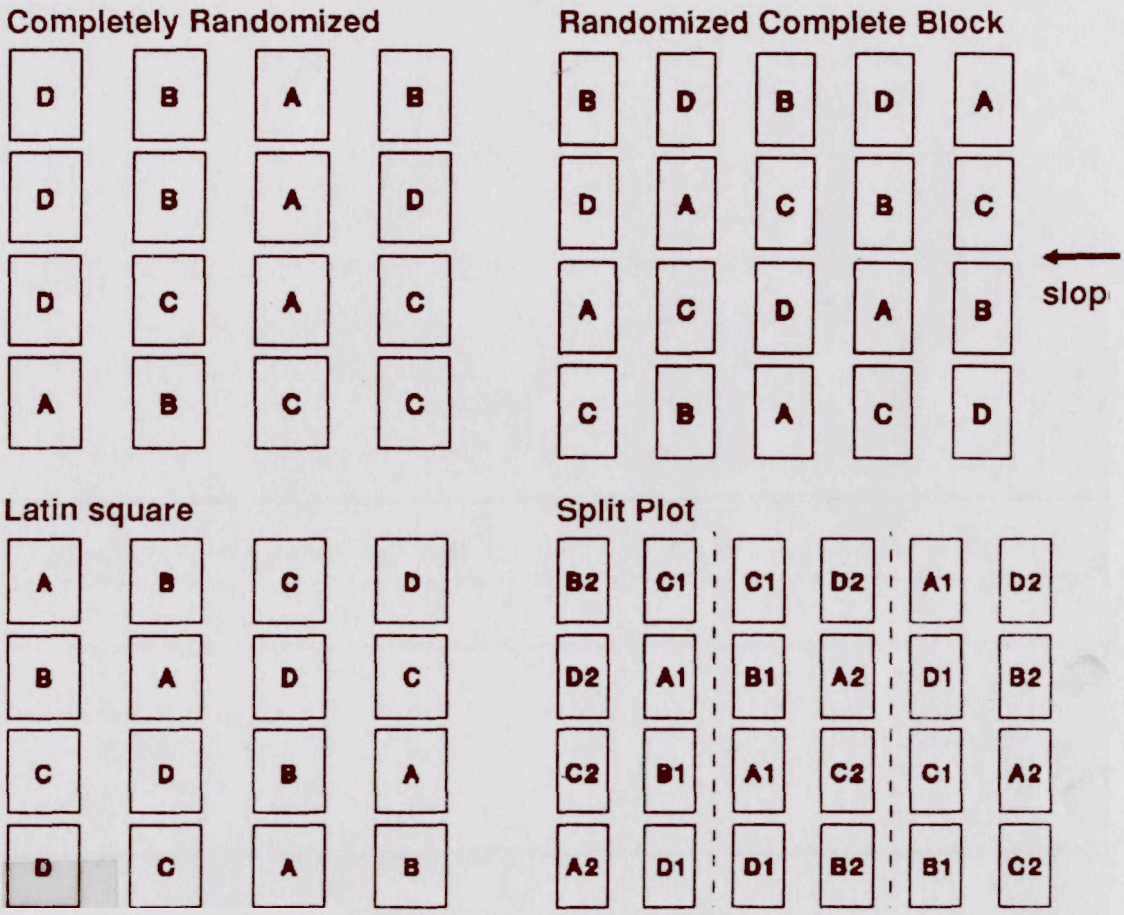
United States
Environmental Protection
Agency

Prevention, Pesticides,
And Toxic Substances
(TS-788)

EPA 700 R-92-008
June 1992

EPA

Monitoring Small-Scale Field Tests Of Microorganisms



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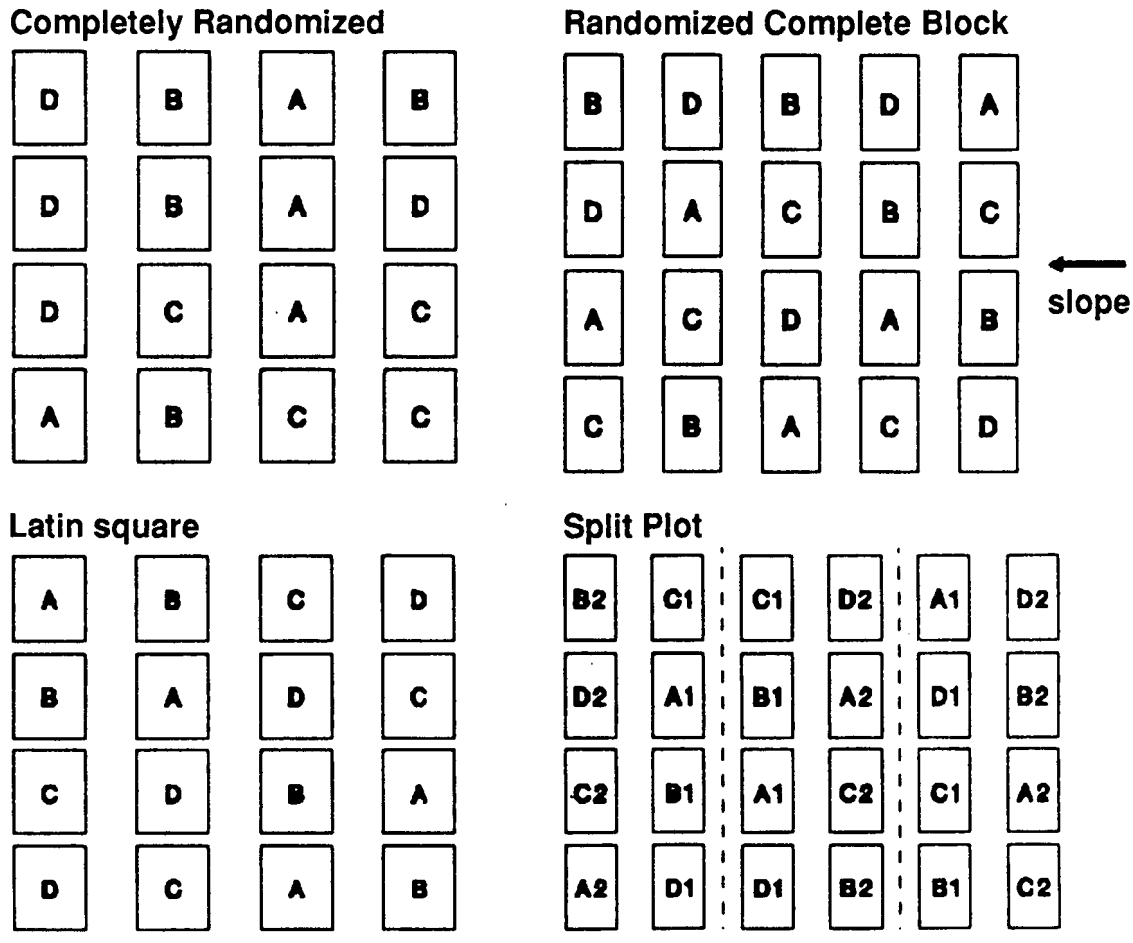

Acknowledgements:

This work was supported in part by USEPA Contract No. 68-02-4296, Work Assignment No. 333, USEPA Contract No. 68-02-4294, Task No. 2-44, and USEPA Contract No. 68-D0-0061, Task No. 1-8.

TABLE OF CONTENTS

INTRODUCTION

This document provides individuals planning small-scale field tests of microorganisms with guidance on the scientific principles and appropriate methodologies for monitoring the environmental fate and impact of microorganisms introduced into the environment. Monitoring of microorganisms can be used for several general purposes including:

1. assessing the performance of a microorganism (including the gathering of data about microbial survival, competitive ability, or yield of crop plants),

2. contributing to an assessment of an adverse human health risks or environmental impact, or

3. elucidating the biology or ecology of the microorganism.

Monitoring procedures vary from qualitative to quantitative, from simple to complex, and with the type of microorganism and environment into which it is introduced. In most cases, monitoring for beneficial environmental impacts is assumed. Monitoring for adverse impacts is also appropriate when uncertainty exists. The primary focus of this document is on monitoring for potential adverse impacts on the field site environment.

A general scheme for designing a program to monitor microorganisms in the environment is shown in Figure 1. The first phase in monitoring program design is to clearly define the program's objectives based on available knowledge of the microorganism to be released (i.e., the microorganism profile), the environment (i.e., the field-site profile), and the field test protocol (i.e., the experimental profile). The integration of information contained in these three profiles for a given small-scale field introduction provides the basis for development of a specific monitoring program.

The second phase in the design of a monitoring program is to determine the monitoring objectives and the appropriate monitoring intensity for the small-scale field test. The monitoring objectives should establish what endpoints are to be characterized. The endpoints might include, for example, population density of the microorganisms in the rhizosphere, gene transfer frequencies, or effects on the plants infected by a microorganism. The appropriate monitoring intensity is determined by the degree of uncertainty and the potential severity of effects associated with the microorganism.

The third phase in the design of a monitoring program is to develop the specific monitoring plan. Development of the monitoring plan involves defining the physical layout of the field test, monitoring zones, and sample collection and analysis strategies.

Throughout the course of the field test, data will be collected and analyzed. The development of a monitoring plan should be a dynamic, iterative process in which modifications to monitoring practices can be made during the field test in response to changing conditions observed in the field or problems associated with sample collection which were unforeseen at the start of the field test.

Before any samples are collected, a quality assurance plan should be in place to ensure that the resulting data will be scientifically sound and unbiased. Quality assurance and quality control procedures assist the researcher in balancing time constraints and procedural costs with the data quality necessary to achieve the monitoring objectives. Finally, appropriate health and safety procedures should be integrated into the monitoring program in order to ensure the overall safety of persons conducting the field test.

These guidelines serve as a starting point for the development and implementation of an effective monitoring plan for the small-scale field testing of a microorganism. More specific guidance on techniques used in monitoring microorganisms in the environment can be found in Levin et al., 1992, Ginzburg 1991, and through the work of international groups (OECD, 1991; Commission of the European Communities et al., 1992).

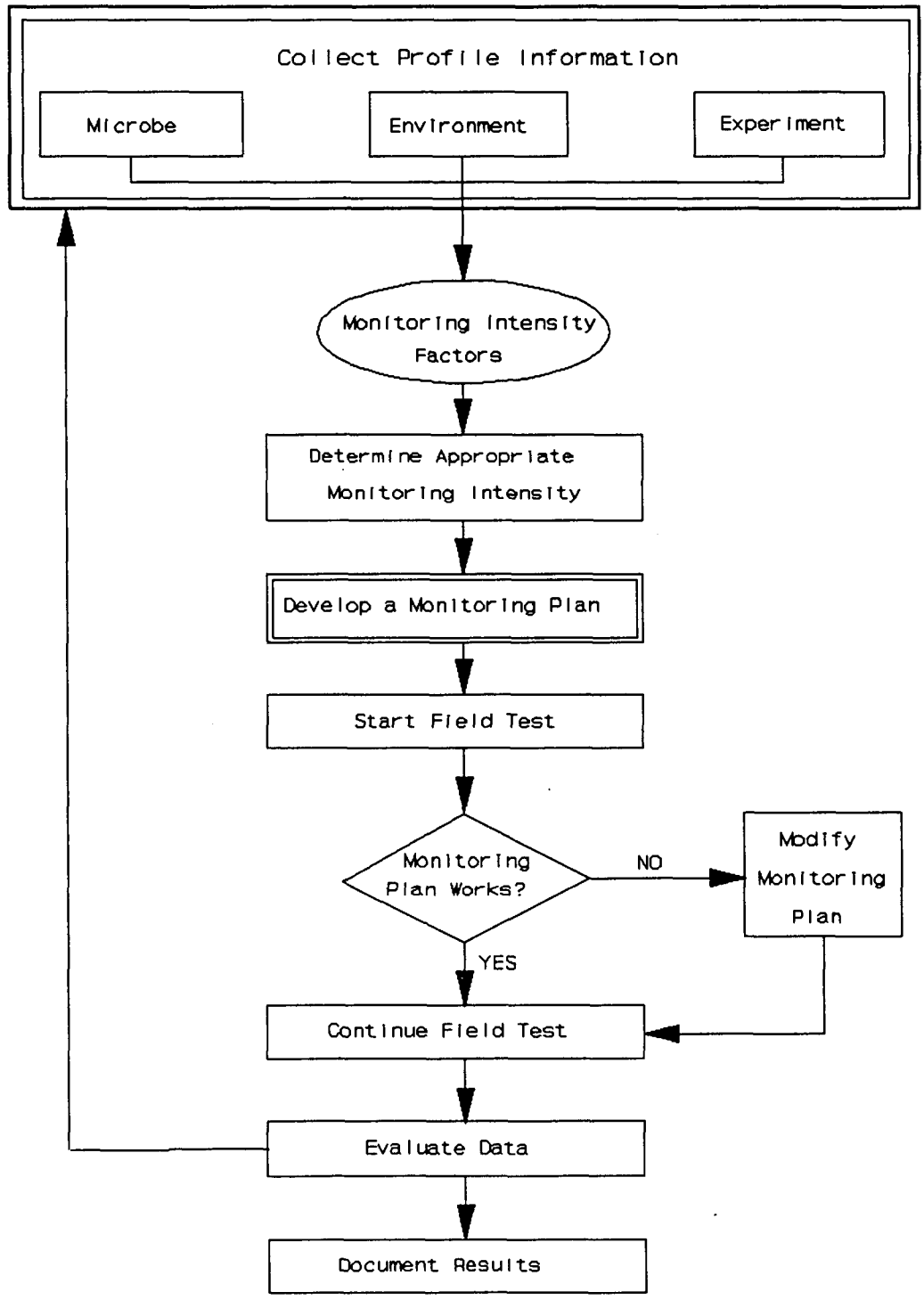

Figure 1. Schematic development of a monitoring program.

CHAPTER 1: COLLECTING INFORMATION

1.1 Gathering Information

A well-designed field test considers the interaction of the microorganism to be tested, the field test site, and the experimental design. Identification and achievement of field test objectives are dependent upon the interaction of all three elements. The kinds of measurements and the precision of those measurements should be based on the understanding of the microorganism and the test site. The possibility of unexpected effects, known adverse impacts, or incorrect conclusions should be considered in this planning step to ensure that appropriate information will be obtained from the field test.

In defining these decisions the investigator would consider the information to be gathered from the field test, the time and resources available for gathering the information, and the consequences of inadequate or erroneous information being gathered.

The objectives of the field test should be classified as major or minor and ranked in importance. Because monitoring designs often result in more precise information gathering for some treatment comparisons than for others, the design should first address those objectives judged to be of greatest importance. At this point in the planning process, consultation with a statistician can contribute significantly to achieving the aims of the investigator.

Appendix A contains lists of various environmental, microbial, and experimental characteristics that might be considered in developing experimental and monitoring designs. Not every characteristic listed in Appendix A will be pertinent or necessary for a given field test.

1.2 The Microorganism Profile

The microorganism profile should contain information on those characteristics that will allow the investigator to predict behavior of the microorganism in the field in terms of fate and survival, and, if desired, to identify the microorganism in the field. This information should also allow for identification of the possible health and environmental effects of the release. The investigator should select methods to effectively identify the microorganism and should determine the sensitivity of the method in terms of minimum detection limits.

The microorganism profile will vary with the microorganism under study and should include considerations such as: the history of environmental use of the microorganism, the microorganism's life cycle and ecology, the phenotype expected in

the environment of use, relevant genetic modifications that may have been made, and the phenotypic consequences of these modifications. Information leading to taxonomic identification is important here. Not only does the taxonomic identity help to formulate hypotheses concerning the direct potential for adverse impacts, but where such data are lacking, taxonomic information may be used to formulate specific monitoring endpoints. In some cases, taxonomic information may direct the investigator to literature on more thoroughly studied organisms that are closely related phylogenetically to the test microorganism.

The degree of uncertainty associated with possible adverse impacts of testing microorganisms in the field is an important consideration. Traits that should be addressed to reduce this uncertainty include pathogenicity, infectivity, toxicity, survival and competitiveness, and the potential for genetic transfer. Other characteristics related to microbial fate that may be considered include microhabitat, nutrient requirements, oxygen requirements, motility, survivability and dormancy (see Appendix A).

Some of the information discussed above may be available in the literature for microorganism being field tested and for the parental organism. If it is unavailable, key data can be obtained through testing in the laboratory or at the field test site. If necessary, this information could then be used to determine specific experimental procedures that should be followed during field testing to limit microorganism dissemination between treatments or outside of the experimental plots during and between treatments. A discussion of such determinations is found in Strauss et al. (1985).

1.3 The Field Site (Environmental) Profile

Selecting an appropriate field test site is also an important factor in the design, implementation, and success of any field test. The investigator should select the field test site taking into account the characteristics of the microorganism to be tested (e. g., ability to survive and disseminate) and the objectives of the experiment. The site should also be evaluated in terms of these same considerations for potential adverse impacts and/or uncertainty. Several considerations to take into account when selecting a test site are described in this chapter. A more thorough listing is contained in Appendix A.

If possible adverse environmental impacts are identified in the microorganism profile, a site that is isolated spatially from inhabited areas or nontarget susceptible species can be selected, or appropriate containment procedures, such as border rows, can be applied. For effective monitoring within a given site, the field site profile should recognize the heterogeneity inherent to the site. It may therefore be useful to characterize the site as to climate, topography, hydrology, soil type and any biotic factors which could affect the planned test (e.g., the presence of populations of

indigenous microorganisms with the same antibiotic resistance being used to identify the test microorganism). The investigator is advised to pay particular attention to environmental characteristics and experimental procedures that might serve as a route for dissemination or might affect survival of the microorganisms within and outside the test site.

In characterizing the site the investigator may use U.S. Geological Survey topographic maps to indicate the location of the site; U.S. Department of Agriculture (USDA) soil survey maps to determine soil type, topography, and surface drainage patterns; maps with information on wind direction, frequency, and strength; aquifer maps for ground-water information; and, if available, aerial imagery for identifying proximity of the site to inhabited areas and any other features that might not otherwise be captured on standard maps. Many of these maps are available from local Federal, State, or county authorities. If they are not readily available, the investigator can contact the National Cartographic Information Center in Reston, Virginia ([703] 959-6045), which serves as a clearinghouse for a variety of maps.

The investigator can check with appropriate federal, state, and local agencies for any other information that may affect the use of the site for a field test. This information might include the presence of threatened or endangered species in the area, migration routes for protected fauna, restrictions on sampling or the transportation of environmental samples, and specific permitting or licensing requirements.

The site profile should be evaluated in light of the microorganism to be tested. This evaluation should consider the characteristics of the microorganism under study, the possibility of adverse impacts, and the degree of uncertainty concerning predicted behavior. If there is a possibility of adverse impacts, then the investigator should carefully examine the field test site characteristics to determine possible consequences of those effects at that site and to identify how such effects could be measured. Much knowledge has been gained from experience with tests of nonindigenous microorganisms, plant or animal pests, and microbial pesticides that have been safely conducted.

Careful attention may be necessary to determine the presence or absence of indigenous biota that (1) have a capacity for readily accepting or transferring genetic information of concern (e. g., antibiotic resistance) from or to the test microorganisms; (2) have been shown to transport microorganisms (either in their intestinal tracts, hemolymph, or salivary glands, or on their body surfaces); (3) are reported to be sensitive to the introduced microorganism and/or its products; (4) are natural hosts for the microorganism to be released; or (5) are listed as endangered, threatened, or of special concern to Federal or State governments. For example, if there is the potential for transfer of genetic material from the test microorganisms, the investigator may

need to include in the field site profile information concerning the indigenous microbial community of the test site environment.

While much of the information needed for the site profile may be readily available from existing sources, some of the information may have to be obtained directly from the intended field site in order to fully characterize it. The intensity of the field site characterization will be inversely proportional to familiarity with the behavior of the microorganism in the test environment. The greater the uncertainty or potential for adverse impacts, the greater the intensity of the field site characterization.

Site characterization can also include surveying nearby areas that could serve as sites for microorganism establishment outside of the designated field test area in order to evaluate possible avenues of dissemination. In many cases microorganism survival and transport considerations indicate distinct zones surrounding a field site in which monitoring may be desirable. These zones may be delineated by topographical considerations (e. g., slope), prevailing wind direction, or may include distinct sampling zones at or below the soil surface. For example, if the microorganism is mobile in ground water and the release site is composed of porous soils, then several vertical sampling horizons down to and including the ground-water aquifers may need to be considered. The definition of monitoring zones includes: the medium to be sampled, the lateral and vertical extent of the zones, and a clear description of the rationale for selecting those zones.

The results of the site characterization can be used to: 1) develop testable hypotheses concerning microbial fate within the site and beyond and potential effects in the environment; 2) provide a measure of site variability as a basis for field test protocol design and selection of analytical techniques (considering precision and accuracy); and 3) establish a "before" picture of the site that may be used for comparison when monitoring for "after" effects.

1.4 The Experimental Profile

The experimental profile is shaped by both of the preceding profiles. The information gathered in the preceding profiles is used to justify the choices made in the actual layout of the test site, how the microorganisms are applied to the test site, and the endpoints measured during the field test. As outlined in Appendix A, the experimental profile is composed of three major parameters contributing to the field test design: the experimental design, the treatment design, and the monitoring design. The experimental design includes: field plot design, physical layout of the treatments, sampling strategy, statistical model for analyzing the data collected, and the choice of environmental factors to be monitored during the experiment. Apart from field tests purely for basic research, experimental design is intended to monitor beneficial impacts or efficacy of a microbial treatment. The treatment design would include: site preparation prior to the experiment and site maintenance during the experiment

(including the test area, border areas, and the monitoring zones), and treatment characteristics such as methods of applying the microorganism (e.g., seed coating or aerosol spray) or different concentrations of the microorganism being applied. The monitoring design includes: identification of monitoring zones (taking into account features of the specific field site), determining the endpoints to be measured within each monitoring zone, and determining the sample collection strategy for each monitoring zone. This document addresses monitoring design for adverse impacts.

Experimental design includes the chosen statistical model which will be used to compare treatment effects. Statistical procedures are used to examine and reduce the effects of experimental error in the measured data. Appendix B briefly outlines common experimental designs for field tests and the statistical models that can be applied to evaluate the treatment effects. Chapter 2 provides an extensive overview of field plot design and statistical analysis. Experimental design should be considered a flexible feature in planning a field test because selection of the statistical model for the experiment is considered in conjunction with the physical layout of treatments and monitoring design. The experimental design should be chosen prior to the initiation of the field test. However, the choice of the experimental design does not limit the options for monitoring and data analysis.

Treatment design, including site preparation and maintenance, may affect the behavior of the microorganism upon release and the necessary monitoring of the microorganism in the field. The method of microorganism application would include: the number of microorganisms to be released per application; the frequency and duration of release; and the manner of the release (e.g., droplet size when using spray application). The investigator can draw upon the literature or the documented results of current research activities that address phenomena associated with specific microorganism application procedures.

Monitoring design is determined by the field test site, the characteristics of the microorganism, and the certainty with which the interaction between microorganism and environment can be characterized. Within the field test site, monitoring zones are chosen with consideration of important field heterogeneities at the site such as soil type, fertility, surface topography, climatology, hydrology and microbial populations. To focus the results on the treatments being tested, test plots are selected to minimize the effects of field heterogeneities. Monitoring design is discussed in the following chapters which describe considerations that would determine monitoring intensity and the sampling strategy.

CHAPTER 2: OVERVIEW OF FIELD PLOT DESIGN AND STATISTICAL ANALYSIS

2.1 Introduction

This section is intended to help scientists work with statisticians to design experiments for the field by highlighting relevant statistical methodology that can be found in detail in agricultural and biological statistical references (Cochran and Cox, 1957; Gomez and Gomez, 1984; Little and Hills, 1978; Steel and Torrie, 1980). Other chapters on statistics relevant to monitoring the environmental release of microorganisms (McIntosh, 1991), or on evaluating chemical controls of plant diseases (Nelson, 1986) may also be useful. This section is not intended to be a cookbook of how to plan and analyze field research on microorganisms, nor is it intended to be a substitute for statistical references.

Field tests involving microorganisms, like all field experiments, are dependent on: 1) the development of a hypothesis that can be tested, 2) a well-designed carefully controlled test, and 3) proper interpretation of the results in the context of existing environmental conditions at the time and location of the trial. Statistical procedures are simply the tools by which experiments can be properly designed and interpreted so that the true effect can be inferred with a degree of certainty. Biological experimentation in particular is greatly aided by the proper use of statistics, because rarely, if ever, will a simple description of a limited number of observations of a biological phenomena yield the same result. Variability is the rule in biology and not the exception. Ideally if a biological experiment could be repeated indefinitely under the same conditions the responses would eventually produce a convincing pattern and the true effect would be known. Statistics provides the means by which this true effect can be inferred from a more economically feasible number of observations.

Proper experimental design and interpretation, however, does not guarantee successful final results from field trials because many unforeseen circumstances may occur in the field that can have profound effects (drought, wind, hail, lightning, allelopathic effects of previous plants, weed competition, soil heterogeneity, etc.). Planning is the most important phase of experimentation that can ensure that the treatments selected will provide relevant estimates for testing hypotheses, and can reduce the impact of unforeseen problems. Careful planning and consultation with a statistician at the initial stages are the most important steps in developing field plot experiments that will yield a maximum return. Biologists who understand some of the principles underlying experimental designs will have more fruitful planning sessions with statisticians.

2.2 Basic Considerations for Statistical Analyses

2.2.1 Analysis of Variance - Introduction

The basic procedure for determining whether or not some treatment has a significant effect on a population (testing a hypothesis) is the analysis of variance (ANOVA). The ANOVA structures the information about the variability of the measurements by grouping them according to the source of variability. For example, one expected source of variability is the treatment, another may depend on the way the treatments are arranged (experimental design), and the remainder is called the error. The error is also known as the random variation or unexplained variation and is critical to the ANOVA. The comparison of the amount of variation that can be assigned a source, e. g., treatment, to the error determines whether the differences between the treatment means are sufficiently distinct from the differences that random variability will cause. The variations among the different sources in an ANOVA are estimated by quantities called the mean squares. The ratio of mean squares produces a statistic called the F-statistic. F can be tested for significance against "critical values" that have been determined for given probabilities that true differences can be assumed to exist. Most researchers typically use a 5% probability level as a scientifically comfortable cutoff for significance. Because field experimentation is inherently more variable than laboratory experimentation, levels of probability between 5 and 10% are commonly used to indicate that something is happening that may require further experimentation to verify. Significance levels greater than 10% should not in general be used.

2.2.2 Power of test

The power of a test is the ability of the test to find two means significantly different from each other. The power of a test can be increased particularly by increasing the number of replications of samples taken. The calculation of the power of the test depends on an understanding of the different types of errors, which is beyond the scope of this overview. This may be an important consideration for a high risk introduction for which a single field trial with a limited number of replications may be attempted. For those cases consultation with a statistician is essential. As a general rule, when no background information on variability is available, repeated trials, or more than one location for a trial that has approximately 10 degrees of freedom for the error mean square increase the likelihood of determining whether or not real differences exist. Degrees of freedom (df) are dependent on the treatment design and numbers of replications used. An examination of an F table, which provides critical levels of F for determining significance, at a 5% probability level where the error has 10 df and a treatment has 1 df (e. g., genetically-engineered microorganism (GEM) vs. no GEM) indicates that the treatment would need to account for approximately 5 times the variation attributable to random error to be significant. Whereas, if the error term had only 3 df, for the same example, the treatment variance

would have to be more than 10 times greater than random variation. The exact meaning for degrees of freedom and formulas for their calculation are available in statistical texts.

2.2.3 Requirements for Analysis of Variance

There are three requirements for F-tests in ANOVA to be valid. The actual statistical requirements for practical purposes are not listed here, only the working requirements are discussed. Two of the requirements are generally satisfied if the treatments are randomized and replicated. The last requirement, that the variances associated with each treatment be similar, depends in part on the type of data being collected and may require that the data be transformed to ensure that the F-test is legitimate.

There can be no estimate of error without replication. Treatments should be applied to more than one experimental unit (plot) to be considered replicated. Repeated measurements (samples) from a single plot do not constitute replication of a treatment. For example, most experiments will be designed to test several hypotheses related to both plant effects as well as soil microbial effects, and therefore are likely to have plot dimensions of 2 - 10 m^2. Yet only 1-10 grams of soil may be necessary for plating out on media to quantify the microbial entity. Soil sampling for a microorganism is necessarily done by taking several samples from a given area because of the natural variability in microbial dispersion. Therefore, each plot would require multiple samples just to estimate the microbial population. The estimate of the population in the one plot, although it required multiple samples, is just one replicate measure of that population for the treatment. In general, increasing the number of replications is the simplest means of increasing the sensitivity of any experiment. Of course, there is a point of diminishing returns as the cost of increasing the size of the experiment with increased replications will eventually outweigh the return in increased precision. There are procedures for determining the minimum number of replications (formulas and tables) which require previous knowledge similar to the methods used to determine sample sizes (Cochran and Cox, 1957). Where no prior knowledge is available, a general guideline as discussed in section 2.3.2 is to adjust the number of replications so that the error mean square has approximately 10 degrees of freedom.

Randomization is particularly important in field studies so that measurements are not inadvertently biased. The field environment often has gradients, e. g., slope of the field, shading from neighboring trees, variability due to differences in soil type, in texture or previous cropping, and especially in moisture and fertility. In order to ensure that these sources of variability do not overlap with treatment patterns and confound the effects of the treatments, assignment of treatments to the plots is done randomly. True randomization is best achieved with random number tables found in statistical texts, or with computer randomization schemes.

Variances associated with each treatment should be similar in order for the F-test to be legitimate. Tests for homogeneity of variance can be done using Bartlett's chi-square or the Fmax test. Transformations of the data are used in cases where variances are heterogeneous. Microbial populations characterized by colony counts often have variances that will increase by orders of magnitude as the mean increases, thus violating the assumption of homogeneous variance. Transformation of the raw data by taking the log of the values (or log of values +1 when there are zero counts) will usually correct for this violation (Steel and Torrie, 1980). Percentage data that cover a range of 30-70% or are calculated as percentages of a control usually does not require transformation. However, percentage data that cover a wide range, 0-100%, 0-50%, or 50-100%, usually need an arcsine transformation. Bimodal percentage data covering 0-20% and 80-100%, is best transformed by taking the square root of the values. When many of the raw data values are less than 10 and especially when there are a large number of zeros, taking the square root of the values and adding 0.5 is helpful. Transformed data should be tested in the same manner as the raw data to determine appropriateness of the transformation.

2.2.4 Regression

Regression is another method of statistical analysis that has two important functions relative to hazard assessment of microorganisms. Regression analysis is the appropriate procedure whenever the treatments that are being tested are a quantitative series, e. g., dosage response experiments. In its simplest form, linear regression analysis can be thought of as a statistical procedure for fitting a line. Regression establishes whether or not the response to the quantitative series of treatments can be adequately described by the line and also provides estimates of the rate (slope) and background level of response (intercept). This analysis can be extended to curvilinear responses and to describing the response to more than one set of treatments (multidimensional response surfaces). Use of regression analysis in this form requires making an assumption about the functional relationship (linear, curvilinear, etc.) between treatment and response, and that the treatment variables are quantitative.

Regression analysis can also be used to provide an ANOVA, and through the proper choice of statistical models produce an equivalent analysis (Neter and Wasserman, 1974). The advantage of the regression procedure (also known as a linear model approach to ANOVA) in obtaining an ANOVA is that the problem of missing data can be effectively handled. Experimental units in field experimentation often are lost during the course of the trial due to unforeseen circumstances. The regression procedure allows for an unbiased estimation of error even when there are a few missing data points. Missing value estimation procedures are also available for use in ANOVA, but are more limited in scope and effectiveness.

2.3 Experimental Designs

An experimental design is the physical way the experimental units are arranged. This provides a means of control over error by reducing the effect of natural variation on the differences between treatments. The simplest of designs is the Completely Randomized Design (CRD) in which all replications of all treatments are randomly assigned to the plots in the field (Figure 2). The CRD is simple, easy to analyze and flexible. However, it is rarely used in field experimentation except when the experiment is very small and the site is known to be very uniform because it provides no control over natural sources of variability (soil heterogeneity, etc.)

2.3.1 Randomized Complete Block Design

The most commonly used basic design is the Randomized Complete Block (RCB). In the RCB, instead of randomly assigning all replicates of all treatments to a plot anywhere in the field, each complete set (replicate) of treatments is assigned to a block of adjacent plots (Figure 2). This arrangement reduces the effect of random error because adjacent plots are usually more alike than nonadjacent plots. Each treatment is still randomly assigned to a plot within each block. Blocks should actually be selected by visiting the field, or based on previous experience with the site, in such a way as to minimize variation within a block. Usually blocks are selected on the basis of environmental sources of variation, particularly slope, shading, moisture, fertility, or soil type. Even if there is no visible evidence of potential problems or differences the field area is probably not uniform. When there are no visible sources of variation it is recommended that blocks be as small as practical and be square. Randomized complete blocks are simple to design and analyze and missing plot values can be readily estimated.

2.3.2 Latin Square

When there is a small number of treatments in the experiment, usually between two and ten, a design that may further increase control over natural sources of variability is the Latin Square (LS). The LS design uses blocking in two directions (Figure 2). In order to use the LS design, the number of replications should equal the number of treatments, and several squares may be needed when only 2-3 treatments are being tested to obtain a good estimate of error. The Latin square is more difficult to arrange, analyze, and estimate missing values. It is usually used where a small uniform experimental site is difficult to obtain, e. g., orchards in hilly production areas.

2.3.3 Split Plot

As the number of treatments in an experiment increases, and thus larger areas are required for establishing a complete set of treatments, experimental error naturally increases. Designs are available to subdivide the complete set of treatments

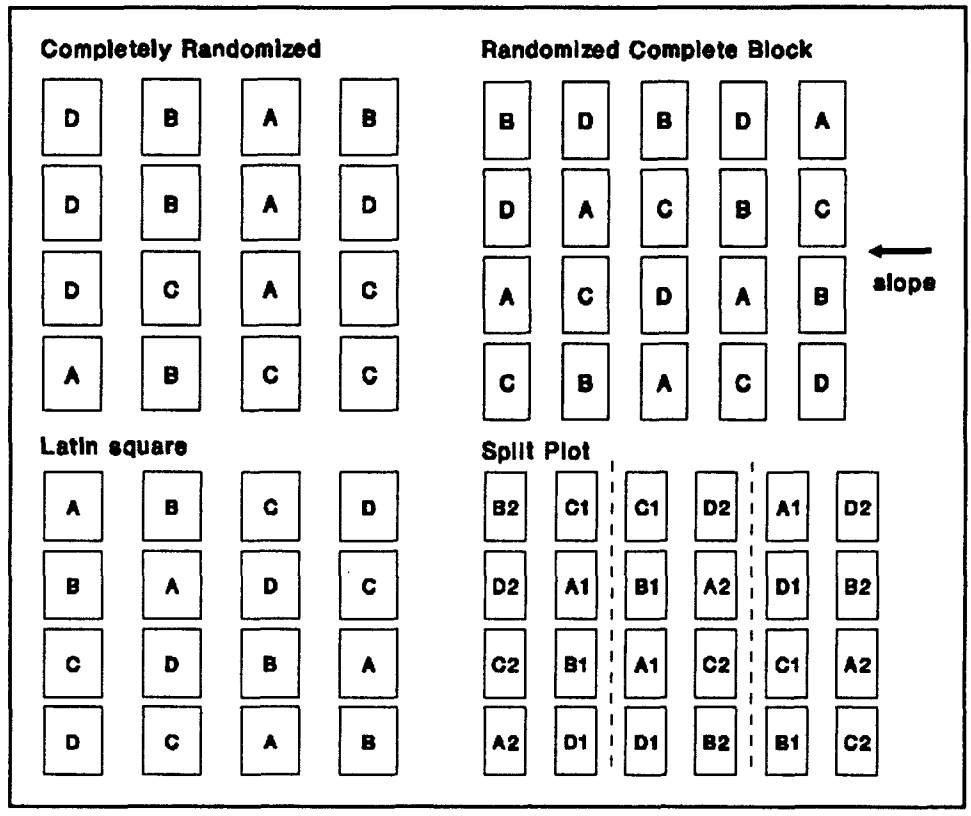

Figure 2 Illustration of four experimental designs: **1) Completely Randomized** with four replications of four treatments (A-D); **2) Randomized Complete Block** design with five replications (blocks) of four treatments (A-D), note blocks are columns and each block has a complete set in random order of treatments (A-D); **3) Latin square** design with four replications and four treatments, blocks in two directions (rows and columns) have a randomized complete set of treatments (A-D); and **4) Split plot** design with two sets of treatments A-D and 1-2 that are replicated three times, blocks are two sets of columns in this case. Note treatments 1-2 in the split plot have experimental units that are 4 times larger than treatments A-D.

according to certain rules so that each incomplete block is more uniform and a good estimate of error is still possible. There are many different incomplete block designs each with different restrictions and increased difficulty in setup, analysis and estimation of missing values (Cochran and Cox, 1957). The most commonly utilized variation of an incomplete block design is the split plot. The split-plot design is used when either the experimental material or mechanical difficulties arise in randomizing all the treatment combinations to the same size plot. For example, experiments involving

tillage or previous cropping history are easier to manage as larger contiguous sections of a block rather than as individually randomized plots scattered throughout a block. The split-plot design, in this case, would use two different sizes of experimental units with two different randomizations, one for the larger units or whole plots and one for the smaller units, the subplots (Figure 2). The effects of the treatments on the whole plots are estimated with less precision than those at the subplot level. Split-plots and variations of these reduce some of the logistical problems of managing the experiment but are more difficult to analyze, and to estimate missing values.

2.4 Factorials

The need to obtain as much information as possible from a given field experiment often dictates the need to investigate several different types of treatments simultaneously. This was alluded to in the split plot example, where one set of treatments (tillage) even required a different size experimental unit. Experiments where all of the treatments can be grouped together in a way that they can be considered qualitative or quantitative levels of the specific groups, e. g., presence or absence of a strain of a microorganism, or 10^3 vs 10^6 cfu's of a microorganism (both examples are 2 levels of one factor), are considered factorial experiments. Factorial experiments can be arranged in any of the standard experimental designs discussed previously, and are thus sometimes referred to as a treatment design. When the objective of the experiment is exploratory work to determine the effects of several different factors over a specified range, and to determine if there is a relationship between the factors, then factorial experiments are very useful. Factorial experiments, particularly those that are designed with two levels of each factor, are a very powerful way to gain a wide range of information. Factorials with only two levels of each factor are analyzed in an ANOVA as separate sources of variability. Associated with each factor is an independent F-test for significance. In comparison to an experiment that had multiple treatments or levels, the F-test for that larger group of treatments would be a test of significance averaged over all real and nonreal differences. In that case we might not be able to detect the real differences that occurred. The major disadvantage of factorials is in their most basic form in which all possible treatment combinations constitute one replication of the experiment, they rapidly become very large and difficult to manage.

2.5 Design Summary

The best design is the simplest design available that provides the desired precision. Several other points to consider that will affect experimental error are size and shape of the plots, collecting quantitative observations on unforeseen sources of variation that become apparent during the course of the experiment or that could not be sufficiently controlled by design, and guard or border rows within a plot. In the field the experimental units upon which the treatments are applied are the plots of land. The size and shape of the plots will also affect the precision and accuracy of the

experiment. In most cases involving row crops, plots should be long and narrow with the length of the plots running in the direction of the gradient that is used as the blocking factor. This will in effect average out some of the variation within a plot, and allow for management with typical row-crop equipment. When tests involve aerosols, or spores that readily move from plot to plot, square plots are preferable with particular attention to using border rows and collecting data from the center portion only. The use of border rows or guard rows in each plot is required when a treatment applied to one plot may affect the neighboring plot. At least one border row on each side of the section of the plot from which data is collected is advisable for row crops even when interplot interference problems are not expected. This ensures more typical growth of plants within a treatment, and allows the researcher to infer what could happen on a larger scale. Data collected quantitatively on factors that develop during the course of an experiment can be used in a mathematical process called covariance analysis to account for the errors not removed or accounted for by blocking.

2.6 Mean Separation Techniques

After the data have been analyzed in an ANOVA and a significant F-test has been obtained, a procedure to determine which treatment differences are real needs to be chosen. An F-test, because it is based on a ratio, has degrees of freedom associated with the numerator (treatment or factor) and with the denominator (error). A significant F-test that has more than one degree of freedom associated with the numerator only indicates that at least one of the pairs of means is different. There are numerous techniques available that help to separate which means are significantly different, but many multiple comparison procedures are also easily abused (Nelson and Rawlings, 1983). The multiple comparison procedures should be reserved for those situations where no obvious treatment structure exists. The preferred method is to plan the treatment comparisons prior to analysis and set up plots with this in mind. The use of orthogonal contrasts to set up single degree of freedom F-tests for those planned comparisons then provides for a more powerful comparison (Steel and Torrie, 1980; Gomez and Gomez, 1984; Little and Hills, 1978).

CHAPTER 3: DEVELOPING MONITORING OBJECTIVES

3.1 Formulating Monitoring Objectives

The monitoring objectives should establish what is to be characterized or determined based on the endpoints that have been identified. Generally, the more uncertainty or greater possibility of adverse health or environmental impacts, the more extensive the data needs associated with the monitoring objective. Where there is greater uncertainty about the potential behavior of the test organism, the investigator should develop additional objectives and a more detailed monitoring design to monitor. If adverse impacts are likely, then specific objectives should be developed to monitor intensively for specific adverse health or environmental impacts. (See examples at the end of this chapter.)

Each scenario of a field test can be defined as a process that begins with the release of the microorganism, continues with the fate of the introduced microorganism, and concludes with an endpoint such as an effect. An endpoint may be a biological, physical, or chemical characteristic measured in the experiment. In determining the monitoring objectives, the investigator defines specific measurable endpoints at critical points in the process.

Survival, transport, and fate considerations are endpoints that help identify areas of likely or potential colonization, both in the field test environment as well as in other environments that may be potentially accessible to the microorganism after its release. The identification of potentially accessible environments is critical in establishing monitoring zones, because then the objectives for each monitoring zone can be established.

There are likely to be several endpoints for any given hypothesis. The endpoints may range from: potentially beneficial (e.g., increased plant production or reduced pest populations), to neutral, or to potentially adverse impacts (e.g., death of nontarget plants). The investigator should select those endpoints which define the monitoring objectives for the field test.

The importance of the planning process cannot be emphasized enough. Without careful planning, it is quite possible that the field test will fail to achieve the desired monitoring objectives. For example, sampling or laboratory analytical errors could lead to data with little value to determine endpoints. Conversely, generation of more extensive or detailed data than is required to address the monitoring objective could result in an unnecessary expenditure of resources.

The collection of environmental data is a complex process of many iterative steps and there is usually more than one way of performing any given step. Every option that exists within a given step carries a different implication in terms of cost, time, data

quality, and the risk of arriving at an incorrect conclusion. These guidelines attempt to allow investigators the flexibility to design their monitoring programs to achieve their specific goal(s) within the bounds of a sound quality assurance program. (Discussion of quality assurance and quality control is found in Chapter 5.)

3.2 Determining the Appropriate Monitoring Intensity

After evaluating the microorganism and field test site profiles and before deciding on an experimental design, the investigator should consider various hypotheses and define specific endpoints associated with efficacy, possible adverse impacts, or the fate of the microorganism or its genetic material.

This step may be complicated by uncertainty concerning microbial ecology of the microorganism in question. The investigator may need to extrapolate from data derived from laboratory experiments. There may be some doubt about whether the assumptions made in the laboratory can be transferred to the field. Here, investigators should use their best professional judgment to determine the likelihood of certain endpoints. Uncertainty surrounding the interaction of the microorganism in a given environment should be viewed not so much as a liability, but rather as a variable in the monitoring design.

The degree of uncertainty in predicting how a microorganism will survive and possibly affect the environment can be combined with the potential for adverse impacts to indicate the appropriate monitoring intensity for a given experiment. The interaction between knowledge about the microorganism to be tested and the potential for adverse impacts in a given environment is illustrated in Figure 3. The greater the certainty that adverse impacts are possible, the greater the monitoring intensity required to confirm that an environmental hazard does not occur. The greater monitoring intensity is justified by the increased risk of obtaining false negative results. For example, field test data generated through a low intensity survey could indicate that no adverse impacts occurred, when indeed adverse impacts did occur and would have been detected through a high intensity sampling plan. The purpose of the monitoring design is to ensure that the probability of such a false negative will be low.

Qualitative sampling can, in some instances, help to identify the dispersal and distribution of microorganisms over a geographical area and provide information on survival during and after the intended period of performance in the test environment. Qualitative samples can also be used to note adverse impacts. For example, the spread or development of plant disease could be characterized by using a qualitative number system to indicate severity of disease. For some low intensity monitoring, however, the objectives may be to quantify microorganism populations, in addition to obtaining qualitative measure of presence or absence. Qualitative endpoints may also be appropriate for detecting the presence of such unintended effects as transconjugants and gene exchange with indigenous microorganisms.

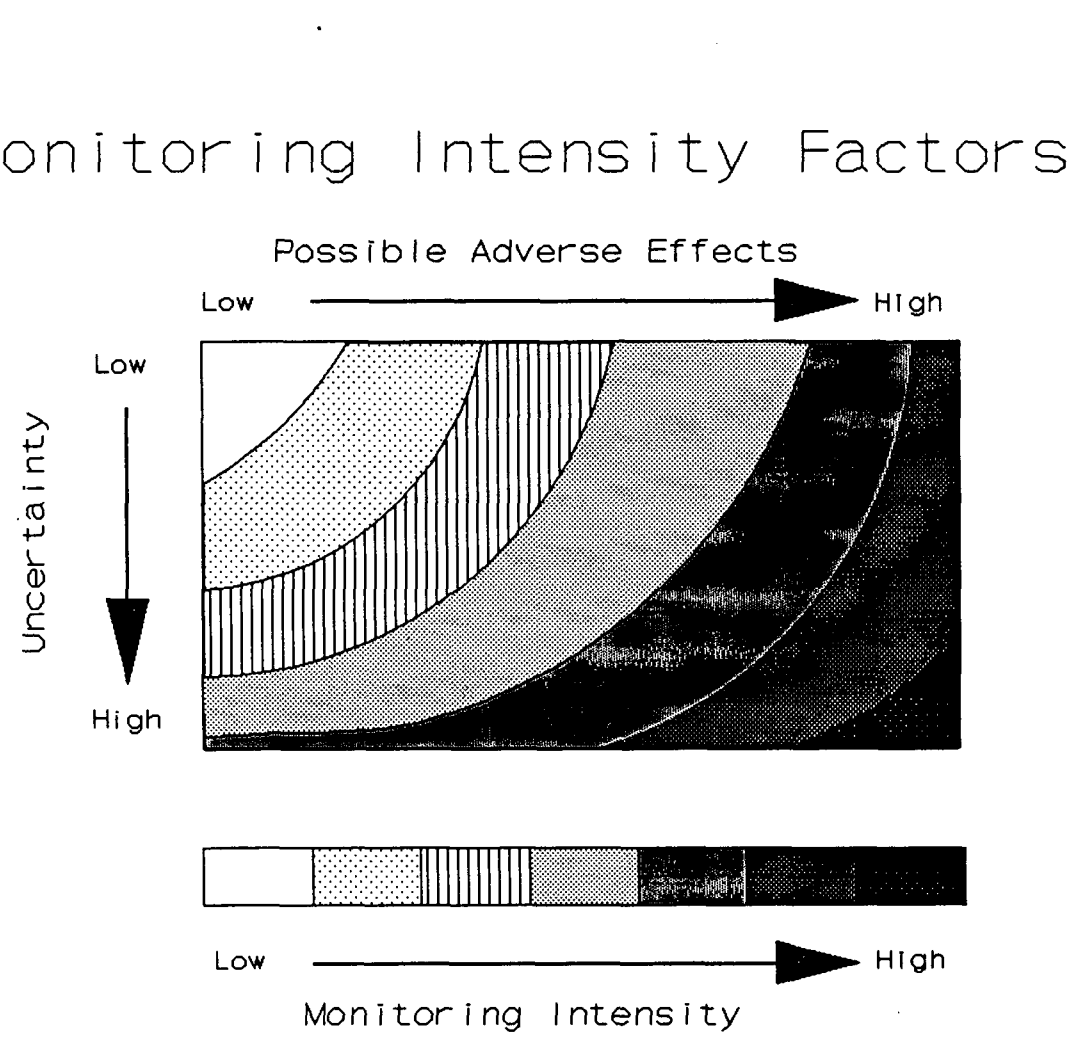

Figure 3. Monitoring intensity factors.

The greater uncertainty concerning characteristics of the microorganisms being tested at a specific field site could coincide with increased monitoring intensity due to the lack of information concerning the interaction between the microorganism and the new environment.

Determining appropriate monitoring intensity is one of the most important steps in constructing the monitoring design. It is also the most difficult to direct by way of generic guidelines because the hypotheses will be site- and microorganism-specific. One approach is for the investigator to create these hypotheses for each field test through an interrogatory process. While not a complete list, the following questions may serve as a useful template for such an approach:

- Will the microorganism under study survive in the area of application? How long and at what population density is the microorganism expected to survive? Are

there selection factors potentially favoring the microorganism or allowing a competitive advantage?

- How could the microorganism be transported outside the release site?

- Could this microorganism cause adverse human health or environmental impacts in or outside of the release site?

- Does the microorganism excrete any metabolites that could alter the environment to which it is released, for example, lowering the soil pH or contributing to eutrophication of aquatic environments.

- How effective will the microorganism be in producing the desired effect (e.g., an increase in nitrogen fixation)?

- What is the probability that the microorganism could transfer genetic material to another organism within or outside the release site? What is the likelihood that such a transfer could cause adverse health or environmental impacts?

3.3 Examples of Monitoring Intensities and Objectives

3.3.1 Low Intensity Monitoring Objectives.

The lowest monitoring intensity would be appropriate for a well-studied microorganism for which information on its behavior in the proposed test site is available, and it is known that adverse human health or environmental impacts are unlikely. This is the most basic design. In its simplest form, the monitoring objective may be satisfied by providing regularly scheduled visual inspections for any out-of-the-ordinary phenomena or conditions that are contrary to what were predicted, such as a sudden die-off of plants in the test site.

Since no adverse impacts are likely, there are no measurable health or environmental effect endpoints around which to design a monitoring effort. Consequently, the primary monitoring objective will be associated with designing a field test protocol to characterize fate and survival of the microorganism in the field. When artificial or natural barriers are part of the release scenario, a monitoring objective might focus on the performance of these barriers. The field test protocol should contain contingency plans for additional monitoring or early termination of the test if unexpected phenomena are observed.

Example: A microorganism, indigenous to the field test site, is to be used for the trial. The field test site has been well characterized: surface water runoff is possible due to the slope of the field test site and ground-water contamination is unlikely due to soil types. The method by which the microorganism is to be applied is by seed coating. Extensive references and data show no adverse human health or environmental impacts and suggest little or no potential for adverse impacts upon release to the field. There may be a slight potential for wind dispersal and surface water runoff. No measurable endpoints for human health or environmental effects have been identified. The monitoring objective is to characterize the fate and survival of the microorganism by measuring the mean population size of the microorganism in various zones in the upper soil layers.

3.3.2 High Intensity Monitoring Objectives.

The need for increased monitoring intensity could be indicated where there is little information about the microorganism's interaction with the proposed environment or where there is some potential for adverse impacts (e.g., competition with indigenous populations, gene transfer). If there is uncertainty about survival or possible adverse impacts, then the investigator should, in addition to performing the procedures outlined above, establish monitoring objectives to measure and test hypotheses about endpoints associated with possible adverse health or environmental impacts, as well as endpoints associated with fate.

The monitoring objectives directed towards possible adverse impacts will address whether more or less monitoring is needed in subsequent field tests. While additional time and resources may be needed to gather data to resolve uncertainties, the effort will be balanced by reducing the risk of allowing possible adverse impacts to go undetected and by gathering additional information concerning the behavior of the organism.

Where data exist to show that the microorganism under study has commonly caused adverse health or environmental impacts, the investigator should design the field test protocol as described above and add an objective aimed at characterizing the nature and extent of the adverse impact. Additional precautions in the field test protocols can be included to attempt to confine the microorganism (e.g., the use of borders, control of insect vectors, or use of dikes and channels to control runoff).

Example: In this case assume that laboratory studies have shown that under the right conditions the microorganism could cause an adverse impact on a nontarget species of plant. A reconnaissance survey reveals that the susceptible nontarget plant species inhabits certain areas surrounding the test site. In addition to the fate and survival objectives stated in the first example, another objective may be added to quantitatively determine adverse impacts on the nontarget plant species outside of the test site.

CHAPTER 4: DEVELOPING A MONITORING PLAN

4.1 Introduction

The monitoring plan should consider both the experimental design and physical layout of the test plots as well as the area surrounding the field test site and the potential for transport of the organisms outside the test plots. The monitoring plan is developed to address the monitoring objectives as identified in the previous chapter. This chapter will describe further steps and considerations in determining how to address the monitoring objectives. Throughout the discussion of the monitoring plan, the investigator should keep in mind that this plan is not static. It should adapt to changing conditions observed in the field or problems with sample collection and analysis often encountered only after the field test is underway and data are evaluated.

4.2 Defining Monitoring Zones

To address the monitoring objectives and endpoints of interest, monitoring zones should be determined. The definition of monitoring zones should include: the medium to be sampled, the lateral and vertical extent of the zones, and a clear description of the rationale for selecting those zones. These zones may be limited to surface sampling, or may include distinct sampling zones below or above the surface. For example, if the microorganism is known to disseminate through porous soils, then several vertical sampling horizons down to and including the ground-water aquifers may need to be considered.

It is essential that each monitoring objective be related to a specific measured endpoint in each monitoring zone. Generally, a variety of physical, chemical, and biological properties can be measured on each collected sample. For example, population counts of the released microorganism, population counts of competing microorganisms, and the presence of genetic sequences from the test microorganism in indigenous microorganisms might all be measured as endpoints from a single soil sample.

In addition to measurements specific to monitoring zones, changes in the ambient conditions (e.g., wind speed and direction, rainfall, sudden temperature changes) during application of the microorganisms could have immediate impact on the field study. Temperature and relative humidity are important factors affecting the survival of airborne cells (Cox, 1987) and data on these parameters would assist in modeling transport of viable microorganisms.

For zones associated with low intensity of monitoring, measurements may often be of a qualitative rather than quantitative nature to inspect for the presence or absence of the test microorganism itself or unspecified effects. For example, monitoring of field

release trials in California in the spring of 1987 demonstrated the usefulness of a qualitative method employing sentinel plants and deposition plates as a passive method to detect the presence of released airborne bacteria (Lindow et al., 1988; Seidler and Hern, 1988).

Figure 4 illustrates a release site with eight potential monitoring zones:

Zones 1 & 2 - The efficacy and strain competition study areas generally contain experimental layouts of sampling locations; however, these areas can also contain monitoring stations.

Zone 3 - The buffer area immediately surrounding the study areas can provide monitoring close to the study areas. This buffer zone may serve as the initial monitoring zone and identification of the test organism in this zone could trigger further sampling in the surrounding areas.

Zone 4 & 5 - In situations where aerial transport is possible or likely, a downwind monitoring area can indicate the presence of microorganisms away from the release site(s). For example with spray releases, wind speed and direction greatly affect particle transport, fate, and the placement of deposition plates and sentinel plants.

Zones 6 & 7 - Monitoring the soil downslope can indicate movement of microorganisms away from the release site(s) particularly if an intensive rainfall washes away dikes or contributes to unexpected runoff.

Zone 8 - For microorganisms that can survive in aquatic environments, monitoring in downslope surface water bodies or downgradient ground-water aquifers may be required.

4.3 Defining a Sample Collection Strategy

After establishing the monitoring zones at the test site, the sampling strategy for each monitoring zone is selected. There are three important considerations: (1) ensure that the sampling is representative of an entire zone at the selected point in time, (2) provide numerical estimates for decision making that have quantifiable error limits, and (3) provide estimates of sampling endpoints that are precise enough for the lowest possible cost.

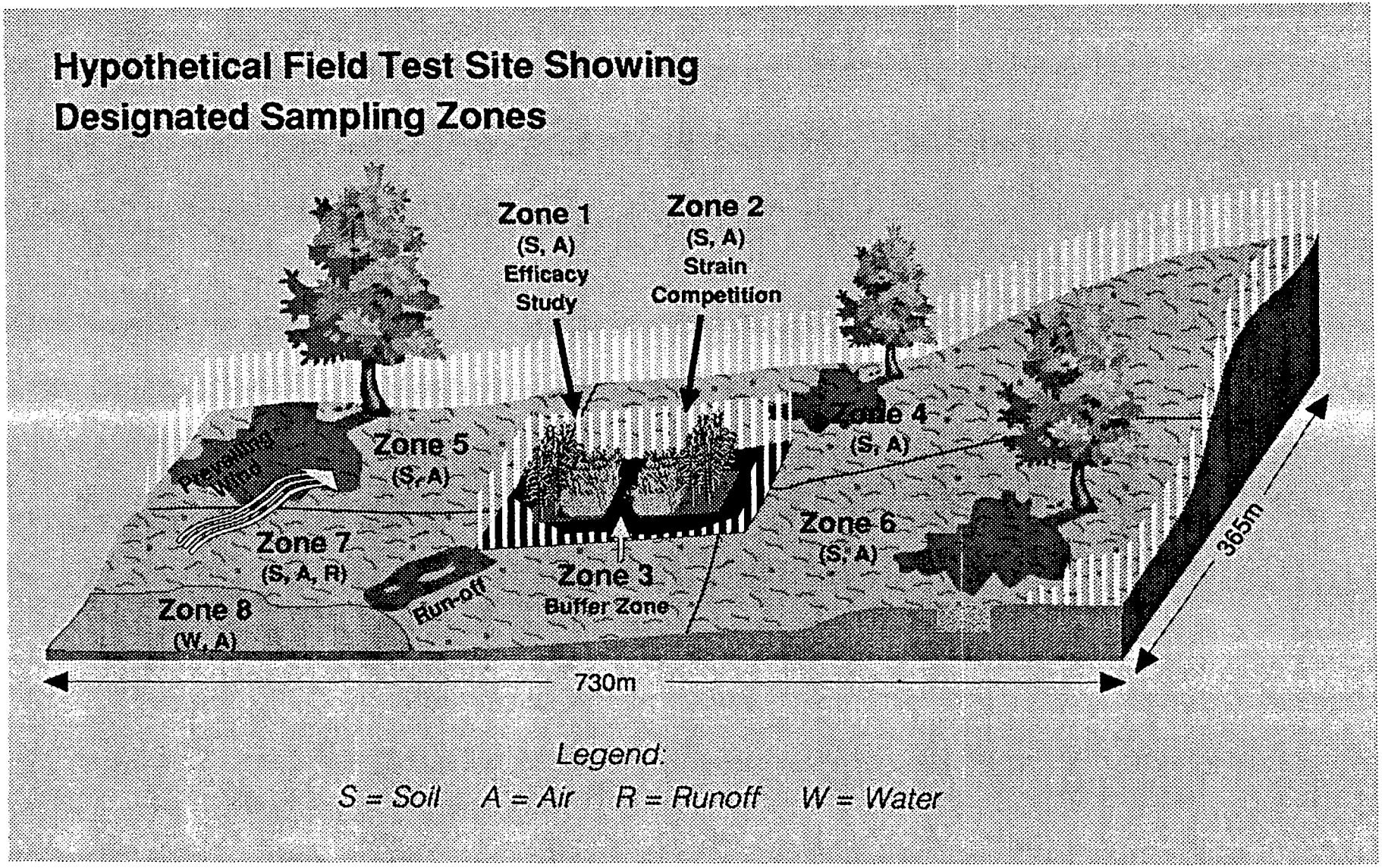

Figure 4. Hypothetical Field Test Site Showing Designated Sampling Zones.

A variety of sampling strategies for monitoring programs have been described by other authors (Borgman and Quimby, 1988; Barcelona, 1988; Gilbert, 1987; Size, 1987). Although most of these discussions deal with the monitoring of chemical contaminants in the environment, the sampling strategies outlined are also applicable to microorganism monitoring programs. Several of the most commonly used strategies are briefly summarized in Appendix C along with their most important advantages and disadvantages.

4.4 Sampling

For every experimental situation there is a target population from which data are collected. The basic procedure, regardless of the experimental design, is to take measurements on a random, representative sample of some population of interest from which estimates of the mean and variability of the larger population are made. Repeated measurements (samples) are needed in all cases in order to calculate any statistic. Precision or sensitivity of an experiment depends ultimately on the amount of information collected. A comparison of two means or treatments becomes more sensitive, and thus can detect smaller differences, as the sample size increases.

4.4.1 Sample Size

The population from which data will be collected should be identified at the planning stage. If possible, its properties should be determined so that appropriate measurements, sample sizes, and sampling techniques can be determined. The number of samples that are necessary for a good estimate of the mean and variability of the population depends in part on the way the organism is dispersed throughout a field (Figure 5). If the organism or population from which data are being collected is randomly dispersed throughout the field (or evenly spaced as in a systematic planting), a few large samples will give results similar to many small samples. Many natural populations of microorganisms, however, are found as clumps of individuals rather than randomly distributed individuals. It is usually better to measure more small samples in this case. There are several different formulas that are available for determining the number of samples to be taken. They all require prior knowledge about the mean and variability of the population. These values can be obtained from prior experiments or the literature, or can be estimated values based on experience. Formulas and tables for determining sample size can be found in statistical texts. The results of such calculations should then be weighed against what is economically feasible.

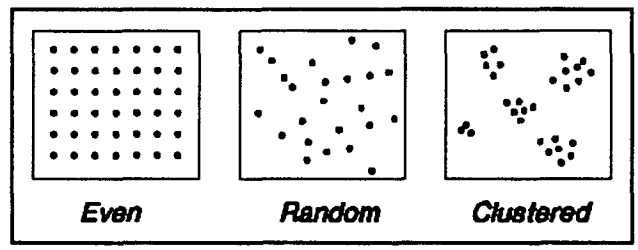

Figure 5. Spatial dispersion patterns. Each dot represents the location of an organism in a field.

Other considerations with respect to sampling include whether or not the samples will be destroyed and whether repeated samples will be taken from the same experimental unit. Destructive sampling may be necessary for many assay procedures (e.g., reisolation of engineered Rhizobium strains from root tissue) but the removal of the plant may influence the environment and subsequent growth of adjacent plants. Care should be exercised so that subsequent measurements, either different assays or repeated measurements on the same population, are minimally affected by the sampling technique. Destructive sampling will also limit the number of samples that can be taken from an experimental unit. Sampling over a period of time may be necessary in order to determine rate or timing of certain processes. Similar to the determination of a mean, the determination of a rate is improved with increased frequency of sampling. Often the shape of the response curve in biological experimentation is curvilinear and thus there is a need for a greater number of samples are needed to produce a reliable estimate of the rate.

4.4.2 Sampling Techniques

Selecting the sampling strategy for a particular monitoring zone establishes the sampling plan in general terms. There are three commonly used sampling techniques in field experimentation: simple random sampling, systematic sampling, and stratified random sampling.

Simple random sampling uses random numbers from a table or computer program to identify which sampling units are actually examined or collected. Use of this procedure removes the possibility of conscious or unconscious bias that may occur. Random sampling ensures that each sampling unit has an equal probability of being selected. Often sampling arbitrarily, where an evaluator picks a plant or a soil sample anywhere in a plot, is done but should not be mistaken for true random sampling. A sampling method that should be avoided is selection of a "typical" sample where the evaluator selects what is thought to typify the population. This inherently leads to biased samples, like the largest greenest plants from a nitrogen fixation study.

Systematic sampling is often employed when the individual sampling units are difficult to identify and number. It is easier to set up and to employ and thus less prone to errors than simple random sampling. The starting point, the first sampled unit within a plot, is randomly determined as before, but then subsequently sampled units are selected at a preset, regular interval e. g., every 7th plant or every 1 m. The interval is also determined randomly within the constraints of the size of the experimental unit and the sample size that needs to be collected. Several different sampling patterns can be used with systematic sampling. The patterns range from a simple diagonal transect across the experimental unit to one with multiple paths across the experimental unit in the shape of an X or W (Figure 6). In general, increase of sample size through increase in the number of paths is best for populations with clumped patterns of dispersion. A simple increase in number of samples along a path is best for randomly distributed populations (Lin et al., 1979).

The third sampling technique is known as stratified sampling. This technique first subdivides the population into predetermined subsets, like distance from an inoculation source, depths of soil, or strips in a field based on a soil characteristic or an environmental gradient. Usually equal numbers of samples are obtained from each subset using either of the two previously described techniques. The advantage of this technique is that additional information and improvement in precision within a subset are gained.

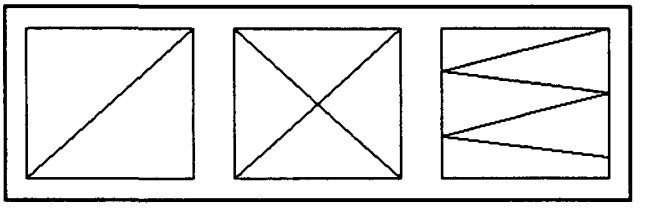

Figure 6. Systematic sampling patterns used in field plots or strata (subsets of field plots).

The different techniques employ different formulas for determination of variability (Cochran, 1977). The techniques should be carefully considered, particularly when the population from which samples are to be collected is large or has an inherent pattern of dispersion in a field. In many cases, particularly for large sampling zones, grid sampling is also easier to implement in the field; moreover, it can be more efficient in some cases, for qualitative monitoring objectives such as a microorganism's presence or absence. To lay out the specific sampling plan in each monitoring zone, the

number and locations of samples need to be clearly defined in terms of the following parameters, described in detail below.

Grid Configuration and Orientation. A variety of sampling grids, such as square, rectangular, triangular, and crossed patterns, are commonly used in monitoring programs. As long as the pattern is constructed to be representative of the monitoring zone, any of these patterns is appropriate for most sampling objectives. The following are some examples where a particular grid configuration and/or orientation can provide a more efficient design:

- When the subsurface is sampled, the vertical grid spacing is often reduced compared with the horizontal spacing because significantly more variation in most endpoints of interest is expected in the vertical direction.

- For a fixed number of sampling locations, a triangular grid supplies more complete coverage of a sampling zone than a square or rectangular grid.

- When looking for small zones of microorganism population or adverse environmental impacts, the choice between a square or rectangular grid for searching purposes may depend on the shape of the target (Gilbert, 1987).

- In situations where transport from the release site is possible, consider sampling downwind, downgradient, and in all four compass directions. Sample distances from the release points may be influenced by modeling of the microorganisms' transport under field release conditions.

- Many field and monitoring endpoints exhibit spatial correlation due to the effects of the original application process, secondary transport processes, or in situ field heterogeneities. Spatial correlation considerations may lead to the use of anisotropic, rectangular, or triangular grids (Flatman et al., 1988).

Number of Sampling Locations. The results from a monitoring program are always subject to uncertainty due to sampling variability, because measurements can only be taken at a limited number of stations and times within the release site. In monitoring programs, the major source of sampling variability is field heterogeneity. For example, soil and water properties such as moisture content and pH can vary greatly across a test site, even over small distances, leading to large variations in the survival and environmental impact of released microorganisms. It is advisable that the number of samples to be collected and analyzed at least meets the minimum necessary to achieve the monitoring objectives and with sufficient redundancy to ensure that the objectives will not be jeopardized if some of the samples cannot be collected or analyzed as planned. The largest reductions in the uncertainty of monitoring results are often realized by taking measurements at a greater number of locations because

too few measurements can hinder the ability to make decisions based on the monitoring results.

Number of Replications. Replication is the statistical concept of independently repeating the same measurement under identical conditions in order to estimate the variance of a set of measurements. The use of replication in sampling and analysis is critical in assessing statistical variations in the monitoring results from sources such as treatment effects, field heterogeneity, and measurement uncertainty.

Measurement Precision. Measurement variability is a result of imprecision in the analytical methods and instrumentation used to identify and measure microbial populations and environmental effects. For instance, variability can arise from different adsorptive properties of sample tubes or variation in mixing via vortex or sonication methods. In some cases this variability can contribute significantly to uncertainty in monitoring results. This uncertainty can be controlled by selecting reliable and reproducible measurement techniques. From a statistical design point of view, the choice between alternative techniques may be based on relative cost, accuracy, and precision. The field test protocol should be designed to supply a quantitative assessment of the measurement precision by including measurement replication as part of the sample collection strategy.

Sampling Times. For certain microbial monitoring studies, it will be necessary to monitor the survival and effects of microbial populations over extended periods of time after release. The following are examples of important parameters to be considered concerning sampling times: 1) initial monitoring measurements taken in conjunction with application of the microorganisms; 2) the time intervals at which measurements are taken subsequent to application; 3) the total time period for monitoring; and 4) the criteria that will be used to determine that no further monitoring is required, such as the limit of detection or a change in season.

Sample Collection Methods. When selecting monitoring methods, it is wise to take the following steps: 1) review the sampling zones and endpoints to be monitored; 2) determine the minimum acceptable detection limit for the microorganisms to be monitored; 3) determine the degree of accuracy and precision needed in the results. With this information, the investigator can then review available methods and select the appropriate method(s) that will meet the study objectives. There are a variety of classical sampling and analytical methods to follow the fate and genetic stability of released microorganisms and new methods are evolving rapidly. These methods have been reviewed extensively in the literature (e. g., see Stotzky et al., 1989, and Fredrickson and Seidler, 1989). The method should be verified in conditions as near to field conditions as possible to ensure that the selected method will perform its intended function.

4.5 Implementation of the Monitoring Plan

The investigator should be prepared to make adjustments in the monitoring plan as implemented during the field test. Numerous factors may require adjustments in order for the field test to provide the desired information. Minor adjustments may be necessary as the field test is implemented due, for instance, to changes in weather conditions subsequent to application of microorganisms at the test site or changes in microbial populations during the year.

Major adjustments to the monitoring plan, such as moving into a new monitoring zone, should be carefully considered so that sampling in the new zone will contribute the desired information. An expansion of the monitoring effort may be warranted during a field test, for instance, in the case where a microorganism has been detected in an area outside of the field test site and concerns dictate the need for further sampling in the surrounding zones.

CHAPTER 5: DEVELOPING PROCEDURES TO ASSURE DATA QUALITY

5.1 Quality Assurance and Quality Control

Every monitoring project should have a quality assurance project plan (QAPjP) to ensure that the data derived during the project will be scientifically sound and unbiased. Quality data will have a greater likelihood of yielding valid conclusions related to the project's principal hypothesis. This section provides guidance in establishing procedures to accomplish these goals. Additional literature on QA principles and practice is referenced at the end of this section.

Quality assurance criteria provide a balance between the constraints of time and procedural costs and the quality of the data necessary to achieve the research project objectives. A QA plan is designed to accomplish the following:

- to establish criteria to control the quality and evaluate the validity of data collected during the project,
- to provide standardized methods for sample preparation and analysis,
- to utilize reference standards or other analysis procedures to verify and assess the quality of the data collected,
- to aid in the maintenance and calibration of analytical instruments used during the project and ensure the equality of the reagents, chemicals and other raw materials employed for the project.

To assist this effort, it is necessary to identify both qualitative and quantitative estimates of the quality of the data needed to fulfill project objectives. Quality assurance guidelines clearly identify the decisions to be made from the research effort and specify the calculations to be applied to the data.

5.2 Measurement Quality Objectives

Measurement quality objectives (MQOs) are specific goals describing the data quality sought for each measurement. The project MQOs are established on the basis of project data needs utilizing appropriate referenced methods to obtain the data. Lower-than-desired data quality could require different data analysis or result in modifications to the levels of confidence assigned to the data. Uncertainty is expected in experimental measurements, however, MQOs for the analytical method used should remain constant for that particular method throughout the project. The MQOs are defined by the following six attributes:

1) detection limit-- the lowest concentration of an organism that a specific procedure can reliably detect

2) precision-- the level of agreement among multiple measurements of the same characteristic

3) accuracy-- the agreement of the observed value with a reference or true value

4) representativeness-- the degree to which the data collected accurately and precisely describe the population of interest

5) completeness-- the quantity of data that is successfully collected with respect to the amount of data intended in the experimental design

6) comparability-- the similarity of data from different sources within individual or multiple data sets or the similarity of data from related projects

The MQOs, as well as specific procedures for assessing them, should be addressed in the project QA plan for sampling, sample preparation and analysis of samples. Detection limits should be discussed for each method used in the project. The reporting units, reporting format, expected range of values and detection limit should be included in the sections on precision and accuracy for each method used. It is important to remember that MQOs should be discussed in terms of the project objectives, not simply in terms of the test method's capabilities.

5.3 QA/QC Procedures for the Field

Besides validation of the quality of analytical measurement, a QA/QC procedure provides for tracking samples effectively from field collection through laboratory analysis and final reporting. There is little use analyzing samples accurately and exhaustively if they have been improperly collected, mislabelled or improperly stored prior to analysis.

QA/QC procedures in the field start with knowledge of sampling equipment operation. A training program should be implemented to ensure that each person responsible for a particular type of sampling understands the proper operation of sampling devices and sample collection protocols. Each person involved in the sampling effort needs to be aware of the overall sampling design and how that person's role fits into the overall design. Common sense dictates that standard operating procedures and maintenance procedures for all sampling devices be available at the field test site to facilitate repairs and other contingencies.

The following is a suggested list of points to consider when developing QA/QC procedures for the field test site.

- All equipment should be adequately inspected, cleaned and maintained. Equipment used for the generation, measurement or assessment of data should be regularly tested and calibrated. A logbook to record these procedures is recommended.

- A sample identification code should be carefully determined to assign a unique combination of numbers or letters to each sample. The sample identification system should permit the sample to be tracked throughout the collection and analysis process.

- Sample data forms should be created to facilitate data collection. Sample data forms should document all relevant information describing the sample.

- All sampling activities should be documented with a description of each day's activities and other relevant information in a bound logbook. Every entry should be dated and initialed.

- Plans for shipping and storage of samples to maintain sample integrity should be established.

5.4 QA/QC Procedures for the Laboratory

Quality assurance and quality control (QA/QC) procedures in the laboratory also start with knowledge of equipment operation. All laboratory personnel need to be trained in the use of their particular analytical instrument. The laboratory should have all equipment manuals and standard operating procedures on hand to address the procedures to be performed. Published literature may supplement standard operating procedures. Many of the same concerns listed above for equipment maintenance and calibration are also applicable here.

The inclusion of reference standards can also aid in assuring the quality of data obtained. This gives an in-process means of confirming the quality of the data, the reproducibility of the instrument and the detection of any bias of the measurements. These samples should be looked on as an addition to, not a substitution for, normal instrument calibration. Spiked samples can also determine if the sample matrix exerts an effect on the measurements. In microbial studies, it is also advisable to subject occasional samples believed to be the organism of interest to confirmatory tests by other methods. This confirmatory backup reinforces the results of the analytical method used and allows for detection of any unexpected microbes exhibiting traits similar to the microbe of interest.

Preparation of reagents used routinely in the project warrants special consideration in terms of quality control. Raw material quality, including such simple components as water and chemicals, can drastically affect results. It is advisable to have certain

criteria for acceptance of raw materials, such as levels of contaminants or performance in physical tests. Chemical products should be traced by lot number. Certificates of analysis for the product should be routinely obtained from the supplier to aid in determining what contaminants are responsible for poor product performance.

Utilizing a well-organized method of QA/QC will greatly aid in the generation of valid data. In addition, a QA/QC system allows one to track product performance more closely, ensures reproducible product production, and facilitates troubleshooting when a product failure occurs.

5.5 References Used to Prepare a QA Plan

The following references may be useful in the preparation of a QA plan:

1) Federal Register. 40 CFR Part 792, Toxic Substance Control Act (TSCA): Good Laboratory Practice Standards; Final Rule. 54 FR 34034. August 17,1989.

2) Taylor, J.K., and T.W. Stanley. 1985. Quality Assurance for Environmental Measurements. American Society for Testing Materials, Philadelphia, PA.

3) U.S. Environmental Protection Agency. OTS Guidance Document for the Preparation of Quality Assurance Project Plans. Office of Toxic Substances, Washington, D.C. September, 1987.

4) U.S. Environmental Protection Agency. OTS Quality Assurance Guidance Document for the Preparation of Field Test Plans for Biotechnology Programs. Office of Toxic Substances, Washington, D.C.

5) U.S. Environmental Protection Agency. Preparing Perfect Project Plans. Risk Reduction Engineering Laboratory, Cincinnati, OH.

CHAPTER 6: DEVELOPING PROCEDURES TO ASSURE HEALTH AND OCCUPATIONAL SAFETY

6.1 General Information

The health and safety of field and laboratory personnel, the general public, and the environment can be ensured through an integrated program of standard operating procedures, personnel training, careful site planning, supervision, and operation. The assistance of an Institutional Biosafety Committee (IBC), an institutional review group, can provide feedback on health and personnel safety issues as well as the overall safety of a study.

In performing certain field tests, there is the potential for exposure to microbiological agents that may be hazardous. Therefore, all personnel involved in these on-site activities should be familiar with the types of hazards that may be present, the ways in which those hazards can be mitigated, and safe practices applicable to the conduct of those activities. Thus, the first step in addressing health and safety considerations for a field test is making sure all personnel on-site during the field test have been adequately trained and fully understand their respective duties.

6.2 Test Site Emergency Procedures

Test site emergency procedures include a plan documenting the corrective actions that will be taken in the event of an accident. Examples of events needing specific emergency procedures are the accidental spill or release of the microorganism, sampler breakdown, compromised samples, or the breach of the test site by unauthorized personnel. Each field test will have unique potentials for accidental releases and concurrent emergency response procedures. It is imperative that all personnel involved in the field test be made aware of these plans and how to implement them. In addition, it is advisable that emergency procedures for halting the field test be in place before the start of the field test so that unforeseen effects can be halted quickly. These procedures may include application of chemical control agents or the controlled burning of the field test site. Examples of unforeseen circumstances that could result in emergency termination are an unexpectedly rapid increase in cell density, detection of the microorganism at greater distances than expected from the test site and unexplained damage to plants or animals in or adjacent to the test site. The investigator, in consultation with the EPA, is responsible for designing this termination "trigger," taking into account the characteristics of the microorganism and the field test site. The investigator is urged to consult with the appropriate State agency about specific policies concerning fumigation or the application of chemical control agents to the site. Many states, for instance, prefer that certain disinfestation procedures be conducted by a State-certified applicator.

6.3 Laboratory Procedures and Reference Documents

Establishing health and safety procedures for the laboratory is also important in the planning of any field test. An excellent reference specific to the safe handling of microorganisms in the laboratory is the Centers for Disease Control (CDC) and the National Institutes of Health (NIH) publication, Biosafety in Microbiological and Biomedical Laboratories (NIH, 1984). Investigators are encouraged to refer to this document for detailed information concerning laboratory practices. If the field test involves a recombinant DNA microorganism, an additional reference is the Guidelines for Research Involving Recombinant DNA Molecules (1986), produced by NIH.

Additional reference documents applicable to health and safety include the Toxic Substances Control Act, Good Laboratory Practices (GLPs) Guidance (1983), the Basic Field Activities Safety Training Manual, the Dioxin Field Sampling Guide, the Occupational Safety and Health Guidance Manual for Hazardous Waste Site Activities, and the Standard Operating Safety Guides developed by EPA. These guidance manuals specify proper techniques and procedure for work involving potentially toxic or hazardous materials.

REFERENCES

Barcelona, M. J. 1988. "Overview of the Sampling Process." In L.H. Keith, (ed.). Principles of Environmental Sampling, American Chemical Society, pp. 3-23.

Borgman, L. E., and W. F. Quimby. 1988. "Sampling for Tests of Hypothesis When Data are Correlated in Space and Time." In L.H. Keith, (ed.). Principles of Environmental Sampling, American Chemical Society, pp. 25-43.

Cochran, W. G. 1977. Sampling Techniques. Wiley, New York. 428 pp.

Cochran, W. G. and G. M. Cox. 1957. Experimental Designs, 2nd ed. Wiley, New York. 611 pp.

Commission of the European Communities, U. S. Department of Agriculture, U. S. E. P. A. 1992. Methods for the Detection of Microorganisms in the Environment. National Technical Information Service, Springfield, VA, Document PB92-1374S4/AS.

Cox, C. S. 1987. Aerobiological Pathways of Microorganisms. John Wiley and Sons, New York.

EPA. U. S. Environmental Protection Agency. 1983. Code of Federal Regulations. Toxic Substances Control Act, Good Laboratory Practices Guidance, CFR November 29, 1983. Vol 48, No. 230, pp. 53922

Flatman, G. T., E. J. Englund, and A. A. Yfantis. 1988. "Geostatistical Approaches to the Design of Sampling Regimes." In L.H. Keith (ed.). Principles of Environmental Sampling, American Chemical Society, pp. 74-84.

Fredrickson, J. K., and R. J. Seidler. 1989. Evaluation of terrestrial microcosms for detection, fate, and survival analysis of genetically engineered microorganisms and their recombinant genetic material. EPA-600/3-89/043.

Gilbert, R. O. 1987. Statistical Methods for Environmental Pollution Monitoring, Van Nostrand Reinhold, New York, 320 pp.

Ginzburg, L. R. 1991. Assessing Ecological Risks of Biotechnology. Butterworth-Heinemann, Stoneham, MA, 379 pp.

Gomez, K. A. and A. A. Gomez. 1984. Statistical Procedures for Agricultural Research. Wiley, New York. 680 pp.

Levin, M. A., R. J. Seidler, and M. Rogul. 1992. Microbial Ecology: Principles, Methods, and Applications. McGraw-Hill, Inc. New York. 403 pp.

Lin, C. S., G. Poushinsky, and M. Mauer. 1979. An examination of five sampling methods under random and clustered disease distributions using simulation. Can. J. Plant Sci. 59:121-130.

Lindow, S. E., G. R. Knudsen, R. J. Seidler, M. V. Walter, V. M. Lambou, P. S. Amy, D. Schmedding, V. Prince, and S. E. Hern. 1988. Aerial dispersal and epiphytic survival of Pseudomonas syringae during a pretest for the release of genetically engineered strains into the environment. Appl. Environ. Microbiol. 54:1557-1563.

Little, T. M. and F. J. Hills. 1978. Agricultural Experi-mentation: Design and Analysis. Wiley, New York. 350 pp.

McIntosh, M. 1991. Statistical techniques for field testing of genetically engineered microorganisms. In: Levin, Morris and Strauss (eds.), Risk Assessment in Genetic Engineering: Environmental Release of Organisms. McGraw-Hill, New York, pp. 219-239.

Nelson, L. A. 1986. Use of statistics in planning, data analysis, and interpretation of fungicide and nematicide tests. Pages 11-23. In: K. D. Hickey (ed.) Methods for Evaluating Pesticides for Control of Plant Pathogens. American Phytopathological Society, St. Paul, MN. 312 pp.

Nelson, L. A., and J. O. Rawlings. 1983. Ten common misuses of statistics in agronomic research and reporting. J. Agron. Educ. 12:100-105.

Neter, J. and W. Wasserman. 1974. Applied Linear Statistical Models: Regression, Analysis of Variance, and Experimental Designs. Irwin, Homewood, IL. 842 pp.

NIH. National Institutes of Health. 1986. Code of Federal Regulations. Guidelines for Research Involving Recombinant DNA Molecules, CFR May 7, 1986, Vol. 51, No. 88, pp. 16958.

NIH. National Institutes of Health. 1984. Biosafety in Microbiological and Biomedical Laboratories, March 1984. HHS Publication No. (CDC) 84-8395, Government Printing Office.

OECD. Organisation for Economic Cooperation and Development. 1991. Report of OECD Workshop on Monitoring of Organisms Introduced into the Environment, OCDE/GD(92)71. OECD, Paris.

Seidler, R. J., and S.C. Hern. 1988. EPA Special Report: The release of ice-minus recombinant bacteria at California test sites. ERL-Corvallis, OR.

Size, W. B. 1987. "Use of Representative Samples and Sampling Plans in Describing Geologic Variability and Trends." In W.B. Size (ed.). Use and Abuse of Statistical Methods in the Earth Sciences, Oxford University Press, New York, pp. 3-20.

Steel, R. G. and J. H. Torrie. 1980. Principles and Procedures of Statistics. McGraw-Hill, New York. 633pp.

Stotzky, G., M. A. Devanas, and L. R. Zeph. 1989. Methods for studying bacterial gene transfer in soil by conjugation and transduction. EPA-600/3-89/042.

Strauss, H., D. Hattis, G. S. Page, K. Harrison, S. R. Vogel, and C. Caldart. 1985. Direct release of genetically engineered microorganisms: A preliminary framework for risk evaluation under TSCA. Report CTPID 85-3, Center for Technology, Policy, and Inductrial Development. Massachusetts Institute of Technology, Cambridge.

The following references may be useful in the preparation of a QA plan:

EPA. U.S. Environmental Protection Agency. OTS Guidance Document for the Preparation of Quality Assurance Project Plans. Office of Toxic Substances, Washington, D.C. September, 1987.

EPA. U.S. Environmental Protection Agency. OTS Quality Assurance Guidance Document for the Preparation of Field Test Plans for Biotechnology Programs. Office of Toxic Substances, Washington, D.C.

EPA. U.S. Environmental Protection Agency. Preparing Perfect Project Plans. Risk Reduction Engineering Laboratory, Cincinnati, OH.

Federal Register. 40 CFR Part 792, Toxic Substance Control Act (TSCA): Good Laboratory Practice Standards; Final Rule. 54 FR 34034. August 17,1989.

Taylor, J.K., and T.W. Stanley. 1985. Quality Assurance for Environmental Measurements. American Society for Testing Materials, Philadelphia, PA.

APPENDIX A.
MICROORGANISM, ENVIRONMENTAL, and EXPERIMENTAL PROFILE VARIABLES

A1.0 MICROORGANISM PROFILE

The following list of variables should be considered for inclusion in the microorganism profile.

A. Detection, isolation, and enumeration procedures
   1. Detection procedures: molecular, immunological, marker, selective cultivation, bioassay
   2. Isolation procedures: selective cultivation, enrichment in liquid cultures
   3. Enumeration procedures: marker, immunological, most probable number techniques

B. Pathogenicity of parent microorganism and introduced microorganism
   1. Mode of pathogenicity: biotroph; necrotroph; toxin producer; opportunistic or frank; etc.
   2. Host affected: human; animal; plant or microbe

C. Physiology (useful for fate and survival of microorganism, and detection and isolation)
   1. Nutritional characteristics
      a. Carbon requirements
         1) Chemoheterotrophic: organic carbon for energy; organic carbon for growth
         2) Chemolithotrophic: inorganic compounds for energy; inorganic carbon (CO_2) for growth
         3) Photoheterotrophic: light for energy; organic carbon for growth
         4) Photolithotrophic: light for energy; inorganic carbon (CO_2) for growth
      b. Other primary nutrient requirements: sulfur, nitrogen, phosphorus, potassium, etc.
      c. Growth factor requirements: amino acids, purine and pyrimidines, vitamins and micronutrients
   2. Oxygen status/E_h requirements
      a. Obligately aerobic
      b. Obligately anaerobic
         1) Anaerobic respiration
            electron acceptor: nitrate, sulfate, CO_2, etc.

   2) Fermentative
c. Facultatively anaerobic
3. pH requirements: optimum and range of tolerance
4. Water requirements; dessication tolerance; halophilic
5. Temperature optimum: psychrophilic; mesophilic; thermophilic
6. Other laboratory growth characteristics
   a. Colony morphology
   b. Doubling times/kinetics/growth constants
   c. Motility
   d. Nutrient diversity
   e. Sporulation/dormant forms
   f. Disinfection profile/inactivation kinetics
7. Role of unmodified parent in nature
   a. Microhabitat
      1) Soils: adsorption; desorption
      2) Water
      3) Sediment: adsorption; desorption
      4) Air
      5) Plants or plant parts: rhizosphere; leaf surface
   b. Environmental function
   c. Persistence: spores or other resistant forms
   d. Responses to environmental stress: seasonality, dormancy
   e. Expected role of introduced microorganism
8. Physical characteristics
   a. Shape
   b. Density
   c. Diameter
9. Genetics
   a. Chromosomal size
   b. Presence or absence of plasmid(s)
      1) Size
      2) Incompatibility group
      3) Host range
      4) Phenotype
   c. Susceptibility to natural mechanisms of genetic transfer
      1) Conjugation
      2) Transformation
      3) Transduction
   d. Mutability to UV irradiation or common chemical mutagenesis
   e. Construction of the modified microorganism
      1) Source of insert DNA
      2) Characteristics of deletion, insertion, and vector (sequence, functions encoded)
      3) Methods of vector and insert construction

4) Method of introduction of the vector into the primary microorganism
5) Amount and nature of vector or donor DNA remaining in the modified microorganism
6) Location of insert to construct: chromosomal or autonomously replicating (e. g. plasmid)
7) Laboratory containment conditions (NIH guidelines) for the modified microorganism

A2.0 ENVIRONMENTAL PROFILE

The following list of variables should be considered for inclusion in the environmental profile.

A. Soils/sediments
   1. Abiotic
      a. Physical
         1) Seasonal parameters
            a) Temperature
            b) Moisture
            c) Oxygen levels
            d) Organic matter
            e) Depth to ground water
         2) Parameters not affected by seasons
            a) Types
            b) Texture/mineralogy/percent sand, silt, clay through the profile
            c) Permeability/porosity through the profile
            d) Bulk density through the profile
            e) Slope
            f) Depth of horizons and to bedrock
            g) Distance from adjacent water bodies
      b. Chemical
         1) pH/E_h through the profile
         2) Nutrients through the profile
         3) Conductivity through the profile
         4) Pollutants such as agricultural chemicals through the profile
         5) Cation exchange capacity
   2. Biotic
      a. Host microorganism/similar species/possible DNA vectors
      b. Predators/parasites
      c. Vectors of microbial movement
      d. Competitiveness of introduced microorganism with indigenous species
      e. Growth characteristics in the field

B. Surface water
   1. Abiotic
      a. Physical
         1) Seasonal parameters
            a) Temperature
            b) Flow velocity
            c) Turbulence
            d) Depth
            e) Density/viscosity
            f) Average suspended sediment concentration
            g) Medium sediment diameters
            h) Maximum tidal velocity
         2) Parameters not affected by seasons
            a) Type (lentic, lotic)
            b) Surface areas
               1. Length
               2. Width
            c) Distance from release source if release susceptible to intermediate transport
      b. Chemical: many are seasonally variable
         1) pH/E_h
         2) Nutrients
         3) Dissolved oxygen
         4) Conductivity
         5) Salinity
         6) Hardness
         7) Pollutants
   2. Biotic
      a. Host organisms/similar species
      b. Predators/parasites
      c. Vectors of microbial movement
      d. Growth characteristics in water
      e. Adsorption to suspended particles/sediments
      f. Competitiveness of introduced microorganism

C. Ground Water
   1. Abiotic
      a. Physical
         1) Seasonal parameters
            a) Temperature
            b) Flow velocity
            c) Dissolved oxygen
            d) Density/viscosity

e) Colloidal materials: dissolved organic matter; suspended sediments
f) Depth to groundwater
2) Parameters not affected by seasons
a) Aquifer type
b) Volume
1. Depth
2. Width
3. Distance to wells from source

b. Chemical
1) pH/E_h
2) Nutrients
3) Conductivity
4) Salinity
5) Hardness
6) Pollutants

2. Biotic
a. Host microorganisms/similar species/DNA transfer vectors
b. Predators/parasites
c. Vectors of microbial movement
d. Competitiveness of introduced microorganism
e. Growth characteristics in the field
f. Interaction with the colloidal substances

D. Air/Ambient
1. Abiotic
a. Physical (seasonal parameters)
1) Wind speed
2) Turbulence
3) Wind direction
4) Humidity
5) Temperature
6) Rainfall
7) Solar intensity

b. Chemical
1) Pollutants
2) Nutrients

2. Biotic
a. Host microorganism/similar species
b. Predators/parasites
c. Vectors
d. Growth characteristics in the field

E. Environmental Use - Presence of:
   a. Well heads
   b. Residences/playgrounds/etc.
   c. Potable water supplies
   d. Endangered or threatened species or other special concerns

A3.0 EXPERIMENTAL PROFILE

The experimental profile is shaped by both of the preceding profiles. Therefore, the experimental profile will frequently repeat information already listed in these. The following list of variables should be considered for inclusion in the experimental profile. Special effort should be made to justify experimental choices considering the features found in the microorganism and environmental profiles.

A. Experimental design: explain the rationale of the treatments and site selection in relation to the objectives of the field test.
   1. Field plot design
      a. Randomized complete block; split plot; etc.
      b. Number of replications
      c. Expected level of variation within treatments
      d. Method of randomization for treatment location assignment
   2. Physical layout of treatments: a schematic diagram is essential
      a. Size of individual treatment plots
      b. Size of overall experimental plot
      c. Aspect of site; slope; prevailing wind; drainage
      d. Presence of border rows and buffer areas
      e. Blocking factors if present or needed
         1) Slope
         2) Soil heterogeneities
         3) Prior treatments or rows
   3. Monitoring meteorology during experiment
      a. Parameters measured: rainfall; temperature; humidity; ensolation; etc.
         1) On-site equipment: type; location in plot
         2) Weather station: directional location; proximity
      b. Measurement frequency and quality: Maximum/minimum; continual
   4. Sampling strategy
      a. Frequency
      b. Duration
      c. Sampling design: grid; stratified random; judgmental
      d. Sample type: plant tissue; soil sample; etc.
      e. Destructive or nondestructive

f. Effect of sampling on remaining plot population

5. Statistical model used to analyze data
   a. Homogeneity of variance prior to ANOVA
   b. Transformation of data prior to analysis
   c. One way or two way ANOVA
   d. Regression models; multivariate analysis
   e. Expected level of confidence to separate means

B. Treatment Design: Consideration of site preparation prior to treatment application, the treatment application(s) and the plot management during the experiment.
   1. Site preparation: experimental plot; border areas; monitoring zones
      a. Cultivation; preplant herbicides, pesticides, fertilizers; etc.
      b. Planting method; seeding rate; row spacing; cultivars; planting date
      c. Border rows: between treatments/around experimental plot; size; plant species/cultivar; row spacing; planting date
   2. Treatment Characteristics: application types, nature of control and other variables
      a. Aerosol, surface or subsurface applications
         1) Concentration; rate
         2) Frequency
         3) Duration
         4) Discharge characteristics: rate, velocity, surface tension, spray angle
         5) Surface and subsurface application: liquid, granular, powder; delivery pressure if liquid
      b. Nature of control treatments: what is being controlled?
         1) Product formulations without active microbe
         2) Fertility levels to determine treatment effect
      c. Other variables: strains; product formulations; etc.
   3. Plot management during experiment
      a. Cultivation after planting; fertilizer applications
      b. Expected weed, insect or disease controls needed
      c. Expected sampling/harvest schedules; sampling methods
      d. Irrigation

C. Monitoring design: for environmental fate/effect
   1. Endpoint determinations for monitoring zones based on release scenario
   2. Establishment of monitoring zones: size; location
   3. Sampling strategy and frequency within each zone

APPENDIX B.
Common Experimental Designs

Experimental Design	Application	Description	Statistical Analysis
Completely Randomized.	One treatment factor. No blocking factors.	Treatments are assigned completely at random so that each experimental unit has an equal chance of receiving any treatment.	One-way ANOVA.
Randomized Complete Block.	One treatment factor. One blocking factor.	All experimental units grouped according to blocking factor. Each block constitutes a complete replication of the experiment. All treatments are applied in every block.	Two-way ANOVA.
Randomized Incomplete Block. a. Balanced b. Partially balanced	One treatment factor at several levels. One blocking factor.	All experimental units grouped according to blocking factor. Each block constitutes only a partial replication of the experiment. Not all treatments are applied in every block. a. Every pair of treatments occurs the same number of times throughout the experiment. b. Not every pair of treatments occurs the same number of times throughout the experiment.	a. Special ANOVA. b. General regression.
Latin Square.	One treatment factor. Two blocking factors. Number of levels is the same for treatment factor and both blocking factors.	All experimental units grouped according to combinations of two blocking factors. Each treatment appears only once for each level of the first blocking factor, and only once for each level of the second blocking factor.	Three-way ANOVA.
Complete Randomized with Factorial.	Two or more treatment factors. All combinations of all factor levels are tested, each combination is a separate treatment.	Treatments are assigned completely at random so that each experimental unit has an equal chance of receiving any treatment.	Factorial ANOVA interactions.
Fractional Factorial.	Two or more treatment factors, each having several factor levels. Too expensive to run all possible treatments.	Ability to estimate higher-order interaction effects is sacrificed by including only specific treatments that allow for estimation of only main factor and lower-order interaction effects.	Factorial ANOVA.
Split Plot.	One treatment factor. One blocking factor	Blocking factor expected to have larger effect is assigned to main plots. Each main plot is divided into subplots to which treatment factor is assigned.	Special ANOVA.

APPENDIX C. COMMON APPROACHES TO SAMPLING

Sampling Strategy	Description	Advantages	Disadvantages
Adaptive Sequential	As the samples are collected and analyzed the perceptions of the sample zone are changed by the analysis of the results. There may be a need for additional stages of sampling to increase coverage of the zone or to focus sampling within subzones. Only a portion of the sampling resources are allocated to the initial stage of sampling; remaining resources are held in reserve for more detailed follow-up sampling.	1) Even where second stage sampling is not anticipated, studies often uncover information that makes follow-up sampling necessary. 2) More efficient allocation of sampling and analysis resources. 3) Information from the initial stage of sampling used to provide precise sampling design for the second stage of sampling.	1) May be more expensive because of intermediate data analysis.
Grid	A grid of lines is used to divide the sampling zone into a regular configuration of subzones called grid cells. Dimensions and orientation of the grid are fixed, but location of the origin of the grid within the zone is randomly selected. Sampling locations are established at intersections of grid lines.	1) Provides representative sampling of entire zone. 2) Provides unbiased characterization of sampling zone. 3) Usually more efficient than simple random and stratified random sampling for some objectives such as qualitative presence or absence sampling.	1) May be less robust against specific departures form assumptions.
Judgmental	Sampling locations are selected by visual inspection of the zone and judgmental decision about those locations that are most appropriate.	1) Easy to conduct in the field. 2) Focuses sampling on locations judged significant.	1) Does not lead to representative sampling of the entire zone. 2) Provides biased characterization of entire zone. 3) Cannot statistically quantify reliability of resulting estimates.

Sampling Strategy	Description	Advantages	Disadvantages
Stratified Random	A grid of lines is used to divide the sampling zone into a regular configuration of subzones called grid cells. Sampling location within each grid cell is selected by simple random sampling.	1) Provides representative sampling of the entire zone. 2) Usually more efficient than simple random sampling. 3) Provides unbiased characterization of sampling zone.	1) Random sampling within call more difficult to conduct in field than grid sampling.
Simple Random	All possible samples are numbered from 1 to N, where N is the total number of samples. A series of n random numbers, each between 1 and N, is drawn, and the samples that bear these n numbers constitute the set of selected samples.	1) Provides unbiased characterization of sampling zone.	1) Does not guarantee a representative sample from the zone; chance selection may result in closely clustered sampling locations. 2) Often less efficient;may not provide the same precision of estimate for a given number of samples as other sampling strategies. 3) Can be more difficult to conduct in the field than more structured strategies.