°C prior to filling the bottles. The supernatant was discarded and the cells were re-suspended in sterile distilled water (4°C). The final concentration of cells was read at OD₆₆₀ and was 0.70; according to plate counts the density was 10⁷ CFU/ml.

The inoculum was immediately transported to the field site. The inoculant volume for each soil plot was 1250 ml of the 10⁷ CFU/ml suspension. The inoculum was dispersed using a watering can for the soil plots. The

rhizosphere plots received 2.5 ml of the 10^7 CFU/ml inoculum by a sterile pipette over the seeded area.

2.6.5 Maintenance of microcosms and field plots

The cores were weighed to recorded weight on alternate days; the field was watered manually according to environmental conditions. The field and microcosms were weeded when needed.

2.6.6 Soil and rhizosphere sampling from the field and microcosms

The inoculum was left for 3 hours to settle in the field and microcosms before the first samples were taken. A bulb planter tool was used when sampling from the FS, FSA, FR and FRA areas, a sample roughly the size of a core was removed and was placed in a zip-lock plastic freezer bag. In the case of the rhizosphere plots, the entire plant and a section of soil around the rhizosphere were removed. One sample per plot was removed at each sampling time. The area from which the sample was removed was marked with a plastic stake to avoid re-sampling in that area at a later date.

Each soil sample was placed through a 4 mm sieve to create a uniform sample and remove large debris and rocks. A 10 g sample of the soil was added to 95 ml of distilled water, this was considered a 10^{-2} dilution as 10 g of soil is approximately 5 cc volume, which is equivalent to 5 ml. This mix was blended in a Waring blender for 1 min, and then allowed to settle for one min

before sampling. The rhizosphere samples were carefully stripped of loose soil adhering to the root system and the plant portion removed at the root crown. The entire root with tightly adhering soil was used as the rhizosphere sample. The weight was recorded and distilled water was added to bring the weight to 100 ml, 10^{-1} dilution, this was blended as above for one min and left to settle for one min. The sampling schedule is given in appendix 4.

Figure 4: Photograph of core removal and GMO release A) Soil core removal prior to field trial, each core contains a intact sample of the top 15 cm of field plot B) Release of GMO to the soil plots on day 0.

A)

B)

Figure 5: Photograph of field plot and microcosms A) Field plot prior to release. Each plot is enclosed with plastic edging and is one m². B) Soil microcosms with canola plants at day 22 in the growth chamber.

A)

B)

2.7 Plating Techniques

The dilution series plated at each sampling time varied from a 10^{-1} , 10^{-2} , 10^{-3} series to a 10^{-2} , 10^{-3} , 10^{-4} series to accommodate the number of bacteria present. These dilutions were used based on preliminary microcosm studies to obtain plates with between 30-300 viable colonies. Each dilution was replicated three times for a total of nine plates, per plate type per sample. The dilution buffer was freshly made before each sampling, 5 ml of stock I [50 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1000 ml of dd H_2O] and 1.25 ml of stock II [34 g KH_2PO_4 in 1000 ml dd H_2O] (note both stock solutions were autoclaved and stored at 4°C).

The plates were inoculated by spreading 100 μl of diluted sample on the plated with a L-shaped glass rod. Incubation time varied with the type of plate, generally the F-agar plates showed substantial growth after 24 hr, the GATZ plates with (GATZ+) and without (GATZ) antibiotics tended to take between 4 and 14 days to show countable colonies clearing atrazine.

2.7.1 Quantification of host survival

Host survival was quantified by plate counts on two types of selective media, GATZ+ and *Pseudomonas* F-agar containing rifampicin (50 $\mu\text{g/ml}$) and naladixic acid (10 $\mu\text{g/ml}$). Only dilution series with 30-300 colonies per plate were counted. Only bacteria showing atrazine clearing as a clear ring around the colony were counted.

2.7.2 Quantification of transconjugants

When plating samples for identification of potential transconjugants the media was as described above for GATZ, without the addition of the antibiotics. Colonies, which grew and showed evidence of the presence of the pADPTel plasmid by a clearing zone around the colony, were re-streaked onto GATZ with antibiotics to rule out the original strain. Any potential transconjugants were kept as a glycerol stock at -80°C and further characterized once the field trial had concluded.

2.7.3 Comparison plating

At the end of the field and microcosm trials there were 150 frozen glycerol stocks of potential transconjugants. The stocks were plated onto various media to compare growth. The frozen samples were grown in two types of liquid media, TYE and GATZ liquid. This was completed as some bacteria did not grow well in the heavy selection of the GATZ media but did appear to contain bacteria with the ability to degrade atrazine. These cultures were then dilution plated onto solid media. The first media was TYE with agar this rich media served to allow pure colonies to grow as individual colonies contamination may have been occurring on the GATZ plates. The colonies were then re-streaked back onto GATZ with and without antibiotics to eliminate strains that appeared to be the host. Selected strains, which

exhibited similar morphology, were then streaked onto minimal media A with atrazine and no carbon source, and onto Kings B media.

2.8 Transconjugant identification

A total of ten potential strains were chosen from the original 150 potential transconjugants to develop a method of accurate identification and characterization. This was carried out by a combination of methods including Biolog, 16S rRNA sequencing and PCR amplification of sections of the ADP-1 plasmid.

2.8.1 Confirming isolates had differing substrate usage patterns

Biolog gram-negative plates were inoculated with the selected potential transconjugants for further characterization to confirm that they exhibited different carbon source usage patterns (BIOLOG Inc., CA, USA). This is visualized as a color produced by the reduction of a tetrazolium dye. The purified bacterial samples were grown overnight in TYE and washed 3X in 1/10 minimal media A. Each sample was read on a spectrophotometer and diluted with MMA to an OD₆₀ of 0.35-0.45. Each of the 96 wells was inoculated with 150 µl of the sample using a multi-channel pipette. Any change in color of each well was noted and recorded against the negative control at 24, 48 and 72 hours. Please note that a positive control was used, the original inoculum, and any samples which exhibited a carbon use pattern which were identical to it was eliminated. One sample did not show growth on

the plates with antibiotics and therefore may have been a indigenous *P. putida* which acquired the plasmid.

2.8.2 16S rRNA sequencing

The 16s rRNA gene was amplified using primers designed corresponding to base 63 to1378 of the 16S rRNA gene of *E.coil* (Marchesi *et al.*, 1998). PCR products were purified and 5 were sent for one pass sequencing to University of Ottawa Biotechnology Research Institute (ON, Canada). The database BLAST was used for sequence comparison and DNAMAN , version 4.11 (Lynnon Biosoft, 1994-9) for manipulation of the sequence information.

2.8.3 PCR amplification for acquired gene characterization

PCR was used to confirm the presence of the three atrazine genes in the potential transconjugants and the presence of the *tfrA-tnpA* region of the pADP-1 plasmid (appendix 5). Since the pADPTel plasmid contains a number of transposable elements, PCR was also used to amplify the junction between IS1071 and *atzA* and *atzB* to check for IS1071 mediated movement of those genes (appendix 5). PCR was completed using both genomic DNA preparations and whole cells. Primers were designed using DNAMAN and synthesized by Sigma Genosys (ON, Canada) unless otherwise indicated. Primers for *atzA*-IS1071 and *atzA* were kindly supplied by Natalie DuCharme (unpublished, 2002), primers for *atzB* and *atzC* are described by Topp *et al* (2001).

The PCR reagents were all obtained from Invitrogen (ON, Canada). The PCR reactions contained DNA (0.05 - 0.15 ng of genomic or a single colony of cells), 0.5 μ l *Taq* DNA polymerase, 2 μ l 10 X PCR buffer, 0.02 mM of each deoxyribonucleotide, 0.3 mM $MgCl_2$ and 0.1 μ M of the forward and reverse primers. The volume was increased to 20 μ l with sterile ddH₂O. The conditions for both were the same except 2 μ l of water (20 μ l total volume) was replaced with 2 μ l of DMSO in the whole cell reactions.

PCR reactions were performed in a Whatman Biometra thermocycler. Each reaction began with an initial 3-min denaturation step at 95°C. Amplification of the *atz* genes and *atz*-IS1071 junction following the manufacturer's recommendations; 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 55°C and 60 sec of elongation at 72°C. There was a 10 min elongation step, 72°C, at the end of the last cycle. Amplification of the 16s rRNA gene was completed over 30 cycles following the above series of steps with an extended elongation of 105 s.

3. Results

3.1 Confirmation of *kilAtelAtelB* genes on the plasmid pADP-1

The pADP plasmid from *Pseudomonas ADP* was transformed into *P. putida*. (figure 6). The presence of the atrazine degrading genes (*kilAtelAtelB*) on the plasmid was confirmed by Southern blot analysis. Pulse field gel electrophoresis (PFGE) was used to confirm the presence of a large plasmid (figure 7). A plasmid of known size was used as a supercoiled marker to determine the approximate size of the plasmid (data not shown) which was estimated to be 111 Kb. PFGE can be used to detect the presence of a large plasmid however deletions of < 10 Kb will not show an evident size difference.

3.2 Selection of appropriate media to detect atrazine-degrading activity

Since the *P. putida* host strain can use a wide range of carbon sources, it was important to determine which media would provide the best selection for our experiments. Carbon sources tested were succinate, glycerol and glucose. The best media to culture *P. putida* and detect atrazine-degrading activity was GATZ with D-glucose, atrazine, potassium tellurite, rifampicin, naladixic acid and cyclohexamide (data not shown). Atrazine degraders grown on this media showed the fastest clearing of atrazine.

Figure 6: Southern blot analysis of *kilAtelAtelB* on pADP. The presence of *kilAtelAB* genes on the plasmid of CR30RNSLL(pADPTel) was confirmed by by agrose gel electrophoresis (a) and Southern blot analysis (b). The Allen method (Cork *et al*, 1995) for mega-plasmid extraction was used to isolate the 111 Kb plasmid. The plasmid can be seen in lane 3 of 7a and hybridization with *kilAtelAB* is shown in lanes 3 and 9 of 7b. λ HindIII (lanes 1 and 7) is used as a molecular weight marker.

a)

b)

Figure 7: Pulse field gel electrophoresis of *P. CR30RNS* carrying pADP (lanes 2,4,6,8) and pADPTel plasmid (3,5,7,9). Lanes 2,3,6 and 7 contain agrose plugs prepared using the method described by Speijer *et al* (1999) and lanes 4,5,8 and 9 agrose plugs prepared according to the method by Ramos-Diaz and Ramos (1998).

3.3 Pre-release and off site monitoring for atrazine degrading microorganisms

Prior to release of the host bacteria, CR30RNSpADPTel, soil samples were taken from between the field plots as well as outside the testing area, to determine if indigenous, atrazine degrading, microorganisms were present. To detect possible long distance movement of the atrazine degrading genes, soil samples were also removed from outside the test area during the trials. These soil samples were taken 4 m away from the field site in each navigational direction as well as from the runoff plain (appendix 3). All samples were tested for the ability to degrade atrazine and reduce tellurite according to the method described for the experimental samples. No atrazine degraders were isolated from any of these samplings (data not shown) but bacteria with the ability to reduce potassium tellurite to tellurium metal were frequently found.

3.4 GMO field and core release

The microcosm cores were removed from the field site 24 hours before inoculation and placed in an alternating pattern in the growth chamber. The weight of the soil from each core ranged from 300-500 g. The atrazine was applied to the soil prior to the core removal. The GEM release was completed on June 30, 2001. The concentration of the GMO released was 10^8 CFU/ml, according to spectrometer reading 10^7 CFU/ml by viable plate count, this

discrepancy was likely due to the presence of small amounts of atrazine precipitate that remained in the GMO suspension. Figure 4 is a picture of the GMO release.

3.5 Microcosm and field sampling

The timeline of sampling is detailed in appendix 1. The dilution series for each sample was adjusted to match the number of bacteria. When bacterial numbers were very low, and 10^{-1} dilutions were used, it was difficult to isolate atrazine-degrading colonies due to the presence of soil particles (figure 8). The parental strain was distinguished from the transconjugants by plating on GATZ+ which contained rifampicin and naladixic acid for which resistance was encoded on the host chromosome. Figure 9 shows the number of atrazine degrading bacteria per g of soil. The number of bacteria increased initially, usually to day 2, then steadily declined. Atrazine degraders could be isolated until day 8 in field untreated soil (FS) and field atrazine treated soil (FSA) and day 21-23 in the microcosms.

The rhizospheres were established by planting canola in field and microcosms at the same time as the GMO release. Typically the seedlings germinated by day 8. By this time, the atrazine degraders had already decreased and were only detected on the first sampling date in the field (day 15) and day 21-23 for microcosms (figure 8). This rapid decline was not expected based on preliminary microcosm studies (data not shown)

3.6 Identification of transconjugants

From each soil sample, the number of potential transconjugants was tested on GATZ plates. Colonies which showed a clear area, indicating atrazine degradation were re-streaked onto GATZ+ and GATZ to distinguish the original introduced GMO. The colonies were difficult to separate as a pure culture and a series of purification streaks were needed for each since many 'single' colonies contained contaminating bacteria which could survive on the media. A total of 150 potential transconjugants were isolated during the trial period and maintained as frozen glycerol stocks. Ten possible transconjugants were chosen for further characterization. Each transconjugant was inoculated into gram-negative Biolog plates and the pattern of substrate produced the identifications listed in table 1. These tests not only indicated the bacterial species but also revealed substrate usage patterns. The final 10 transconjugants were selected based on differences within them and also in comparison to the inoculum. In cases where a high degree of similarity of biolog and morphological patterns were found one representative strain was chosen. Consideration was also given to possible transconjugants isolated over the time frame of the trials.

3.6.1 16s rRNA Sequencing

The area of the 16S rRNA gene amplified corresponded to the 63rd base and 1378th base on the 16S rRNA gene on *E. coli*. The sequencing reaction was not able to produce a continuous sequence for the 1.3 Kb PCR product,

therefore the sequence obtained from each end was separately compared to published 16S rRNA sequences at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, Maryland (appendix 6) using BLAST function. All sequences could be matched to a species level, according to Organization for Economic Co-operation and Development (OECD 1997). The 16S rRNA gene sequence cannot yet differentiate between *P. putida*, *P. fluorescens* and *P. chlororaphis* and a number of intermediate strains such as *P. pseudoalcaligenes*.

Figure 8: *Brassica napus* plants in a microcosm core and field plot and atrazine degrading bacteria on selective plates. *Brassica napus* plants in a microcosm core (a) and field plot (b) day 23. Examples of bacterial growth on GATZ plates from dilution series c) an example GATZ plate from a 10^{-3} dilution of a sample removed from an atrazine-treated field soil plot. The atrazine degrading bacteria are clearly seen with a clear ring around the colony. D) a plate with a 10^{-1} dilution of a soil atrazine treated field plot from day 8. This plate was typical of plates from the samples taken after day 3, many of the colonies did not show evidence of atrazine clearing.

a)

b)

c)

d)

Figure 9: Number of atrazine-degrading bacteria in the soil as determined by selective plating on GATZ media. The samples were taken from field soil (FS), field atrazine treated soil (FSA), microcosm soil (LS) and microcosm atrazine-treated soil (LSA).

3.7 Characterization of potential transconjugants

The potential transconjugants were tested for the presence of the atrazine degradation genes, the junction between the IS1071 elements and *atzA* and *atzB* as well as a signature region of the ADP plasmid (figure 11, figure 12 and table 2). Four of the ten potential transconjugants (1/SA, 1/FSA, 15/FR and 15/FRA) had the same PCR profile as CR30RNSpADP-1, *atzA* was not present in three transconjugants (15/LRA, 64/LRA and 95/LRA) and *atzB* and *atzC* were absent in one of the potential transconjugants (64/LRA). To ensure that an *atzA* or *atzB* gene did not reside in the bacteria, a Southern blot carrying chromosomal DNA from 15/LRA, 64/LRA and 95/LRA was hybridized with *atzA* (15/LRA, 64/LRA and 95/LRA) and *atzB* (64/LRA). This confirmed the lack of the *atzA* and *atzB* gene in those samples which had a negative result for PCR (appendix 7). The junction between IS1071-*atzA* and IS1071-*atzB* was not amplified in three of the transconjugants (15/LR, 64/LR and 95/LR) and the signature region of the pADP-1 plasmid was not amplified in three of the ten (3/FS, 15/LR and 64/LRA). These results indicate rearrangement of the atrazine degradation genes in the transconjugants.

Figure 10. Colonies grown on GATZ media from each potential transconjugant group. Atrazine degradation, seen as a clear ring around the colony, was visible on all plates in natural light.

Sample ID	Biolog	63F	1378R
1/FSA	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>
1/LSA	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>
2/FSA	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>
3/FSA	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>
15/FR	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>
15/FRA	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>
15/LR	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>
15/LRA	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>
64/LRA	<i>Pseudomonas</i>	<i>Arthrobacter</i>	<i>Arthrobacter</i>
95/LRA	<i>Aquisprillium</i>	<i>Arthrobacter</i>	<i>Arthrobacter</i>
CR30RNSpADP-1	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>

Table 1: Transconjugant identification according to Biolog and 16s rRNA sequencing.

Figure 11: Areas amplified on plasmid ADP for characterization of potential transconjugants. Arrows indicate primer binding site and direction.

Figure 12. PCR amplification of *atzA* and *atzB* in the 10 potential transconjugants. A) PCR of the 10 potential transconjugants with primers designed to amplify a portion of *atzA* (A) and *atzB* (B) of pADP-1. The PCR products obtained were electrophoresed on a 1.5% agarose gel for 1.5hr at 60V. The lanes are; 1, 15/FRA, 2, 15/FR, 3, 15/LR, 4, 15/LRA, 5, 64/LRA, 6, 95/LRA, 7, 1/SA, 8, 2/FSA, 9, 1/FSA, 10, 3/FS, 11, CR30RNSpADP-1. B) PCR of the 10 potential transconjugants with primers designed to amplify a portion of *atzC* from pADP-1. The PCR products obtained were electrophoresed on a 1.5% agarose gel for 1.5hr at 60V. The lanes are; 1, 15/FRA, 2, 15/FR, 3, 15/LR, 4, 15/LRA, 5, 64/LRA, 6, 100bp ladder, 7, 95/LRA, 8, 1/SA, 9, 2/FSA, 10, 1/FSA, 11, 3/FS, 12, CR30RNSpADP-1.

a)

b)

Figure 13. PCR amplification of IS1071-*atzA* and IS1071-*atzB* junctions in the 10 potential transconjugants. PCR of the 10 potential transconjugants with primers designed to amplify the IS1071-AtzA (A)a)) and IS1071- AtzB (A)b)) junction of pADP-1. The lanes are; 1, 15/FRA, 2, 15/FR, 3, 15/LR, 4, 15/LRA, 5, 64/LRA, 6, 95/LRA, 7, 1/SA, 8, 2/FSA, 9, 1/FSA, 10, 3/FS, 11, CR30RNSpADP-1. PCR amplified TfrA-TnpA junction of pADP-1, (B). The lanes are; 1, 15/FRA, 2, 15/FR, 3, 1/FSA, 4, n/a, 5, 15/LR, 6, 3/FS, 7, 64/LRA, 8, 95/LRA, 9, 15/LRA, 10, 1/SA, 11, 2/FSA, 12, CR30RNSpADP-1, 13, Ps. ADP, 14, Ps. ADP, 15, CR30RNSpADPTel. The PCR products obtained were electrophoresised on a 1.5% agarose gel for 1.5hr at 60V.

A)

A**B**

1 2 3 4 5 6 7 8 9 10 11 1 2 3 4 5 6 7 8 9 10 11

B)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Sample ID	atzA	atzB	atzC	IS1071-atzA	IS1071-atzB	tfrA-tnpA
1/FSA	✓	✓	✓	✓	✓	✓
1/LSA	✓	✓	✓	✓	✓	✓
2/FSA	×	✓	✓	✓	✓	✓
3/FSA	✓	✓	✓	✓	✓	×
15/FR	✓	✓	✓	✓	✓	✓
15/FRA	✓	✓	✓	✓	✓	✓
15/LR	✓	✓	✓	×	×	×
15/LRA	×	✓	✓	✓	✓	✓
64/LRA	×	×	×	×	×	×
95/LRA	×	✓	✓	×	×	✓
CR30RNSpADP-1	✓	✓	✓	✓	✓	✓

Table 2. PCR results from amplification of selected possible transconjugants using six sets of primers

4.0 Discussion

The purpose of this research was to validate the use of microcosms for the routine survival assessment and rate of gene transfer between a genetically modified bacterium and indigenous soil bacteria. This project monitored the transfer of herbicide-degradation genes located on a conjugative (self-transmissible) plasmid. A research project has previously validated the use of soil microcosms for predicting field survival of genetically modified organisms (Gagliardi *et al.*, 2001). This project sought to further validate the use of the soil cores with a different GEM and to determine the prediction capabilities of the cores in relation to the horizontal gene transfer.

4.1 Creation of the inoculating GMO and stability of all genetic modifications

The inoculating bacterium was modified to carry the large ADP plasmid with the atrazine degradation genes. This plasmid posed some technical challenges due to its large size, which were overcome with the use of pulse field gel electrophoresis (PFGE). Initial testing of the inoculum however, revealed that the bacterium was not able to reduce potassium tellurite to tellurium metal suggesting an instability of the *kilAtelAtelB* genes. Subsequent purification and southern blot analysis of the plasmid confirmed that some of the genes, specifically the *kilAtelAtelB* genes, were not present. Because the bacterium was not maintained in potassium tellurite, due to accumulation of insoluble tellurium, this may have contributed to the loss of these genes.

However, the host bacteria, *Pseudomonas putida* CR30 still showed some resistance to potassium tellurite and therefore does not need to maintain these genes. This is contrary to previous studies that suggest that resistance to potassium tellurite in gram-negative bacteria is not common (Sanchez-Romero *et al.*, 1998). Sanchez *et al.*, (1998) inserted the same genes into *P. putida* and found them stably maintained when present on the chromosome, but plasmid-encoded genes were lost at a rate of 1-2% per generation in the absence of selection. Therefore, during transconjugant and host isolation, the tellurite-related phenotypes were not considered.

4.2 Background testing for indigenous atrazine degraders

To ensure there were no organisms with the ability to degrade atrazine already present in the soil, samples were taken surrounding the field site were grown in atrazine-enriched media before the field trial. This tested whether there were indigenous atrazine degraders in the soil. Topp *et al.* (2000) found plasmid-borne atrazine-degrading genes in diverse bacteria from Canadian and French soils. Samples were also taken surrounding the field site during the field trial to ensure no movement of atrazine degrading genes to indigenous bacteria. We found no evidence of atrazine degraders from any of these background tests.

4.3 The use of selective plating to identify atrazine degraders

Selective plating on atrazine-enriched media was chosen as the method for isolating the host and potential transconjugants from the field and microcosm trials. Selective plating permits the use of high levels of selective agents, is fairly easy to carry out, and requires relatively inexpensive materials. The drawbacks of this approach are that the bacteria must be culturable on the chosen media, long incubation times are sometimes required, only phenotypic expression (i.e. clearing of atrazine surrounding the colony) can be detected, and the method is only suitable when large populations of bacteria are being tested. Because of these drawbacks, selective plating as a means to enumerate survival of the introduced GMO and persistence of foreign genes is inherently selective. Thus, the numbers of persisting host and potential transconjugants revealed by this method is likely underestimated.

The selective plating technique worked well for identifying colonies able to express the atrazine degradation genes when there were many atrazine degraders in the sample. When the number of atrazine degraders began to decrease, it was necessary to use a lower dilution of the sample, which also included a higher amount of soil particles and other contaminants, which made the plates extremely difficult to analyze. The contamination was caused by the presence of fungi that could rapidly overgrow a plate, the growth of non-atrazine degrading bacteria, the growth of colonies near an atrazine degrader, and the presence of the host. These factors were compounded by

the fact that bacteria, with the ability to degrade atrazine, grew very slowly and the atrazine clearing was often not evident on the plate until after seven days or more of incubation.

To counter these contaminants discussed above, several approaches were used. To alleviate fungal growth, high levels of cyclohexamide were used, but this did not stop all growth. Some fungi grew from a small black colony that was difficult to separate from potential transconjugants or host bacterium. A number of non-atrazine degrading bacteria were able to grow on the plates most likely due to the presence of minute amounts of nutrients from the dilution sample. When there were few bacteria in the sample, low dilution factors (10^{-1} dilution) were needed and this resulted in a significant amount of soil particles being smeared on the plate, thus providing a source of nutrient for competitive bacteria. Many bacteria also appeared to have a mild to high resistance to potassium tellurite since they were able to reduce it to tellurium metal as evidenced by the gray to black color of colonies. Bacteria, naturally resistant to potassium tellurite, have previously been identified by Yurkov *et al.* (1996), who found seven strains of obligate aerobic photosynthetic bacteria from fresh water samples. Colonies, which appeared to degrade atrazine were sub-cultured and often were not pure since a number of contaminating bacteria were also evident. This contamination was probably due to the growth of bacteria living near an atrazine degrader on areas of the media where atrazine had been broken down. Because the GATZ plates,

used for transconjugant selection, also allowed the original host to grow, individual colonies were re-plated simultaneously on GATZ and GATZ+ (plates containing the antibiotics rifampicin and naladixic acid) plates. The presence of the antibiotics in the GATZ+ plates identified the host bacteria because the antibiotic resistance genes were on the host chromosome. This plating allowed accurate separation of the host and transconjugants. The combination of these strategies increased the accuracy of transconjugant isolation.

The selective plating method used for estimating the survival of a GMO was limited to those bacteria culturable under the same conditions as the host. The selective nature of the media also requires atrazine degradation to be phenotypically evident, thus this strategy would only identify bacteria that contained all three atrazine degrading genes. The phenotype of atrazine degradation was easily distinguished as a clear ring around the bacteria. Therefore the estimation of the number of potential transconjugants is underestimated.

4.4 Collection of atrazine degraders from soil samples

Soil samples were removed from the field and microcosms according to the schedule in appendix 1. The schedule of sampling was based on earlier microcosm studies that indicated that the first three days were critical for horizontal transfer. The number of atrazine degrading bacteria initially

increased (at 48 hr), then decreased to undetectable levels by day 8 in the field and day 21-23 in the microcosms (figure 9). This trend of an initial increase then decrease in bacterial numbers agrees with Dupler and Baker (1984) and Filonov *et al.* (1999) who attributed this to an adaptation to the new environment. At this point, we followed the survival of total atrazine degraders because it was impractical to separate transconjugants from the original host. The host strain was expected to survive longer in the field, but in our experiment it was undetectable soon after inoculation, so very little information about survival in soil. The most obvious additional trend was a higher number of CFU/g of atrazine degraders in soil of atrazine-treated microcosm and field plots than their untreated counterparts (figure 9), suggesting that the atrazine provided a positive selective pressure for atrazine degraders.

Two main changes would have improved the soil survival data collection effort. A phenotypic marker (preferably colorimetric, not antibiotic) on the host chromosome would have allowed simultaneous identification and separation of transconjugants and host bacteria. It would still be necessary to purify the individual colonies to eliminate contaminants as described above. Secondly, more sampling times, especially in the first eight days, would have created a more informative set of data on the growth of these bacteria and would have permitted statistical data analysis.

4.4.1 Collection of atrazine degraders from rhizosphere samples

In these experiments, we were testing whether the presence of a rhizosphere, in this case a canola seedling, would enhance the environment for *Pseudomonas* survival. Rhizosphere sampling began on day 15 and at this point in the experiment the number of atrazine degraders in the soil were very low in the microcosms and undetectable in the field except for the atrazine-treated plot in which very few atrazine degraders remained present. Although the presence of a rhizosphere did show to increase the number of *Pseudomonads* in the soil (pretrial experiments, data not shown), the numbers of atrazine degrading bacteria were below detectable levels (by selectable plating) in our field plots and were only evident in the microcosms after 21-23 days. Therefore, the rhizosphere experiments were unable to contribute to our evaluation of whether microcosms could reflect the trends seen in the field trials.

The rhizosphere experiment may have provided more valuable information had the seeds been allowed to germinate and establish the rhizosphere before the plot was inoculated. In the present study, a “true” rhizosphere did not develop for several days once the canola seed germinated. By inoculating an established rhizosphere, the effect on the GMO could have been assessed at the first time point. It is likely that the time required for the rhizosphere to develop allowed the bacteria to move away from the seed by water movement, and to be out competed by existing bacteria. Another method,

which might have improved the rhizosphere experiment is to soak the seeds in the inoculum before planting (Espinosa-Urgel *et al.*, 2000). This method would have ensured a close association between the seeds and inoculated bacteria before planting. Additional experiments would be necessary to test which of these methods would provide the best experimental system.

4.4.2 Enrichment for atrazine degraders following the field and microcosm trials

Once the number of atrazine degraders in the field and microcosms were undetectable by selective plating, enrichment of soil and rhizosphere samples was carried out to see if viable atrazine degraders were present. The entire rhizosphere or soil sample was incubated in an atrazine rich liquid media for 14 days and samples were plated on to GATZ plates. No atrazine degraders were found from enrichment of soil samples, nor from the rhizosphere field plots (data not shown). Two atrazine degraders were found by enrichment from the atrazine treated microcosm rhizosphere thus indicating there were low numbers of viable atrazine degraders maintained on the rhizosphere. This suggests the rhizosphere in combination with the selective pressure of atrazine may have provided a better environment for the atrazine degraders.

4.5 The contribution of water movement in the soil to microcosm and field plot experiments

Pseudomonads are not motile bacteria in soil, however water movement can flush the cells through the soil. Trevors *et al.*, (1990) studied the movement of *P. fluorescens* through soil and rhizosphere microcosms and the movement was shown to be dependant on the rate and volume of water flow through the system (Trevors *et al.*, 1990). Also the rate of cell division decreases when water levels are too high, which would have a negative impact on the survival of the Pseudomonads (OECD, 1997). Although dry conditions have been shown to have a negative impact on *P. putida* survival, Winstanley *et al.* (1993) found the rate of decline of *P. putida* in soil microcosms was significantly greater at 35% than at 50% field capacity water content. The cores used in this study were watered to a designated weight, approximately 60% capacity, on a regular basis and the field plot was watered during dry spells. This watering regime may have caused too much movement of the bacteria, however it was required so as not to let water levels sink to a level detrimental to the survival of the inoculated *Pseudomonas*.

4.5.1 The contribution of atrazine as a selective agent in field and microcosm trials

Atrazine was applied to the field and microcosm prior to the release of GMO. The availability of viable recipient cells in treated (atrazine), compared to untreated conditions is an important consideration. It is possible that the

atrazine may have reduced the number of recipient cells since it could kill organisms that could not tolerate it in the environment. Therefore, there could have been several potential recipients that were killed by the atrazine before they had the chance to take up the plasmid. This is similar to the finding of Ramos-Gonzalez *et al.*, (1991) who found that the presence of the selective agent decreased the number of potential recipients before conjugation took place. In addition, the atrazine degradation products can also be toxic. The presence of the ADP plasmid would allow the atrazine to be degraded to cyanuric acid and N-isopropylamine, both can be metabolized by a variety of soil bacteria (De Souza *et al.*, 1998a). For example, our host bacteria, *P. putida* can degrade cyanuric acid and N-isopropylamine to CO_2 and NH_4^+ . However if the atrazine was only partially degraded (i.e. as far as hydroxyatrazine by *atzB*, see figure 1) the products formed could be toxic to some members of the indigenous population.

In the untreated plots, the entire indigenous microbial population would be present in the soil, which would create a more competitive environment for the bacteria. The main competition for the introduced GMO in the soil environment was probably from other Pseudomonads (Lindow, 1992). These bacteria would occupy the same niche in the environment, require the same nutrition, and would be able to compete with the GMO. Compeau *et al.*, (1988) demonstrated this by looking at the survival of *P. fluorescens* and *P. putida* in sterile and non-sterile soil. When sterile soil was sequentially

inoculated with the two *Pseudomonad* strains, the first inoculated strain colonized the soil and prevented the colonization of the second. Similarly, Al-Achi *et al.*, (1991) found that, when introduced as pairs into irradiated, sterile soils, a *P. fluorescens* strain prevented optimum colonization by a *P. putida* strain. The addition of *P. putida* to sterile soil already populated by *P. fluorescens* impeded growth of *P. putida* in that soil. Competition and competitive niche exclusion likely also occurred in our experiments on untreated soil and rhizosphere.

4.6 The effect of a large plasmid on bacterial fitness

It has been shown that the increased metabolic load of a large plasmid decreases the fitness of a GMO as well as a transconjugant which acquired the plasmid (Grabherr, 2002; Paulsson, 2002; Tiedje *et al.*, 1989). Transconjugants were selected from the soil and rhizosphere plots based on ability to express the atrazine genes on atrazine selective media (i.e. not for the presence of the entire ADP-1 plasmid). Therefore, a rearrangement of the genes required for atrazine degradation may have occurred to suit the individual transconjugant. In theory the ADP-1 plasmid also contains insertion sequences, IS1071, which have been shown to facilitate gene movement (figure 2). Insertion sequences and transposons are mobile within the genome of gram-negative bacteria, and can act as new promoters or as terminators (Wyndham, 1994). If two IS elements are located near each other in the appropriate orientation, they can be transposed to a second genome as

a unit, along with any intervening genes (OECD, 1997). Nakatsu and Wyndham (1993) found the IS1071 element to be connected with transposition events. The ADP-1 plasmid would likely have caused the transconjugants to grow more slowly (due to the increased metabolic load) unless a genetic rearrangement relieved this pressure (for example, a transposition of the atrazine degrading genes to the genome).

There are three ways that genes may have re-arranged to reduce the metabolic load imposed by the large ADP-1 plasmid. They may have integrated into the chromosome of the transconjugant, moved onto smaller plasmids, or the bacteria could have worked as a consortium where a number of bacteria were involved in the degradation of atrazine (De Souza et al., 1998c). For the purpose of this study, only the first two rearrangements will be considered as in the last case the bacteria would not be selected for due to an inability to degrade atrazine alone. These rearrangements could account for the apparent loss of the large plasmid in some transconjugants, as well as the loss of the junction from IS1071 to *atzA* and/or IS1071 to *atzB* (figure 13 and table 2). Topp *et al.* (2000) found that the atrazine genes from soil bacteria were separated on 2 or 3 plasmids, and generally the *atzB* gene was accompanied by an IS1071 element. De Souza *et al.* (1998b) found the atrazine clearing phenotype of *P. ADP* was unstable during continued culturing on laboratory media, especially in the absence of atrazine as the sole nitrogen source. They also found upon repeated sub-culturing on

atrazine media, that the phenotype did not reappear and subsequent genotype analysis confirmed the loss of *atzABC* genes (De Souza *et al.*, 1998). Our finding combined with finding of previous studies suggest the ADP-1 plasmid is unstable and gene rearrangement can occur.

Studies have shown that the fitness of the host is adversely affected when genetic modifications have been made. Winstanley *et al.* (1993) studied the survival of *P. putida*, with a visible marker inserted, in soil and lake water microcosms. When these bacteria were released into these microcosms, the marked *P. putida* died due to the increased metabolic load. Similarly, Iwasaki *et al.* (1994) reported that the density of *P. putida* containing a plasmid coding for mercury resistance decreased rapidly to less than 10^2 to 10^3 CFU/gm within five days in soil microcosms. These studies concur with our findings that the fitness of the introduced host was adversely affected when it was genetically modified in both soil and rhizosphere experiments (figure 9).

4.7 Transconjugant identification

Potential transconjugants were isolated during the experiment from the atrazine selective plates. These were maintained as frozen glycerol stocks and re-plated at the end of the field and microcosm trials. The 150 potential transconjugants were purified and plated onto different types of media to characterize growth characteristics, for example TYE and KingsB. Only 10 transconjugants were chosen for further identification from a variety of

conditions and sampling dates. Two of the possible transconjugants were detected only upon growth of bacteria on enrichment media (i.e. not found by dilution plating). The number of potential transconjugants I chose to characterize was limited for two reasons; financial constraints, and also since a reliable method was being designed for future work, a small sample was chosen to determine the most appropriate technique.

The identification of the ten potential transconjugants was completed by biolog and 16s rRNA sequencing from PCR products obtained by amplification using primers corresponding to base 63 to 1378 of the 16s rRNA gene of *E. coli* (Marchesi *et al*, 1998). Biolog was used primarily to ensure the potential transconjugants had a different substrate use pattern (table 1).

4.7.1 Identification of the 8 transconjugants isolated from field and microcosm experimental trials

BLAST comparisons with the NCBI database, of the 16S rRNA sequences obtained for the potential transconjugants, isolated during the field and microcosm trials, indicated all were Pseudomonads (table 1). According to a report by OECD (1997) 16S rRNA sequencing cannot conclusively differentiate between *P. putida*, *P. fluorescens* and *P. chlororaphis*. Gene transfer between Pseudomonads in the environment has been shown to occur, as well. Ravatn *et al.* (1998) described the horizontal transfer of chlorocatechol degradation genes between *P. B13* to *P. putida* in activated

sludge. A sequence alignment and phylogenetic tree analysis shows the sequence similarity between the strains and shows that none have the same composition as the inoculated bacteria (figure 14). Interestingly, the first transconjugants isolated (during the first 24 hr) were 100% similar (indicating they are likely the same strain) and highly similar to the host bacteria. Most of the strains isolated from day 3 and day 15, also showed a high similarity to the inoculated bacteria, indicating they are closely related strains to the inoculum and the transconjugants isolated from the earlier sampling dates. One sample isolated from the rhizosphere of an atrazine treated microcosm on day 15 is identified as a *Pseudomonas* by sequence identification, but according to a sequence alignment appears to be distantly related to the other *Pseudomonas* transconjugants. The identification results found for the transconjugants isolated during the field and microcosm trials indicate that the genes were often transferred, stably maintained and expressed by bacteria closely related to the GMO.

4.7.2 Identification of the 2 transconjugants isolated through enrichment experiments

Two potential transconjugants were isolated from enrichment of a rhizosphere from an atrazine-treated microcosm after the experimental trials were finished. These were identified by BLAST comparison of their 16S rRNA sequence as *Arthrobacter*. Isolating a gram-positive transconjugant was an unexpected result, although a previous prerelease trial also found the plasmid

was transferred to gram-positive bacteria (J. Germida, personal communication). *Arthrobacter* are obligate aerobes, catalase positive, chemo-organotrophic bacteria. They form rod and coccial cells during their growth cycle and are common in soil environments, with an optimum growth temperature of 25-30°C. Although they are gram positive, de-colorization during gram staining can easily occur and can be misidentified as gram-negative bacteria. They are able to utilize a wide range of carbon sources, including herbicides, which makes them very competitive bacteria. They are also highly resistant to desiccation and starvation. The ubiquitous nature of *Arthrobacter*, coupled with their ability to degrade herbicides like glyphosate (N-phosphomethyl-glycine) and pentachlorophenols (PCPs), suggests that they could become useful in environmental cleanup. Strong *et al.* (2002) recently discovered an *Arthrobacter* with the ability to use atrazine as a sole carbon and nitrogen source. The isolation of a transconjugant with very little relation to the host indicates that the genes can be transferred, maintained and expressed in bacteria with a low similarity to the host GMO.

Figure 14: 16s rRNA sequence comparison for atrazine-degrading transconjugants isolated from the field and microcosm (refer to section 2.8.2).

4.8 Genetic characterization of transconjugants

The isolation of the transconjugants was based on their ability to degrade atrazine on selective media and their identification was based on 16S rRNA sequences. This does not give information about the status of the plasmid and atrazine-degrading genes. To confirm the presence of the ADP-1 plasmid, as well as determine genetic rearrangements of the *atz* genes, segments of the plasmid were amplified using PCR. The areas that were amplified included a section of each of the atrazine genes required for degradation. Movement of the atrazine genes is often associated with a loss of a copy of the IS1071 element (Topp *et al.*, 2000); therefore, the junction between *atzA* and *atzB* with IS1071 was also amplified. To check for the presence of the large ADP plasmid, a signature region at the junction of the *tfrA-tnpA* sequence was amplified and the presence of the plasmid was checked by PFGE. These experiments were carried out to identify genetic rearrangements of the introduced genes (figure 12, figure 13 and table 2).

Four of the isolated transconjugants showed the same results for all PCR reactions as were generated with the host bacteria, as well as a large plasmid by PFGE. These are from day 1 field (1/FSA) and microcosm (1/LSA) soil treated with atrazine and day 15 from the field, both treated (15/FRA) and untreated (15/FR) with atrazine. In these four transconjugants it appears that the ADP-1 plasmid was transferred completely and stably maintained.

In one transconjugant (3/FSA), isolated on day 3 from a field soil plot, a rearrangement of the atrazine genes was evident since no large plasmid was detected by PFGE and there was no amplification of the *tfrA-tnpA* region. But the atrazine genes and IS elements were present according to the production of an amplification product by PCR of equal size to the host strain. It is likely that the genes moved to the chromosome or to smaller plasmids. Another transconjugant (15/LR) from the rhizosphere in a microcosm on day 15, also lacked a large plasmid and the signature region of pADP. However, for this transconjugant the junction regions between IS1071 and *atzA*, and IS1071 and *atzB* were also not amplified. This sample was isolated from a microcosm containing no selective pressure. In this case, the *atz* genes may have been transferred to the chromosome or a smaller plasmid but lost some of the genes during the transposition. The apparent loss of two IS elements indicates the movement of the genes may have been transposon mediated.

Two *Arthrobacter* were identified as potential transconjugants. Both were isolated by enrichment from the rhizosphere of an atrazine-treated microcosm --one on day 64 (64/LRA), and the other on day 95 (95/LRA). The 64/LRA did not show an amplification product with any of the PCR reactions used for characterization. In contrast the 95/LRA contained the *atzA* and *atzB* genes; all other tested regions were missing. Both of these *Arthrobacter* lacked a large plasmid. As previously mentioned, the field site did not have bacteria that could degrade atrazine. Therefore the 95/LRA most likely picked up the

genes from the pADP plasmid, but because the 64/LRA did not appear to contain these genes, the source of its atrazine degradation ability is unknown. The 95/LRA transconjugant appears to be similar to a recent discovery by Strong *et al.* (2002) who found their isolated *Arthrobacter* was able to degrade atrazine and showed sequence similarity to *atzB* and *atzC* but not *atzA*.

The discovery of the *Arthrobacter* with the ability to degrade atrazine without containing a gene similar to *atzA* provides evidence that there is another *atzA*-like gene which can complete the first stage in the degradation pathway (figure 1). This *atzA*-like gene must also carry out the dechlorination of atrazine to hydroxyatrazine, and this gene may also be present in two other transconjugants (2/FSA, 15/LRA, table 2) which did not have the *atzA* gene.

DeSouza *et al.* (1998a) hypothesized that the ADP-1 plasmid was derived from smaller plasmids, which had contained the *atz* genes individually, and that it had been formed by the transposon-mediated movement of the genes onto the one plasmid. Topp *et al.* (2000) found that the loss of the *atzA* and *atzB* genes from the pADP was due to homologous recombination between the IS1071 insertion sequence elements. It was therefore expected that genetic rearrangement in our transconjugants would be transposon mediated. However, of the 10 transconjugants studied, only two showed evidence of

IS1071 mediated gene movement (15/LR, 95/LRA) as reflected by the lack of amplification product for the junctions of IS1071 to *atzA* and *atzB*. The transconjugant from day 15 also appeared to have lost the plasmid, thus indicating that the genes had transferred to another plasmid or plasmids, or may have been transposed onto the chromosome. In contrast the 95/LRA still contained the signature region of the ADP-1 plasmid, but lacked the full sized plasmid as revealed by PFGE (table 2).

The results from this set of experiments indicated that although the transconjugants appeared closely related (by 16S rRNA sequencing, figure 14) their transconjugation events were likely independent as revealed by the divergent PCR profiles. For example, 1/FSA and 2/FSA show high sequence similarity of their 16S rRNA but 2/FSA does not show presence of the *atzA* gene. Conversely, sample 15/LRA shows very little sequence similarity of the 16s rRNA to 2/FSA but shows the same PCR profile. For example, a smaller plasmid which did not contain transposable elements would have been easier to monitor in the environment.

4.9 Horizontal transfer of the atrazine degradation genes

The three common systems for gene transfer in bacteria, namely conjugation, transduction and transformation, have been observed among members of the genus *Pseudomonas*. All three gene transfer mechanisms have been

observed under laboratory and natural conditions (OECD, 1997). Gene transfer by all three mechanisms is affected by biological factors such as the nature and host range of the mobile genetic element, the concentrations of recipient and donor organisms, and the presence of other organisms which prey on donors and recipients. Abiotic factors such as temperature, moisture, and soil composition affect the gene transfer frequency (Sayre and Miller, 1990).

Conjugation is generally accepted as the most common method of horizontal gene transfer in the environment. But transformation of both chromosomal and plasmid DNA has also been shown to occur in soil environments (Lorenz *et al.*, 1988; Lorenz *et al.*, 1991; Paul *et al.*, 1987; Paul *et al.*, 1991; Stewart, 1990; Stewart and Sinigalliano, 1991). Studies have shown that soil environments provide protection from nuclease activity due to the interaction between the DNA and clay and other soil particles (Lorenz and Wackernagel, 1991; Khanna and Stotzky, 1992; Romanowski *et al.*, 1991). During natural transformation, the DNA is lost from the donor cell and must be in close proximity to a naturally competent cell. The foreign DNA must then be able to integrate into the new hosts DNA without being degraded. To our knowledge no previous studies have demonstrated that *Pseudomonads* can conjugate with *Arthobacter*, therefore, it is more likely that the *Arthobacter* transconjugant (95/LRA) acquired the ability to degrade atrazine through transformation of the introduced genes.

4.9.1 Potential transconjugants not isolated

Because this research is a risk assessment initiative, it is important to identify the limitations of the study. De Souza *et al.* (1998c) found evidence that a bacterial consortium can degrade atrazine. In this study, many of the frozen potential transconjugant cultures were actually composed of two or more bacteria that alone could not degrade atrazine (data not shown). The separation of the atrazine degrading genes among several bacteria would alleviate the metabolic load on a single bacterium. For example, if atrazine were partially degraded to hydroxyatrazine by one bacterium, another containing the *atzB* gene would be able to de-aminatate hydroxyatrazine to form N-isopropylammelide (figure 1). These consortiums would not be identified as transconjugants in our experimental system.

4.8 Future Studies

The objective of this study was to isolate bacteria which expressed the ability to degrade atrazine, but, because we were detecting only those with complete atrazine degrading ability, there were likely other bacteria that contained only a portion of the atrazine degrading genes. Although it is important to have a method to monitor expression of foreign genes (in this case atrazine-degradation) released into the environment, a complete risk assessment should also detect just the presence of the genes. Using PCR to characterize the DNA in a soil sample may be a better way to monitor the persistence of the genes.

There have been several techniques developed to isolate DNA directly from soil samples (Thiem *et al.*, 1994; Zhou *et al.*, 1995). These have been used to quantitatively assess gene persistence in natural environments and to apply DNA diagnostic techniques to accurately identify a bacteria (Allison *et al.*, 2001; Brumlik *et al.*, 2001; Ferris *et al.*, 1996; MacNaughton *et al.*, 1999; Muyzer *et al.*, 1993; Rolleke *et al.*, 1996; Teske *et al.* 1996a; Teske *et al.* 1996b; and Wawer *et al.*, 1995). Dejonghe *et al.* (2000) has used one of these DNA diagnostic techniques, known as denaturation gradient gel electrophoresis to follow the movement of foreign genes, as well as the change in the indigenous bacteria community structure.

One of the main difficulties with the selective plating method was that it was optimized for the host, and may not have been suitable for all potential recipients. But even direct PCR extraction of DNA from soil may not be suitable for all transconjugants. A new promising method described by Spiro and Lowe (2002) uses an affinity bead-based method for quantifying target DNA in the environment, but it has not been extensively tested. All DNA based technologies are more expensive than selective plating, but offer more reliable results.

The ultimate goal of this type of validation study is to develop a method which provides a true representation of the recipient bacterial community. This

would enable the establishment of a mathematical model to evaluate gene transfer frequency in the environment. This project tested a potential model system for the quantitative assessment of horizontal gene transfer.

5.0 Conclusions

In this project we set out to explore if the simple PVC soil microcosm, validated in the previous study (Gagliardi *et al.*, 2001), can be used to predict the field behavior of a GMO containing the catabolic plasmid ADP-1. The three questions posed in the hypothesis section are addressed below.

1. Did the GMO find a niche in the parental strain environment?

Yes, the bacteria did appear to initially find a niche in the soil plots and microcosms; however, the increase in atrazine degraders was short lived. The rhizosphere plots did not indicate that the introduced bacteria had formed an association and were not detectable by the second rhizosphere sampling.

2. Can transconjugants be cultured using a selective plating method?

Transconjugants were isolated using the selective plating method. However, they probably represent only a small fraction of the transconjugants. This method likely only selected for bacteria similar to the host.

3. Was the plasmid stably maintained in the transconjugants?

The atrazine genes were transferred to transconjugants in the environment. Four of the eight transconjugants isolated during the field and microcosm trials stably maintained the ADP-1 plasmid. Two showed a loss of the *atzA* genes, while two others lost the ADP-1 plasmid, but maintained the atrazine degradation genes.

4. Do trends from the microcosms trials reflect the results from the field studies?

Yes there were similar trends of atrazine degrader survival in the soil microcosms and field trials and both were *Pseudomonas* species.

On a final note, the most important finding from this experiment was the isolation of the *Arthrobacter* atrazine degrading strain on day 95. Its isolation indicates that foreign genes are able to horizontally transfer across a wide range of bacteria. Clearly horizontal gene transfer is an important consideration for any environmental release of a genetically modified micro-organism.

7. References

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Appendix 1. Minimal media A composition

352 ml	ddH ₂ O
40 ml	Phosphate (10X)
4ml	MgSO ₄ (100X)
4 ml	Trace Elements (100X)
Total volume 400ml	

Trace Elements

Na ₂ EDTA 2H ₂ O	12 g
NaOH	2 g
ZnSO ₄ 7H ₂ O	0.4 g
MnSO ₄ 4H ₂ O	0.4 g
CuSO ₄ 5H ₂ O	0.1 g
FeSO ₄ 7H ₂ O	3.0 g
pH 7.2	
Na ₂ SO ₄	5.0 g
NaMO ₄ 2H ₂ O	0.1 g

Appendix 2. Creation of the GMO. Genetic modifications

Genetic Element	Size & Location	Function	Source
Antibiotic resistance rifampicin, naladixic acid, streptomycin	Chromosome of <i>P. putida</i> CR30	Antibiotic resistance	Selective pressure experiments
pADP-1	108 kb strain CR30RNS	Contains genes for atrazine degradation	enrichment of atrazine contaminated soil
<i>kilAteIAB</i> <i>mini Tn5 transposon</i>	2.958 kb inserted in pADP-1	Tellurite (TeO ₃) resistance	plasmid RK2 of <i>Klebsiella</i> <i>aerogenes</i>

Appendix 3: Field site layout, the squares represent the plots with each in triplicate, samples for off site monitoring and gene movement were removed from between the plots as well as 4 m in each navigational direction from the site

Appendix 4. Field and microcosm sampling schedule

Time	Sample	Test
3hr	FS, FSA, LS, LSA	Survival
1d	FS, FSA, LS, LSA	Gene transfer
2d	FS, FSA, LS, LSA	Gene transfer
3d	FS, FSA, LS, LSA	Gene transfer
7d	FS, FSA, LS, LSA	Survival
8d	FS, FSA, LS, LSA	Gene transfer
14d	FS, FSA, LS, LSA	Survival
14d	FR, FRA, LR, LRA	Survival
21d	FS, FSA, LS, LSA	Survival
21d	FR, FRA, LR, LRA	Survival
22d	FS, FSA, LS, LSA	Gene transfer
22d	FR, FRA, LR, LRA	Gene transfer
35d	FS, FSA, LS, LSA	Survival
35d	FR, FRA, LR, LRA	Survival
36d	FS, FSA, LS, LSA	Gene transfer
36d	FR, FRA, LR, LRA	Gene transfer

Appendix 5. Primers used in the characterization of the ADP plasmid

AtzA- F	GTTGTGAGCAATGCCTACCTCGG
AtzA- R	AGTGTCCACCTCATTGCCGTAAGC

(Topp *et al.*, 2001)

AtzB- F	TCACCGGGGATGTCGCGGGC
AtzB- R	CTCCCCGCATGGCATCGGG

(Topp *et al.*, 2001)

AtzC- F	GCTCACATGCAGGTACTCCA
AtzC- R	GTACCATATCACCGTTTGCCA

IS1071- F	CCGCGCCAGTGCTAGCTTTGCGTAC
AtzA – R	GGACTCTGGGTACTGATCCA

(Natalie DuCharme, unpublished)

IS1071- F	CCGCGCCAGTGCTAGCTTTGCGTAC
AtzB – R	GCTGGTGAAACCCGGTGTA

TfrA- F	AACGACGACCTGGTGCATTGCA
TnpA – R	CTGCGAGCAGAAACCCTCAAT

63-F	CAGGCCTAACACATGCAAGTC
1378- R	GGGCGGAGTGTACCAAGGC

(Marchesi *et al.*, 1998)

Appendix 6. Sequences for 16s rRNA identification of transconjugants.
The 16s rRNA gene was amplified using primers designed corresponding to base 63 to1378 of the 16s rRNA gene of *E.coil* (Marchesi *et al.*, 1998).

1FSA

TGATT	CAGCG	GCGGACGGGT	GAGTAATGCC	TAGGAATCTG	CCTGGTAGTG	GGGGACAACG
TTTCG	AAAAG	AACGCTAATA	CCGCATACGT	CCTACGGGAG	AAAGCAGGGG	ACCTTCGGGC
CTTGCG	CTAT	CAGATGAGCC	TAGGTCGGAT	TAGCTAGTTG	GTGGGGTAAT	GGCTCACCAA
GGCGAC	GATC	CGTAACTGGT	CTGAGAGGAT	GATCAGTCAC	ACTGGAACTG	AGACACGGTC
CAGACT	CCTA	CGGNAGGCAG	CAGCGGGGAA	TATTGGACAA	TGGGCGAAAAG	CCTGATCCNG
CCNTG	CCGCG	TGTGTGAAGA				

1SA

GCCTG	GAGT	GGGGGACAAC	GTTTCGAAAAG	GAACGCTAAT	ACCGCATACG	TCTACGGGA
GAAAG	CAGGG	GACCTTCGGG	CCTTGCGCTA	TCAGATGAGC	CTAGGTCGGA	TTAGCTAGTT
GGTGG	GGTAA	TGGCTCACCA	AGGCGACGAT	CCGTAACTGG	TCTGAGAGGA	TGATCAGTCA
CACTG	GAACT	GAGACACGGT	CCAGACTCCT	ACGGGAGGCA	GCAGTGGGGA	ATATTGGACA
ATGGG	CGAAA	GCCTGATCCA	GCCATGCCGC	GTGNGTGAAG	AAGGTCTTCG	GATTGTANAG
CAC	TTTAA	GTGGAGGAAG	GGCACCAAGC	TAATACCTTG		

2FSA

TGATT	CAGCG	GCGGACGGGT	GAGTAATGCC	TAGGAATCTG	CCTGGTAGTG	GGGGACAACG
TTTCG	AAAAG	AACGCTAATA	CCGCATACGT	CCTACGGGAG	AAAGCAGGGG	ACCTTCGGGC
CTTGCG	CTAT	CAGATGAGCC	TAGGTCGGAT	TAGCTAGTTG	GTGGGGTAAT	GGCTCACCAA
GGCGAC	GATC	CGTAACTGGT	CTGAGAGGAT	GATCAGTCAC	ACTGGAACTG	AGACACGGTC
CAGACT	CCTA	CGGNAGGCAG	CAGCGGGGAA	TATTGGACAA	TGGGCGAAAAG	CCTGATCCNG
CCNTG	CCGCG	TGTGTGAAGA				

3FS

NAGTAATGCC	TAGGAATCTG	CCTGGTAGNG	GGGGACAACG	TTTCNAANGG	AACGCTAATA
CCGCATACGT	CCTACGGGAG	AAAGCAGGGG	ACCTTCGGGC	CTTGCGCTAT	CAGATGAGCC
TAGGTCGGAT	TAGCTAGTTG	GTGGGGTAAT	GGCTCACCAA	GGCGACGATC	CGTAACTGGC
TGACAGGATG	ATCANTCACA	CTGGAACCTGA	GACACGGTCC	ANACTCCTAC	GGGAGGCAGC
AGTGGGGAAAT	ATTGGACAAT	TGGGCTGAAA	GCCTGATCCN	NCCATGCCGC	GTGNGTGAAG
AAGGTCTTCG	GATTGTNAAG	CAC	TTTAA	GTGGAGGAAG	GGCANTAAGC
TAATACCTTG	CTGTTTTGAC	GTTACCGACA	GAATAAGCAC	CGGCTAACTC	TGTGCCAGCA
GCCC	CGGTA				
ATACAGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCGNCG	TCANGNGGTN
CNTTAAGNTG	NATGNGAAAG	CCCCGGGCTC	AACCTGGGAA	CTGCATCCAA	AACTGGCGAN
CTAGAGTACN	GTNGAGGGTG	NCTGGAATTT	CCTGNGTANC	GGTNAAATGC	

15FR

CTGCGTTCNA	CGGGNNANTA	ATGCCTANGA	ATCNGCCTGN	NAGTGGGGGA	CAACGTTTCN
AAAGGAACGC	TAATACCGCA	TACGTCCTAC	GGGAGAAAAGC	AGGGGACCTT	CGGGCCTTGC
GCTATCAGAT	GAGCCTAGGN	CNGATTACCT	ANNTGGTGGG	GTAATGNTCA	CCAAGGCGAC
GATCCGTAAC	TGGTCTGAGA	GGATTGATCA	GTCACACTGG	AACTGANACA	CGGTCCAGAC
TCCTACGGGA	GGCAGCAGTG	GGGAATATTG	NACAAATGGGC	GAAAGCCTGA	TCCAGCCATG
CCGCGTGTGT	GAANAAGGNC	TTCGGATTGT	AAAGCACTTT	ANGTTGGGAG	GAAGGGCAGT
AAGCTAATAC	CTTGCTGTTT	TGACGTTACC	GACAGAATAA	GCACCGGCTA	ACTCTGTGCC
ANCAGCNCGC	GGTAATACAG	AGGGTGCAAG	CGTTAATCGN	NAATTACTGG	GNGTAAAGCG
CGCGTAGGTG	GTTCNTTAAN	NTGGATGTGA	AAGCCCCGGG	CTCAACCTGG	GA
CANA	ACTGGC	GAGCTAGAGT	ACNGTAGAGG	GTG	

15FRA

AGTAATGCCT AGGAATCTGC CTGGTAGNGN GGGACAACGT TTCGAAAGGA ACGCTAATAC
 CGCATACGTC CTACGGGAGA AAGCAGGGGA CCTTCGGGCC TTGCGCTATC ANATGAGCCT
 AGGTCGGATT AGCTAGTTGG TGGGGTAATG GCTCACCAAG GCGACGATCC GTAACCTGGT
 TGAGAGGATG ATCAGTCACA CTGGAACTGA GACACGGTCC AGACTCCTAC GGGAGGCAGC
 AGTGGGGAAT ATTGGACAAAT GGGCGAAAAGC CTGATCCAGC CATGCCGCGT GTGTGAAGAA
 GGTCTTCGGA TTGTAAAGCA CTTTAAGTTG GGAGGAAGGG CAGTAAGCTA ATACCTTGCT
 GTTTTGACGT TACCGACAGA ATAAGCACCG GCTAACTCTG TGCCAGCAGC CGCGGNAATA
 CAGAGGGTGC AAGCGTTAAT CGGAATTACT GGGCCGTAAA GCGCGCGTAG GTGGTTCGTT
 AAGTTGGATG TGAAAGCCCC GGNTCAACC TGGGAACGTC ATCCAAAACCT GGCGAGCTAG
 AGTACGGTAN AGGNTGNTGG AATTTCCCTGT GTAGCGGTGA AATGCGTAGA TATNGGAAGG
 AACACCAGTG GCCGAAGGCC

15LR

CTGCGTTCNA CGGGNNANTA ATGCCTANGA ATCTGCCTGG NAGTGGGGGA CAACGTTTCG
 AAAGGAACGC TAATACCGCA TACGTCCCTAC GGGAGAAAGC AGGGGACCTT CGGGCCTTGC
 GCTATCAGAT GAGCCTAGGT CGGATTACCT AGTTGGTGGG GTAATGGCTC ACCAAGGCGA
 CGATCCGTAA CTGGTCTGAG AGGATGATCA GTCACACTGG AACTGACACA CGGTCCAGAC
 TCCTACGGGA GGCAGCAGTG GGGAAATATTG GACAATGGGC GAAAGCCTGA TCCAGCCATG
 CCGCGTGTGT GAAGAAGGTC TTCGGATTGT AAAGCACTTT ACGTTGGGAG GAAGGGCAGT
 AAGCTAATAC CTTGCTGTTT TGACGTTACC GACAGAATAA GCACCGGCTA ACTCTGTGCC
 AGCAGCCGCG GTAATACAGA GGGTGCAAGC GTTAATCGGA ATTACTGGGN GTAAAGCGCG
 AAACCTGGCGA GCTAGAGTAC GGTAGAGGGT G

15LRA

GGATTACTAN CTGATTCCGA CTTACGCAG TCGAGTTGCA GACTGCGATC CGGACTACNA
 TCGGTTNTGC GAGATTAGCT CCACCTCGCG GCTTGGCAAC CCTCTGTACC GACCATTGTA
 GCACGTGTGT AGCCCAGGCC GNAAGGGCCA TGATGACTTG ACGTCATCCC CACCTTCCTC
 CGGNTTGTCA CCGGCAGTCT CTTACAGTG CCCACCATAA TGTGCTGGTA ACTAAGGACA
 AGGGTTGCGC TCGTTACGGG ACTTAACCCA ACATCTCACG ACACGAGCTG ACGACAGCCA
 TGCAGCACCT GNGNCAGAGT TCCCGAAGGC ACCAATCCAT CTCTGGAAAG TTCTCTGCAT
 GTCAAGGCCT GGCAANGCTC TTCGCGNTGC TTCGAATTAA ACCACATGCT CCACCGCTNG
 TGCGGGCCCC CGTCAATTCA TTTGAGTTTT AACCTTGCGG CCGNACTCCC CNGGCGGTCA
 ACTTAATGCG TTAGCTGCGC CACTAAAATC TCAAGGATTC CAACGGCTAG TTGACATCGC
 TTACGCCCCG

64LRA

CGGGTGANTA ACACGTGAGT AACCTGCCTT GACTCTGGGA TAAGCCTGGG AAACCTGGGTC
 TAATACCGGA TACGACCATC TGGCGCATGT CATGGTGGTG GAAAGCTTTT GTGGTTTTGG
 ATGGACTCGC GGCCATCAG CTTGTTGGNG GGGTAATGGC CTACCAAGGC GACGACGGGT
 AGCCGGCCTG AGAGGGTGAC CGGCCACACT GGGACTGAGA CACGGCCCAG ACTCCTACGG
 GAGGCAGCAG NGGGGAATAT TGCACAATGG GCGCAAGCCT GATGCAGCGA CGCCGCGTGA
 GGGATGACGG CTTTCGGGTT GTAAACCTCT TTCANTAGGG AAGAAGCGAA AGTGACGGTA
 CCTGCAGAAG AAGCGCCGCG TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGGCGCAA
 GCGTTATCCG GAATTATTGG GCGTAAAGAG CTCGNAGGCG NTTTGTNCNG TCTGCTGTGA
 AANACCGGGG CTCAACTCCG GTTCTGCAGC

95LRA

ACGGGTGACT AACACGTGAG TAACCTGCCC TTGACTCTGG GATAAGCCTG GGAACTGGG
 TCTAATACCG GATACGACCA TCTGGCGCAT GTCATGGTGG TGGAAAGCTT TTGTGGTTTT
 GGATGGACTC GCGGCCTATC AGCTTGTTGG TGGGGTAATG GCCTACCAAG GCGACGACGG
 GTAGCCGGCC TGAGAGGGTG ACCGGCCACA CTGGGACTGA GACACGGCCC AGACTCCTAC
 GGGAGGCAGC AGTGGGGAAT ATTGCACAAAT GGGCGCAAGC CTGATGCAGC GACGCCGCGN
 GAGGGATGAC GGCCCTTCGGG TTGTAAACCT CTTTCAGNAN GGAAGAAGCG AAAGTGACGG
 TACCTGCAGA AGAAGCGCCG GCTAACTACN TGCCANCAGC CGCCGGCAAT ACGTAGGGCG
 CAAGCGCTAT CCGGAATTAT TGNNGCGTAA CGAGCTCNTA TCNNCNTTTG

CR30RNSpADPte1

GATTCAGCGG	CGGACGGNTG	AGTAATGCCT	AGGAATCTGC	CTGGTAGTGG	GGGACAACGT
TTCGAAAGGA	ACGCTAATAC	CGCATACGTC	CTACGGGAGA	AAGCAGGGGA	CCTTCGGGCC
TTGCGCTATC	AGATGAGCCT	AGGTCGGATT	AGCTAGTTGG	TGGGGTAATG	GCTCACCAAG
GCGACGATCC	GTAAC TGGTC	TGAGAGGATG	ATCAGTCACA	CTGGAACTGA	GACACGGTCC
AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	ATTGGACAAT	GGGCGAAAGC	CTGATCCAGC
CATGCCCGGT	GTGTGAAGAA	GGTCTTCGGA	TTGTAAAGCA	CTTTAAGTTG	GGAGGAAGGG
CAGTAAGCTA	ATACCTTGCT	GNITTTGACGT	TACCGACAGA	ATAAGCACCG	GCTAACTCTG
TGCCAGCAGC	CGCGGTAATA	CAGAGGGTGC	AAGCGTTAAT	CGGAATTACT	GGGCGTAAAG
CGCGCCGTAG	GNGGCTCGTT	AAGTTGGATG	TGAAAGCCCC	GGGCTCAAAC	CTGGGAACTG

Appendix 7. Chromosomal digest of DNA from transconjugants not showing presence of the *atzA* or *atzB* gene from pADP by a negative PCR result. This digest was southern blotted and probed with DIG-labelled *atzA* and *atzB*. No hybridization occurred.