Evaluation of Terrestrial Microcosms for Assessing the Fate and Effects of Genetically Engineered Microorganisms on Ecological Processes

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EVALUATION OF TERRESTRIAL MICROCOSMS
FOR ASSESSING FATE AND EFFECTS OF
GENETICALLY ENGINEERED MICROORGANISMS
ON ECOLOGICAL PROCESSES

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EXECUTIVE SUMMARY

The principal objective of this project was to evaluate and modify the existing U.S. Environmental Protection Agency's Office of Pesticides and Toxic Substances (EPA/OPTS) terrestrial microcosm test system and test protocols such that they can be used to determine the environmental fate and ecological hazards of genetically engineered microorganisms (GEMs). The intact soil-core microcosm represents terrestrial ecosystems, and when coupled with appropriate test protocols, such microcosms may be appropriate to define and limit risks associated with the intentional release of GEMs.

The terrestrial microcosm test system was used to investigate the survival and transport of two model GEMs (*Azospirillum lipoferum* and *Pseudomonas* sp. Tn5 mutants) to various trophic levels and niches and through intact soil cores. Subsequent effects on nutrient cycling and displacement of indigenous microorganisms were evaluated. The model organisms were a diazotrophic root-colonizing bacterium (*A. lipoferum*) and a wheat root growth-inhibiting rhizobacterium (*Pseudomonas* sp.). The transposable element Tn5 was used as a genetic marker for both microorganisms in two separate experiments. The organisms were subjected to transposon mutagenesis using a broad host-range-mobilizable suicide plasmid.

The transposon Tn5 conferred levels of kanamycin resistance up to 500 µg/ml (*Pseudomonas* sp.), which allowed for selection of the bacteria from environmental samples. The presence of Tn5 DNA in the genome of the model GEMs also allowed the use of Tn5 gene probes to confirm and enumerate the microorganisms in different samples from the microcosms. Two types of root growth-inhibiting *Pseudomonas* sp. Tn5 mutants were obtained and used in microcosm studies: those that lacked the ability to inhibit either wheat root growth or the growth of other microorganisms in vitro (tox⁻) and those which retained these properties (tox⁺).

Intact soil cores for use as microcosms were obtained from the Palouse region of eastern Washington State and the Columbia Basin of south-central Washington State. The GEMs were either inoculated directly onto seeds (*A. lipoferum*) or mixed with the plow layer of soil (*Pseudomonas* sp.). GEM colonization of the rhizosphere, rhizoplane, and bulk soil was analyzed with respect to depth. Transport of the bacteria through the soil cores with percolating water, and the potential for bacterial transport by insects and earthworms, were analyzed. The concentration of soluble nutrients in microcosm leachates was analyzed to assess any impacts on soil nutrient fluxes caused by introduction of the bacteria. Potential ecological impacts in the form of niche displacement of indigenous rhizosphere microorganisms were also measured.

The studies with the two model GEMs, which had contrasting results, supported the usefulness of this microcosm design for studying fate and transport of a variety of genetically engineered bacteria that are intended for environmental release. The *A. lipoferum* mutants were relatively noncompetitive in the rhizosphere and were not detected in bulk soil, insect gut, or microcosm leachate. In contrast, the *Pseudomonas* sp. Tn5 mutants extensively colonized the rhizosphere and rhizoplane and were present in the bulk soil throughout the entire soil core (60 cm long). The Tn5 mutants were also detected in earthworms that were introduced into the microcosms. Colonization of the wheat seedling rhizoplane by the *tox*⁺ mutants was greater than colonization by the *tox*⁻ mutants, indicating that toxin production by the *Pseudomonas* sp. mutants provided a competitive advantage in the early stages of plant growth. In neither study were significant impacts on nutrient fluxes (N, C, P, S) or indigenous rhizosphere microflora (*Rhizobium leguminosarum*, nitrifying bacteria, total heterotrophic bacteria, or asymbiotic N₂-fixing bacteria) observed from inoculation with the transposon mutants.

The contrast between the colonization and transport of these two microorganisms in intact soil-core microcosms indicates that this microcosm design is useful for testing GEMs to assess their environmental fate and potential ecological effects. Results from this research are being used to help develop guidelines and test protocols that can decrease risks associated with intentional release of GEMs.

This report is organized into three sections with two appendices to provide guidelines and protocols for using intact soil-core microcosms in Biotechnology Risk Assessment. Section 1.0 describes the results of the initial experiment using *A. lipoferum* Tn5 mutants as model GEMs. Section 2.0 focuses on nutrient dynamics in the soil cores and the potential for using nutrient efflux as a tool to monitor potential ecological effects from the release of genetically engineered microorganisms. Because of the limited transport and detection of *A. lipoferum* mutants used in the initial experiment, a second experiment was designed and conducted, using a root colonizing *Pseudomonas* sp. (Section 3.0) to obtain additional data and modify protocols to refine the utility of this microcosm design for Biotechnology Risk Assessment. This second experiment, although not included in the original proposal, was necessary to complete the principal objective, which was to modify the existing EPA/OPTS protocol (EPA/600/3-85/047) for terrestrial microcosms to allow the evaluation of genetically engineered microorganisms.

Appendix A includes the Standard Operating Procedures that were developed for this project. Appendix B includes the designs for a microcosm containment system (MCS) that was

included as an option with the original proposal. A microcosm containment system was constructed from the first design presented in this appendix, and the second design reflects the changes deemed necessary after construction and testing of the prototype.

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1.0 INTACT SOIL-CORE MICROCOSMS FOR EVALUATING THE FATE OF GENETICALLY ENGINEERED MICROORGANISMS IN THE ENVIRONMENT*

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SUMMARY

Intact soil-core microcosms were studied to determine their applicability for evaluating the transport, survival, and potential ecosystem effects of genetically engineered microorganisms before they are released into the environment. Microcosms were planted with wheat and maize seeds and inoculated with *Azospirillum lipoferum* SpBr17 and SpRG20a Tn5 mutants, respectively. Heat-killed cells served as controls. Above-ground plant biomass and leaf tissue nitrogen were measured to determine inoculation effects on plant growth. Microcosm leachate, rhizosphere soil, plant endorhizosphere, and xylem exudate were sampled for *A. lipoferum* Tn5

*Portions of this work were previously published in *Applied and Environmental Microbiology*, 55:198-202.

populations. Aphids and European corn borers were evaluated as potential vectors for *A. lipoferum* Tn5 dissemination. Whole pGS9 DNA probe hybridized to non-*Azospirillum* colonies in leachate, necessitating the use of a more specific DNA probe constructed from the *npII* region of Tn5. *Azospirillum lipoferum* Tn5 mutant populations, determined by most-probable-number DNA hybridization, varied from undetectable to 10^5 per gram of dry root in the rhizosphere, with smaller populations detected in the endorhizosphere. Maize plants with established *A. lipoferum* Tn5 populations had higher nitrogen concentrations, but plant yield was unaffected. Rhizosphere populations of nitrifying bacteria (10^4 to 10^5 /g dry soil) and *Rhizobium* (10^2 to 10^4 /g dry soil) were unaffected by inoculation with *A. lipoferum* Tn5 mutants. These results indicate that microcosms maintain a high degree of ecosystem complexity and can serve as a representative model system to examine transmission through the ecosystem, rhizosphere and endorhizosphere colonization, potential displacement of other rhizosphere microorganisms, and effects on plant growth by genetically engineered microorganisms.

INTRODUCTION

Methods are needed to evaluate genetically engineered microorganisms (GEMs) in representative ecosystems to assess their fate and potential effects before they are released into the environment. Laboratory-contained microcosms that are composed of intact soil cores have potential as tools for risk assessment before field testing. Current philosophy in regard to the release of GEMs into the environment is that each organism should be regulated on the basis of the risk posed by that individual organism, rather than by how that organism was altered (27).

A standard test system must be adaptable to evaluate the release of a wide variety of microorganisms as well as detect possible detrimental effects on the ecosystem. Microcosms serve these purposes and can also be used to determine the efficacy of GEMs in a field-like situation without regulatory constraints. Intact soil-core microcosms have been used to study ecosystem stability (44), nutrient cycling (45), and the effect of utility wastes or other toxic substances on ecological processes (43,13,42). Microcosms have also facilitated the study of microorganism and pollutant interaction in aquatic (31) and sediment ecosystems (14). Chemical substances are dispersed, diluted, or degraded following their introduction into the ecosystem, but microorganisms can reproduce and increase in concentration under favorable conditions. Therefore, microorganisms have the potential to spread and colonize habitats that may be unique to each genotype.

Azospirillum lipoferum, a motile, diazotrophic root-colonizing bacterium that has been shown to increase plant biomass and nitrogen uptake when inoculated onto wheat (21) and maize (15,28), was chosen as the test bacteria for this study. *Azospirillum* spp. are widely distributed in soils and can form asymbiotic associations with plant roots (32). The effects of *Azospirillum* inoculation on root development and function, which are variable, have been recently reviewed (29). The strains used in this study colonize the rhizosphere, endorhizosphere, and xylem of maize (24) and wheat (22).

Azospirillum Tn5 mutants were selected to enhance their detection and enumeration in the microcosms. The presence of Tn5 in the bacterial genome confers kanamycin resistance (18), which can be used as a selective marker and also as a unique DNA sequence to positively identify mutants by DNA hybridization with a Tn5-specific probe (11). In addition, Tn5 mutants have been shown to have a competitive advantage over their parent wild type, even in the absence of any apparent selective pressure (4).

Transposon mutants can serve effectively in studies to evaluate environmental fate and effects from the release of GEMs because they have been widely used as cloning vectors (23,3), for mutagenesis studies of bacterial symbiosis with plants (36), for plant pathogen interactions (7,9), and for determining bacterial survival in soil (40). Moreover, transposon mutants of *Pseudomonas syringae* have potential use as biocontrol agents against Dutch elm disease (33). This study was initiated to determine the applicability of intact soil-core microcosms for evaluating transport of microorganisms through the ecosystem, their colonization of the rhizosphere and endorhizosphere, displacement of other indigenous rhizosphere microorganisms, and effects on plant growth.

MATERIALS AND METHODS

Bacterial Cultures

Escherichia coli MV12 containing the suicide plasmid pGS9 (11) was used as the Tn5 donor in matings. *Azospirillum lipoferum* strains SPRG20a [20a] and SpBr17 [17], ATCC 29708 and 29709, were purchased from the American Type Culture Collection, Rockville, Maryland.

Culture Media and Maintenance

Luria-Bertani (LB) broth (25), containing 25 ppm each kanamycin sulfate (Sigma Chemical Co., St. Louis, Missouri) and chloramphenicol (Sigma), was used for maintenance of *E. coli* MV12. Nitrogen-free *Azospirillum* medium was used as the basal medium (1). *Azospirillum* cultures were maintained on *Azospirillum* medium (AM), which consisted of nitrogen-free *Azospirillum* medium amended with 1.0 g NH_2Cl and 1.0 g yeast extract (Difco, Detroit, Michigan) per liter. *Azospirillum* Tn5 mutants were routinely cultured on AM amended with 25 ppm kanamycin (Km) sulfate. Stock cultures of all bacteria were maintained at -80°C in 50% glycerol.

Bacterial Matings

Log-phase *A. lipoferum* cultures were centrifuged; cells were washed in 0.85% sterile saline and resuspended in sterile saline to obtain approximately 10^9 cells/ml. For matings, 0.1 ml *E. coli* MV12 donor and 0.4 ml *A. lipoferum* 20a or *A. lipoferum* 17 recipients were mixed, then transferred onto a 47-mm-diameter, 0.01- μm filter (Sartorius, Göttingen, F.R.G.) on 1.5% Luria-Bertani agar. After mating, cells were suspended in 0.5-ml sterile saline and spread onto 150 x 15 mm plates of AM containing 25 ppm Km to select for transconjugates.

Confirmation and Characterization of Tn5 Insertion

Putative *Azospirillum* Tn5 transconjugates were confirmed by spot-blotting an equal number of cells onto GeneScreen Plus (New England Nuclear Corp., Boston, Massachusetts) and hybridizing them with whole pGS9 ^{32}P probe. Plasmid DNA was labeled with ^{32}P -dCTP (New England Nuclear Corp.) using a nick translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Maryland). Plasmid gel electrophoresis was done by the procedure of Kado and Liu (19). Isolation of chromosomal DNA and restriction digests with *Eco*RI and *Hind*III (International Biotechnologies Incorporated, New Haven, Connecticut) were achieved as described by Sadowsky (35). Transfer of restricted DNA onto GeneScreen Plus was by vacuum blot (American Bionetics, Emeryville, California) by the manufacturer's protocol.

Microcosms and Inoculation

Intact soil cores for use as microcosms were obtained as described by Van Voris (42) using a steel coring apparatus containing polyethylene pipe (Phillips Petroleum Co., Dallas, Texas). The

intact soil cores, held inside the plastic pipe after removal from the driving tube, were placed in a housing on Buchner funnels [24-cm polypropylene (Dyna Lab, Rochester, New York) or 20-cm porcelain (Coors, Golden, Colorado)]. Two soil-plant combinations common to agricultural regions of eastern Washington were used. Cores (17.8 x 60 cm) of Palouse silt-loam soil (fine-silty, mixed, mesic Pachic Ultic Haploxeroll) from the Palouse region were planted with spring wheat (*Triticum aestivum*); cores (25 x 60 cm) of Burbank sandy loam soil (sandy, skeletal, mixed, xeric Torriorthent) common to the Columbia Basin were planted with maize (*Zea mays*). Common local seed varieties [NC+3390 maize (Wilbur-Ellis Seed Co., Pasco, Washington)] and Edwall spring wheat (USDA Research Station, Pullman, Washington)] were used.

Tn5 mutant cultures were washed in 0.85% sterile saline and suspended in sterile deionized water to obtain a density of 9×10^8 and 4×10^8 CFU/ml for *A. lipoferum* 17 or 20a, respectively. Burbank soil microcosms were planted with four maize seeds per microcosm, each seed receiving 1.0 ml of an equal mixture of three *A. lipoferum* 17 Tn5 mutants. Palouse microcosms were planted with five seeds per microcosm with each seed receiving 1.0 ml of a single *A. lipoferum* 20a Tn5 mutant. Heat-killed suspensions of the same cells served as controls. There were six replicates for each treatment in a completely randomized design, and one-way analysis of variance tables were calculated to determine if statistically significant differences ($p \leq .05$) existed between measured parameters in inoculated versus control microcosms. Microcosms were maintained at a water potential of approximately -0.03 MPa and temperature of 22°C, as monitored by soil temperature and moisture probes on line to a datalogger (21x, Campbell Scientific Co., Logan, Utah). Photoperiod was set for 12 h with initial light intensity at the soil surface 400 microeinsteins/m² (4100 K Ultralume Lamps, Phillips Lighting Corp., Bloomfield, New Jersey).

Insects

Insects were introduced onto plants after the first sampling. Ten European corn borers (*Ostrinia nubilalis*) (French Agricultural Research Service, Lamberton, Minnesota), at the third to fourth instar, were placed on individual maize plants, and were confined to the lower stalk by loosely wrapping with chiffon. Approximately 50 to 100 aphids (*Schizaphis* sp.) indigenous to eastern Washington State were introduced onto wheat plants. After 12 and 21 days for corn borers and aphids, respectively, insects were sampled, macerated in sterile deionized water, and assayed for *A. lipoferum* Tn5 mutants. Approximately 25 aphids were removed from the wheat leaf surface, and 4 to 9 corn borers were removed from the maize stem interior.

Sampling Frequency and Methods

Three replicate microcosms from each treatment were sacrificed at 4 and 9 weeks to determine bacterial concentration and movement. Soil cores were removed from the pipe intact, and root samples with adhering soil were taken randomly throughout the core with care to avoid sampling those growing at the interface between the core and the pipe. Standard methods were used for sampling rhizosphere soil (47). *Rhizobium leguminosarum* rhizosphere populations were enumerated by the most-probable-number (MPN) plant infectivity method of Weaver and Frederick (46) using peas (*Pisum sativum*) as the host plant. Nitrifying bacteria in the rhizosphere were enumerated by the MPN microtechnique of Rowe et al. (34). Endorhizosphere analysis for Tn5 mutants (30) was accomplished by placing washed wheat and maize root sections in 1.0% chloramine T (Sigma) for 15 and 60 min, respectively, followed by a 30-min wash in sterile 0.1 M, pH 7.0 phosphate buffer. Roots were then rinsed three times with sterile deionized water, macerated, and evaluated for the presence of *A. lipoferum* Tn5 mutants.

Xylem exudate was collected after cutting plants 5 cm from the base of the stem with sterile scissors and letting fluid collect on the stem surface for several minutes; the fluid was then removed with a sterile pipette tip and spread-plated immediately. Leachate was collected in sterile, acid-washed, deionized water-rinsed, 2-liter Erlenmeyer flasks after saturating the cores with deionized water. Leachate was placed at 4°C within 12 h of breakthrough and entry into the flasks. Plant sampling for top biomass was conducted according to standard methods (17). Leaf nitrogen concentration was determined by Kjeldahl analysis (5) before maize tasseling and wheat booting.

Enumeration of *Azospirillum* Tn5 Mutants

Rhizosphere, endorhizosphere, xylem exudate, insects, and leachate samples were analyzed for the presence and population of *Azospirillum* Tn5 mutants. Colony blots (12) as well as MPN-DNA hybridization (11) were used for enumeration. All blots were then hybridized with ³²P-labeled probe and autoradiographed for enumeration. Because whole pGS9 probe proved nonspecific, a 30-base-pair sequence in the *nptII* region of Tn5 was used as a probe. The *nptII* probe was synthesized (Model 380A, Applied Biosystems, Foster City, California) and purified by polyacrylamide gel electrophoresis with 15% polyacrylamide and 7 M urea according to the manufacturer's protocol. Kinase end-labeling (25) with [³²P]ATP (New England Nuclear) routinely achieved 10⁸ dpm/μg DNA.

Soil Recovery

To determine the optimal medium for enumeration of *A. lipoferum* Tn5 cells in soil, *Azospirillum* Tn5 cultures were washed and spiked into Palouse soil at 10^3 and 10^5 cells/g soil. Soils were incubated at 4°C for 1 h. *A. lipoferum* Tn5 cells were enumerated by plate count colony blot and MPN-DNA hybridization, as described previously, using nitrogen-free *Azospirillum* medium and AM amended with 150 and 200 ppm Km and cycloheximide (Sigma).

RESULTS

Azospirillum Tn5 transconjugates were obtained at a frequency of approximately 10^{-9} . All Tn5 mutants were stable after 50 generations of growth in AM broth without Km (data not shown). Both *A. lipoferum* 20a and 17 wild-type strains harbor two plasmids (Figure 1.1A). The *A. lipoferum* Tn5 mutants chosen for this study harbored only the two indigenous plasmids, ensuring that pGS9 was not present as an extrachromosomal element (Figure 1.1A). Two of the mutants [20a-2 and 17-6], however, maintained Tn5 in the smaller of the two indigenous plasmids (Figure 1.1B). These plasmids may be present in multiple copies and can be mechanically broken when subjected to the detection protocol used (19). Whole chromosomal DNA restriction digests (Figure 1.2) with *Eco*RI and *Hind*III, which give 0 and 2 cuts in Tn5, respectively (18), demonstrated that each mutant had a single copy of Tn5 inserted uniquely in the genome (Figure 1.2). The one exception was mutant 17-6, which possessed pGS9 vector sequences, as demonstrated by the increase in the total number of *Hind*III-generated fragments hybridizing to pGS9, three of which correspond to the same size as *Hind*III-generated fragments of pGS9.

The optimal medium for the recovery of *Azospirillum* Tn5 mutants when spiked into Palouse soil was AM containing 150 ppm Km and 200 ppm cycloheximide. Higher concentrations of antibiotics inhibited the Tn5 mutants. When 150 ppm streptomycin was added in addition to 150 ppm Km, growth of the mutants was also inhibited; therefore, streptomycin was not used as an additional selective marker for the Tn5 mutants. The use of nitrogen-free medium selected against nonnitrogen fixers but also limited the growth of *Azospirillum* and therefore the amount of DNA available for hybridization when compared to AM, which provided nitrogen and yeast extract. All Tn5 mutants used in this study were able to grow on nitrogen-free medium, indicating that nitrogen fixation functions were not affected by the transposon insertion. As many as 3.0% of the *Azospirillum* Tn5 mutants spiked into soil were enumerated by colony blots; the limit of detection was approximately 10^3 CFU/g dry soil. Although not evident in the initial soil recovery

Figure 1.1. Plasmid Gel Electrophoresis (A) and Autoradiogram of Vacuum-Transferred DNA Hybridized with a pGS9 DNA Probe (B). Lanes contain: *Pseudomonas putida* mt-2 with the TOL plasmid (117 Kb) as a size reference (1); *A. lipoferum* 17 wild type (2); and Tn5 mutants 17-1 (3); 17-3 (4); 17-6 (5); and 20a-2 (6).

experiment, leachate from microcosms receiving heat-killed cells contained bacteria other than *Azospirillum* that were homologous to pGS9. To determine if the homology resulted from Tn5 sequences in indigenous soil bacteria or from vector sequences, a 30-base-pair sequence in the *nptII* region of Tn5 was used as a probe. Isolated bacteria homologous to the probe when spot-blotted and hybridized with pCU101 (pGS9 without the Tn5 DNA sequence) gave a strong hybridization signal, while a duplicate blot did not hybridize with the 30-mer *nptII* probe, indicating homology was with vector sequences (data not shown).

Rhizosphere populations of the *Azospirillum* Tn5 mutants in the inoculated microcosms were variable, ranging from undetectable to 10^5 /g dry weight (Table 1.1). Endorhizosphere populations as determined by MPN-DNA blots at midpoint sampling were below the detection limit for wheat roots, ranging from 5.5×10^3 to 6.5×10^3 CFU/g dry maize root in the Burbank soil microcosms. Populations determined by plate count and MPN manifold blot were generally in agreement, although the latter gave more consistent and slightly higher (typically 50%) values. The background of bacteria with the ability to grow on AM amended with antibiotics ranged from nearly none (on insect samples) to 10^6 /g soil in the Palouse rhizosphere; thus, the detection limit of mutant populations in rhizosphere samples was lower. No *Azospirillum* Tn5 mutants were detected in leachate, xylem exudate, insects, or earthworms found fortuitously during microcosm sampling.

Figure 1.2. Agarose Gel Electrophoresis of Chromosomal Restriction Digests of *A. lipoferum* Tn5 Mutants (A) and Autoradiogram of Vacuum-Transferred DNA Hybridized with a pGS9 DNA Probe (B). Lanes 1 through 5 contain *HincIII* restriction digests of Tn5 mutants 17-1 (1); 17-3 (2); 17-6 (3); 20a-2 (4); and *A. lipoferum* 17 wild type (5). Lanes 6 through 10 contain *EcoRI* restriction digests of Tn5 mutants 17-1 (6); 17-3 (7); 17-6 (8); 20a-2 (9); and *A. lipoferum* 17 wild type (10).

The mean plant leaf tissue nitrogen concentration (Table 1.1) was higher for the inoculated maize plants than for the controls. This difference was not statistically significant, although there was a relationship between the presence of *Azospirillum* and tissue nitrogen concentration in the replications (Table 1.1). Available nitrogen, as reflected in the nitrate concentration in the initial leachate from control microcosms, was significantly higher ($p \leq .05$) for Palouse soil ($x = 287$ ppm) than for the Burbank soil ($x = 130$ ppm). There was no significant difference in the number

Table 1.1. *A. lipoferum* Rhizosphere Populations, Plant Biomass, and Leaf Tissue Nitrogen Concentration, Before Plant Grain Fill

Treatment/ Replicates	<i>A. lipoferum</i> Rhizosphere (log MPN/g dry soil)	Plant Biomass Dry Weight (g)	Leaf Tissue N (%)
Maize/ Burbank soil			
1 - L ^a	4.55	6.78	3.02
2 - L	3.79	8.21	3.23
3 - L	bd ^b	12.42 (9.14 ±2.93)	2.64 (2.97 ±0.30)
1 - HK ^c	bd	8.06	3.21
2 - HK	bd	8.51	1.98
3 - HK	bd	14.11 (10.23 ±3.37)	1.96 (2.38 ±0.72)
Wheat/ Palouse soil			
1 - L	6.43	2.39	5.22
2 - L	bd	3.04	5.39
3 - L	bd	2.27 (2.57 ±0.41)	5.31 (5.30 ±0.09)
1 - HK	bd	2.65	5.17
2 - HK	bd	2.97	5.45
3 - HK	bd	2.86 (2.83 ±0.16)	5.00 (5.21 ±0.23)

^a Replicates receiving live *Azospirillum* Tn5 mutants.

^bbd, below detection.

^c Replicates receiving heat-killed cultures.

of tillers per wheat plant in the inoculated microcosms (1.32) versus control microcosms (1.29), or between plant top biomass of maize or wheat at either sample point (Tables 1.1 and 1.2). Although plant top biomass means of wheat in control and inoculated microcosms were not significantly different, a decrease in top biomass of maize plants with established rhizosphere *Azospirillum* populations was observed as each of the individual replications with measurable *Azospirillum* populations had lower plant biomass (Tables 1.1 and 1.2).

Table 1.2. *A. lipoferum* and *Rhizobium leguminosarum* Rhizosphere Populations and Plant Biomass After Plant Grain Fill

Treatment/ Replicates	<i>A. lipoferum</i> Rhizosphere Population (log MPN/g dry soil)	Plant Biomass Dry Weight (g)	<i>Rhizobium</i> Rhizosphere Population
Maize/ Burbank soil			
4 - L ^a	3.78	47.4	--
5 - L	bd ^b	64	--
6 - L	4.23	47.9 (53.1 ±9.4)	--
4 - HK ^c	bd	73.1	--
5 - HK	bd	57.4	--
6 - HK	bd	59.4 (63.3 ±8.6)	--
Wheat/ Palouse soil			
4 - L ^a	4.40	20.3	2.58
5 - L	3.75	18.5	3.23
6 - L	bd	13.4 (17.4 ±3.6)	2.42 (2.74 ±0.43)
4 - HK ^c	bd	15.6	3.31
5 - HK	bd	20.5	2.84
6 - HK	bd	14.7 (16.9 ±3.1)	4.30 (3.48 ±0.75)

^a Replicates receiving live *Azospirillum* Tn5 mutants.

^b bd, below detection.

^c Replicates receiving heat-killed cultures.

Rhizobium leguminosarum populations were present in the wheat rhizosphere of the Palouse soil microcosms, but were not influenced by inoculation with the *A. lipoferum* 17 Tn5 mutant (Table 1.2). Rhizosphere populations of *Nitrosomonas* spp. were similar for all treatments, while *Nitrobacter* spp. populations were consistently higher in wheat rhizosphere than in the maize rhizosphere (Figure 1.3). Neither *Nitrosomonas* spp. nor *Nitrobacter* spp. rhizosphere populations were influenced by *A. lipoferum* inoculation in either the Palouse or Burbank soil microcosms (Figure 1.3).

Figure 1.3. Rhizosphere Soil Populations of *Nitrosomonas* spp. (A) and *Nitrobacter* spp. (B) in Microcosms Inoculated with *A. lipoferum* Tn5 Mutants or Receiving Heat-Killed Cultures. Error bars represent 5% confidence intervals.

DISCUSSION

The frequency of Tn5 insertions (10^{-9}) into the *A. lipoferum* strains, although low in comparison with other gram-negative bacteria (37), are similar to those previously observed for *Azospirillum* (41). Media and antibiotic concentrations used were not completely selective, allowing growth of *Azospirillum* but not suppressing growth of all indigenous soil microorganisms. This limited the detection of *A. lipoferum* Tn5 mutants in soil to approximately 10^3 /g soil.

Xylem exudate and insects had low background populations of kanamycin-resistant (Km^r) bacteria, as did Burbank microcosm leachate samples; hence, the Tn5 mutants, if present and culturable, would have been readily detected by colony hybridization or MPN-DNA hybridization. Because of a high background of Km^r bacteria in Palouse soil able to grow on AM, the relatively slow growing *A. lipoferum* population may have been outcompeted, limiting its detection in the rhizosphere populations to approximately 10^3 CFU/g dry soil. Endorhizosphere samples and insect macerates from the maize plants contained a fungus that grew on AM agar with 200 ppm of cycloheximide. This fungal growth may have masked Tn5 mutant populations in those samples.

MPN-DNA hybridization was more effective for enumerating *A. lipoferum* Tn5 bacteria when high background levels of Km^r bacteria were present. In some samples, positive signals were obtained at low dilutions in microdilution plate blots while no positive colonies were detected on colony blots of dilution plates of the same samples. Presumably, the static broth provided *Azospirillum* more favorable conditions for growth and competition with other Km^r bacteria. *Azospirillum* seemed to be especially overgrown by the indigenous Km^r bacteria on solid media. Typically, the indigenous Km^r bacteria would appear after 2 days of incubation at 27°C where a minimum of 3 days was required for *Azospirillum* colonies. Additionally, not all colonies blotted efficiently, requiring their purification and isolation before hybridization with Tn5 probe for confirmation.

The *nptII* probe was used because preliminary studies with whole pGS9 probe showed a high background of indigenous bacteria with homology to the suicide vector. Studies with *Pseudomonas putida* Tn5 mutants added to dried and remoistened Palouse soil enumerated by MPN-DNA hybridization did not reveal homology of indigenous bacteria with whole pGS9 probe (11). In that study, either homologous bacteria were not able to grow on the media used for *P. putida* Tn5 selection, or the bacteria were not present because of air-drying of soil. Probe

specificity and preliminary studies with appropriate controls are essential before usage for enumeration of specific bacterial populations in environmental samples.

Although *Azospirillum* rhizosphere populations were not uniform among replicate microcosms, a pattern was evident. Of the three replicate Palouse and Burbank microcosms receiving live inoculum at each sampling, two microcosms had detectable rhizosphere populations of *A. lipoferum* Tn5, while populations were below detection levels in one microcosm.

Variability of *Azospirillum* Tn5 rhizosphere populations between replicates probably resulted from inherent differences in the microcosms. Intact soil cores, because they are removed as discrete pieces from the field, contain inherent variation that represents the complexity of that ecosystem. Examples of macroscopic differences observed in some microcosms and not others include visible layers of Mt. St. Helens ash from the 1980 eruption, the emergence of mushrooms, and the presence of earthworms. Microscopic differences could include variable distribution of indigenous soil bacteria (e.g., antibiosis) and distribution of nutrients.

The potential for niche displacement of rhizosphere populations of nitrifying bacteria and *Rhizobium* in the microcosms was also evaluated because of their involvement in biochemical nitrogen cycling and the ability of *Rhizobium* sp. to be stimulated in the rhizosphere of nonhost plants. These bacteria occupy the same physical medium (rhizosphere and bulk soil) so that any antibiosis or other deleterious effect on indigenous microbial populations from inoculation of *Azospirillum* Tn5 mutants would likely be evident on those species. Although changes in the rhizosphere populations of nitrifying bacteria and *Rhizobium leguminosarum* were not observed in the inoculated microcosms, their consistent presence and low variability between replicates make them good indicators of ecosystem stability.

Plant growth effects from inoculation with *Azospirillum* have been attributed to N₂ fixation (26), production of plant growth hormones (39), and increased nutrient uptake at the roots (26). The expression of these traits and limited root colonization likely contribute to the variability in plant growth response common to *Azospirillum* studies. This suggests that the extent of *Azospirillum* root colonization may be the main factor controlling plant growth response.

Edaphic factors have been shown by others to strongly influence *Azospirillum* interaction with plants. *Azospirillum* inoculation of wheat in soils high in nitrogen or with high levels of nitrogen applied as a fertilizer have shown decreased effect on plant dry weight (20,21) and nitrogen incorporation into plant tissue (2) in comparison with those grown under low nitrogen

conditions. Maize grown in Burbank soil, low in nitrogen relative to Palouse soil, showed a greater response to inoculation as indicated by an increase in plant leaf nitrogen in those replicates with detectable *Azospirillum* Tn5 mutants (Table 1.1), although top dry weight was not affected. However, O'Hara et al. (28) tested seven nonindigenous strains of *A. brasilense* under greenhouse conditions and found that none extensively colonized the endorhizosphere and that yields were relatively constant, regardless of population size and location on roots.

Bacterial strain plant specificity must also be considered when examining the growth response of *Azospirillum*-inoculated plants. Soil and plant combinations used in this study may not have been conducive to expression of those bacterial genes that promote plant growth, such as N₂ fixation (8), or production of plant growth factors (39,16), by the specific *Azospirillum* strains used. As with wheat, plant genotype-*Azospirillum* interactions have been shown to be strain specific for maize (15).

Effects on plant growth (10) as well as on ecosystem processes (13) are likely to be dependent on the size of the microbial population. The inoculum concentration in this study was relatively small (10⁸-10⁹ CFU/seed) in comparison with most other studies. An optimum *Azospirillum* inoculation concentration has been shown for maize that was 10 fold less than the competing *Pseudomonas* population (10). Increasing the concentration of *Azospirillum* past the optimum population can offset beneficial effects to the plant.

In field plot experiments spanning 3 years, significant increases in wheat grain yield between treatments were not found even though there were increases as great as 31% in individual plots (2). This observed field variability is consistent with the variability in root colonization and plant growth effects observed in the microcosms, indicating that the intact cores have retained some degree of field complexity.

Undisturbed soil samples have been shown to allow some bacterial passage through the macropores (38), suggesting the potential for microbial leaching and subsequent transport in groundwater. The close association and adhering of *Azospirillum* with roots and low levels of inoculum likely limited their leaching through the soil cores in this study. *A. lipoferum* strains were chosen for their reported xylem mobility (24,22); hence, there was a potential for transmission from the plant vascular system to phytophagous insect vectors. In the specific *Azospirillum* soil-plant combination used in this study, extensive bacterial movement and colonization was not observed. Although the possibility exists that mutations incurred by the insertion of Tn5 influenced the competitive ability of the bacteria, and therefore xylem mobility, it

is more likely that the migration of *Azospirillum* mutants into the xylem is uncommon and occurs only under specific conditions. Transposon insertions have been shown to impair the relative competitiveness of *Rhizobium* (6) and, therefore, could have had a negative impact on the competitive ability of the *A. lipoferum* mutants used in this study. However, this is less likely for Palouse soil-core microcosms because a mixture of three different mutants was used for inoculation. Additionally, Tn5 mutant bacteria have been provided a selective advantage in comparison with the wild type (4).

We have demonstrated that intact soil-core microcosms maintain many of the features useful for evaluating GEM transmission through the ecosystem, rhizosphere and endorhizosphere colonization, potential displacement of other rhizosphere microorganisms, and effects on plant growth. This is illustrated by the detection problems realized during hybridization of pGS9 to indigenous Km^r bacteria, whereas no such problem was encountered when using dried stock soil for preliminary recovery studies. This phenomenon is indicative of the higher order of ecosystem complexity maintained by use of intact soil-core microcosms (44). Other aspects of environmental release not evaluated in this study that may be evaluated using soil-core microcosms include GEM persistence and gene transfer. A major advantage of microcosms for GEM evaluation is their easy replication, necessary to determine the probability of finding a given effect or population for risk assessment. The use of intact soil cores allows the testing and comparison of GEM fate and effects in a number of different soil types, including those at the site intended for field testing.

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2.0 NUTRIENT EFFLUX FROM INTACT SOIL-CORE MICROCOSMS FOR EVALUATING THE IMPACTS OF GENETICALLY ENGINEERED MICROORGANISMS ON ECOSYSTEM PROCESSES

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SUMMARY

Nutrient export from intact soil-core microcosms in leachate or by plant uptake was evaluated as a means to assess the ecosystem impacts from the environmental release of genetically modified root-colonizing bacteria. Intact cores of two soil types, including a Burbank sandy loam and a Palouse silt loam, were excavated for use as microcosms, seeded with corn or wheat, respectively, and inoculated with *Azospirillum lipoferum* Tn5 mutants. Microcosms were leached at 33 and 67 days after seeding, and the leachate was analyzed for sulfate, phosphate, ammonium, nitrite, nitrate, dissolved organic carbon, and inorganic carbon. Statistically significant differences in leachate nutrient concentrations were observed between soil types, but differences caused by inoculation with the genetically altered bacteria were not observed. The one exception was a significant difference in the amount of dissolved organic carbon (and total carbon), probably because heat-killed cells were added to the control. Plant uptakes of N, P, S, B, Cu, Fe, Mg, Mn, and Zn were also not affected by inoculation, but the coefficients of variation tended to be lower than for the leachate analyses. The measurement of nutrient export from microcosms, in particular

that of carbon and nitrogen, may be used as a holistic indicator of ecosystem impacts resulting from the release of genetically engineered microorganisms.

INTRODUCTION

One major concern regarding the release of recombinant microorganisms to the environment is the potential for unforeseen ecosystem impacts (20). An ecological impact can be considered as a measurable detrimental effect on either the organisms or the processes within the ecosystem. Microorganisms can therefore impact an ecosystem through, for example, pathogenicity of component flora and fauna or through displacement or disruption of other microorganisms that carry out ecologically important functions or processes. Some of the most important ecosystem functions catalyzed by microorganisms involve nutrient cycling. Efficient nutrient cycling in an ecosystem may be determined by measuring nutrient fluxes and concentrations in various ecosystem pools (e.g., soils, microorganisms, plants) or by measuring specific biochemical activities directly involved in nutrient cycling (decomposition, microbial respiration, N₂ fixation, nitrification, denitrification).

Field testing of genetically engineered microorganisms currently requires a permit, and applications are reviewed on a case-by-case basis. On the basis of past experience, the potential for introduced microorganisms, recombinant or otherwise, to have a negative impact on an ecosystem appears low. Past attempts to introduce *Rhizobium* spp. (17), microbial biocontrol agents, or microbial fertilizers to benefit crop growth have demonstrated the difficulty of establishing introduced microorganisms in soils or the rhizosphere. However, once microorganisms are released into the environment, they are not readily recalled or eliminated, and an a priori assessment of zero risk obviously cannot be made. Introduced organisms can also compete with indigenous organisms or fill an unoccupied niche. Microorganisms have been introduced into ecosystems in which they had not previously existed and then have survived, multiplied, and had negative impacts (11,20). In addition, there are examples of single gene changes in microbial plant pathogens that have allowed them to overcome the host's natural defense mechanisms (5). Therefore, it is desirable to determine the potential for ecosystem impacts from genetically engineered microorganisms before their release to the environment.

Microcosms have been used for a variety of purposes in environmental studies, including organism toxicity testing (16), the fate of chemicals (15), chemical ecosystem impacts (18), and nutrient cycling (24), and have been suggested for use in biotechnology risk assessment (3).

Recently, a standardized procedure from the ASTM intact soil-core microcosm test protocol (23) was modified to assess the fate of and potential ecological effects from the release of genetically engineered microorganisms. This procedure also allows for measurement of nutrient efflux in leachate as a means for evaluating nutrient cycling processes within the intact soil cores.

Azospirillum lipoferum, a motile, diazotrophic root-colonizing bacterium, was chosen as the test bacteria for this study because it has been shown to increase plant biomass and nitrogen uptake when inoculated onto wheat (9) and corn (7) roots. The objectives of this study were to determine the impact from inoculation with *Azospirillum lipoferum* Tn5 mutants on the efflux of nutrients from agricultural soil-core microcosms through leaching and uptake by wheat (*Triticum aestivum*) and corn (*Zea mays*) and the usefulness of these measurements for monitoring ecological effects from the release of genetically engineered microorganisms.

MATERIALS AND METHODS

Soil-Core Microcosms

Intact soil cores 17.8 cm in diameter and 60 cm deep (23) were obtained from the U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS) Palouse Conservation Field Station near Pullman, Washington, for use as microcosms. The cores consisted of a Palouse silt loam soil (fine-silty, mixed, mesic Pachic Ultic Haploxeroll) taken from a field low on the landscape. The cores were taken by pushing a steel coring device with Driscopipe® liner into the soil by means of a hydraulic ram mounted on the back of a tractor with dual flotation tires filled with water for added weight. The steel coring barrel moved easily through the upper 30 cm, which had been moistened by recent rains; however, the rest of the core was taken with more difficulty as the B horizon was high in clay and dry. Following their removal, soil cores were transported back to the Pacific Northwest Laboratory (PNL) at Richland, Washington, moistened with 1 liter deionized water, and covered until used.

The 25.5-cm-diameter (60-cm-deep) intact soil cores were obtained from a native sagebrush-bunchgrass plant community near West Pasco, Washington. The soil is classified as a Burbank sandy loam (sandy, skeletal, mixed, xeric Torriorthent) a soil type commonly used for irrigated agriculture in the Columbia Basin of south-central Washington State. Cores were

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removed as previously described except that a large front-end-loading tractor was used to drive the steel core barrel into the soil. Moisture had penetrated to a depth of approximately 15 cm in the sandy loam soil but the core barrel readily penetrated the soil. The dry unconsolidated material at the bottom of the core did not remain in the polyethylene pipe liner upon removal, requiring backfilling the bottom of the soil cores with the material that remained in the hole. This soil was homogeneous and unconsolidated with no distinct soil horizons and therefore heterogeneities among the replicate cores from the uneven recovery of subsoil were unlikely. Cores were transported back to PNL where they received 1 liter of deionized water. Table 2.1 lists the physical and chemical characteristics of the 0- to 15-cm depth of the two soils used in this study.

The soil cores were placed on Buchner funnels (24-cm polypropylene from Dyna Lab, Rochester, New York; or 20-cm porcelain from Coors, Golden, Colorado); the porous plates were lined with glass wool placed in plywood boxes with slots for six microcosms. The Buchner funnels were connected to sterile, acid-washed, deionized-water-rinsed, 2-liter Erlenmeyer flasks for leachate collection. Common local seed varieties [NC+3390 field corn (Wilbur-Ellis Seed Co., Pasco, Washington) and Edwall spring wheat (USDA Research Station, Pullman, Washington)] were seeded into 25.5- and 17.8-cm-diameter intact soil cores, respectively. Soil cores were

Table 2.1. Selected Properties of Soil Types Used as Intact Soil-Core Microcosms

Soil Property	Burbank Sandy Loam	Palouse Silt Loam
% sand	45	1
% silt	51	78
% clay	4	21
pH	7.4	5.6
Organic carbon (%)	0.5	2.0
Total sulfur (%)	0.05	0.04
Total N (%)	0.06	0.16
Total P ($\mu\text{g/g}$)	2400	3770
Phosphate-P ($\mu\text{g/g}$)	4.8	5.8
Ammonium-N ($\mu\text{g/g}$)	6.1	18.3
CEC (meq/100 g)	5.5	23.8

inoculated with two strains of *Azospirillum lipoferum* or heat-killed cells as a control. These microorganisms and the details of inoculation are described elsewhere (1). The four treatments were replicated six times in a completely randomized design, and two-way analysis-of-variance tables were calculated to determine if statistically significant differences ($p \leq .05$) existed between measured parameters in inoculated versus control microcosms and between soil types.

Microcosms were maintained at a water potential of approximately -0.03 MPa and temperature of 22°C as monitored by soil temperature and moisture probes on line to a datalogger (21x, Campbell Scientific Co., Logan, Utah). Photoperiod was set for 12 h with initial light intensity at the soil surface 400 microeinsteins/m² (4100 K Ultralume Lamps, Phillips Lighting Corp., Bloomfield, New Jersey). Soil-core leachate was collected at 33 and 67 days after seeding. Deionized water was added slowly over time to the microcosms to saturate and leach the soil cores. Leachate was collected in acid-washed, 250-ml Erlenmeyer flasks. Leachate was decanted into sterile acid-washed containers, its volume determined, and placed at 4°C within 12 h after soil breakthrough and entry into the flasks.

LEACHATE NUTRIENT ANALYSIS

Filtrates were analyzed for the inorganic ions PO₄³⁻, SO₄²⁻, NO₃¹⁻, and NO₂¹⁻ by ion chromatography. Samples were analyzed on AS1 anion-exchange columns (Dionex Corp., Sunnyvale, California) using 3mM NaHCO₃ and 2.4 mM Na₂CO₃ as eluents of at flow rates of approximately 2.3 ml/min. Normal configuration of the ion chromatograph (Dionex Model 10) consisted of the anion exchanger followed by a membrane eluent suppressor using 0.025 N H₂SO₄ to neutralize the basic eluent stream, followed by conductivity detection over the 1- to 100-μS ranges (full scale) with detection limits of about 0.06 ppm or better for NO₂¹⁻, NO₃¹⁻, PO₄³⁻, and SO₄²⁻. Ammonium was determined by ion-specific electrode (ISE) using the Orion model 9512 gas-sensing electrode (Orion Research Inc., Cambridge, Massachusetts) after pH adjustment according to the manufacturer's protocol.

Dissolved organic carbon was determined by acidifying filtered leachate to pH 2 with phosphoric acid, sparging to remove inorganic carbon as CO₂, and injecting the sample into a Dohrmann Model DC-80 Carbon Analyzer (Xertex, Santa Clara, California). Total carbon (TC) was determined on filtered leachate by direct injection into the carbon analyzer, and inorganic carbon was calculated as the difference between TC and organic carbon. The loss of purgeable organics during the sparging of acidified aliquots was assumed to be negligible.

PLANT NUTRIENT ANALYSIS

Plant sampling and preparation of tissue for analysis followed the methods of Jones and Steyn (8). Corn was sampled at the tasseling stage by removing the first fully developed leaf below the whorl from two plants in each microcosm. For spring wheat, the four uppermost leaves were removed before heading from four plants in each microcosm. Plant leaves were dried at 60°C for 48 h and then ground (40 mesh) using a mechanical mill with stainless steel surfaces to minimize contamination with trace metals.

Ground plant material was ashed using a modified wet procedure in which 0.1 g of dried plant material was placed in a Teflon bomb with 2.5 ml of concentrated HNO₃ and put in an oven at 140°C for 6 h to overnight. The ashed tissue was brought to 20 ml with deionized water and analyzed directly for P, S, Zn, Fe, Mn, and Mo by inductively coupled plasma spectrometry (Applied Research Laboratories Model 3560). Total nitrogen content of plant tissue was determined by Schwartzkopf Microanalytical Laboratories (Woodside, New York).

RESULTS AND DISCUSSION

Microcosm Leachate Analysis

Between 750 ml and 3 liters of sterile deionized water were required to leach the soil cores; the larger Burbank soil cores (25.5-cm diameter) required more water than the Palouse soil cores (17.8-cm diameter). Because the Burbank soil microcosms were twice the volume of the Palouse soil-cores, twice as much soil volume was leached, therefore requiring a greater volume of water. The volume of leachate that was collected from the first leaching (t = 33 days) ranged from 40 to 380 ml in the Palouse soil cores and from 30 to 270 ml in the Burbank soil cores. The nutrient analysis from the first leaching is shown in Table 2.2. The values are presented as concentration (µg/ml) rather than the total amount of nutrient leached because both representations showed similar significant differences between soil types or inoculation treatment. A two-way ANOVA table was calculated for the individual nutrient analyses; the two factors were soil type and bacterial inoculation (live versus heat-killed cells). For all nutrients except ammonium and nitrite, there was a statistically significant difference ($p \leq .05$) between the two soil types at the 33-day leaching (Table 2.2). Leachate ammonium and nitrite concentrations were low for both soils, and

Table 2.2. Leachate Analyses from First Sampling (33 days)

Nutrient	Maize, Burbank Soil <i>A. lipoferum</i>		Spring Wheat, Palouse Soil <i>A. lipoferum</i>		<i>p</i> > F	
	Br17 Tn5	Control	RG20a Tn5	Control	Soil Inoculum	
SO ₄	41.02 (58.6) ^a	52.20 (62.2)	18.02 (37.9)	18.89 (34.9)	0.00	0.45
PO ₄	1.90 (25.3)	2.34 (47.4)	0.83 (60.2)	0.96 (67.7)	0.00	0.12
NH ₄	0.04 (50.0)	0.02 (50.0)	0.09 (200)	0.03 (33.3)	0.49	0.14
NO ₂	0.04 (150)	0.02 (50)	0.04 (75.0)	0.05 (60.0)	0.43	0.14
NO ₃	89.94 (28.2)	130.03 (47.6)	279.73 (44.2)	287.31 (50.0)	0.00	0.61
TC ^b	59.31 (25.3)	68.70 (12.9)	12.51 (39.6)	17.60 (42.4)	0.00	0.04
DOC ^c	16.36 (25.9)	21.42 (32.6)	5.37 (24.0)	6.91 (36.2)	0.00	0.05
DIC ^d	42.95 (29.2)	47.28 (74.3)	7.14 (60.5)	10.69 (50.7)	0.00	0.11

^a Data are in µg/ml. In parentheses, coefficient of variation, for n = 6.

^b TC, total carbon.

^c DOC, dissolved organic carbon.

^d DIC, dissolved inorganic carbon.

approached the analytical detection limits. In addition, because ammonium is a cation, it would be held on cation-exchange sites in the soil and would not leach to a great extent with the water percolating through the soil profile.

Microcosm inoculation with the *Azospirillum* transposon mutants had no significant effect on the levels of soluble nutrients in the microcosm leachates at day 33 or 67 (Tables 2.2 and 2.3) with the exception of total carbon and dissolved organic carbon at day 33 (Table 2.2). The concentrations of total carbon and dissolved organic carbon were significantly higher in the controls than in the microcosms receiving the live inoculum. This difference likely results from the addition of dead microbial biomass to the microcosms as heat-killed *Azospirillum* cultures. Between 4 and 5 ml of 8.5×10^8 cells/ml of heat-killed *Azospirillum* culture were added to each microcosm. If we assume that the average hydrated cell mass for *Azospirillum* is 0.01 ng, the cells

Table 2.3. Leachate Analyses from Second Sampling (67 days)

Nutrient	Maize, Burbank Soil <i>A. lipoferum</i>		Spring Wheat, Palouse Soil <i>A. lipoferum</i>		<i>p</i> > <i>F</i>	
	Br17 Tn5	Control	RG20a Tn5	Control	Soil Inoculum	
SO ₄	70.46 (77.6) ^a	23.50 (153)	22.04 (150)	3.57 (106.7)	0.07	0.09
PO ₄	1.04 (40.4)	2.94 (40.1)	1.51 (65.6)	0.78 (43.6)	0.11	0.68
NH ₄	0.15 (133)	0.04 (25.0)	0.04 (50.0)	0.11 (1090)	0.68	0.93
NO ₂	0.01 (100)	0.02 (50)	0.14 (136)	0.03 (66.7)	0.17	0.17
NO ₃	0.77 (129)	0.53 (81.1)	25.98 (113)	2.24 (114)	0.12	0.10
TC ^b	86.05 (41.6)	93.25 (30.9)	28.10 (65.2)	17.00 (127)	0.00	0.70
DOC ^c	53.30 (55.6)	61.02 (41.3)	17.00 (74.4)	9.10 (129)	0.00	0.80
DIC ^d	32.75 (26.2)	32.23 (18.2)	11.11 (52.7)	7.89 (126.4)	0.00	0.56

^a Data are in µg/ml. In parentheses, coefficient of variation, for n = 6.

^b TC, total carbon.

^c DOC, dissolved organic carbon.

^d DIC, dissolved inorganic carbon.

are 60% water, and the typical dry cell is composed of 50% carbon (2), then approximately 7 mg of cell carbon was added to the soil cores. For the Burbank soil cores, the difference in organic carbon concentration in the leachate is approximately 5 µg/ml and the average volume of leachate collected was 250 ml; thus a total of 1.25 mg of carbon was leached from the soil cores. The amount of carbon added as cell biomass could therefore have accounted for the observed differences in organic carbon content of the leachates from control and inoculated microcosms.

The soil-core microcosms required between 5 and 6 liters of sterile deionized water at the second leaching (t = 67 days). After the first leaching, the wheat and corn plants rapidly removed moisture from the soil cores, requiring significant quantities of moisture to maintain them at a water potential above wilting point. Between 100 and 700 ml of leachate was recovered from the Burbank microcosms and between 15 and 300 ml from the Palouse soil cores. Total, organic, and

inorganic carbon concentrations in the leachate from the 67-day leaching (Table 2.3) were significantly different between the two soil types as with the first sampling; however, none of the other elements analyzed differed significantly. A comparison of nutrient concentrations in leachate from the first and second leachings (Tables 2.2 and 2.3) indicates the most soluble nutrients were removed by the first leaching and/or by plant uptake. This is clearly the case for nitrate; the concentration in the 33-day leachates were 10- to 100 fold higher than in the 67-day leachates. There was no significant difference in the concentration of soluble nutrients in the 67-day leachate between microcosms receiving live *Azospirillum* and those receiving heat-killed cells.

The significant difference between the two soils in inorganic carbon in microcosm leachates at the two samplings (Tables 2.2 and 2.3) would be expected because the Burbank soil typically has CaCO_3 coatings on sand and pebble particles in the upper soil horizons while the Palouse soil does not. The organic carbon level of the Palouse surface soil was higher than the Burbank (Table 2.1), although greater amounts of soluble organic carbon were present in the Burbank microcosm leachates (Tables 2.2 and 2.3). This inconsistency likely results from root exudation and sloughing of cells, which contribute greatly to this leachable soluble C pool. Extremely soluble carbon compounds are released by plant roots into soil, and their composition and quantity depend on plant species, growth conditions, rooting medium, and stage of plant development (6). Corn and wheat grown for ≈ 25 days in solution culture released 1200 and 342 μg polysaccharide per plant, respectively (22). Thus corn released greater amounts of polysaccharides, which contribute greatly to the soluble carbon compounds in corn (10) and wheat root (21) exudates. Finally, root-derived carbon was 26 fold more extractable with a mild percolation technique than native soil organic components (12), indicating root-derived carbon is more soluble in soil. The Burbank soil-corn combination may have had greater root carbon produced than the Palouse soil-wheat combination, resulting in the higher soluble organic carbon levels detected in the Burbank corn microcosms (Tables 2.2 and 2.3).

The detection of statistically significant differences between soil types as well as inoculum or organic carbon in the leachate at both sampling times suggests that this would be a sensitive method for evaluating nutrient processing within the soil-core microcosm. Because available organic carbon can be a major factor influencing or limiting the growth of heterotrophic microorganisms in the environment, the efflux of soluble carbon from soil-core microcosms as leachate combined with appropriate controls could be used to evaluate potential ecological effects from the release of genetically engineered microorganisms. The measurement of DOC concentrations has also been suggested as a means of calibrating microcosm function with the field (14). It would also be useful to include, in future studies, fractionation of the DOC to obtain

general information on the classes of organic compounds (e.g., humic materials, carbohydrates, amino acids) that contribute to the DOC, indicating the source of the carbon as well as its availability to the microbial heterotrophic population.

The coefficients of variation (CV) tended to be lower for the analyses from the first than for the second sampling, ranging from 5% to 200% (mean, 51%) (Table 2.2) as compared to CVs from 18% to 153% (mean, 80%) (Table 2.3). This variation may have resulted partially from differences in the volume of leachate collected from the individual microcosms, and may also reflect real field variability. O'Neill et al. (13) also found large variations (CV = 14% to 100%) in nitrate leaching from replicate soil-core microcosms but the much lower CVs for calcium (6% to 10%) indicated that variability was nutrient dependent. This variability suggests that only large differences in concentration for some nutrients, as was observed between soil types, will be statistically significant. The high CVs of the analyses of some nutrients may limit the sensitivity of measurements of nutrient efflux from soil-core microcosms as indicators of GEM environmental impact. However, because of the importance of carbon and nitrogen to ecosystem function and the extensive information available concerning the processing of these nutrients in ecosystems, the export of DOC and inorganic nitrogen species from soil-core microcosms should be included in microcosm analysis of ecosystem impacts from GEM introduction. These impacts could occur by means of antagonism of microorganisms involved in mineralization of nutrients from soil organic matter or through deleterious effects on plant growth limiting uptake of nutrients from the soil.

Plant Tissue Nutrient Concentrations

Macro- and trace element concentrations in maize and wheat leaf tissue were not influenced by inoculation with the *Azospirillum* cultures, but significant differences were detected, as would be expected, between the two soil-plant combinations (Table 2.4). The CVs were typically lower than for nutrient concentrations in the microcosm leachates; the mean CV for all plant tissue analyses was 16.7. Therefore, nutrient analysis of plant tissue may be useful as a indicator of GEM impacts on plant nutrient assimilation. Plant-beneficial bacteria are organisms that can be improved for use in agricultural biotechnology (4), and monitoring of plant assimilation of nutrients in microcosms may be one means of evaluating the organisms for potential deleterious impacts on target and nontarget plants. Tolle et al. (19) found that microcosms were sensitive predictors of the field for evaluating plant uptake of trace elements from fly ash-amended soil.

Table 2.4. Nutrient Concentration in Leaf Tissue of Maize and Spring Wheat at 33 Days

Nutrient	Maize, Burbank Soil <i>A. lipoferum</i>		Spring Wheat, Palouse Soil <i>A. lipoferum</i>		<i>p</i> > F	
	Br17 Tn5	Control	RG20a Tn5	Control	Soil Inoculum	
N	2.97 (10.1) ^a	2.38 (30.3)	5.30 (1.7)	5.21 (4.4)	0.00	0.16
B	21.6 (9.7)	20.4 (15.7)	18.5 (22.7)	17.1 (17.5)	0.17	0.16
Cu	9.28 (7.3)	9.2 (37.0)	35.6 (85.1)	19.0 (53.2)	0.14	0.48
Fe	153.2 (12.6)	181.3 (14.0)	282 (48.9)	260 (70.3)	0.19	0.97
K	37149 (6.8)	38559 (7.2)	53748 (7.2)	52155 (7.1)	0.00	0.97
Mg	2790 (7.4)	2735 (13.6)	3214 (3.9)	3020 (4.1)	0.06	0.47
Mn	80.7 (4.3)	89.7 (16.2)	74.1 (4.7)	79.1 (5.9)	0.15	0.20
P	2930 (7.5)	2873 (6.6)	3200 (2.8)	2550 (9.9)	0.02	0.40
S	2954 (8.4)	2785 (11.5)	3821 (2.6)	3808 (0.9)	0.00	0.47
Zn	15.73 (12.1)	13.9 (12.9)	37.3 (44.8)	32.2 (18.6)	0.21	0.44

^a Data are in µg/ml of dry plant tissue. In parentheses, coefficient of variation, for n = 6.

The rate at which nutrients are leached from soil has been suggested as a method for environmental impact assessment because it is a holistic measurement, representing the functions of numerous microbial groups as well as abiotic processes (13). In a study to evaluate nutrient leaching from microcosms as means for monitoring environmental impact, O'Neill et al. (13) found that effects from the addition of toxic chemicals to microcosms could be detected in nutrient export in leachate from the microcosms but not by changes in population/community parameters. The major factor limiting the usefulness of microcosms for evaluating the nutrient-related ecosystem impacts from the release of plant associated GEMs is likely to be the bulk volume of soil available for root exploration. In the later stages of crop growth of both maize and wheat, moisture was removed rapidly from the microcosm soil by the plants; in the field, roots typically explore and withdraw water from a larger volume of soil. A high density of roots will also result in the extraction of a greater concentration of available nutrients, and the nutrient dynamics in the microcosm soil at the later stages of plant growth will likely be different from that which would occur in the field. Therefore, the length of time intact soil-core microcosms can mimic field

situations in which GEM interactions with plants are being evaluated may be limited, dictated by the soil volume available, the plant species, and plant growth rate.

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3.0 FATE OF ROOT GROWTH-INHIBITING *PSEUDOMONAS* SP. Tn5 MUTANTS IN INTACT SOIL-CORE MICROCOSMS

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SUMMARY

Transposon Tn5 mutants of a wheat root growth-inhibiting nonfluorescent *Pseudomonas* sp. were inoculated into intact soil cores to determine the fate and potential impacts from the release of this organism into agricultural ecosystems. Transposon mutants that no longer inhibited the growth of wheat roots or *Escherichia coli* through the production of a toxin (tox⁻) as well as Tn5 mutants similar to the wild type (tox⁺) were inoculated into the surface soil of intact soil-core microcosms; the soil was spring wheat planted, and Tn5 mutant populations were monitored over time in the bulk soil, rhizosphere, and rhizoplane with depth. Populations were also monitored in core leachate and in the gut of earthworms. The population of the introduced Tn5 mutants declined over time in the surface soil but was present in significant numbers in the wheat rhizosphere and rhizoplane throughout the 60-cm soil-core depth. Rhizoplane populations of the tox⁺ Tn5 mutants were significantly higher than populations of tox⁻ mutants at the seedling stage but were not significantly different at the flowering stage. The Tn5 mutants were transported through the core with percolating water and were present in the gut of earthworms. Plant growth as well as indigenous rhizoplane populations of *Rhizobium leguminosarum*, associative N₂-fixing bacteria, and nitrifying bacteria were not affected by inoculation with the Tn5 mutants. These experiments

indicate that intact soil cores may have utility for assessing the fate and potential ecosystem impacts from the release of genetically engineered microorganisms to the environment.

INTRODUCTION

Plant-associated, and in particular rhizosphere-associated, bacteria have great potential for biotechnological applications in agriculture for biological control of plant diseases and for improving plant nutrition and providing plant growth factors (10). Microorganisms used in these applications may be improved through genetic engineering; however, concern about the release of genetically engineered microorganisms (GEMs) to the environment requires that the environmental risks be evaluated before a large-scale release. Microcosms have been identified as potential tools for both research and risk assessment concerning the release of GEMs to the environment (7). Microcosms have previously been used to assess the fate and impacts of released chemicals to the environment (40,19) and the interactions between pollutants and microorganisms (30). The intact soil-core microcosm was developed to evaluate the fate and ecological effects of chemicals in the environment (39) and, more recently, modified to evaluate the fate and environmental impacts from the release of GEMs (3). This research investigates the use of intact soil-core microcosms for evaluating the fate and potential environmental impacts from a genetically altered root-colonizing pseudomonad.

The use of no-till cultivation for wheat, and cool, wet weather in the spring months, favors the growth of nonfluorescent *Pseudomonas* sp. that inhibit wheat root growth (13). These bacteria can heavily colonize the rhizoplane of winter wheat and stunt root growth (15,16) and can achieve high population densities on crop residues (17,36). These wheat root growth-inhibiting *Pseudomonas* sp. have been shown to produce a low molecular weight toxin that inhibits the growth of wheat plants as well as some bacteria and fungi (4). This toxin appears to be a secondary metabolite, because activity can be detected in cultures grown under a wide range of conditions (5). These bacteria extensively colonize peas, oats, and lentils, but are not deleterious to these plants (17). Mutants that no longer produce the toxin (15) may have potential as a biocontrol agent to displace the wild type, as has been accomplished for ice nucleation-minus *Pseudomonas syringae* for control of frost damage (24). Also, other root-colonizing bacteria have been isolated that specifically inhibit the growth of weeds (8). Because these bacteria can exhibit a deleterious effect to a narrow host range and are aggressive root colonizers, they may also have potential for biotechnological applications in agriculture.

The specific objectives of this study were to measure the colonization of *Pseudomonas* sp. Tn5 tox^+ and tox^- phenotypes in the wheat rhizoplane, rhizosphere, and bulk soil with depth; determine the potential for transport of the introduced bacteria through the intact soil cores with percolating water and root growth; determine the potential for antagonism of several physiological groups of rhizosphere bacteria; and to determine if earthworms can serve as vectors of the introduced bacteria in soil.

MATERIALS AND METHODS

Bacterial Strains and Media

Toxin-producing (tox^+) and toxin-deficient (tox^-) Tn5 mutants of wheat root growth-inhibiting *Pseudomonas* sp. isolate RC1 were obtained from Dr. Lloyd F. Elliott [U.S. Department of Agriculture, Agriculture Research Service (USDA-ARS), Pullman, Washington]. The tox^- phenotype indicates the mutant is unable to inhibit root and *E. coli* growth. Described elsewhere are the procedures and vectors used for generation of transposon mutants (6), screening for toxin production (5), mutant growth characteristics (4), and chromosomal location of Tn5 insertion in the bacterial genome (45). Three mutants of each phenotype (tox^+ or tox^-) with unique Tn5 insertions were selected for inoculation of soil cores to increase the probability of introducing an organism that was not severely debilitated in its competitive ability (3). The medium of Sands and Rovira (31) amended with 500 $\mu\text{g}/\text{ml}$ of kanamycin (Sands Km⁵⁰⁰) was used for enumeration of mutants in microcosm samples and for routine culturing. Tryptic soy agar (TSA) (27) was used to enumerate total bacterial numbers.

Microcosms and Inoculation

Intact cores (17.5 x 60 cm) of Palouse soil (fine-silty, mixed mesic Pachic Ultic Haploxerolls) were obtained for use as microcosms and maintained as previously described (3). Briefly, intact soil cores were collected using a steel coring apparatus containing polyethylene pipe (Phillips Petroleum Co., Dallas, Texas). The intact soil cores, held inside the plastic pipe after removal from the driving tube, were placed in a housing on 20-cm-diameter Buchner funnels that were connected to sterile, acid-washed, deionized water-rinsed, 2-liter Erlenmeyer flasks for leachate collection. Six replicates of each treatment, including tox^+ , tox^- , and uninoculated control, were arranged in a completely randomized design. The top 15 cm of soil was removed from each of six replicate microcosms and combined. The soil was mixed for 15 min in a rotating soil mixer

then placed at 4°C until inoculated. Six 150 x 25 mm Sands Km⁵⁰⁰ agar plate cultures of each mutant were harvested separately, 48 h after inoculation, by adding 10 ml of sterile deionized water and dislodging the cells with a glass rod. The bacterial suspensions were transferred to a sterile bottle, diluted with sterile deionized water, mixed, and placed on ice. Optical density (600 nm) of each inoculum was measured and adjusted to obtain the same relative cell concentrations for each culture. Inoculated treatments consisted of a mixture of three tox⁺ mutants (A4-6, B4-7, I3-4) or a mixture of three tox⁻ mutants (RC-D, RC-E, RC-F). The three different mutants for each phenotype (tox⁺ or tox⁻) were mixed in equal portions and diluted to 200 ml final volume before soil inoculation. The control treatment received 200 ml of sterile deionized water.

Soils were inoculated and mixed in a rotating mixer for 30 min, stopping twice to break up the soil aggregates, to ensure a thorough mixing of soil and inoculum. Equal amounts (approximately 2.3 kg) of inoculated soil were returned to the replicate microcosms. Six seeds of spring wheat (*Triticum aestivum* L. Edwall) were planted in each microcosm and thinned to four plants after germination. Microcosms were watered with sterile deionized H₂O to maintain a water potential of approximately -0.03 MPa with a growth temperature of 22°C, as monitored by soil temperature and moisture probes on line to a datalogger (21x, Campbell Scientific Co., Logan, Utah). Photoperiod was set for 12 h with an initial light intensity at the soil surface of 400 microeinsteins/cm (4100 K Ultralume Lamps, Phillips Lighting Corp., Bloomfield, New Jersey). Two earthworms (*Lumbricus* sp.) were introduced into microcosms after 3 weeks of wheat growth to assess their potential as vectors of the introduced bacteria.

Nondestructive Microcosm Sampling

Pseudomonas sp. Tn5 mutant populations in the surface soil were measured over time. Approximately 15 g of soil was removed from the top 15 cm of the microcosms at each sampling point, taking care not to remove or disturb plant roots; 10 g was used for bacterial enumerations and 5 g for moisture content analysis. Two wheat seedlings with intact roots were removed on day 21 to determine the extent of root colonization by the Tn5 mutants. Methods used for rhizosphere and rhizoplane sampling are described in detail elsewhere (15). Briefly, for rhizosphere soil samples, the soil adhering to roots was washed off the root into a sterile dilution blank. The washed roots were then macerated with a mortar and pestle for the rhizoplane samples. Initial dilutions were shaken for 20 min on a gyratory shaker at 250 rpm. Rhizosphere populations of native *Rhizobium leguminosarum* were determined by the most-probable-number infectivity method using *Pisum sativum* as the host plant (41).

Twenty-five days after inoculation, microcosms were leached with sterile deionized water (250 ml) applied twice daily (9 to 12 h apart) until leachate was observed in 2-liter Erlenmyer leachate collection flasks. Leachate was decanted into a sterile container and placed at 4°C within 12 h of soil breakthrough and entry into the flasks. Leachate samples were serially diluted and plated onto Sands Km⁵⁰⁰ to enumerate *Pseudomonas* Tn5 mutant populations.

Destructive Microcosm Sampling

Microcosms were sacrificed on day 54, or the boot stage of wheat. Wheat plants were cut at the soil surface and above-ground plant biomass determined. Two cuts were made along the length of the polyethylene pipe enclosing the intact soil cores; the pipe was removed, and the intact core was separated into five sections according to depth (0-15, 15-25, 25-35, 35-45, and 45-55 cm). The outer 1 cm of soil, which was next to the pipe, was pared to remove soil and roots at the pipe interface. Roots with adhering soil were removed from each section and processed as previously described for day 21, except rhizoplane sample roots were macerated for 90 sec in a tissue homogenizer (Sorvall Omni-Mixer, E. I. Du Pont De Nemours and Co., Newtown, Connecticut). At each depth the soil remaining in the soil core after rhizoplane and rhizosphere sample collection was mixed and retained as a bulk soil sample.

Pseudomonas sp. Tn5 mutant populations in the rhizoplane, rhizosphere, and bulk soil were determined at each depth by dilution plating on Sands Km⁵⁰⁰ medium. Total bacterial numbers in the rhizoplane were determined by dilution plating on TSA. Rhizoplane populations of N₂-fixing bacteria in the soil-core surface layer (0 to 15 cm) were enumerated by the MPN-acetylene reduction method (22), and *R. leguminosarum* was enumerated as described earlier (day 21). Ammonium-oxidizing bacteria on the rhizoplane were determined by the MPN method of Schmidt and Belser (32).

Earthworms were removed from the soil cores during sampling and placed in autoclaved (40 min, 2 consecutive days) Palouse soil containing 1% ground wheat straw (a water potential of -0.03 MPa) and incubated at 4°C to enrich (17) for the introduced bacteria. *Pseudomonas* sp. Tn5 mutant populations were measured in this soil on Sands Km⁵⁰⁰ after a 7-day incubation. Earthworms were removed after 23 days in the wheat straw-amended soil, washed, surface disinfected by placing in 1% Chloramine T (Sigma Chemical Co., St. Louis, Missouri) for 1 min., and dissected to sample the gut for the presence of the introduced bacteria.

Data were analyzed as a completely randomized design with six replicates. A protected least significant difference (LSD) was conducted at the $p \leq .05$ level of significance for all treatment mean comparisons (35).

RESULTS

The insertion site of Tn5 into the genome was unique for each of the three tox^+ or tox^- mutants selected for each treatment (data not shown), thereby increasing the probability of including mutants that were not severely impaired in competitive ability. Sands Km⁵⁰⁰ provided excellent selection for the Tn5 mutant bacteria because no growth occurred on plates from dilutions of the control microcosms. This level of kanamycin was chosen because it was the highest level that could be used without reducing the plating efficiency of the transposon mutants (data not shown). The stability of Tn5 in these bacteria was not investigated in this study; however, previous studies have shown that Tn5 was relatively stable with regards to excision and loss from the chromosome of *P. putida* and *R. leguminosarum* cv. *phaseoli* grown in soil (18).

Bulk surface soil (0 to 15 cm depth) populations of the *Pseudomonas* Tn5 mutants declined slowly from approximately 1×10^8 CFU per dry gram of soil at the time of inoculation to 4×10^5 CFU/dry g after 30 days and 3×10^4 CFU/dry g after 54 days (Figure 3.1). Populations of tox^+ and tox^- mutants declined in the surface bulk soil at an identical rate. The LSD of the mean bulk soil populations of the Tn5 mutants in the inoculated soil layer at the day 54 sampling (Figure 3.2) were considerably larger than the same value in Figure 3.1. This difference in the LSD value occurred because all depths were statistically analyzed together for Figure 3.2. A marked increase in variability occurred below the inoculated 0- to 15-cm layer; some bulk soil samples in the lower sections of the soil core had populations less than the detection level of 200 CFU/dry g soil.

At the day 21 sampling, the wheat seedling rhizoplane population of *Pseudomonas* Tn5 mutants was more than 10 fold higher than in the rhizosphere, which was 10 fold higher than in the bulk surface soil (Figure 3.3). The mean tox^+ populations in the bulk or rhizosphere soil were not significantly different from tox^- populations (Figure 3.3). However, mean rhizoplane populations of tox^+ bacteria were significantly higher than tox^- populations. Leachate appeared in the collection flasks after approximately 5 days of leaching. Between 2.5 and 3.5 liters of sterile water was used to leach each microcosm, and the volume collected was consistently between 90 and 150 ml. Eleven of 12 inoculated microcosms had detectable populations of Tn5 mutants in their leachate, which averaged more than 10^3 CFU/ml (Figure 3.3).

Figure 3.1. Populations of *Pseudomonas* sp. Tn5 Tox⁺ and Tox⁻ Mutants in the Inoculated Surface Soil of Soil-Core Microcosms Over Time. Error bars represent significant differences ($p \leq .05$).

Distribution of *Pseudomonas* Tn5 mutants by depth in the rhizoplane, rhizosphere, and in bulk soils at day 54 is shown in Figure 3.2. Differences in rhizoplane populations of tox⁺ or tox⁻ Tn5 mutants were less than 1.5 log units over the length of the soil core. The rhizosphere populations of the Tn5 mutants at each depth paralleled those of the rhizoplane but were approximately 10 fold lower. Bulk soil populations declined sharply from approximately 3×10^4 CFU/g in the inoculated surface soil to approximately 10^2 CFU/g in the four deeper sections. There was no significant difference between Tn5 mutant tox⁺ and tox⁻ rhizoplane, rhizosphere, or bulk soil populations with depth except in the rhizosphere at the 15- to 25-cm depth. At microcosm harvest (day 54), there was no significant difference in plant biomass between treatments. Average shoot biomass was 7.83 g (dry weight) per microcosm for control, 8.41 for tox⁻, and 9.08 for tox⁺ treatments with an LSD value ($p \leq .05$) of 1.89 g. Because all wheat roots were not recovered, a total root mass could not be calculated. However, bacterial inoculation caused no obvious stunting of root growth.

Total bacterial populations (on TSA) in the rhizoplane tended to follow a pattern similar to that observed for the transposon mutants (Figure 3.4). Populations declined 10 fold or less on roots from the surface to those sampled from the lower sections. The total population tended to be lower in the tox⁻ treatments at all depths, but significant differences were found only at the 15- to 25-cm depth. Extreme variability was found when the tox⁺ and tox⁻ populations on the rhizoplane

Figure 3.2. Rhizoplane, Rhizosphere, and Bulk Soil Populations of *Pseudomonas* sp. Tn5 Tox⁺ and Tox⁻ Mutants with Depth in the Intact Soil Cores at Day 54. Error bars represent significant differences ($p \leq .05$).

Figure 3.3. Populations of *Pseudomonas* sp. Tn5 Tox⁺ and Tox⁻ Mutants in Microcosm Leachate, Bulk, and Rhizosphere Soil and in the Rhizoplane at the Wheat Seedling Stage (day 21). Error bars represent significant differences ($p \leq .05$).

represented as a percent of the total bacterial population as determined on TSA. Values ranged from 8% to virtually 100% for both, demonstrating that both inoculated bacteria constituted a significant portion of the total bacterial population on the rhizoplane.

Inoculation with the different inhibitory pseudomonad Tn5 mutants had no significant ($p \leq .05$) effect on rhizoplane population sizes of *R. leguminosarum*, N₂-fixing bacteria, or ammonium-oxidizing bacteria in the surface (0 to 15 cm) soil. Rhizoplane populations of *Rhizobium leguminosarum* biovar *viceae* ranged between 7.23 ± 0.43 and 7.43 ± 0.35 log MPN/g dry root, free-living N₂-fixing bacteria ranged between 4.82 ± 0.29 and 5.05 ± 0.51 log MPN/g dry root, and populations of ammonium-oxidizing bacteria ranged from 4.25 ± 0.24 to 4.66 ± 0.20 log MPN/g dry root. In addition, *R. leguminosarum* populations in the rhizosphere of tox⁺ treatments (7.17 log MPN/g dry soil) were not significantly different (LSD = 0.52; $p \leq .05$) from populations in the tox⁻ treatments (7.37) or in the uninoculated microcosms (6.91) at the day 21 sampling.

In the autoclaved and wheat straw-amended soil, populations of tox⁺ and tox⁻ Tn5 mutant bacteria were 10^5 /g dry soil or greater 7 days after earthworms from microcosms were added. Three of six earthworms sampled after 23 days had *Pseudomonas* Tn5 mutants present in their gut. No growth was observed on Sands Km⁵⁰⁰ receiving dilutions from earthworms sampled from uninoculated microcosms or their straw-amended soil.

Figure 3.4. Rhizoplane Populations of Total Bacteria (on TSA) with Depth at Day 54 in Soil-Core Microcosms Inoculated with Tn5 Mutants and Uninoculated Controls. Error bars represent significant differences ($p \leq .05$).

DISCUSSION

Inoculation of plow layer soil with the three genotypically distinct Tn5 mutants of tox^+ or tox^- root inhibitory pseudomonads resulted in colonization of the wheat rhizosphere and rhizoplane of roots sampled from all depths. The use of Sands Km⁵⁰⁰ provided excellent selection for the *Pseudomonas* Tn5 mutants, thus eliminating background growth even at the lowest dilutions. There was no difference between tox^+ and tox^- mutant populations in the bulk soil at any sampling time. Populations of tox^+ mutants colonizing the wheat rhizoplane were significantly higher than populations of tox^- mutants 21 days after seeding and inoculation. Rhizosphere populations of tox^+ and tox^- mutants were not statistically different at either sampling. The rhizoplane represents the region of the root zone where the density of inhibitory pseudomonads is highest (15) and the concentration of the antibiotic produced by these bacteria would probably be greatest. Thus, the greatest potential for antagonism of the indigenous microflora would be in the rhizoplane.

The root-colonizing pseudomonads introduced at high population densities in the surface soil of the microcosms declined more than 1000 fold over a 54-day period (see Figure 3.1). These results agree with the decline of *Pseudomonas* sp. in bulk soil after the introduction of large populations of laboratory-cultured bacteria into uncropped soil in other studies (38,28). In the later stages of plant growth, that is, the boot stage, the rhizoplane as well as the rhizosphere population

densities of the Tn5 mutants in the surface layer had declined by 10 fold or greater (see Figures 3.2 and 3). The rhizosphere bacterial population of wheat has been shown to decrease with the age of the plant and to decline sharply during flowering (33). A rifampicin- and nalidixic acid-resistant fluorescent pseudomonad mutant introduced as a seed inoculum on wheat declined from approximately 10^8 to less than 10^4 CFU/g root from germination to harvest (42). However, it cannot always be predicted a priori that bacteria introduced into soil will rapidly die off (12,9), indicating that bacterial decline in soil is dependent on the type of bacteria as well as environmental conditions.

Results of this study indicate that inoculation of soil with either tox^+ or tox^- wheat root growth-inhibiting *Pseudomonas* sp. did not affect *R. leguminosarum* rhizosphere populations at the seedling stage or rhizoplane populations of *R. leguminosarum*, N_2 -fixing bacteria, or ammonium-oxidizing bacteria at boot stage. The *Pseudomonas* sp.-produced toxin has been shown not to affect the growth of *Rhizobium* sp. in vitro (unpublished data).

Toxins have long been assumed to provide a selective advantage to microorganisms that produce them. While there have been many demonstrations of antibiosis in vitro, experimental evidence for the selective advantage of toxin-producing bacteria in natural systems is, with a few exceptions (1,23), limited. The use of intact soil cores as microcosms provides a means to evaluate such interactions in the laboratory while retaining complexity analogous to the field. Evaluation of intact soil cores as microcosms to study the effect of fly ash on plant uptake of trace elements demonstrated that soil-core microcosms could serve as reasonable analogs of the field (37,40).

The Tn5 mutant bacteria introduced into the surface soil layer moved through the soil core with percolating water, as evidenced by the presence of these bacteria in the leachate (see Figure 3.3). Because the cores were gradually saturated by the application of small quantities of water over a long period of time, it is unlikely that significant volumes of water channeled along the soil-pipe interface and into leachate collection flask. If channeling had occurred, water would likely have been observed after the first few volumes (250 ml) of water had been added rather than after the fourth day when 2.5 to 3.5 liters of water had been added to the various microcosms. This evidence, plus the tight fit of the soil core in the polyethylene pipe and the fact that the soil expands as it absorbs water, indicate bacterial transport through the soil core. In addition, distribution with depth of *Pseudomonas* Tn5 mutants in bulk soil (see Figure 3.3) indicates bacterial movement occurs through the core itself. At sampling, soil and roots at the soil-polyethylene pipe interface were pared to eliminate any influence from bacteria transported by channeled water. Smith et al. (34)

concluded that *E. coli* and Cl^- moved through the center of intact cores faster than at core edges and that transport potential through intact soil cores is greater than through soil that has been mixed and packed.

Experiments using sieved, packed soil columns demonstrate that pseudomonads are more susceptible to water-mediated transport than other bacteria. Madsen and Alexander (25) found no vertical dispersal of bacteria occurred below the inoculated layer without some transporting agent (i.e., water) and that *P. putida* was transported farther and in greater numbers than *R. japonicum*. Other studies have demonstrated *Pseudomonas* spp. to be more prone to movement through soil than other bacterial species such as *Bacillus* and *Azotobacter* (44). However, even the movement of *P. fluorescens* through sterile soil was restricted in all but very wet conditions (44).

Populations of nonfluorescent Tn5 mutants found in leachates of the intact soil-core microcosms are comparable to those observed by Martin (26) in a study evaluating the leaching of bacteria from wheat roots. That study also found fluorescent pseudomonads to be partitioned into leachate to a greater degree than other rhizosphere bacteria. Earlier work with intact soil-core microcosms of the same soil type used in this study as well as with a more coarsely textured soil showed that *Azospirillum lipoferum* Tn5 mutants introduced as a seed inoculum on wheat or corn roots were not detected in leachate. However, the size and method of the inoculum and the extent of root colonization was considerably less in that study (3).

The pseudomonad Tn5 mutants colonized roots in the lower section of the soil core (55 cm); however, the mode of transport to roots in this zone is unknown. Because the soil cores were leached at day 25 and the bacteria were present in the bulk soil of the lower core sections at day 54, bacterial colonization of roots in these zones may have resulted from percolating water. In a field study with potato plants, irrigation increased rhizosphere populations of *P. fluorescens* 10 fold whereas no increase was found in nonrhizosphere soil (2). The decline in populations of the introduced bacteria on wheat roots with depth is typical of that observed in other studies and is not indicative of the mode of movement of bacteria to a particular root segment. Bacteria introduced as seed inoculants on wheat were present in progressively lower numbers with increasing depth from the seed in the absence of vertical water movement in a laboratory system designed to control soil matric potential (21). A similar decline with depth of inoculated root-colonizing fluorescent pseudomonads has been observed in the field (43).

Deleterious effects on plant growth were not observed in this study because population densities were considerably less than the 10^6 CFU/mg dry root necessary for growth inhibition

(15). In addition, other factors that contribute to optimal root colonization and growth inhibition, such as low temperature (14), susceptible wheat cultivar (13), or the presence of crop residues (18), were not provided.

The presence of the *Pseudomonas* Tn5 mutants in the digestive tract of earthworms present in the soil cores suggests that earthworms may act as vectors in disseminating bacteria introduced into soils. Also, the population of introduced bacteria could be significantly increased upon passage through the earthworm; Parle (29) found that bacterial and actinomycete numbers increased by more than 100 fold over numbers found in the soil during passage of food through the worm gut. Earthworms have been shown to increase the transport of bacteria in soil as well (25).

The transposon Tn5 has been shown in this study and others (38) to provide an excellent phenotypic tag for tracking the fate of microorganisms in the environment. Tn5 could provide a genotypic tag as well, and may be used for direct (20) and indirect (18) analysis of populations in soils using DNA-hybridization techniques. Further, transposons can create clean mutations that may be of use in studying the effect of specific mutations on environmental competency or pathogenicity of a microorganism (23,11,45). Such transposon mutants could also have potential for commercial applications in agricultural biotechnology (23).

This study has demonstrated that toxin production can influence the rhizoplane population of a root-colonizing *Pseudomonas* sp. The fact that no differences in rhizoplane, rhizosphere, or bulk soil populations of tox^+ or tox^- root growth-inhibiting *Pseudomonas* sp. Tn5 mutants were observed except in the rhizoplane of wheat seedlings suggests that the tox^- phenotype may be used as a biocontrol agent to suppress such deleterious bacteria in the rhizoplane.

Intact soil cores have been used in this study as analogs of the field to study microbial fate and effects of root-associated bacteria on indigenous bacterial populations in an agricultural ecosystem. The dissemination, survival, and impacts of an introduced genetically altered microorganism should be of value in future research involving genetically engineered microorganisms in intact soil-core microcosms intended for release to the environment. Calibration of microcosm with the field environment would improve the utility of microcosms for predicting behavior in the field (30), and, thus, risk assessment.

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APPENDIX A

STANDARD OPERATING PROCEDURES

STANDARD OPERATING PROCEDURE - TD 2369 - SOP1

Sampling and Equilibration of Soil-Core Microcosms

Project Title: Evaluation of Terrestrial Microcosms for Assessing the Fate and Effects of Genetically Engineered Microorganisms on Ecological Processes

Project Manager: Dr. Peter Van Voris

Principal Investigator: Dr. James K. Fredrickson

INTRODUCTION

Terrestrial microcosm systems have been developed for assessing the transport, fate, and ecological impacts associated with the release of toxic and hazardous chemicals to the environment. It has been suggested that microcosms may be ideal ecosystem-level test systems for assessing these same parameters after the environmental release of genetically engineered microorganisms (GEMs). A set of recently published terrestrial microcosm test protocols and guidelines suggests procedures for performing a multitiered screening of the potential toxicity of a chemical or hazardous substance (Van Voris 1988; Van Voris et al. 1984, 1985a,b). These protocols for sampling and maintenance of microcosms will be followed for this project.

Microcosm Collection

Microcosm soil cores are extracted from a homogeneous field so that soil properties are not highly variable among the different cores. Steel driving tubes are used to extract the 17.8- and 25.5-cm-diameter by 60-cm-long cores. Polyethylene (Driscopipe®), tubes line the steel driving tubes (Figure A.1). The cutting edge of the driving tubes is slightly smaller in diameter than the Driscopipe to allow for soil expansion during the coring process. A backhoe is used to push the driving tube into the soil and then to remove the cores by wrapping a chain from the backhoe bucket around the handles and pulling the tube out. The soil cores and polyethylene tube are removed as a single unit. Topsoil from the field is then added to fill the top 10 cm of the microcosms before they are transported back to the laboratory.

®Phillips Petroleum Co., Bartlesville, Oklahoma.

Microcosm Maintenance

The microcosm cores rest on Buchner funnels covered with a thin layer of glass wool. These units are placed in carts or in the microcosm containment systems (MCS) described elsewhere. Spring wheat (*Triticum aestivum*) and corn (*Zea mays*) are seeded into 17.8- and 25.5-cm-diameter intact soil cores, respectively. Soils are periodically brought to a water potential of approximately -0.3 bar, near optimum for plant and microbial growth. Measurement of water potential is monitored with tensiometers or gypsum blocks placed in several of the soil cores, and filtered (0.2- μ m), ion-free water is added when the potential declines to -1 bar.

MATERIALS

Polyethylene Pipe

Driscopipe[®], a product of the Phillips Petroleum Company, is the material of choice for the soil-core microcosms. Composed of ultrahigh molecular weight, high-density, nonplasticized polyethylene, it is impermeable, lightweight, tough, rigid, and highly resistant to acids, bases, and biological degradation. It is used commercially for underground gas pipelines and can be buried for 50 years with no expected structural changes. Driscopipe[®] can be purchased from Maskell-Robbins, Inc., of Mountlake Terrace, Washington. Specifications for the 17.8- and 25.5-cm-diameter microcosms are listed below:

1. 25.5-cm Driscopipe 8600, ultrahigh molecular weight, high-density polyethylene pipe, SDR 29.5, 56 psi, OD 10.75 in., ID 10.022 in., wall thickness 0.364 in.; each piece 60 cm long and edges smoothed.
2. 17.8-cm Driscopipe 1000, high-density, high molecular weight polyethylene pipe, SDR 26, 65 psi, OD 7.125 in., ID 6.577 in., wall thickness 0.274 in.; each piece 60 cm long and edges smoothed.

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STANDARD OPERATING PROCEDURE - TD2369 - SOP2

Plant Productivity and Tissue Analysis

Project Title: Evaluation of Terrestrial Microcosms for Assessing the Fate and Effects of Genetically Engineered Microorganisms on Ecological Processes

Project Manager: Dr. Peter Van Voris

Principal Investigator: Dr. James K. Fredrickson

INTRODUCTION

Soil microorganisms are integral catalysts in the cycling of essential plant nutrient elements in soils. Ecological impact upon an ecosystem from introduction of genetically engineered or novel microorganisms could result in changes in nutrient flow processes. Changes in ecosystem nutrient status may be reflected in plant productivity or yield and plant nutrient content. To determine if introduced bacteria have influenced ecosystem nutrient dynamics, the productivity and nutrient content of the above-ground portion of spring wheat (*Triticum aestivum*) and corn (*Zea mays*) from both inoculated and control microcosms can be measured.

PROCEDURES

Nutrient Analysis

Standard procedures are used for plant sampling and analysis (Jones and Steyn 1973). Corn is sampled before tasseling by removing the entire leaf fully developed below the whorl from two plants in each microcosm; the four uppermost leaves of spring wheat are sampled before heading from four plants in each microcosm. Plant leaves are dried at 60°C for 48 h before grinding. Dried plant material is ground (40 mesh) using a mechanical mill with stainless steel surfaces to minimize contamination with trace metals.

Ground plant material is ashed using a modified wet ash procedure in which 0.1 g of dried plant material is accurately weighed and placed in a Teflon container with 2.5 ml of concentrated HNO_3 at 80°C for 12 h. The ashed tissue is then brought to 20 ml and analyzed directly for P, S, Zn, Fe, Mn, and Mo by inductively coupled plasma (ICP) spectrometry. Total nitrogen content of plant tissue is measured by standard Kjeldahl procedure (Bremner and Mulvaney 1982) or, alternatively, by a Leco CHN analyzer.

Productivity Analysis

Plant biomass production is assessed by harvesting the above-ground portion of the plant, drying it at 60°C , and weighing. Plant biomass is expressed as grams of dry weight per plant.

REAGENTS AND MATERIALS

Concentrated HNO_3 (Mallinckrodt Inc., of Paris, Kentucky)

Teflon bombs

Inductively Coupled Plasma Spectrometry (Applied Research Laboratories Inc., Sunland, California & Jarrell-Ash) (Thermo Jarrell-Ash Corp., Franklin, Maine)

Mettler PL1200 electronic balance, reading range 0.01 g (Mettler Instruments Corp., Highstown, New Jersey)

Mettler AE 163 electronic balance, dual weighing range, readability 0.0001

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STANDARD OPERATING PROCEDURE - TD2369 - SOP3

Rhizosphere and Endorhizosphere Sampling

Project Title: Evaluation of Terrestrial Microcosms for Assessing the Fate and Effects of Genetically Engineered Microorganisms on Ecological Processes

Project Manager: Dr. Peter Van Voris

Principal Investigator: Dr. James K. Fredrickson

INTRODUCTION

More than 80 years ago, Hiltner (1904) described the rhizosphere as the zone of soil around roots where microbial growth is stimulated by organic compounds released by roots. Since then, the region of plant root interaction with microorganisms has been extended to include the rhizoplane or root surface and the endorhizosphere, in and between cells of the root cortex (Patriquin et al. 1983). *Azospirillum* spp. are preferentially enriched in the rhizosphere and rhizoplane and appear to readily establish in the endorhizosphere of maize and wheat (Patriquin and Dobereiner 1978). The rhizosphere is inherently a zone not physically well defined, and thus there are a variety of procedures for obtaining rhizosphere samples. The main objective of this Standard Operating Procedure is to clearly delineate the procedures that will be used for rhizosphere and endorhizosphere sampling.

PROCEDURES

Rhizosphere Sampling

Rhizosphere samples for microbiological analysis are obtained following a standard soil analysis procedure described by Wollum (1982). Corn (*Zea mays*) and spring wheat (*Triticum*

aestivum) roots and rhizosphere soil are carefully excavated with a hand trowel from soil-core microcosms. Samples are placed in sterile whirl-pak bags, transported on ice to the laboratory, and processed the same day.

Roots are shaken lightly to remove loosely adhering soil. For each sample, approximately 5 g of root with adhering soil is transferred to a 99-ml blank dilution bottle containing sterile physiological saline. Because variability within each bulk sample is possible, numerous subsamples are used in the first dilution. Dilution bottles are shaken on a mechanical reciprocating shaker at 150 rpm for 10 min; 10 ml of diluent is immediately removed and transferred to a 90-ml sterile saline dilution blank (alternatively, 1 ml of diluent is added to 9 ml of sterile saline) to establish the 10^{-3} dilution. The dilution series is then continued to 10^{-8} or to extinction. The GEM models are then enumerated according to GEM Detection and Enumeration Standard Operating Procedure 835-SOP5. Bulk soil samples are handled in a similar manner; however, 10 g of soil is placed in a 95-ml dilution blank for the 10^{-1} dilution.

Endorhizosphere

Endorhizosphere samples are taken from the same bulk samples obtained for rhizosphere soil samples. Roots are washed free of soil with sterile deionized water. Nodal roots are then surface sterilized (Patriquin and Dobereiner 1978) by immersing the roots, except the proximal 2 cm of cut ends, in 1% chloramine-T (Sigma) solution for 1 h. The root pieces are then immersed for 30 min in sterile 0.05 M pH 7 phosphate buffer and rinsed three times with sterile deionized water. Approximately 3 g of fresh root is macerated in 99 ml of sterile physiological saline, or variations thereof with the same weight-to-volume ratio, in a blender to free bacteria residing in the endorhizosphere. A dilution series of the root macerate is prepared in sterile saline blanks as described above.

REAGENTS

Purity of all reagents is analytical grade, and all solutions are made with deionized water.

Sterile Physiological Saline

Dissolve 8.5 g of NaCl (J.T. Baker, Phillipsburg, New Jersey) in 1000 ml of deionized water; dispense appropriate volume into dilution bottles, and autoclave at 121°C and 15 psi for 20 min.

Chloramine -T

Dissolve 1 g of chloramine-T (*N*-chloro-*p*-toluenesulfonamide; Sigma) in 100 ml of deionized water.

Phosphate Buffer

Phosphate buffer pH 7 is prepared by mixing 195 ml of stock 0.1 M NaH₂PO₄ (J.T. Baker) with 305 ml of 0.1 M Na₂HPO₄ (J.T. Baker) and diluting to 1 L with distilled water. Buffer is sterilized by autoclaving as described earlier.

Equipment

Instruments and equipment used for these procedures include: Mettler PL1200 electronic balance, reading range 0.01 g; Mettler AE163 electronic balance, dual weighing range, readability 0.01 mg; New Brunswick portable reciprocating shaker Model G-2 (New Brunswick Scientific Co., New Brunswick, New Jersey); Osterizer blender Model 854-04 (John Oster, Manufacturing, Milwaukee, Wisconsin).

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STANDARD OPERATING PROCEDURE - TD 2369 -SOP4

Leachate Analysis

Project Title: Evaluation of Terrestrial Microcosms for Assessing the Fate and Effects of Genetically Engineered Microorganisms on Ecological Processes

Project Manager: Dr. Peter Van Voris

Principal Investigator: Dr. James K. Fredrickson

INTRODUCTION

Microbiological and chemical analysis of microcosm leachates provides information as to the movement of mutant constructs through the soil/plant continuum and their influence on soil nutrient cycling processes. Binding of microorganisms to soil clay and organic particles can limit their movement through the profile. Empirical observations such as the lack of leaching of microbes through soil during periods of heavy rain, failure to wash substantial numbers of cells from soils in perfusion or leaching experiments, and increased release of microbes upon sonication or treatment with surfactants are cited as evidence for binding of microorganisms to soil particles (Stotzky and Burns 1982). However, movement of genetically engineered microorganisms through soil and into groundwater could be an important pathway for their environmental dissemination and potential exposure to humans. Hypothesized ecological impacts resulting from the release of genetically engineered microorganisms to the environment include effects upon nutrient cycling. Changes in ecosystem nutrient cycling processes may be reflected in soluble nutrient (PO_4^{3-} , SO_4^{2-} , NO_3^- , soluble organic carbon) levels in soil.

PROCEDURES

Leachate Collection

Leachate from microcosms are collected in sterile containers and transported on ice to the laboratory. Aliquots of leachate are analyzed for microorganisms on the same day, and leachates for chemical analysis are immediately filtered through a Millex®-GV 0.22- μ m filter unit (Millipore Co., Bedford, Massachusetts) and frozen (-20°C) until analyzed.

Microbiological Analysis

Tenfold serial dilutions (to 10^{-8} or extinction) of aliquots of microcosm leachates are prepared in sterile physiological saline. Enumeration of *A. lipoferrum* Tn5 mutant constructs are conducted according to GEM Detection and Enumeration Standard Operating Procedure TD 2369 - SOP5.

Chemical Analysis

Filtrates are analyzed for inorganic anions, PO_4^{3-} , SO_4^{2-} , and NO_3^{1-} by ion chromatography (Dionex Model 10, Dionex Corp., Sunnyvale, California). Samples are routinely analyzed on AS1 (Dionex Corp., Sunnyvale, California) anion-exchange columns having a wide working range for these common anions using an eluent of 3 mM NaHCO_3 and 2.4 mM Na_2CO_3 at flow rates of about 2.3 ml/min. Normal configuration of the IC (Dionex Model 10) consists of the anion exchanger followed by a membrane eluent suppressor that uses 0.025 N H_2SO_4 to neutralize the basic eluent stream, and is followed by conductivity detection over the 1- to 100- μ S ranges, full scale, with detection limits (twice baseline noise) of about 0.06 ppm or better for NO_3^{1-} , PO_4^{3-} , and SO_4^{2-} . The elution order and detection limits can be altered or improved as necessary by supplemental use of other available columns (AS3, AS4a), eluents (commonly carbonate-bicarbonate-hydroxide ratios), and LC systems (Models 16, 2120 IC systems, Dionex). The routine system employs a Wisp (Waters Associates, Milford, Massachusetts) autosampler using volumes up to 200 μ l. Usually no sample pretreatment other than filtration is necessary, unless low-level fluoride analyses are required; in that case, adjustment of the sample to the eluent composition is needed. External standardization using bracketing of sample concentrations is done routinely, with calibration curves constructed by linear power-curve fits, as appropriate. Standard stock solutions (1-2 g/L) are prepared from the appropriate reagent-grade chemicals (oven-dried and stored under desiccation, as necessary), and the stock solutions are stored at 4°C in the dark.

Working standards are prepared by dilution of stock solutions. If the sample matrix is suspected to cause alteration of response, standards may be prepared into a background solution simulating the sample matrix; however, the wide working range of the AS1 column usually makes matrix matching unnecessary for most samples.

As necessary, the IC analysis may be supplemented by alternative specific detection methods. Nitrate/nitrite may be determined colorimetrically using the automated cadmium reduction method (APHA/AWWA/WPCF 1975), which determines nitrite directly and nitrate plus nitrite after reduction. Detection limit is 0.01 µg/ml as nitrite. Fluoride may be determined by ion specific electrode (ISE), with a detection limit of about 0.02 µg/ml. Analysis of F⁻ with Orion model 9609 (Orion Research Inc., Cambridge, Massachusetts) combination electrode involves addition of commercial total ionic strength adjustment buffer (TISAB III, Orion) according to the manufacturer's recommendations, followed by direct comparison of the millivolt sample reading to a standard curve. In addition, a standard curve can be used to monitor for matrix effects. Typical minimum volume required for analysis is 0.05 ml, plus 0.05 ml TISAB III.

Ammonia is determined by ISE using the Orion model 9512 gas-sensing electrode after adjustment of pH according to manufacturer's recommendations. Direct reading comparison to a standard curve and standard addition techniques will be used. Typical minimum volumes required are 2 ml of sample and 0.025 ml of 10 M NaOH.

Dissolved organic carbon (DOC) are determined by acidifying the filtered leachate to pH<2 with phosphoric acid, sparging to remove inorganic carbon as CO₂, and injecting the sample into a Dohrmann (Santa Clara, California) Model DC-80 Carbon Analyzer, which uses UV-enhanced acidified persulfate oxidation of the organics to CO₂, followed by infrared detection. Total carbon is determined by direct injection of the filtered leachate, with inorganic carbon quantified by the difference between total and organic carbon. If purgeable organics are suspected, inorganic carbon may be determined directly by injection into an acidified solution, bypassing the UV-oxidation step, and proceeding to the detector. NBS-grade ground, oven-dried potassium hydrogen phthalate (KHP) is used to make the 2-g DOC/L stock, which is preserved by adding 1 mM NaN₃ and storing at 4°C in the dark. Dilutions of the stock solution are made into 18-megaohm water (Milli-Q system, Millipore Co., Bedford, Massachusetts), with the concentration of the dilutions corrected for any contribution by the water (normally ≤0.25 µg/ml). Dilutions below 50 µg/ml are prepared fresh daily, and dilutions of 50- to 100 µg/ml are kept for no more than 4 days. Although the instrument possesses single-point calibration, time and use cause some deterioration of the electronic calibration, so a standard curve bracketing the observed sample values is always used.

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STANDARD OPERATING PROCEDURE - TD 2369-SOP5

Genetically Engineered Microorganism (GEM) Detection and Enumeration

Project Title: Evaluation of Terrestrial Microcosms for Assessing the Fate and Effects of Genetically Engineered Microorganisms on Ecological Processes

Project Manager: Dr. Peter Van Voris

Principal Investigator: Dr. James K. Fredrickson

INTRODUCTION

The assessment of risks involved in the release of genetically engineered microorganisms to the environment requires tracking the environmental fate of the organism to determine its survival, growth, and dissemination. A variety of standard methods have been applied to the detection and enumeration of microorganisms in the environment including direct plating, enrichment cultures, identification of characteristic cell components (e.g., fatty acids, proteins, rRNAs), direct microscopy, immunofluorescence, and immunoradiography. The advantages and disadvantages have been the subject of much discussion and are reviewed by McCormick (1986). Recently, DNA probes have been used to detect *Salmonella* spp. (Fitts et al. 1983) and *Yersinia* spp. (Hill et al. 1983) in foods and microorganisms with specific catabolic genes in sediments (Sayler et al. 1985). The advantage to using gene probes for environmental detection is that they are very specific and have the potential for detection of gene transfer. Described herein is a procedure for DNA hybridization we have used to successfully enumerate *Pseudomonas putida* and *Rhizobium* spp. transposon (Tn5) mutants in soil (Fredrickson et al. 1988).

PROCEDURES

Spot-Blot DNA Hybridization

Enumerations of *Azospirillum lipoferum* Sp Br17 (ATCC 29709) and *A. lipoferum* RG 20a (ATCC 29708) model GEMs (mutants expressing Tn5 insertion-associated kanamycin

resistance) inoculated into microcosms are made by a spot-blot most-probable-number (MPN) technique (Fredrickson et al. 1988). After inoculating microcosms with the model GEMs, samples are obtained from the rhizosphere and endorhizosphere (TD2369-SOP3), leachate (835-SOP4), plant xylem fluid (TD2369-SOP6), and insect gut (TD2369-SOP8). Initial sample dilutions are made as described in TD2369-SOP3, except that modified *Spirillum* Nitrogen Fixing medium (MNFM) is used for the diluent, and aliquots of 277.5 μ l are removed and added to five wells in a sterile microtiter plate. Serial 1:10 dilutions (27.5 μ l) are made to extinction (10^{-8}) to obtain a total volume of 277.5 μ l MNFM broth per well. MNFM medium is amended with 100 μ g/ml each kanamycin and cyclohexamide for added selection against indigenous microorganisms.

After incubation of microtiter plates at 27°C for 4 days, the entire contents of each well is quantitatively transferred to individual wells of a microsample filtration manifold (Schleicher & Schuell Co., Keene, New Hampshire), taking care to avoid disturbing and transferring sedimented soil particles, and filtered onto GeneScreenPlus (New England Nuclear Corp., Boston, Massachusetts). Cells are lysed, DNA denatured, fixed by addition of 250 μ l of 0.5 *N* NaOH to the individual wells, and filtered two times, followed by identical treatment with 1 *M* Tris pH 7.5 and air-drying of filters. After DNA-DNA hybridization and autoradiography, positive blots indicating the presence of target DNA are scored for each dilution and the MPN value for a five-replicate 10-fold dilution series is calculated (Alexander 1982). Uninoculated microcosm samples serve as controls to discern any background homology or nonspecific binding of probe. Alternatively, colony blots from appropriate sample dilutions on MNFM agar plates amended with kanamycin and cyclohexamide can be used to identify the model GEMs. The procedures used for colony blots with Whatman #541 filter paper are described by Gergen et al. (1979).

Plasmid DNA and Probe Preparation

E. coli MV12, harboring the suicide vector plasmid pGS9 containing Tn5, is cultured in LB broth with 50 μ g/ml each chloramphenicol and kanamycin (Sigma), which selects for the plasmid pGS9 and maintenance of the transposon Tn5. Large-scale plasmid DNA preparations were performed as described by Maniatis et al. (1982). Purified plasmid DNA for use as probes is prepared by ultracentrifugation of plasmid preparations on CsCl (1.0 g/ml) density gradients with 0.5 mg/ml ethidium bromide at 55,000 rpm for 17 h at 20°C followed by 1 h at 45,000 rpm at 20°C. Plasmid bands are removed and extracted four times with isopropanol:5 *M* NaCl (1:1) in Tris pH 8.0 buffer to remove ethidium bromide. DNA is recovered by ethanol precipitation. [32 P] DNA probe is made using a nick translation kit (Bethesda Research Laboratories, Gaithersburg, Maryland) following the suggested protocol. [32 P]dCTP at 3000 Ci/mmol (New England Nuclear)

is used as the labeling nucleotide. Labeled probe is separated from unincorporated nucleotides by using a spun-column procedure (Maniatis 1982), and activity is quantified by liquid scintillation counting.

DNA-DNA Hybridization

GeneScreenPlus filters are placed back to back in a heat-sealable plastic bag with 15 ml of the manufacturer's suggested prehybridization solution and incubated for 6 to 8 h at 65°C. Whatman 541 filter colony blots are saturated with 6x SSC (SSC is 0.15 M NaCl, 0.15 M sodium citrate) and washed for 1 to 2 h in 50 mM Tris pH 8, 1 M NaCl, 1 mM EDTA, and 0.1% SDS at 37°C with constant agitation to remove cell debris. Prehybridization is done as described previously for GeneScreenPlus with 5x SSC substituting for the prehybridization solution. Salmon sperm carrier DNA and [³²P] DNA probe are denatured by heating in boiling water for 10 min; they are cooled and added to plastic bags containing filters and solutions at 150 µg/ml and 10⁵ cpm/ml hybridization solution, respectively. Plastic bags are resealed and incubated at 65°C for 12 to 14 h with agitation.

Filters are washed according to manufacturer's directions for GeneScreenPlus filters. Alternatively, Whatman 541 colony blots are washed three times in 2x SSC at 65°C (20 min each) followed by one wash in 0.2x SSC at 55°C for 20 min. Autoradiography is carried out at -70°C using XAR-5 x-ray film (Eastman Kodak Co., Rochester, New York).

REAGENTS

All reagents and media used in this program are prepared from chemicals of analytical grade or of the highest purity available. A detailed chemical inventory listing chemical lot number and manufacturer stock number is kept on a computer file. Water for preparation of all solutions is distilled and deionized by the Milli-Q (Millipore Co., Bedford, Massachusetts) water purification system.

Modified Spirillum Nitrogen Fixing Medium (MNFM)

Medium #838 ATCC Culture Handbook

KH ₂ PO ₄	0.4 g	Modified: NH ₄ Cl 1.0 g
K ₂ HPO ₄	0.1 g	Difco yeast extract 1.0 g
MgSO ₄ · 7H ₂ O	0.2 g	
NaCl	0.1 g	
CaCl ₂	0.02 g	
FeCl ₃	0.01 g	
NaMoO ₄ · 2H ₂ O	0.002 g	
Na malate	5.0 g	
Distilled water	1.0 L	

Adjust pH to 7.2-7.4 and autoclave for 20 min. Kanamycin sulfate and cycloheximide (Sigma Chemical Co., St. Louis, Missouri) are made as stock solutions (25 mg/ml) in distilled water, filter sterilized (0.2 µm), and added to cooled media to achieve the desired final concentration.

Luria-Bertani (LB) Broth

Tryptone (Difco Labs., Detroit, Michigan)	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
Distilled water	1.0 L

Adjust pH to 7.5 and autoclave for 20 min. Chloramphenicol (Sigma) is made as a stock solution in ethanol and stored at -20°C.

0.5 N NaOH

20.0 g NaOH (J. T. Baker)/L

1.0 M Tris-pH 8.0

Dissolve 121.1 g Tris base in 800 ml of water. Adjust pH to 8.0 by adding approximately 42 ml concentrated HCl and raise the final volume to 1 L.

Ethidium Bromide

Add 1 g of ethidium bromide (Sigma) to 100 ml of water. Stir on a magnetic stirrer for several hours until dissolved; store in the dark at 4°C.

Isopropanol: 5 M NaCl in Tris pH 8.0

10 mM Tris base (Sigma) is prepared by dissolving 0.121 g Tris in 80 ml water, adjusting the pH to 8.0 with concentrated HCl, and diluting to 100 ml; 100 ml isopropanol and 29.22 g NaCl are added to the 10 mM Tris and the solution is shaken.

Nick Translation Kit (Bethesda Research Laboratories)

[³²P] dCTP 3000 Ci/mmol (New England Nuclear)

SSC

Stock 20x SSC is prepared by dissolving 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of water, adjusting the pH to 7.0, and bringing the volume to 1.0 L.

Salmon Sperm DNA

A solution of 10 mg/ml salmon sperm DNA is prepared by adding 100 mg salmon sperm DNA (Sigma) to 10 ml water and placing on a magnetic stirrer for several hours until dissolved. Aliquots of 1 ml are dispensed into microfuge tubes and frozen at -20°C.

50 mM Tris pH 8.0, 1 M NaCl, 1 mM EDTA, 1% SDS

With distilled water, 50 ml of 1 M Tris pH 8.0, 200 ml of 5 M NaCl, 1 ml of 1.0 M EDTA, and 100 ml of 10% SDS are made to 1 L.

MATERIALS AND EQUIPMENT

GeneScreen Plus hybridization membrane (New England Nuclear Corp., Boston, Massachusetts)

XAR-5 x-ray film (Eastman Kodak Co., Rochester, New York)

Microsample filtration manifold (Shleicher & Schuell Co., Keene, New Hampshire)

Whatman #541 filter paper (Fischer Scientific, Pittsburgh, Pennsylvania)

Centrifuge, 21,000 rpm, Beckman J2-21M (Beckman Instrument Corp., Palo Alto, California)

Ultracentrifuge, 60,000 rpm, Beckman L8-80M

-80°C Biofreezer, Model 8236 (Forma Scientific, Marietta, Ohio)

pH meter, Model 701A (Orion Research Inc., Cambridge, Massachusetts)

Water bath shaker/incubator, ambient to 85°C (Precision Scientific Co., Chicago, Illinois)

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STANDARD OPERATING PROCEDURE - TD2369 - SOP6

Plant Xylem Exudate Sampling

Project Title: Evaluation of Terrestrial Microcosms for Assessing the Fate and Effects of Genetically Engineered Microorganisms on Ecological Processes

Project Manager: Dr. Peter Van Voris

Principal Investigator: Dr. James K. Fredrickson

Subtask Investigator: Dr. Robert J. Fellows

INTRODUCTION

The phenomenon of sap (xylem) exudation from cut or broken stems was observed and chronicled even before Hales (1927) quantitated root pressure in trees. The origin of this fluid is usually attributed to an influx of water into the roots, which act as an osmometer through the accumulation of solutes within their sap (Kramer 1983).

Flux rates for exudation from the xylem vary widely both between species and diurnally in a single plant (Parsons and Kramer 1974). Environmental effects such as soil water potential and root temperature also alter the rate of flow (Clarkson 1976). To maximize the amount of material to be collected, it is therefore important to standardize the procedures and sampling times to be employed.

PROCEDURES

Collection of Xylem Exudate

The basic technique employed in these experiments is a modification of that of Cataldo et al. (1978). Samples are collected from wheat (*Triticum aestivum*) and corn (*Zea mays*) at two

stages of development. Wheat is sampled during the boot stage (vegetative) and during mid-grain fill (reproductive); corn is sampled when the plants are 1 to 2 ft tall (30-45 days after planting) (vegetative) and at tasseling (reproductive).

A minimum of three plants is used at each sampling period. Sampling is begun within the first 2 h of the light period when maximum flow rates can be expected (Clarkson 1974; D.A. Cataldo, personal communication). To further enhance the potential flux, the plants are watered with filtered (0.2- μ m), ion-free water just before the dark period immediately preceding sampling.

In chamber plants selected for sampling from both species, the leaf blade and sheaths are removed from the lower one-third of the shoot using sterile scalpels, forceps, and gloves. The exposed stem is then fitted with a circular (presterilized) cuvette through which ethylene oxide (C_2H_4O , 99.7% min.) is passed at a flow rate of 500 ml/min for 30 sec for surface sterilization. The gas is vented out of the chamber during this time and is followed by a 1-min flush with sterile filtered air before cuvette removal. The stem is then cut with a sterile scalpel blade and a presterilized 2.0-cm length of gum rubber tubing (the tubing diameter depends on the stem diameter and is chosen to ensure a secure fit). The open end of the gum rubber tube is fitted with a sterile 18-1/2-gauge needle to which is attached a 30.0-cm length of presterilized 1.0-mm-ID polyethylene tubing. The tubing is placed in a sterile 5.0-ml vial cooled to 4°C either on a cooling plate or in an ice bucket.

Collection of exudate proceeds for a minimum of 4 h dependent on the flow rate from the cut stem. During this time the collection vial is covered with sterile foil to minimize potential airborne contamination. The vial is flushed with sterile air just before capping at the conclusion of the experiment. The samples are then analyzed for GEM content as described elsewhere.

EQUIPMENT

Equipment to be used for these procedures includes presterilized gloves, scalpel blades, and needles (18-1/2 gauge) (VWR Scientific). Latex rubber tubing (1/8- to 1/2-in. ID), polyethylene tubing (1-mm ID), and 5-ml vials (VWR Scientific, Seattle, Washington) are sterilized by autoclaving. Ethylene oxide (Matheson Gas Products, Portland, Oregon) is regulated

with a Union Carbide two-stage regulator (UPC 1 25 510). Vials are cooled on a Thermoelectric Unlimited cooling plate while filling. The design for the stem sterilization cuvette is given elsewhere.

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STANDARD OPERATING PROCEDURE - TD2369 - SOP7

Selection of Microbial Genotypes, Maintenance of Cultures, and Laboratory Safety Procedures

Project Title: Evaluation of Terrestrial Microcosms for Assessing the Fate and Effects of Genetically Engineered Microorganism on Ecological Processes

Project Manager: Dr. Peter Van Voris

Principal Investigator: Dr. James K. Fredrickson

INTRODUCTION

To follow the movement and fate of GEM models introduced to microcosms, the *A. lipoferum* strains are genetically modified in vivo by transposon mutagenesis using the broad host range mobilization plasmid pGS9 containing the transposable element Tn5. Transposons are specific DNA elements with the ability to move as a unit and integrate more or less randomly in the bacterial chromosome. Insertion of a transposon into a gene can cause a complete loss of gene function accompanied by drug resistance. This system has been used successfully to isolate at Tn5 insertion mutants of *A. lipoferum* (Vanstockem et al. 1985, 1987). Insertion mutants obtained from conjugation with pGS9 harboring donor strains are positively identified by hybridization with pGS9 DNA probe (see TD2369-SOP5). Mutants are also fully characterized for growth rate in enriched and N-free minimal salts malate medium in comparison to the wild type to ensure against using a mutant that is severely debilitated.

PROCEDURES

Bacterial Strains and Plasmids

Two strains of *Azospirillum lipoferum*, *A. lipoferum* SpRG20a (ATCC #29708) and *A. lipoferum* SpBr17 (ATCC#29709), were purchased from American Type Culture Collection

(ATCC) (Rockville, Maryland) for use as recipients for transposon mutagenesis. Bacterial isolates to be used as conjugative suicide plasmid donors (pGS9) are *Escherichia coli* MV12 (Leu⁻, Thr⁻, Thi⁻, Thy⁻, Trp⁻) from the laboratory of Dr. M. Kahn (Washington State University) and *E. coli* WA803 (Met⁻, Thi⁻), a gift from N. Iyer (Carleton University). *A. lipoferum* strains are grown at 27°C and maintained in nitrogen-fixing medium (NFM) as described in Media #838 in the ATCC catalog (1980). *E. coli* strains are grown at 37°C on Luria-Bertani (LB) medium amended with 25 ppm kanamycin (Sigma). Stock bacterial cultures are preserved by freezing in 40% glycerol.

Construction of *Azospirillum* Tn5 Inserts

The method of Vanstockem et al. (1985, 1987) is employed to isolate 5 mutants. *Azospirillum* recipient and *E. coli* (containing pGS9) donor cells are grown to log phase. Donor and recipient cultures are pelleted by centrifugation (1500 x g for 5 min.). Cells are washed twice in sterile physiologic saline and resuspended in saline solution to obtain approximately 10⁹ cells/ml. Recipient cells are heat shocked at 51°C in a water bath for 5 min, mixed with donor cells, and placed on sterile 0.01-µm nitrocellulose membrane filters (Sartorius Co.) on LB agar plates. Matings are incubated for 24 h at 28°C. Filters containing only recipient or donor cells serve as controls. Following mating, cells are dislodged from filter membranes and resuspended in 0.5 ml sterile saline solution and spread on NFM agar plates (150 x 15 mm) amended with 25 µg/ml kanamycin for the selection of *Azospirillum* Tn5 inserts. Control cells are resuspended, diluted, and spread on appropriate selective media. *Azospirillum* Tn5 inserts are purified by streaking for isolated colonies on NFM agar plates amended with 25 µg/ml kanamycin.

Laboratory Safety

Although the microorganisms used in these studies are relatively innocuous, safety precautions are used when handling the recombinant bacteria. The NIH Guidelines for Research Involving Recombinant DNA Molecules (Federal Register of May 7, 1986, 52FR16958) are routinely followed. This includes autoclaving or incinerating all recombinant cultures used in the study in either laboratory media or soil or plant samples.

REAGENTS AND MEDIA

Purity of all reagents is analytical grade, and all solutions are made with distilled water.

Sterile Physiological Saline

Dissolve 8.5 g NaCl (J.T.Baker Co.) in 1 L distilled water and autoclave at 121°C and 15 psi for 20 min.

Luria-Bertani (LB) Medium

Mix 10.0 g tryptone, 5.0 g yeast extract, and 10.0 g NaCl in 1 L distilled water. Adjust pH to 7.5 with 1 N NaOH. Add 15 g agar for solid medium. Autoclave at 121°C and 15 psi for 20 min.

Spirillum Nitrogen-Fixing Medium (ATCC Media #838)

Mix 0.4 g KH_2PO_4 , 0.1 g K_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1g NaCl; 0.02 g CaCl_2 ; 0.01g FeCl_3 ; 0.002 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; 5.0 g Na-malate; 1.0 L distilled water. Adjust pH to 7.2-7.4 with 5 N NaOH. For solid medium, add 15 g Bacto agar (Difco Labs., Detroit, Michigan). Autoclave at 121°C and 15 psi for 20 min.

Nitrogen-Fixing Medium with 25 ppm Kanamycin

Dissolve 250 mg Kanamycin sulfate (Sigma # K1377) in 10 ml distilled water, filter through a Millix®-GS 0.22- μm filter (Millipore Co., Bedford, Massachusetts), and store aliquots in sterile containers at -20°C. Add 1 ml to 1 L of cooled NFM medium for liquid culture or to lukewarm (~48°C) agar medium before pouring plates.

Modified NFM

NFM is amended with 1 g NH_4Cl and 0.005 g biotin per liter of medium for more luxuriant growth of *Azospirillum spp.*

80% Glycerol

Add 20 ml of distilled water to 80 ml of glycerol (Difco Lab., Detroit, Michigan) and autoclave at 121°C and 15 lb pressure for 20 min. Aseptically dispense 1 ml aliquot into sterile polypropylene freezer vials.

EQUIPMENT

Instruments and equipment used for these procedures include: Mettler PL1200 electronic balance, readability 0.01g; Mettler AE163 electronic balance, dual weighing range, readability 0.01 mg; Beckman J2-21M centrifuge, 21,000 rpm; Brinkmann Eppendorf microcentrifuge, 5,000 rpm (Brinkmann Instruments, Westburg, New York); dark field Quebec Colony counter (American Optical Co., Buffalo, New York); New Brunswick Model G-27 Controlled Environment Incubator Shaker (0-60°C) (New Brunswick Science Co., New Brunswick, New Jersey); Environ Clean Hood by Environmental Air Control Inc.; walk-in cold room maintained at 2°C; Forma model 8326 deep freezer (-85°C), (Forma Scientific, Marietta, Ohio); Milli-Q water purification systems (Millipore Co., Bedford, Massachusetts); Orion Model 701A pH meter (Orion Research Inc., Cambridge, Massachusetts); Cole-Palmer International (Chicago, Illinois); model R1253-00 immersion water circulator-heater (20-85°C); Shaker-Incubator Water Bath (20-85°C) (Precision Scientific Co., Chicago, Illinois). (The first two items are serviced regularly by Hanford Standards Laboratory, and the others are usually calibrated by the user.)

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STANDARD OPERATING PROCEDURE - TD2396-SOP8

Insect Rearing and Sampling for Microbial Analysis

Project Title:	Evaluation of Terrestrial Microcosms for Assessing the Fate and Effects of Genetically Engineered Microorganisms on Ecological Processes
Project Manager:	Dr. Peter Van Voris
Principal Investigator:	Dr. James K. Fredrickson
Subtask Investigator:	Dr. Lee Rogers

INTRODUCTION

Phytophagous insects commonly feed on a variety of grass-related plants including wheat (*Triticum aestivum*) and corn (*Zea mays*). The likelihood of insects acquiring microorganisms from feeding on colonized plants may be dependent on the type of feeding mechanism employed. Representatives from two different insect orders, the Orthoptera and Homoptera, are used in this study. Members of the order Orthoptera have chewing mouth parts, and the Homoptera have piercing-sucking mouth parts.

PROCEDURES

After 12 and 21 days for corn borers and aphids, respectively, insects were sampled, macerated in sterile deionized water, and assayed for *A. Lipoferum* Tn5 mutants. Approximately 25 aphids were removed from the wheat leaf surface, and 4 to 9 corn borers were removed from the maize stem interior.

Orthoptera Experimental Procedures

European corn borers (*Ostrinia nubilalis*) (French Agricultural Research Service, Lamberton, Minnesota) at the third to fourth instar were placed on individual maize plants and were confined to the lower stalk by loosely wrapping with chiffon. All microcosms receiving insects are covered with insect netting to prevent the movement of individual insects between treatments. Individuals are then removed from the plants and their gut contents are removed according to procedures described by Kindler and Spomer (1986). The foregut or crop of grasshoppers contains undigested materials. The crops are removed from five grasshoppers from each microcosm to which the insects were introduced and placed in sterile blender cups containing 0.85% saline. Grasshopper crops are macerated by blending for 2 min, and a dilution series is prepared for each microcosm sampling as described in SOP 3. Analysis of macerated crops for *A. lipoferum* Tn5 mutants is executed according to SOP 5.

Homoptera Experimental Procedures

Approximately 50 to 100 aphids (*Schizaphis* sp.) indigenous to eastern Washington State were introduced onto wheat plants. Aphids (15-20 individuals) are placed on the plants within the microcosms; aphids are sampled from the microcosms after 2 days and macerated as described earlier. Initially, the entire insect bodies are macerated to analyze for *A. lipoferum* Tn5 mutants. If analysis (SOP 5) indicates the presence of the model genetically engineered microorganisms (GEMs), then an attempt is made to dissect out individual insect guts to determine if the model GEMs were ingested.

REFERENCE

Kindler, S. D., and S. M. Spomer. 1986. Biotypic status of six greenbug (Homoptera: Aphididae) isolates. *Environ. Entomol.* 15:567-572.

STANDARD OPERATING PROCEDURE - TD2396-SOP9

Operation of Microcosm Containment Chamber (MCS)

Project Title: Evaluation of Terrestrial Microcosms for Assessing the Fate and Effects of Genetically Engineered Microorganisms on Ecological Processes

Project Manager: Dr. Peter Van Voris

Principal Investigator: Dr. James K. Fredrickson

Subtask Investigator: Mr. Michael W. Ligojke

INTRODUCTION

One of the major benefits of using microcosms for the initial testing of GEMs for potential ecological effects is that a greater degree of containment can typically be achieved in comparison to the field. However, in some situations test facilities such as growth chambers or greenhouses may not provide the degree of containment that may be required or desired for a particular GEM. Therefore, a prototype chamber was developed, as part of a project option, that may be used to achieve a greater degree of containment for recombinant bacteria being tested in soil-core microcosms. The main purpose of the initial design was to develop a chamber that could be used for soil-core microcosm evaluations of the fate and ecological impacts from a GEM, yet limit the transport of the test organisms outside of the microcosm, rather than to achieve a certain level (i.e., P3) of containment. On the basis of the initial design and construction exercise, the prototype MCS design was reevaluated and plans were modified (Appendix B consists of MCS revised plans). This SOP is intended to provide guidance on operation of the MCS and on safety concerns and potential areas for QA/QC consideration. Because the MCS was not used in any experiments as part of this project, modifications will likely be necessary as experience is gained with its use and modifications in its design are made.

PROCEDURES

The prototype chamber was designed to contain up to 6.25- or 18.8-cm-diameter by 60-cm-long intact soil cores. The cores are placed on the Buchner funnels that rest in the slots near the base of the chamber. Leachate collection vessels are then attached to the funnels with Tygon or Teflon tubing, and the front panel of the chamber is then replaced and sealed. Entry to the interior of the chamber after sealing the front panel is through an airlock mounted on the side of the chamber. The interior of the airlock can be disinfected with ethylene oxide, in a manner analogous to an anaerobic chamber airlock, to limit the contamination of the exterior of the chamber with the test organism during sampling, inoculation, etc. All manipulations in the interior of the MCS are conducted through the three glove ports, which are located at positions on the face of the chamber such that there is access to all areas within the chamber.

At the completion of a particular experiment the cores can be bagged and sealed, and the chamber flushed with ethylene oxide for disinfestation. The process for bagging the cores requires a pulley or winch to be installed on the interior of the chamber frame to allow the raising and bagging of the individual cores. Once the cores have been bagged and the chamber disinfested, the front panel can be removed and the bagged cores removed for incineration.

Chamber Integrity Tests

The containment chamber(s) can be evaluated in several ways to determine the integrity of the chamber. Pressure or vacuum tests can be performed to determine if the chamber has been adequately sealed. These tests can be conducted under normal operating conditions (slight negative pressure) or under extremes to determine the pressure at which there is significant failure. A second type of test that can be conducted would involve the release of a particulate tracer material within the chamber and then monitoring for release of the particles during normal operation. This could be done by operating the chamber in a clean room equipped with a particle analyzer and monitoring for particle transport from the chamber. This can serve as both a check on the probability of microbial contamination of the outside atmosphere and for training purposes. A material such as zinc sulfide, highly fluorescent under a black light, has been used in these types of tests. These types of tests are suggested as areas for QA/QC pertaining to chamber operation.

Leachate Collection

Although not included on the prototype chamber, a system was designed for the aseptic collection of leachate from the soil cores while they reside in the chamber. This requires a line of Teflon tubing from the base of the interior of the individual leachate collection vessels to a manifold on the side of the chamber. At the manifold, a stainless steel hose connector exits to the exterior of the chamber. At the exterior face is a stainless steel plug valve (NUPRO P4T) with a hose connector to attach a vacuum flask. This flask is then connected to the leachate collection line with Teflon tubing, and the vacuum line attached to the sidearm which is stopped with cotton to prevent the movement of microorganisms with the air flow. This allows the syphoning of leachate from the individual microcosms into a sterile vessel. After disconnection, the valve can then be removed, replaced by a sterile valve, and autoclaved. The residual material that is left in the lines and leachate collection vessel in the bottom of the chamber presents a problem for sampling the leachate more than once during the course of the experiment. However, it may be possible, by means of several rinse cycles with a disinfectant solution and sterile water, to disinfest the lines to prevent cross-contamination of leachate between sampling times.

APPENDIX B

MICROCOSM CONTAINMENT CHAMBER DESIGN

APPENDIX B

MICROCOSM CONTAINMENT CHAMBER DESIGN

B.1 PROTOTYPE MCS CHAMBER

In ecological testing of GEMs, one priority is to provide a means for containing the recombinant microorganisms being tested in facilities such as growth chambers and greenhouses that were not specifically designed for containing microorganisms. In fulfillment of an optional task in the original project proposal, a microcosm containment system (MCS) was designed and constructed. Construction of the first prototype chamber was successful in that it completes the major goal of this task listed in the project proposal. However, the process of design, construction, and postconstruction review led to the development of a list of several important design modifications that were not described in the original proposal but which are thought to be important for inclusion in the revised designs.

It should be noted that the important design modifications recommended for inclusion in the planned second chamber, which would increase costs significantly, were recommended because experience and insights gained during design and construction indicated that a greater degree of microbe containment and system usefulness might be achieved by incorporating additional features into a second-generation MCS system.

The prototype MCS was designed to contain as many as six large (25-cm diameter) and 18 small (17.8-cm diameter) soil cores, each about 60 cm long, for GEM tests. Because standard glove boxes cannot provide support for the estimated 140- to 230-kg (300- to 500-lb) total soil microcosm mass, all parts of the new chamber were especially constructed, with the exception of the sample interchange, which was purchased from a manufacturer of glove boxes. In addition to containing the large soil microcosms, the chamber provides sufficient free space over the tops of the soil cores to permit growth of tall plants such as grains and corn. Air passing into and exhausting from the chamber is filtered using double sets of HEPA (high-efficiency particulate air) filters achieving filtration effectivenesses of greater than 99.97%. Three glove ports allow working access to the chamber. Ethylene oxide gas may be injected into the chamber and interchange to sterilize equipment and samples being removed.

A three-dimensional sketch of the constructed MCS is shown in Figure B.1. Construction drawings are included in Section B.3. The system was constructed of painted steel and Lexan® in two parts. The top section may be removed to facilitate chamber modification, relocation, and cleaning. Eye bolts were installed on the top to provide support for lifting the top section off the base. Connection between the top section and the base may be quickly disassembled by slitting the seal and removing eight bolts. Additional access to the inside of the system may be obtained with or without the top section in place by removing the soil-column top stabilizer grid and placing it on the lower soil-column support grid. The inside of the chamber may then be accessed via the front panel on the base.

The top section was constructed of Lexan® supported by a steel base consisting primarily of 1-in.² tubing. Lexan® was selected for durability and its resistance to corrosive materials. Some solvents such as xylene can degrade the transparency of the material, however. Three glove ports provide working access to all parts of the upper chamber, and a transfer box (Coy Laboratory Products Inc., Ann Arbor, Michigan) is connected to the lower right-hand side of the chamber. Because Lexan® filters ultraviolet light, the top window of the chamber may be replaced with UV-transparent plexiglass, or UV lamps may be operated within the chamber. Flanges for HEPA filters are located on each upper side panel of the top section.

The base of the chamber provides housing and structural support for soil microcosms, and is mounted on four multidirection, heavy-duty casters to allow the chamber to be easily moved. The base consists of a steel tube frame and 16-gauge sheet metal panels. All steel surfaces were coated with an epoxy paint. A grid of 1.5-in.-thick steel tubing supports soil columns; six panels placed on the grid are used to support a variety of soil column sizes, numbers, and configurations. A large front panel may be removed for easy access to the soil columns. Space is provided beneath the soil columns for leachate collectors, and leachate may be collected via valved ports near the bottom of the chamber.

Drawings and detailed construction instructions are provided in Section 1.3 for the prototype MCS. As experience was gained designing and constructing the first prototype chamber, a list of important modifications was prepared and is included in Section 1.2. Designs for a second MSC will include these suggested modifications.

®Lexan is a registered trademark of General Electric Corporation, Mount Vernon, Indiana.

Figure B.1. Prototype microcosm containment chamber.

B.2 PROPOSED MODIFICATIONS FOR THE PROTOTYPE MCS

During and after construction of the prototype GEM containment chamber, a list of important design considerations was prepared. These modifications will improve flexibility of use; sterilization of large soil columns; chemical resistance of chamber materials; electrical power supply; climate control; and special instrumentation systems.

B.2.1 Proposed Modifications

1. The chambers should be self-contained, rather than exhausted through a filter or other system. Thus, the chambers could be used for greater hazard levels. At a minimum, the chambers should be constructed with sealable ports for future connection to climate-control and special instrumentation systems. The second GEM containment chamber should support closed-loop flow ducts, air conditioning, a negative pressure line with automatic sterilization, and a double HEPA filter inlet. A schematic drawing of this design concept is shown in Figure B.2.
2. Use stainless steel construction. The use of a paint coating on the first prototype chamber, although the paint was a good grade of epoxy, revealed that paint will soon wear from or be scraped off the steel frame. Furthermore, the extra materials and construction costs of stainless steel would actually be less than the costs of painting and paint maintenance. In addition, use of stainless steel would provide superior chemical resistance, a noncorroded base for special coatings (such as epoxy, silicon, or teflon), and reduced costs of chamber cleaning.
3. Add stronger support members to the top of the steel frame to support core isolation and sterilization procedures. Attach pulleys above each core.
4. Provide electrical power (approximately 20 A) within the chamber. Power is required for UV lamps, electric winches, and other devices. Seal the electrical connection using vapor-tight sealers, and use high-temperature silicon sealant on the leads (to block microbe migration through the wire insulators).
5. Purchase of interchange boxes from manufacturers of glove boxes will probably remain cost-effective. Prepare designs in accordance with interchange mounting and operation requirements.

Figure B.2. Flow system in microcosm containment chamber.

6. Limit the windows as much as is feasible. At a minimum, portions of the two side windows and all the back windows should be replaced with stainless steel sheet metal. Attach instrumentation to the back side of the chambers to allow free access to work areas.
7. Continue to use studs welded to the steel frame, but frame all windows in stainless steel. Laminated safety glass (tempered) should be used instead of plastics, such as Lexan, only if Lexan fails impact or chemical compatibility tests. Impact tests should be performed to fail the Lexan and ensure that no cracks form. The Lexan should fail without fracturing--as would the glass. Chemical compatibility tests should include 20% bleach, ethylene oxide, and chlorine gas. Impact tests should be performed on Lexan aged in contact with the chemicals and UV light.
8. Do not use UV-transparent windows. Operate a UV lamp within the chamber when UV radiation is required.
9. Modify funnel design (base of large soil cores) to allow cores to be sealed at both top and bottom. To sterilize cores, first harvest plants, then lift cores, remove funnel bottoms, seal with end cap and sealant, replace core, and seal top with cap and sealant. Disinfect chamber with ethylene oxide. End caps will reduce surface area and trap microbes within the sealed cores. Open chamber after sterilization and incinerate all cores and other wastes.

B.3 DETAILED CONSTRUCTION PLANS: FIRST PROTOTYPE GEM CHAMBER

The prototype GEM containment chamber was built to the following specifications. Instrumentation and air flow control was deferred for inclusion on the planned second prototype chamber. Dimensions in inches unless otherwise specified. Design drawings are located after the construction specifications.

B.3.1 Part 1: Frame

1. Obtain materials:

Steel tubing 1/8 x 2 x 2, length = 20 ft.

Steel tubing 1/8 x 2 x 1, length = 40 ft.

Steel tubing 1/8 x 1 x 1, length = 100 ft.

Angle iron 1/8 x 1 x 1, length = 20 ft.

Sheet metal 1/16 gauge, area = 50 ft².

Lexan 1/4, area = 64 ft².

Epoxy paint, special brand.

Bolts, nuts, washers.

Metal plate, light-duty to cap tubing, heavy-duty for casters.

Casters, 4 each, heavy duty (approx 500-lb capacity each).

Bar stock, 1/4 in. x 2 in. x 20 ft.

2. Cut tubing for chamber legs.

Four each, 2 x 2 x 36.5.

3. Cut angle iron for Elevation A.

Angle [1 x 1]: two each 26.0 in., two each 42.0 in.

4. Cut tubing for Elevation B.

Tubing [2 x 1]: 2 each 26.0 in., 2 each 42 in., 3 each 14.0 in., 2 each 28.0 in.

5. Cut tubing and angle iron for Elevation C.

Tubing [2 x 1]: 2 each 28.0 in., 2 each 46.0 in.

Tubing [1 x 1]: 2 each 28.0 in., 3 each 14.0 in.

Angle [1 x 1]: 8 each 6.0 in.

6. Assemble base of prototype chamber.

Do not assemble the inner five pieces of Elevation C at this time.

Do not attach the eight 6.0-in. angle iron supports at this time.

Weld together outer four pieces of Elevation C.

Cap both ends of all four chamber base legs with 3/16-in. sheet metal.

Weld these caps in place so that the total length of each chamber leg is

36.5 in.

Weld this part of Elevation C to the tops of the four 2 x 2 legs, position outer four pieces of Elevation B to ensure proper alignment of legs.

Weld outer four pieces of Elevation B to the legs. The tops of the members of Elevation B should be 22.5 in below the tops of the 2 x 2 base legs (24.5 in below top of elevation C).

Weld the inner four 2 x 1 pieces of Elevation B into place.

Weld the four angle iron pieces of Elevation A into place.

The angle iron should be oriented with the two flat faces to the outside and to the bottom. The bottom of the angle iron should be 4.0-in. above the bottom of the 2 x 2 legs.

7. Assemble inner parts of Elevation C.

Cap all six tube ends without changing their lengths. Attach inner and outer parts using six bolts (tap thread into tube caps). The top of the inner part of Elevation C shall be 0.5 in. below the top plane of Elevation C. (Upon removal of the six bolts, the inner part of Elevation C will be lowered to rest on top of Elevation B.) Weld the eight 6.0-in. angle iron supports into place. One flat face should be located against the 2 x 1 tubes and the other should be oriented on top with its top elevation 0.5 in. below the top plane of Elevation C. Spacing is shown in Drawing D.

8. Cut tubing for top legs.

Tubing [1 x 1]: 4 each 48.75 in.

9. Cut tubing for Elevation D.

Tubing [1 x 1]: 2 each 28.0 in., 2 each 46.0 in.

10. Cut tubing for Elevation E.

Tubing [1 x 1]: 3 each 26.0 in., 2 each 44.0 in.

11. Assemble top section of prototype containment chamber.

Weld the four pieces of Elevation D together.

Weld the five pieces of Elevation E together.

Cap the bottom end of each of the four legs as was done for the base legs (see No. 6).

Weld the four top legs to the inside corners of Elevation D.

The bottom of Elevation D should be 1.75 in. above the bottom of the legs.

Weld Elevation E to the top legs.

The upper ends of the legs should be attached exactly at the bottom of the four corners of Elevation E. The vertical distance between the bottom of Elevation E and the top of Elevation D should now be 46.0 in.

12. Verify fit of top into base sections.

Place the top section into the chamber base by inserting the lower 1.75 in. of the four top section legs into the inside corners of Elevation C so that they rest on the tops of the 2 x 2 chamber legs. Grind the ends of the 1 x 1 legs if Elevation D does not rest directly on top of Elevation C. Elevation D should rest directly on top of Elevation C. After fit is verified, remove the top section from the chamber base.

13. Cut tubing [1 x 1] for top section structural supports.

Back face: 1 each 46.5 in.

Front face: 1 each 46.5 in., 2 each 20.5 in.

Left side: 1 each 26.0 in., 2 each 22.5 in.

Right side: 1 each 26.0 in., 2 each 22.5 in., 1 each 23.0 in.

14. Cap one end of both 46.5-in. and 23.0-in. lengths of tubing.

These tubes will be used as the vertical structural supports for the back, front, and right sides of the top section of the chamber.

15. Make three each metal flanges using 1/4 x 2 bar stock, or 1/4-in. plate.

Outer dimensions: 14 in. x 14 in.

Inner dimensions: 10 in. x 10 in.

Round edges by grinding.

Weld four each 7.5-in. studs (3/16-in.-dia., threaded) perpendicular to one side of two of these flanges. Position these studs in the center of each side and 1.50 in. from the inside face.

16. Assemble top section structural supports.

Back Face:

Weld the 1 x 1 support between elevations D and E. The tube should be positioned vertically and welded to the bottom of Elevation E and on the inside face of Elevation D. The bottom of this tube should then be located 0.5 in above the bottom face of Elevation D.

Front Face:

Repeat the procedure listed above for the Back Face, and then weld two each 20.5-in. 1 x 1 tubes horizontally between the top section legs and the 1 x 1 mid-face support. The bottom side of the 20.5-in. tubes should be positioned parallel to and 11.0 in. above the top face of Elevation D.

Left Side:

Weld one 26.0-in. 1 x 1 tube horizontally between the top section legs. The bottom face of the tube should be positioned parallel to and 22.5 in. above the top face of Elevation D. Next weld two each 22.5-in. tubes from the top face of the 26.0-in. tube to the bottom of Elevation E. These vertical tubes should be positioned with their inside faces 14.0 in. apart. Spacing is shown in Drawing G. Next weld a square flange (with the 7.5-in. studs facing outward) between the two 22.5-in. tubes. The top of the flange should be welded to the bottom face of Elevation E. Tack weld the flange into place and then weld (continuous) the two side and the top of the flange to the 1 x 1 tubes. The outer face of the flange should be on the same

plane as the outer faces of all 1 x 1 tubes on the left side. All welds are to be located on the inside face of the flange (i.e., not the face with the studs).

Right Side:

Repeat the procedure listed above for the Left Side.

Next, weld a 23.0-in. 1 x 1 tube vertically between the 26.0-in. tube and the inside face of Elevation D. The distance between the back side of the right-side front leg and the front face of the 23.0-in. tube should be 14.0 in (see Drawing G). Next weld (continuous) the flange that was constructed without studs to the inside of the right-side front leg, the 23.0-in. vertical tube, and the inside face of Elevation D. The outer face of this flange should be in the same plane as the outer faces of the 1 x 1 tubes of the Right Side and the outer face of the upper flange.

17. Attach the Top Section to the Base.

Lift the Top Section into place on the Base. Connect the two sections together using two bolts at the base of each leg.

B.3.2 Part 2: Sheet Metal

Approximately 1/16-in.-thick sheet metal will cover the base of the containment chamber between Elevation A and Elevation C. Sheet metal covering the bottom (of Elevation A) and the back and sides (between Elevations A and C) will be welded in place. The sheet metal covering the front face between Elevations A and C will be held in place by studs to allow occasional removal. Drawing H shows the correct sizes of all five sheet metal parts.

1. Cut five pieces of sheet metal.

Bottom: 1 each, 45.0 x 29.0.

Sides: 2 each, 27.0 x 32.5.

Back: 1 each, 43.0 x 32.5.

Front: 1 each, 44.0 x 33.5.

2. Install Bottom.

Notch approximately 2 in. square out of each corner to fit around the base legs. If feasible, place the sheet metal on top of the angle iron comprising Elevation A. Otherwise, invert the base section and attach the sheet metal to the bottom of the flat portion of Elevation A. Tack weld the sheet metal in place and then continuous weld all edges of the metal to Elevation A and the base section 2 x 2 legs.

3. Install Back and Sides.

Position the sheet metal on the outside of the base section. The sheet metal should be positioned so that it covers the lower 0.5 in. of Elevation C, and the upper 0.5 in. of Elevation A. In the horizontal plane, the sheet metal should cover 0.5 in. of the base section leg on each side. Weld all seams as done for the bottom piece of sheet metal. Tack weld the sheet metal to Elevation B as required for support.

4. Install Front Sheet Metal.

The border of this piece should be positioned so that the outer 1.0 in covers the base legs, Elevation A, and Elevation C. Weld 3/4-in. studs (stainless steel, approximately 1/4 x 20) to the base legs, Elevation A, and Elevation C to fit the pattern shown on Drawing H. Drill holes for the studs and verify fit by placing the sheet metal piece onto the base section. Next, prepare a stiffening frame using 3/16 x 1 bar stock. The outer dimensions of this frame should be 33.5 x 44. Place the frame over the sheet metal and tack weld the pieces together so that the outer edges are positioned squarely over each other. Drill the stud holes through the stiffening frame. Prepare a rubber gasket the same size as the stiffening frame. Glue the gasket to the inside face of the sheet metal (i.e., not the same side as the stiffening frame). Attach the front face to the chamber base by positioning the sheet metal with the gasket side in and the stiffening frame out, and fix into place using a washer and nut on each stud.

B.3.3 Part 3: Lexan Windows

Lexan sheets of 1/4-in. thickness will cover the top section of the containment chamber. These sheets will be cut to size as shown in Drawing I by the manufacturer. Widely spaced studs will be used to hold the Lexan sheets in place. Silicone sealant will be used to provide a seal and will be applied from the inside as a last step.

1. Verify that the Lexan sheets were cut to the appropriate sizes and tolerances as shown in Drawing I.
2. Drill stud holes in all five Lexan sheets. Take care not to fracture the sheets.
3. Attach 3/4-in. studs (stainless steel, approximately 1/4 x 20) to the top section legs, Elevation E, and the top section structural supports. The studs should be positioned to fit the stud holes, this will be approximately 3/8 in. from the inside edge of steel tubes.
4. Drill five holes in the top Lexan sheet, as shown in Drawing I. Tap 1/4-in., 3/8-in., or 1/2-in. NPT threads into Elevation E at the locations of these five holes.
5. Assemble the Lexan sheets using an **untightened** nut and a washer on each stud. The sheets should rest on top of Elevation D at the bottom, and should overlap all edges by approximately 3/4 in. **Do not seal with silicone at this time.**
6. Next, disassemble the Lexan sheets to prepare for painting.

B.3.4 Part 4: Painting

Paint all exposed metal surfaces with two coats of a spray epoxy paint. To paint the front door of the base section, remove it and cover the flat face of the gasket material with masking tape.

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