ADVANCES IN
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Selected Methods for the Detection and Assessment of Ecological Effects Resulting from the Release of Genetically Engineered Microorganisms to the Terrestrial Environment

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I. Introduction

The use of microorganisms as alternatives to traditional chemical and physical technologies is being explored in such areas as agriculture, pest control, and bioremediation of toxic wastes. These applications of biotechnology rely on the expression of useful genetic traits in both naturally occurring microorganisms and microorganisms genetically modified by recombinant DNA techniques. In the latter, the merging of the fields of molecular biology and microbial ecology is providing exciting alternative technologies, as well as new uncertainties. These uncertainties are associated with (1) the environmental uses of genetically engineered microorganisms (GEMs) capable of expressing traits not present in the unmodified parent microorganism; (2) the probability of the transfer of these genetic traits to other microorganisms indigenous to the environment; and (3) the possibility of the new traits having a deleterious effect on the environment.

The potential risks to public health and the environment from a deliberate or accidental release of GEMs to the environment are the most urgent concerns, both scientifically and with respect to public policy, associated with this aspect of biotechnology. Questions about the probabilities of survival, colonization, and function of released GEMs and their novel DNA in natural habitats and the ability to predict the consequences of their release will be answered only by applying the knowledge derived from the study of the ecology and molecular interactions among microbes in these habitats. Both biotic and abiotic environmental characteristics affect the survival, perpetuation, efficacy, and risk associated with the release of novel DNA in GEMs to any natural habitat. Moreover, the survival of novel genetic information and its potential effects on the homeostasis of an ecosystem may be greater if the information is transferred to indigenous species that are more adapted to the specific habitat than the introduced GEMs.

The purpose of this article is to summarize the methods and concepts developed and used by the authors to study the potential effects of GEMs on microbial populations and microbe-mediated ecological processes in soil. The potential impacts of GEMs, unrelated to the purposes for which they were engineered, on the structure and function of the natural environments into which they are introduced constitute the bottom-line concern about the release of GEMs to the environment. If a GEM survives in the habitat into which it is introduced and does the job for which it was designed, and even if the novel gene(s) is transferred to indigenous microorganisms, there should be little cause for concern unless the novel gene(s), either in the introduced GEM or in an

indigenous recipient(s), results in some unexpected impacts on the environment.

This concept is easy to state but difficult to translate into an effective experimental design. What effects (i.e., environmental perturbations) should be sought, especially if the novel gene(s) codes for a limited function(s) and the GEM has been selected or programmed for poor survival in a specific habitat? Considering the current state of the art and the paucity of data on detection, enumeration, survival, growth, and transfer of genetic information (both intra- and interspecifically) by microorganisms in natural habitats, the detection, measurement, and evaluation of potential effects of an introduced GEM on ecological processes is akin to finding a needle in a haystack. However, this is the most pertinent concern about the release of GEMs to the environment, and more studies on this aspect must be conducted. Nevertheless, as insufficient basic knowledge is available about the fate of introduced microorganisms, whether genetically engineered or not, in natural habitats, data from studies, especially in microcosms rather than in the field, on the ecological effects of GEMs must be interpreted and applied cautiously to avoid establishing far-reaching and long-lasting policies, criteria, and regulations that may be based on incomplete or erroneous data.

The microbe-mediated ecological processes that should be evaluated before the release of a GEM to the environment should be those for which techniques are well established, that cover a broad spectrum of relevant microbial activities, and that have been successfully used to study the perturbation of the soil environment by chemical and physical factors: for example, (1) metabolic activity and carbon mineralization, as measured by CO_2 evolution or other respiratory techniques; (2) transformations of fixed nitrogen by perfusion techniques; (3) dinitrogen fixation, using the acetylene-reduction technique; (4) species diversity of the microbiota, using selective and differential media; and (5) activity of selected enzymes, such as acid and alkaline phosphatases (to provide a measure of the cycling of P), arylsulfatases (to provide a measure of the cycling of S), and dehydrogenases (to provide another measure of overall metabolic activity). These processes should be monitored for extended periods after the introduction of a GEM, whose fate, as well as that of its novel gene(s), should be concurrently followed. Some desirable characteristics of methods for assessing the ecological effects of GEMs are presented in Table I.

In addition to evaluating the potential effects of GEMs on these defined ecological processes, the investigator should be alert to the possible occurrence of unanticipated effects that cannot be predicted from

TABLE I

DESIRABLE CHARACTERISTICS OF METHODS FOR ASSESSING ECOLOGICAL EFFECTS OF GENETICALLY ENGINEERED MICROORGANISMS

Relevance
Representative of the microbial community
Sensitivity
Reproducibility
Ease (facility; rapidity)
Cost-effectiveness
Interlaboratory validation
Predictiveness (transferability; modeling)
Ecological versus statistical significance

the information encoded on the novel DNA (i.e., pleiotropic effects). For example, the acquisition of a plasmid carrying genes for dinitrogen fixation and antibiotic resistance by various species of phytopathogenic bacteria apparently resulted in a spectrum of unrelated and unpredicted biochemical and physiological alterations (Kozyrovskaya *et al.*, 1984). Other pleiotropic effects resulting from the insertion of novel genes have been reported (e.g., Stotzky, 1989; Stotzky and Babich, 1986). If pleiotropic effects are indicated, the battery of tests for ecological effects should be extended, as such unanticipated alterations could affect ecological processes in soil and other environments. Furthermore, the growth rates of the GEMs, as well as of the homologous microorganisms without the novel gene, and their ability to compete with indigenous microbes in soil should be determined (e.g., by the soil replica-plating technique).

The purpose and function of the introduced novel genes must be considered in the design of the studies. For example, if a GEM carries a novel gene(s) that codes for a catabolic function (e.g., the degradation of a xenobiotic), the soil should be amended with the appropriate "substrate" on which the products of the novel gene(s) act, to determine whether the gene provides ecological advantages to the GEM, whether intermediates are produced, and whether and how these advantages and intermediates affect ecological processes. When GEMs resistant to the toxicity of heavy metals or other antimicrobial agents are used, the soil should be stressed with the specific agents to which the novel genes confer resistance. When GEMs containing *nif* genes are used, nitrification rates and attendant decreases in pH should be monitored, as enhanced dinitrogen fixation could increase nitrification and the accumulation of protons, which could affect numerous ecological processes.

To verify that the novel gene(s) is responsible for any changes in the ecological processes evaluated, all studies should be conducted in parallel in the same soils inoculated with equal amounts of the GEM or the homologous microorganism without the novel gene(s). The existing numbers of the introduced GEM [or its novel gene(s) in another recipient] should be related, over time, to the magnitude of perturbation of any of the ecological processes. The duration of these effects should also be determined, especially after the GEM or its novel gene(s) can no longer be detected.

These studies should be conducted concurrently in several laboratories, to obtain interlaboratory validation and to enhance the development of appropriate procedures with which to study the effects of GEMs on ecological processes in soil. A major goal of the studies should be the development of a standard battery of assays that will most clearly, rapidly, easily, and inexpensively detect *any* ecological effect of introduced GEMs and that can be used in the assessment of the risk of introducing *any* GEMs, either purposely or inadvertently, into soil and other natural habitats.

The studies should be conducted initially in the laboratory, because the potential risks associated with the introduction of a GEM to the environment are unknown. A variety of terrestrial microcosms that purportedly simulate field conditions have been developed. These microcosms range from extremely simple systems that inoculate a GEM into sterile soil added to a sterile nutrient broth in test tubes (e.g., Walter *et al.*, 1987); to sterile or nonsterile soil in a test tube, flask, or other container (e.g., Stotzky *et al.*, 1990); to multiple containers of nonsterile soil enclosed within a larger container (e.g., Fig. 1); to more complex systems that involve undisturbed soil cores of varying sizes brought into the laboratory with minimum disturbance of the structure and biotic composition of the soil (e.g., Bentjen *et al.*, 1989; Fredrickson *et al.*, 1989; Hicks *et al.*, 1990; Van Voris, 1988); to either disturbed or undisturbed soils that are cropped and maintained within chambers that enable the control of temperature, relative humidity, light/dark cycles, and other environmental variables (Fig. 2) (e.g., Armstrong *et al.*, 1987; Gile *et al.*, 1982; Knudsen *et al.*, 1988).

Examples of microcosms with different degrees of complexity and the rationales for their use have been discussed (e.g., Atlas and Bartha, 1981; Cavalieri, 1991; Gillett, 1988; Greenberg *et al.*, 1988; Hicks *et al.*, 1990; Johnson and Curl, 1972; Pritchard, 1988; Pritchard and Bourquin, 1984; Stotzky *et al.*, 1990). Guidelines for the use of soil core microcosms, with descriptions of various core designs, sampling procedures, and statistical analyses, have been published (Van Voris, 1988).

The microcosms and techniques used by the authors to study the

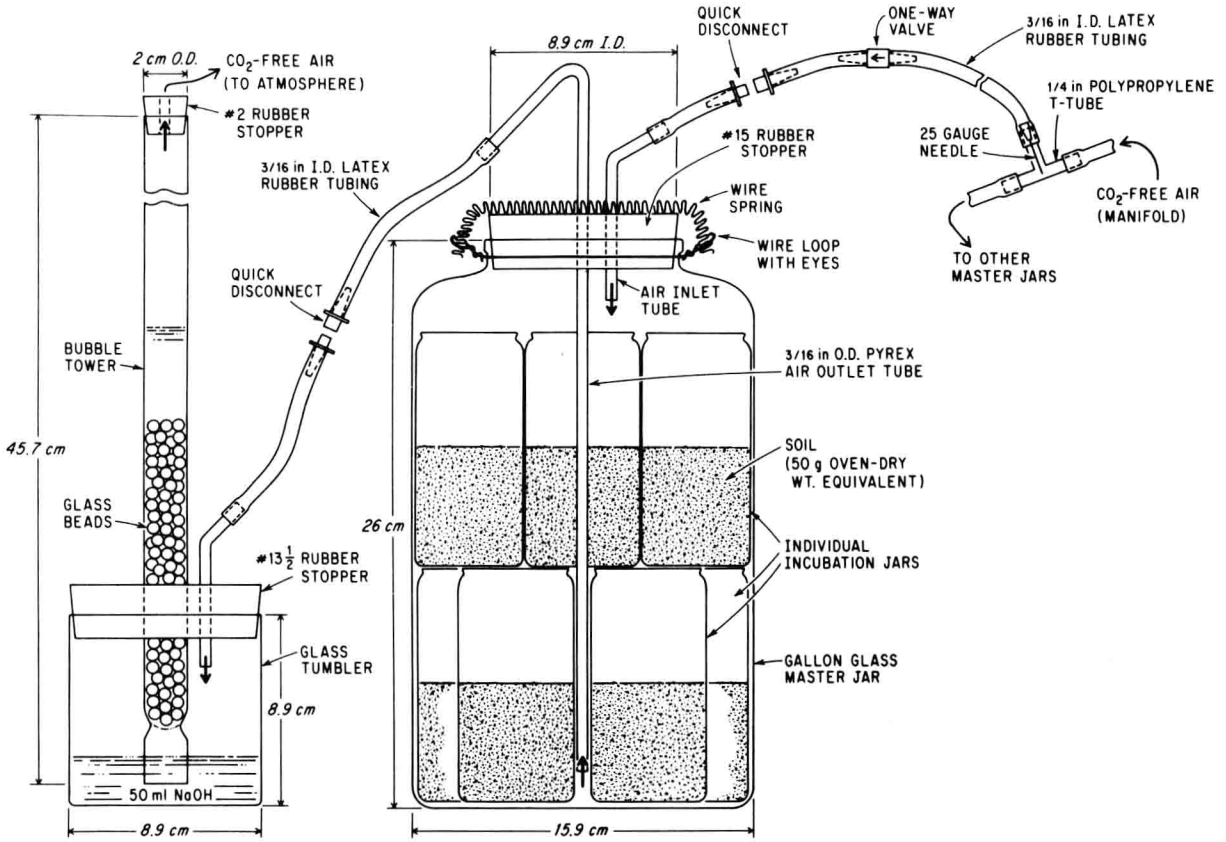

FIG. 1. Incubation unit for measuring CO_2 evolution from soil. The unit is used when subsamples of soil are to be removed during incubation. When this is not required, soil is placed directly into the master jar, which can then be smaller (Stotzky, 1965a).

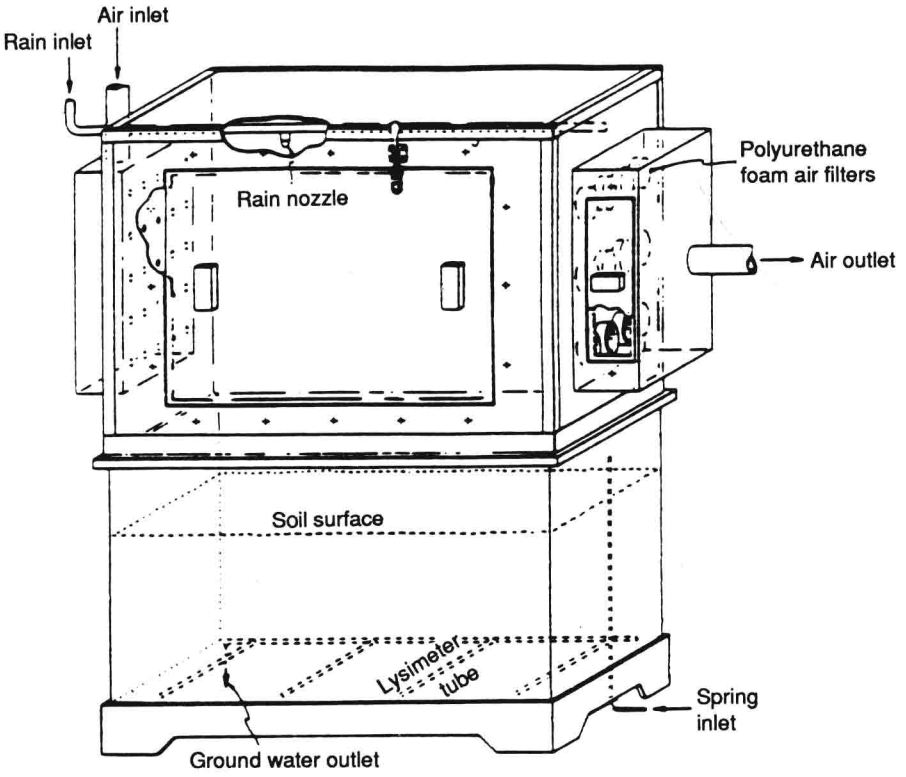

FIG. 2. Microcosm in which environmental variables (e.g., temperature, relative humidity, light/dark cycles) can be controlled (Gile *et al.*, 1982).

effects of GEMs on some microbial populations and processes in soil are described herein. Although other microcosms and techniques are available (e.g., Page *et al.*, 1982; Nannipieri *et al.*, 1990; and as referenced above), only procedures with which the authors have hands-on experience with GEMs in soil are discussed.

II. Methods of Study

A. Soil Preparation

Sieve soil (top ~5 cm) collected in the field through a broad-mesh screen (e.g., 1 cm) to remove stones and plant debris and to disrupt large soil aggregates. Mix the sieved soil thoroughly to provide as uniform and representative a sample as possible. The sieved soil can be

used immediately after collection or it can be stored. Although soil used immediately probably better reflects the microbiological conditions that exist in the soil *in situ*, there are disadvantages: for example, if the same soil is to be used in subsequent experiments, collection from adjacent sites and in different seasons can result in both biotic and abiotic variability. Moreover, if the desired soil is located some distance from the laboratory, considerable time will be consumed in collection. The collection, sieving, and storage of quantities of soil from the same site sufficient for numerous experiments eliminate these disadvantages. Changes in the microbiota as the result of storage of the soil can be rectified to some extent (Stotzky *et al.*, 1962). For example, soils can be maintained in wooden flats (e.g., 55 × 30 × 15 cm) in a greenhouse under a regime of intermittent cropping and fluctuating temperatures and moisture (Stotzky, 1973). If a greenhouse is not available, soils can be stored at room temperature in large plastic or metal garbage cans lined with plastic garbage bags. Two weeks before the initiation of a study, pass the soil through a 2-mm sieve, and rejuvenate the soil by bringing it to −33-kPa water tension and adding glucose (1%, w/w, in a mineral salts solution) and approximately 20 mg of fresh garden soil per gram soil, oven-dry equivalent. Maintain the soil at room temperature, and mix every few days (Devanas *et al.*, 1986).

When soil is to be amended with clay minerals, mix the soil after the initial sieving with the appropriate mined clay mineral. Use an electric-powered cement mixer for uniform and rapid mixing. The desired ratios of clay and soil can be achieved on a weight/weight or a volume/volume basis, although the latter (using buckets) is more conveniently conducted in the field, especially with large volumes of soil.

B. Metabolic Activity

The overall metabolic activity of microbes in soil can be determined with respirometric techniques that monitor either CO_2 evolution or O_2 consumption. These methods, especially when CO_2 evolution is measured, probably provide the best and most easily measured index of the gross metabolic activity of mixed microbial populations in soil (Anderson, 1982; Stotzky, 1960, 1965a, 1972). The "master jar" system (Fig. 1) (Stotzky, 1965a; Stotzky *et al.*, 1958) enables the removal of subsamples of soil during an extended incubation for various analyses (e.g., transformation of substrates, species diversity, enzyme activities, survival of introduced microorganisms, including the GEMs and their novel genes) without disturbing the remaining soil. Sampling without disturbance

eliminates artifactual peaks in CO_2 evolution resulting from the physical disturbance of the soil (Stotzky and Norman, 1961a,b, 1964). The soils are incubated at controlled temperatures and maintained at their −33-kPa water tension by continuous aeration with water-saturated, CO_2-free air. The amount of CO_2 trapped in NaOH collectors is determined, after precipitation of the CO_2 with $BaCl_2$, by automatic potentiometric titration with HCl. The amount of CO_2 evolved from the master jars during an incubation is expressed on the basis of a constant amount of soil, usually 100 g, oven-dry equivalent, which normalizes the respiration rate regardless of the amount of soil present in the master jars.

The potential gross metabolic activity, both aerobic and anaerobic, of the heterotrophic soil microbiota can be measured by the addition of a nonspecific substrate (e.g., glucose), and the potential activity of specific populations can be evaluated by the addition of specific substrates (e.g., celluloses, starches, lipids, proteins) whose mineralization is dependent on the ability of these populations to synthesize the appropriate enzymes. In particular, aldehydes, which are highly selective substrates, can be used (Bewley and Stotzky, 1984; Kunc and Stotzky, 1974, 1977). Ratios of the gross metabolic activity (with glucose or other nonspecific substrates) to that of specific metabolic activities (e.g., with aldehydes or other selective substrates) can be used to indicate whether the presence of a GEM exerts an effect on the metabolism of all components of the indigenous microbial population or only on certain segments of the microbiota. These ratios will also sharpen comparisons between uninoculated control soils and soils inoculated with either a GEM or the homologous parental strain without the novel gene.

When aldehydes are used as specific substrates, the data from the aldehyde studies should be correlated with those from nitrification studies. Both nitrification (an autotrophic process) and mineralization of aldehydes (a heterotrophic process) are restricted to certain but different microbial species, and both processes show similar kinetics in soil, especially when soils are stressed (e.g., with heavy metals or acid precipitation) or altered (e.g., amended with different clay minerals) (Stotzky, 1980, 1986).

The soils should also be amended with the specific substrate (e.g., toluene, xylenes, 2,4-dichlorophenoxyacetate) on which the products of the novel gene(s) in a GEM function, to determine whether the substrate provides an ecological advantage to the GEM, whether intermediates are produced from the substrate, and how any advantages or intermediates affect both nonspecific and specific metabolic activities, as well as other microbe-mediated ecological processes (Doyle *et al.*,

1991; Short *et al.*, 1991). When the GEM contains genes that confer resistance to the toxicity of an antimicrobial agent, the soil should be amended with the appropriate agent, to determine whether such a stress (simulated worst-case scenario) confers an ecological advantage on the GEM and whether this advantage, in turn, influences the activity and population dynamics of the indigenous microbiota.

Measurement of Respiration (Carbon Dioxide Evolution)

Reagents

NaOH (~1.5 *N*): Dissolve, with swirling, approximately 60 g NaOH per liter of distilled water in a 20-liter borosilicate carboy. Fit into the mouth of the carboy a two-hole rubber stopper containing an air inlet tube and a solution outlet tube that is attached to a constant-volume 50-ml stopcock-type automatic pipetter. Connect the air inlet tube to a gas-drying tube containing Drierite and Ascarite, to prevent ambient water vapor and CO_2 from entering the NaOH, and attach a rubber bulb to enable pressurization of the carboy. During the course of the study, adjust the normality of the solution to the amount of CO_2 produced.

NaOH (2.00 *N* standard): Commercially available.

HCl (~7.5 *N*): Dilute approximately 625 ml of concentrated HCl to 1 liter with distilled water (add the HCl to the water). As the normality of the NaOH in the CO_2 collector is adjusted to reflect decreasing or increasing (e.g., following pulsing with a carbon source) respiration rates, the normality of the HCl must also be changed to reflect the 5-fold difference between the normality of the NaOH and that of the HCl. (The CO_2 collector contains 50 ml of NaOH, and the self-filling burette on the automatic titrator has a capacity of only 10 ml, to enhance precision.) Determine accurately the normality of the HCl by titrating it against the 2.00 *N* NaOH standard. Attach a Drierite–Ascarite column and rubber bulb to the air inlet tube, as described for the NaOH carboy. Attach the solution outlet tube to the self-filling burette.

$BaCl_2$ (~3.5 *M*): Dissolve approximately 855 g $BaCl_2 \cdot 2H_2O$ per liter of distilled water. Place the solution in a glass carboy equipped with a Drierite–Ascarite column and rubber bulb on the air inlet tube, and connect the solution outlet tube to a 50- or 100-ml self-filling burette.

Distilled water: Fill a glass carboy with freshly distilled water. Attach a Drierite–Ascarite column and rubber bulb to the air inlet tube and an eyedropper tube to the outlet tube. The flow of water is controlled with a pinch clamp on the outlet tubing.

KOH (~4.0 *N*): Dissolve approximately 224 g KOH per liter of distilled water in either a borosilicate flask or carboy. Transfer the solution to the appropriate glass carboys in the scrubber system of the respiration train.

(*N.B.*: The dissolution of NaOH and KOH is an exothermic reaction and requires the use of a borosilicate container. The solutions must be allowed to cool before transferring to other containers that are not heat-tolerant.)

Procedure. Grow the GEMs and the homologous parental strains as batch cultures to a population of around 10^9 colony-forming units (CFU) per milliliter in an appropriate liquid medium (e.g., L-broth) containing the selection factors necessary to maintain the genotypic and phenotypic characteristics unique to the GEMs and the parental strains and necessary for their selective recovery from soil. Prepare a standard curve for each GEM and homologous parental strain by plotting the absorbance against the numbers of either total (determined microscopically, e.g., with a hemacytometer) or viable bacteria (determined by plating) for a dilution series. Determine the concentration of the bacteria spectrophotometrically (e.g., with a Bausch & Lomb Spectronic 20) at the same wavelength used for the preparation of the standard curve, using sterile medium as a blank. Dilute the bacteria with a sterile substrate (e.g., glucose) solution or sterile water and add to the soil with sufficient water to adjust the soil water tension to -33 kPa and to yield the desired inoculum density per gram of soil, oven-dry equivalent (e.g., one that approximates the density to be used in a field release), and the desired substrate concentration.

Mix the soil in a thin-walled plastic bag by kneading, and store for 48 hours at 4°C, with additional kneading at 24 hours, to enhance the uniform distribution of water, substrate, and cells. After the 48-hour equilibration period, weigh 50 g of soil, oven-dry equivalent, into 100-cm^3 glass vials. If cold-intolerant microorganisms are used, add the cells just before weighing the soils, and mix well. Keep the vials of soil cool until all have been filled. Place the vials into a wide-mouth gallon jar (master jar) (Fig. 1) (pickle and mayonnaise jars are ideal and can be obtained inexpensively). Attach the master jar, via the air inlet tube, to the manifold of a respiration train that contains a scrubber system for removing oil, ambient CO_2, various nitrogen compounds, and other contaminants and then resaturates with water the air that continuously flushes the master jar (Fig. 3). Connect the air outlet tube of the master jar to the CO_2 collector (Fig. 1) (see below for details). At specified in-