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SURVIVAL OF BACTERIA INTRODUCED INTO SOIL:

THE INFLUENCE OF INOCULATION CONDITIONS, PARTICLE ASSOCIATION, EXTRACTABLE SOIL COMPONENTS, AND INOCULUM DENSITY

A Thesis Presented

by

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CHAPTER I

LITERATURE REVIEW

Recombinant nucleic acid technology, or "genetic engineering", has stimulated an industry with vast potential. The earliest commercial products, available for about fifteen years now, were metabolic products of genetically altered microorganisms. Monoclonal antibodies for disease diagnoses and therapeutic hormones including human growth factor and insulin have been produced by large-scale fermentations of bacteria into which the genes coding for these compounds have been cloned. Production of these commodities is inherently contained and controlled (Fowle, 1987; Kingsbury, 1988).

As the industry has developed, the microorganism itself has become the product. The potential to employ microorganisms for a wide variety of environmental functions has evolved in recent years. Numerous agricultural applications, remediation of pollutants, extraction of ores, and enhancement of oil recovery are all areas of intense interest. Included in the development of these field technologies is the utilization of genetically engineered microorganisms (GEMs). These microorganisms are designed to carry out a wider array of useful functions and to do so more efficiently than naturally-occurring microorganisms.

The advent of genetically engineered organisms (plants, animals, and microorganisms) has engendered a great deal of concern about the possibility of an environmental disaster. To predict what might happen upon the introduction of these organisms into the environment, efforts have been made to extend what has been learned

from past accidental, intentional, or natural introductions of new species (or new genes in an indigenous species) into an ecosystem. Simberloff (1985) describes introductions of change into a biotic community in terms of a continuum that ranges from spontaneous mutation in an indigenous species to immigration of a totally exotic species into the extant community. He goes on to point out that we have very little information regarding the impact of these changes because most often they are uneventful, in human terms, and go unnoticed. However, notorious examples of dramatic impact on human concerns remind us that not all introductions are innocuous: kudzu, the gypsy moth, the Japanese beetle, the Chinese chestnut blight, the starling. Changes do not have to be anthropogenic or on the scale of an exotic species, to have great impact. For example, the apple maggot, *Rhagoletis pomonella*, has caused considerable hardship for Northeastern apple growers. This pest extended its feeding range from hawthorne species to apple after a probable two-gene mutation (Simberloff, 1985).

A compelling question concerning the impact of an introduced microorganism is its potential to affect ecosystem properties such as productivity, consumption, decomposition, water fluxes, nutrient cycling and loss, soil fertility, and erosion (Suter, 1985; Vitousek, 1985). Tiedje *et al.* (1989) contended that because microbial species are frequently "functionally equivalent" in terms of such processes as nitrogen fixation, lignin decomposition, or pesticide degradation, in many cases there would be little cause for concern over displacement of native species caused by the introduction of a genetically engineered organism. In contrast, Suter (1985) offers a scenario in which a bioengineered organism might be introduced to increase nitrification in sewage treatment plants, escape, and become established in polluted waters, wetlands, or even some large

fraction of the environment where changes in nitrification rates could affect ecosystem productivity because nitrate and ammonia have different partitioning characteristics and plant uptake rates. This issue remains a conundrum as such effects are extremely difficult to study experimentally.

Numerous conferences have met in the past decade to address issues relating to the deliberate or accidental introduction of genetically engineered organisms into the environment, and the potential risks involved have been the subject of much debate. Tiedje *et al.* (1989) argued that unprecedented environmental impact is a legitimate concern in that biotechnology has combined traits in a single organism that in nature evolved in distinct phylogenetic lines. By their assessment, organisms possessing novel, cross-phylogenetic combinations of genetically determined traits are more likely, on the average, to play novel ecological roles then are organisms with combinations of traits from their own or closely related species.

The genetically engineered microorganism has been of particular concern because precise monitoring and containment of populations in the environment is extremely difficult, if not impossible. The GEM itself has been the subject of a great deal of risk assessment research. Considerable work has been done to elucidate the biological and genetic characteristics of GEMs and potential GEM strains, *e.g.* their possible pathogenicity (Falkow, 1988; Goebel *et al.*, 1984; Suter, 1985) and their genetic stability (Graham and Istock, 1979; Ramos-Gonzalez *et al.*, 1991; Richaume *et al.*, 1989; Smit *et al.*, 1991; Trevors and Odie, 1986; Walter *et al.*, 1987; Wickam and Atlas, 1988). Much attention has also been devoted to developing methods for the detection, monitoring, and recovery of these organisms subsequent to their introductions into the

environment (Amy and Hiatt, 1989; Atlas and Sayler, 1988; Ford and Olson, 1988; Holben *et al.*, 1988; Lindow *et al.*, 1988; Steffan and Atlas, 1988).

The impact of an introduced organism on the indigenous biotic community and its effects on ecosystem processes pose very difficult research problems. While these are among the most critical questions concerning the release of GEMs into the environment, very little work has been done in this area due to the immense complexity of habitats and their indigenous communities. Tiedje (1987) reasoned that there are too many biological processes and indigenous organisms to measure. Changes caused by an introduced organism would likely be very difficult to detect in the whole array of processes operating in a natural habitat, and that extrapolation from model studies to natural systems cannot be assumed to be reliable. Very recently, a few studies have been published that report attempts to monitor ecosystem reactions to the introduction of GEMs (Jones *et al.*, 1991; Short *et al.*, 1991). Jones *et al.* (1991) monitored the impact of introduced GEMs on ammonification, nitrification, and denitrification as well as the population dynamics of the indigenous bacteria that carry out these processes. They observed no statistically or ecologically significant effects. However, it is important to note that the GEMs employed did not carry novel genes relevant to nitrogen transformation processes, nor were the test soils made selective for the GEMs by amendment with the heavy metals and antibiotics to which the GEMs possessed novel resistance genes. Short *et al.* (1991) determined that a 2,4-Dichlorophenol (2,4-D) metabolizing GEM produced a toxic metabolite, 2,4-dichlorophenol (2,4-DCP), in soil that caused a 400-fold decline in the numbers of fungal propagules and a marked decrease in the rate of CO_2 evolution. The parent strain produced neither 2,4-DCP nor

the effects on fungal populations and CO_2 evolution. This outcome would, however, seem a likely consequence of any biological degradation of 2,4-D by means of the oxygenase that catalyzes conversion of 2,4-D to 2,4-DCP, regardless of the origin of the responsible gene.

While ecosystem response to introduced microorganisms remains a largely intractable research question, valuable information about the potential impact of an exotic microorganism on indigenous populations in a natural ecosystem can be gained by monitoring the fate of the introduced microorganism itself. A central concern associated with the deliberate release of a GEM is the competitiveness of the introduced organism in the target environment because the microorganism will generally have to survive and multiply in order to perform its intended function (Alexander, 1985; Olson, 1986). The response of the indigenous community to an invader is, of course, intrinsically related to the competitiveness of that invader.

Generalizable principles and quantitative measures of the competitiveness of an invader relative to established community members could lead to the development of predictive indices useful for risk assessment. The research project presented here was motivated by the need to acquire the survival kinetics data necessary for mathematically modeling persistence of an introduced organism. A working definition of such an "index of persistence" for the quantification of microbial survival can be found in Appendix A.

This study focused on the survival kinetics of a model organism introduced into soil. Soil provides a variety of subhabitats (detritus, rhizosphere, soil fauna, anaerobic microsites, etc.) in which an introduced organism could survive and also represents the environment where GEMs are likely to be most commonly utilized (Tiedje, 1987). To

acquire quantitative data on the survival kinetics, the model organism must first be inoculated into soil, then quantitatively recovered from the soil after desired intervals for enumeration.

Soil is a particularly complex habitat where the survival of microorganisms is affected by a broad array of biotic and abiotic factors. Abiotic factors such as temperature, O_2 tension, water activity, pH, Eh, soil structure and texture, and nutrient status play significant roles in the density and composition of the bacterial community in soil. (Alexander, 1977; Peña-Cabriales and Alexander, 1983a and b; Richaume *et al.*, 1989; Stacey, 1985; van Veen and van Elsas, 1986; West *et al.*, 1985). Biotic factors affecting bacterial survival include the lytic and toxic capabilities of other organisms such as bacteriophages, bdellovibrios, and antibiotic-producing actinomycetes, and fungi (Acea *et al.*, 1988; Acea and Alexander, 1988); intrinsic growth rate (Acea and Alexander, 1988; Hartel and Alexander, 1987); predation by protozoa (Acea *et al.*, 1988; Danso *et al.*, 1975; Habte and Alexander, 1975, 1977 and 1978); susceptibility to starvation (Acea *et al.*, 1988; Liang *et al.*, 1982; Sinclair and Alexander, 1984; West *et al.*, 1985); and competition for carbon (Jannasch, 1968; West *et al.*, 1985). While bacteriophages, bdellovibrios, and lytic and antibiotic-producing organisms do not appear to have a significant effect on the survival rates of introduced organisms (Acea *et al.*, 1988; Habte and Alexander, 1975), predation by protozoa and resistance to starvation are apparently critical factors (Acea *et al.*, 1988; Acea and Alexander, 1988; Liang *et al.*, 1982; Postma *et al.*, 1990b), as may be competitive competence (Jannasch, 1968; West *et al.*, 1985).

Beyond the rather large differences between subhabitats described above and the multitude of factors affecting microbial life, the actual physical matrix of the soil is of

salient importance to the survival and activity of microorganisms in soil. As characterized by Hattori and Hattori (1976), and Stotzky (1972), as well as van Veen and van Elsas (1986), soil is a heterogeneous, discontinuous, and structured environment. The particles of the solid phase range from minute, highly reactive clays to large, relatively inert gravels. This vast variability in the physical structure of the soil makes for enumerable, distinct microbial habitats and, therefore, enumerable, distinct microbial communities.

Many of the abiotic and biotic environmental characteristics mentioned above, such as water activity, surface interaction phenomena, pH, nutrient flux, vulnerability to predation, *etc.*, are determined by or significantly influenced by particle size and pore size. Therefore, microbial populations are very much affected by soil texture. For microbes, the most important component of soil texture is the clay content. Finer textured soils of high clay content almost always have larger populations of indigenous microorganisms than do sandier soils (Alexander, 1977); introduced microorganisms survive at higher levels in fine or heavy textured soils (Macnaughton *et al.*, 1992; Postma *et al.*, 1990b; van Elsas *et al.*, 1986; van Veen and van Elsas, 1986); and introduced organisms also survive at higher levels in soil that has been amended with additional clay (Heijnen *et al.*, 1988; Heijnen and van Veen, 1991).

The chemical reactivity of clays greatly influences microorganisms in the soil. A large body of literature addresses the reactivity of clays and the interaction between clay minerals and soil microbes. Surfaces with considerable negative charge such as many crystalline clays are likely to facilitate adsorption of dissolved macromolecules and adhesion of bacteria, and the cation exchange capacity of clays can influence the

buffering capacity of a system and the availability of mineral nutrients (Fletcher, 1991). Clays affect microbial growth, respiration, and metabolism (Stotzky, 1986), and have been shown to protect bacteria from toxins (Habte and Barrion, 1984), and desiccation (Bushby and Marshall, 1977).

The clay content of a soil determines the physical matrix, or porosity, of that soil. The multitude and size of pores are critically important, as are the aggregates that form in clay-containing soils. Hattori and Hattori (1976) describe the role of soil clay colloid coagulation in the formation of soil aggregates and emphasize the primacy of soil aggregates as sites for the occurrence of bacteria in soil. The internal structure of an aggregate of clay, larger mineral particles, and organic matter includes various types and sizes of pores. Capillary pores with neck sizes of $< 6\ \mu$m were determined by Hattori and Hattori (1976) to be the optimal microhabitats for bacteria, and aggregates $> 10\ \mu$m, and especially $> 20\ \mu$m were ascertained to be of sufficient size to include such capillary pores.

A crucial abiotic factor affecting bacterial survival and activity is water, and water retention in soil is determined by soil porosity. In several investigations by Postma and coworkers (Postma *et al.*, 1988; Postma *et al.*, 1990a; Postma *et al.*, 1990b) survival of an introduced rhizobium was greater in a silt loam than in a loamy sand. In these experiments the same moisture potential of -10 kPa was maintained in both soils which resulted in 40 to 45% moisture in the silt loam but only 16 to 20% moisture in the sandy loam. While other characteristics of these soils may have played a role in the differential survival observed in these experiments, the greater soil moisture content of the silt loam was interpreted to be very significant.

The distribution of bacteria throughout the pore spaces in and between aggregates greatly affects the vulnerability of those bacteria to predation as well as desiccation. Bacteria established in pore spaces too small to be accessible to protozoa are protected in these "refugia" (van Veen and van Elsas, 1986; Vargus and Hattori, 1986). Predation has been determined to be the most influential biotic stress on both indigenous and introduced bacterial populations (Acea and Alexander, 1988; Acea *et al.*, 1988). Elliott *et al.* (1980) demonstrated that soil porosity influences trophic interactions in soil ecosystems: growth of bacteria, nematodes, and amoebae was greater in fine-textured soil. Higher respiration rates noted when bacteria were grown with both predators together rather than with either predator alone were interpreted to be the result of amoebae entering smaller pores than nematodes could penetrate and thereby making more nutrients (bacteria) available to the system as a whole. This phenomenon was more pronounced in finer rather than coarser soil.

The significance of both predation and soil moisture content for bacterial life in the soil and the generally held assumption that most cells are attached to soil particles and rarely occur free in the soil solution (Hattori and Hattori, 1976), suggest that the location within the soil matrix *where* cells occur is of critical importance. It is reasonable to assume that introduced bacteria will, at least initially, occur in relatively open spaces and be more vulnerable to predation and desiccation than are indigenous bacteria. Postma and coworkers (Postma *et al.*, 1989; Postma *et al.*, 1990a) attempted to manipulate the spacial distribution of introduced bacteria in the soil matrix by adjusting the initial moisture level of the soil into which their model organism was inoculated. At lower soil moisture levels, more capillary pores will be empty of water.

Their results indicated that a higher percentage of cells were associated with aggregates $> 50\ \mu m$ when the inoculation was made into drier soil. In addition, cells associated with aggregates $> 50\ \mu m$ exhibited higher rates of survival than free-living cells or cells associated with smaller particles. Several investigations have established that the percentage of introduced cells associated with particles increases over time (Balkwill and Casida, 1979; Ozawa and Yamaguchi, 1986; Postma *et al.*, 1989; Postma *et al.*, 1991). Apparently the cells that survive for considerable periods of time are those that are strongly associated with particles. Bacterial attachment to or enclosure in soil particles/aggregates may serve to enhance cell survival in several ways. Protection from predation is one of these ways. After determining the increasing association of introduced bacteria with large aggregates in response to decreasing initial soil moisture levels as previously described, Postma *et al.* (1990b) also observed increasing numbers of introduced rhizobia were associated with aggregates $> 50\ \mu m$ (at decreasing initial soil moisture levels) when both rhizobia and predators were introduced into previously sterilized soil. The difference was maintained throughout the experiment, and survival of cells associated with aggregates $> 50\ \mu m$ was greater than that of free or small-particle associated cells. In contrast, when rhizobia were introduced into sterile soils (no predators) of different initial moisture levels, differences in percentage of particle association disappeared over time and there was no enhancement of survival due to particle association. Their investigation illustrates the survival-enhancing protection from predation that association with aggregates provides bacteria.

Along with protection from predation, association with soil particles and aggregates may offer bacteria chemical and physical advantages (in addition to water

retention) that enhance survival. Among these are solid-surface adhesion phenomena. Solid surfaces, such as the surfaces of soil particles, exhibit properties that can influence microbial activity (Fletcher, 1991; van Loosdrecht *et al.*, 1990). These can influence adsorption (therefore, availability) of electrolytes, nutrients, growth factors, and inhibitors (Fletcher, 1991). Morphological and physiological differences between attached and free-living bacteria in given experimental systems have been observed. The review by Fletcher (1991) tends to promote the view that bacterial activity may be increased as a direct results of the cells' adhesion to solid surfaces. She supports this hypothesis by presenting studies such as that by Keen and Prosser (1987) who reported higher specific growth rate and lesser response to pH change in attached compared to free *Nitrobacter* sp. cells studied in a continuous culture experiment. Fletcher also presented the work of Remacle (1981) who reported that in a chemostat enrichment of freshwater bacteria, attached cells showed increased mineral uptake as evidenced by greater cadmium accumulation than was seen in free cells. In contrast, van Loosdrecht *et al.* (1990) have contended that there is, to date, no conclusive evidence that adhesion directly influences bacterial activity. They offer instead as an interpretation of the available studies that observed differences in attached and free bacteria may result from bacterial response to the environmental conditions created by solid surface characteristics rather than changes in the cells themselves. Surface-association phenomena have largely been studied in the laboratory by means of cell associations with glass vessels, slides, or beads. The extent of the influence of soil particle surfaces on cellular metabolism *in situ* is, at this time, a matter of speculation.

It has been demonstrated that as introduced cells adjust to oligotrophic environments such as soil or seawater, morphological changes take place that may be related to the adhesion process. Kjelleberg *et al.* (1983) report fragmentation and dwarfing of marine isolates upon starvation after culture in rich medium. The organisms that they worked with adhered to surfaces better as they starved. This phenomenon was interpreted as a survival mechanism enabling them to scavenge nutrients adsorbed to the surfaces. Van Veen and van Elsas (1986, data unpublished) described a pseudomonad isolated from soil that maintained viability under starvation conditions for 55 days while undergoing a 40% reduction in cell size. This organism also produced minute forms upon inoculation into soil. Fletcher (1991) suggests that adhesion to solid surfaces may play a role in bacterial adaptation to nutrient depletion, and that dwarfing and adhesion may be related.

The present investigation involved an attempt to ascertain the influence of inoculum density on survival of bacteria introduced into soil. This information is needed for the development of the index of persistence described in the Appendix A. The interactivity of the influence of inoculum density with the influence of inoculation conditions and laboratory recovery conditions were studied. Mindful of the complexity of soil as a microbial habitat it was considered desirable to manipulate or accommodate various inoculation and recovery factors in order to enhance the survival potential of the model organism and prolong its recoverability from the soil. Chapter II describes the

manipulations of inoculation procedures. In Chapter III, variations in cell recovery protocols are examined and analyzed, and Chapter IV reports the influence of inoculum density as determined within the inoculation and recovery regimens studied here.

CHAPTER II

INOCULATION CONDITIONS

Introduction

To accomplish the intent of their application, specially selected or engineered bacteria introduced into the environment must survive in sufficient numbers to become established and multiply (Alexander, 1985; Olson, 1986). Field trials of bacterial introductions designed to enhance crop yield, suppress soil-borne pathogens, and degrade pollutants have been carried out with some degree of success. However, van Veen and van Elsas (1986) contend that the reproducibility of the results reported has been poor and the variability unacceptably large. They suggest that the main reason for these inconsistent results is the varying extent to which introduced bacteria survive and become established in soil ecosystems. Bacteria introduced into soil are vulnerable to an array of abiotic and biotic factors such as temperature, desiccation, nutrient availability, pH, predation, antagonism, and competition that affect survival (Alexander, 1984; Postma *et al.* 1990a; Stacey, 1985; West *et al.*, 1985). Bacteria can be expected to be more or less affected by these stresses depending upon the location in the soil matrix where these organisms occur (Hattori and Hattori, 1976; Postma *et al.* 1989; Postma *et al.* 1990a, Postma *et al.*, 1990b). It is difficult to monitor and predict the survival of introduced microorganisms (Stacey, 1985), in part because of the heterogeneity and complexity of the soil matrix (van Veen and van Elsas, 1986). Postma *et al.* (1989, 1990a) determined

that introduced rhizobia associated with soil particles or aggregates >50 μm exhibited higher rates of survival than those ascertained to be free-living or associated with smaller particles and aggregates. Postma *et al.* (1989) also determined that the initial moisture level of the inoculated soil influenced the distribution of the introduced cells into pores of different sizes. A higher percentage of the rhizobia were associated with particles and aggregates >50 μm and survival was greater when the cells were inoculated into relatively dry soil.

Soil texture has been demonstrated to have a significant impact on the survival and establishment of introduced bacteria. Several studies have reported markedly greater survival of bacteria introduced into finer-textured, high-clay content soils, in comparison to survival in coarser soils (Macnaughton *et al.*, 1992; Postma *et al.*, 1990b; van Elsas *et al.*, 1986). Heijnen and coworkers (Heijnen *et al.*, 1988, Heijnen and van Veen, 1991; and Heijnen *et al.*, 1991) achieved greatly enhanced survival of an introduced rhizobium by incorporating a bentonite clay amendment into the soil.

The present investigation was motivated by the need to develop an optimized inoculation protocol for studies of survival kinetics. We investigated the effect of minimizing the additional wetting of the field-moist soil upon inoculation, and compared these results to inoculations into dried soil. We also tested the effect of the bentonite clay amendment described by Heijnen *et al.* (1988) on *Agrobacterium tumefaciens* A6.

Materials and Methods

Soil.

The soil used was a Ninigret very fine sandy loam from Amherst, Massachusetts, gathered from the top 15 cm under a sod layer. For experiments involving "field-moist" soil, the soil was protected from drying, forced through a 2-mm sieve, and stored in a closed jar at 4°C. For all other experiments, the soil was air-dried for 24 h, sieved (2 mm), air-dried for 24 h more until weight loss due to water loss was 25%, and stored in a closed jar at 4°C.

Bacterial Strains and Culturing Media.

Agrobacterium tumefaciens A6, resistant to 100 μg ml^{-1} rifampicin, and *Pseudomonas* sp. BS, resistant to 800 μg ml^{-1} ampicillin, 10 μg ml^{-1} rifampicin, and 5 μg ml^{-1} Chloramphenicol were obtained from James M. Tiedje, Michigan State University. *Agrobacterium tumefaciens* resistant to 1000 μg ml^{-1} streptomycin and 50 μg ml^{-1} erythromycin was obtained from Martin Alexander, Cornell University. *A. tumefaciens* A6 was grown in half-strength Tryptic Soy (T-Soy) broth (Difco Laboratories, Detroit, Michigan) for 24 hours in the dark at 30°C in 200 ml of media in 500-ml flasks on a rotary shaker operating at 180 rev min^{-1}. Cells were collected by centrifugation at 8500 × g for 12 min at room temperature, washed, and resuspended in sterile buffer containing 1.1 g Na_2HPO_4 and 0.6 g KH_2PO_4 per liter of distilled water, centrifuged again and resuspended in fresh buffer to the desired inoculum density.

The pseudomonad was cultured in 10% peptone tryptone yeast extract glucose broth (10% PTYG containing the following per liter of distilled water: Peptone (Difco Laboratories, Detroit, Michigan) 0.5 g, Tryptone (Difco) 0.5 g, Yeast Extract (Difco) 0.25 g, glucose 0.25 g, $MgSO_4 \cdot 7H_2O$ 0.03 g, and $CaCl_2 \cdot 2H_2O$ 0.0035 g). The $strp^r$ and $eryth^r$ agrobacterium was cultured in Yeast Extract Mannitol (YEM containing the following per liter of distilled water: Yeast Extract (Difco) 1.0 g, mannitol 5.0 g, K_2HPO_4 0.5 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, $CaCl_2 \cdot 2H_2O$ 0.12 g, NaCl 0.1 g). The pseudomonad and the $strp^r$ and $eryth^r$ agrobacterium were grown up and collected in the same manner as the *A. tumefaciens* A6.

Enumeration of Introduced Bacteria.

Recovered cells were enumerated by means of the pour plate method. As was determined in a previous study (Hoben and Somasegaran, 1982) and demonstrated by a preliminary study with *A. tumefaciens* A6 in our lab, cell recovery in pour-plates was not significantly different from cell recovery on spread-plates. In our results, slightly higher but insignificantly different counts were observed in pour plates (data not shown). The plating medium for the *A. tumefaciens* A6 was half-strength Tryptic Soy Broth with 1% (w/v) agar and amended with 5 μg ml^{-1} crystal violet, 100 μg ml^{-1} rifampicin (Sigma Chemical Co., St. Louis, MO), 100 μg ml^{-1} nystatin (Sigma), and 300 μg ml^{-1} cycloheximide (Sigma). The medium for *Pseudomonas* sp. B8 was 10% PTYG with 1% (w/v) agar amended with 5 μg ml^{-1} crystal violet, 800 μg ml^{-1} ampicillin, 10 μg ml^{-1} rifampicin, and 5 μg ml^{-1} chloramphenicol and fungicides as above. The agrobacterium resistant to streptomycin and erythromycin was grown on YEM, 1% (w/v) agar, 5 μg

ml^{-1} crystal violet, 1000 μg ml^{-1} streptomycin (Sigma), 50 μg ml^{-1} erythromycin (Sigma), and fungicides. The concentrations of antibiotics were sufficient to completely suppress background growth from slurries of one part soil in 9 parts of water for at least three days and allowed detection of the introduced bacteria at densities of $< 10^2$ g^{-1} of soil.

Inoculation of Soil.

Because preliminary studies with the *A. tumefaciens* A6 found no change in survival or replicability when 10-g soil samples were used, 2-g soil samples were employed for this investigation. For the "field-moist" soil experiments, 2-g portions of the never-dried soil were placed into 110 × 20 mm screw-top glass test tubes. All inocula were introduced as suspensions of cells in buffer with the inocula of the field-moist soil prepared to deliver the desired cell density in 0.1 ml of liquid. The cell suspensions were slowly dripped over the surface of the soil and mixed into the soil by gentle rotation and tapping of the tubes. This small inoculum volume was designed to change the soil moisture content as little as possible. For the inoculations of "air-dried" soil, 1.5-g portions of the air-dried (48 h) soil were placed into 110 × 20 mm screw-top glass test tubes. The inoculum was prepared to deliver the desired cell density in a 0.5-ml volume of liquid. In this way the individual soil samples were restored to the moisture level exhibited in the field while being inoculated with the desired density of cells. In the case of the experiment involving "remoistened" soil, the tubes were prepared as above with the dried soil, then wetted with 0.25 ml of sterile water before inoculation with the appropriate density of cells suspended in 0.25 ml of phosphate buffer. Approximately constant moisture levels were maintained by adding 0.1 ml

sterile water to each tube whenever 0.1 g of weight loss was detected: approximately every 10 days.

Clay Amendment.

A 10% dry weight to total weight amendment of bentonite clay (obtained from Peter L. M. Veneman, University of Massachusetts, Amherst, MA) was added to the field-moist soil for the clay experiment and mixed in well. Two-gram portions of this mixture were loaded into tubes and inoculated as in the "field-moist" protocol described above.

Sampling Procedure.

For each experiment, duplicate tubes were sampled for each organism on each sampling day. The inoculated soil in each of the duplicate tubes was suspended in 18.5 ml of sterile phosphate buffer to achieve a 1:10 dilution. A preliminary study revealed no difference in the recovery of cells from suspensions made with the Na/K phosphate buffer and recovery from suspensions made with 0.1% sodium pyrophosphate. Each suspension was vortexed for 60 s, then allowed to settle for 60 s. After the settling period, supernatant subsamples were withdrawn from approximately the top 3 cm of the suspension and transferred to milk dilution bottles of sterile phosphate buffer for serial 10-fold dilution. From each appropriate dilution of each of the duplicate tubes, 3 to 10 replicates (determined according to the number of colonies per plate that was anticipated) were plated on the medium designated for each organism as described in the earlier

section on enumeration of introduced bacteria. Plates were incubated at 30°C for 4 days for the agrobacteria and 2 days for the pseudomonads.

Results

Inoculation into Field-Moist Soil.

Three different trials with three different organisms were carried out using the inoculation method that involved a minimized liquid inoculum volume into soil that was held at field moisture level ($\approx$ 25% water w/w). All three organisms, the streptomycin and erythromycin resistant *A. tumefaciens*, *P.* sp. B8, and *A. tumefaciens* A6 showed very little or no initial burst of growth under this regimen (Fig. 2.1). Survival of these organisms, inoculated into soil in this manner, was brief, particularly at the lower inocula. The two lower inocula of the strp^r and eryth^r *Agrobacterium* (Fig. 2.1A) were no longer detectable after 20 days, and although the two higher inocula were still detectable at 28 days, the experiment was discontinued after that sampling day. The pseudomonad died out very rapidly at all inoculum sizes, and was no longer detectable after 11 days (Fig. 2.1B). *A. tumefaciens* A6 was monitored for the longest period of time. Although the 10^9 inoculum was still detected after 50 days, none of the three lower inocula could be recovered after 40 days (Fig. 2.1C). This method of inoculation proved successful in avoiding the temporary bloom of growth that is often observed during the first 5 to 6 days of incubation of an introduced organism.

A 10^5 Inoculum under Three Soil Moisture Conditions.

A 10^5 inoculum of *A. tumefaciens* A6 introduced into three different soil moisture conditions exhibited three very different survival patterns (Fig. 2.2). The inocula were derived from the same cell suspension which was adjusted only for liquid volume, therefore the physiological status of the organisms should have been essentially the same. The inoculation into "very dry" soil (Fig. 2.2, upper curve) resulted in a small initial increase in CFUs per gram of soil, and at the end of the 32 day monitoring period, the cell density was approximately the same as it was at the time of inoculation. Cells that were inoculated into soil that had previously been air-dried and then partially re-moistened with sterile water before inoculation (Fig. 2.2, middle curve) demonstrated moderate decline in population. Cells that were inoculated via a minimal liquid volume into soil maintained at field moisture level (Fig. 2.2, lower curve) using the same method as that which produced the results shown in Fig. 2.1 showed the most rapid decline in population. The "field-moist" regimen led to decline of almost 3 orders-of-magnitude over the 32-day incubation period.

Clay Amendment.

A 10% bentonite clay amendment improved survival of the *A.tumefaciens* A6 when inoculated at either 10^7 or 10^5 cells per gram of soil. Monitoring of the 10^7 inoculum was ended at day 32, at which point cell recovery from the clay-amended samples was ten times greater than that from non-amended samples (Fig. 2.3, upper curves). In the case of the 10^5 inoculum, survival was monitored for 27 days, and cell recovery was more than two orders of magnitude greater from the clay-amended samples

(Fig. 2.3, lower curves). The clay amendment had an obvious, positive effect on the survival of *A. tumefaciens* A6 in soil.

Three Inoculum Densities under Two Soil Moisture Conditions.

Fig. 2.4 allows comparison of the long term survival curves of *A. tumefaciens* A6 inoculated (at three densities) into field-moist soil (Fig. 2.4A) to the survival curves of the same organism inoculated into air-dried soil (Fig. 2.4B). The curves from field-moist soil exhibit rapid initial decline followed by a period either of much slower decrease in cell numbers (for the higher two inoculum densities) or of no significant decrease in the case of the smallest inoculum. However, cells could no longer be recovered after 63 days even from soil originally inoculated with 10^9 agrobacteria per gram. The results from the air-dried soil differed markedly. Slow decline of cell numbers from the middle and smallest inocula followed a pattern that seemed almost linear on semi-logarithmic axes. The lowest inoculum in this trial was detectable for as long a period of time as the highest inoculum in the field-moist trial (63 days). The middle and highest inocula were still recoverable at the end of the investigation (98 days). Cells from the largest inoculum in the air-dried experiment declined for the first week somewhat more rapidly than during the remainder of the incubation. In these experiments, the long-term survival kinetics of the *Agrobacterium* were greatly affected by the moisture content of the soil into which it was inoculated.

Discussion

Using soil that was maintained at the moisture level it had possessed in the field (approximately 25% water by weight) and delivering the inoculum in a minimal volume of liquid seemed to eliminate the burst of microbial growth that typically follows the wetting of soil (Lund and Goksøyr, 1980; Lynch, 1982). However, the rapid death rate of the organisms inoculated into soil in this fashion was not suitable for the long-term survival studies that we intended to pursue.

Our investigation of the effect of initial soil moisture level on the survival of *A. tumefaciens* A6 produced results similar to the findings of Postma *et al.* (1989). Their work, and ours, indicate that introduced cells survive better when inoculated into soils with a low initial moisture content. The Postma *et al.* (1989) investigation described the influence of initial moisture level on the immediate and ultimate spacial distribution of introduced bacteria in the soil matrix. They concluded that an inoculum introduced into drier soil will be carried by capillary suction into the small-neck pores that provide protected habitats. At very low soil moisture levels, optimally-sized pore spaces are available to the inoculum, whereas at higher moisture levels these spaces are water-filled. At moderate water flux an inoculum will not penetrate pores that are already water-filled. When cells from the inoculum are deposited in larger pore spaces and remain associated with the outside surfaces of aggregates and soil particles, they are more vulnerable to both predation (Vargus and Hattori, 1986) and abiotic stresses (Hattori and Hattori, 1976). Our results from field-moist soil, very dry soil, and partially re-wetted soil (Fig.

2.2) (which after inoculation were incubated at the same moisture level) suggest that the survival of introduced bacteria may be directly related to the extent to which the cells are transported to available capillary pores. In our experiment we re-moistened soil that had been dried to our "very dry" level with one half the amount of water needed to restore it to the moisture level it had in the field. It can be assumed that a sizeable portion of the capillary pores that had been emptied by drying were re-filled by this pre-inoculation wetting, thereby making them inaccessible to the inoculum. The reduced survival of the cells inoculated into the remoistened soil (Fig. 2.2, middle curve) would appear to be related to reduced accessibility to habitable pore spaces at the time of inoculation. It could be assumed that desiccation reduced the populations of protozoan predators in the air-dried soil, and that the improved survival of *A. tumefaciens* A6 in air-dried relative to field-moist soil was the result of fewer predators rather than physical protection from predators by enclosure in capillary pores. However, the reduced survival of the agrobacteria in "re-moistened" soil, which had been subjected to the same degree of desiccation as the air-dried soil, provided support for the hypothesis that protection in capillary pores greatly enhances survival.

The survival-enhancing effect of the bentonite clay amendment that we see here is consistent with the results achieved by Heijnen and coworkers (Heijnen *et al.*, 1988, Heijnen and van Veen, 1991; and Heijnen *et al.*, 1991). The positive influence of clay minerals on the survival of microorganisms has been recognized by Stotzky (1980, 1986), Marshall (1975), van Veen and van Elsas (1986) and others. Clay minerals can provide protection from heat, desiccation, and toxins (Marshall, 1975; Stotzky, 1980, 1986). Roper and Marshall (1978) suggested that clay "envelopes" may form around bacteria.

Although the work presented here does not provide any information as to how the clay amendment improves survival of the introduced bacterium, the work of Heijnen and her coworkers strongly suggests that the clay primarily provides protection from predation by protozoa. Their work has demonstrated that amendment with bentonite, a swelling clay, enhances survival significantly more than amendment with kaolinite, a non-swelling clay (Heijnen and van Veen, 1991). This phenomenon along with the conclusions of another experiment that cast doubt on the importance of clay-coating of bacteria and/or protozoa as significant (Heijnen *et al.*, 1991), suggest that clay amendment enhances survival of introduced organisms by creating protective microhabitats.

The 98-day study (Fig. 2.4) at three inoculum densities emphasizes the markedly different survival kinetics exhibited by the introduced agrobacterium when initial soil moisture level was the only experimental component that was changed. The primary effect of initial soil moisture level appears to be its influence on the spacial distribution of the introduced organisms in the physical matrix of the soil. An introduced cell's fate may be largely determined by the whether or not it is transported to a protective microsite.

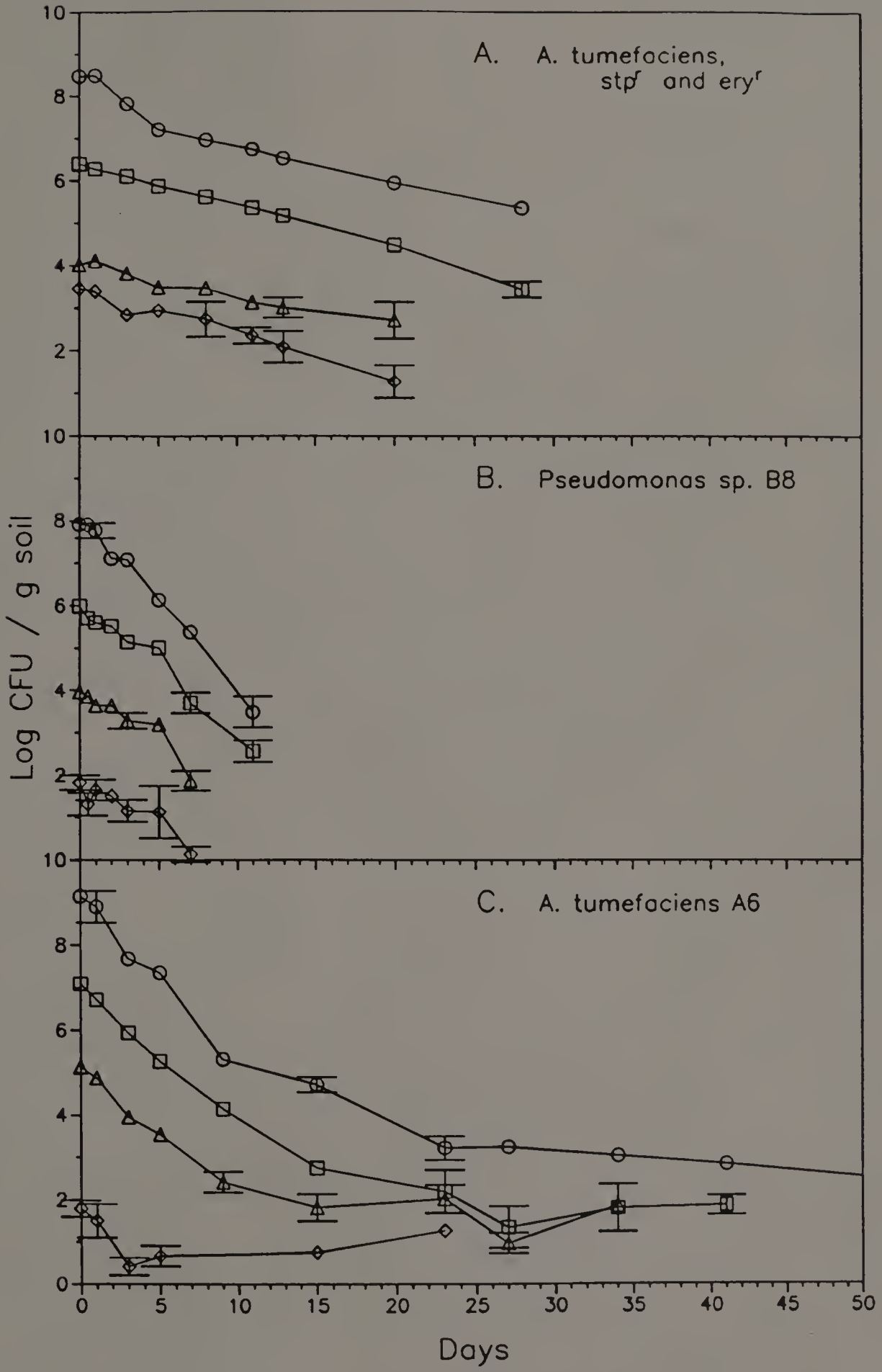

Fig. 2.1 Survival patterns of three different organisms inoculated into "field-moist" soil (soil maintained at the moisture level it possessed in the field, ≈ 25% water w/w) at several inoculum densities. Liquid volume of inoculum was 0.1 ml and was introduced into 2 g of the field-moist soil, thereby changing moisture level of soil to ≈ 30%.

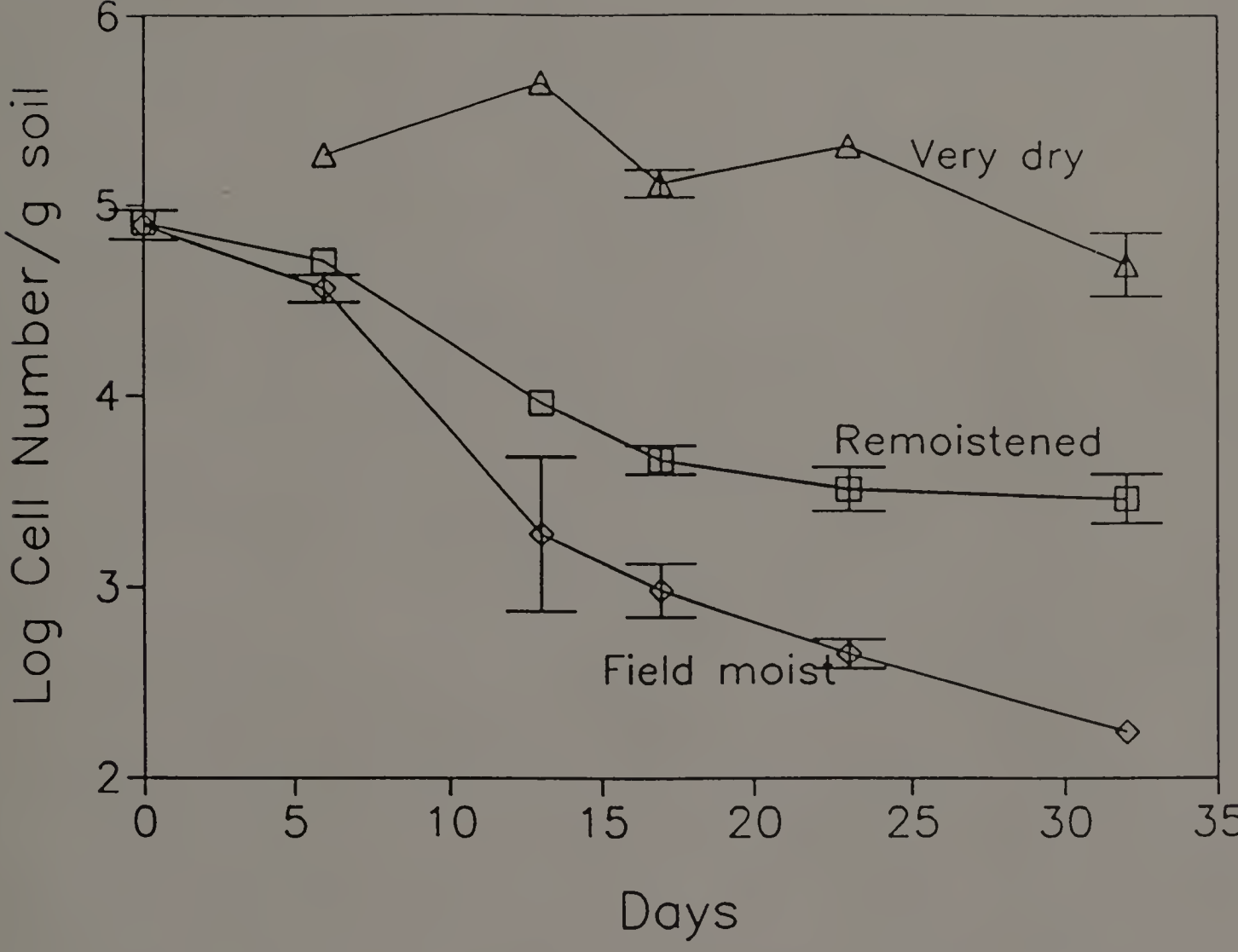

Fig. 2.2 *A. tumefaciens* A6 inoculated into soil at three initial soil moisture levels. "Very dry" was air-dried 48 h and lost 25% of its field-moist weight to evaporation of water. "Remoistened" was "very dry" soil to which half of the water lost to evaporation was restored before inoculation. "Field-moist" was soil held at the moisture level it possessed in the field.

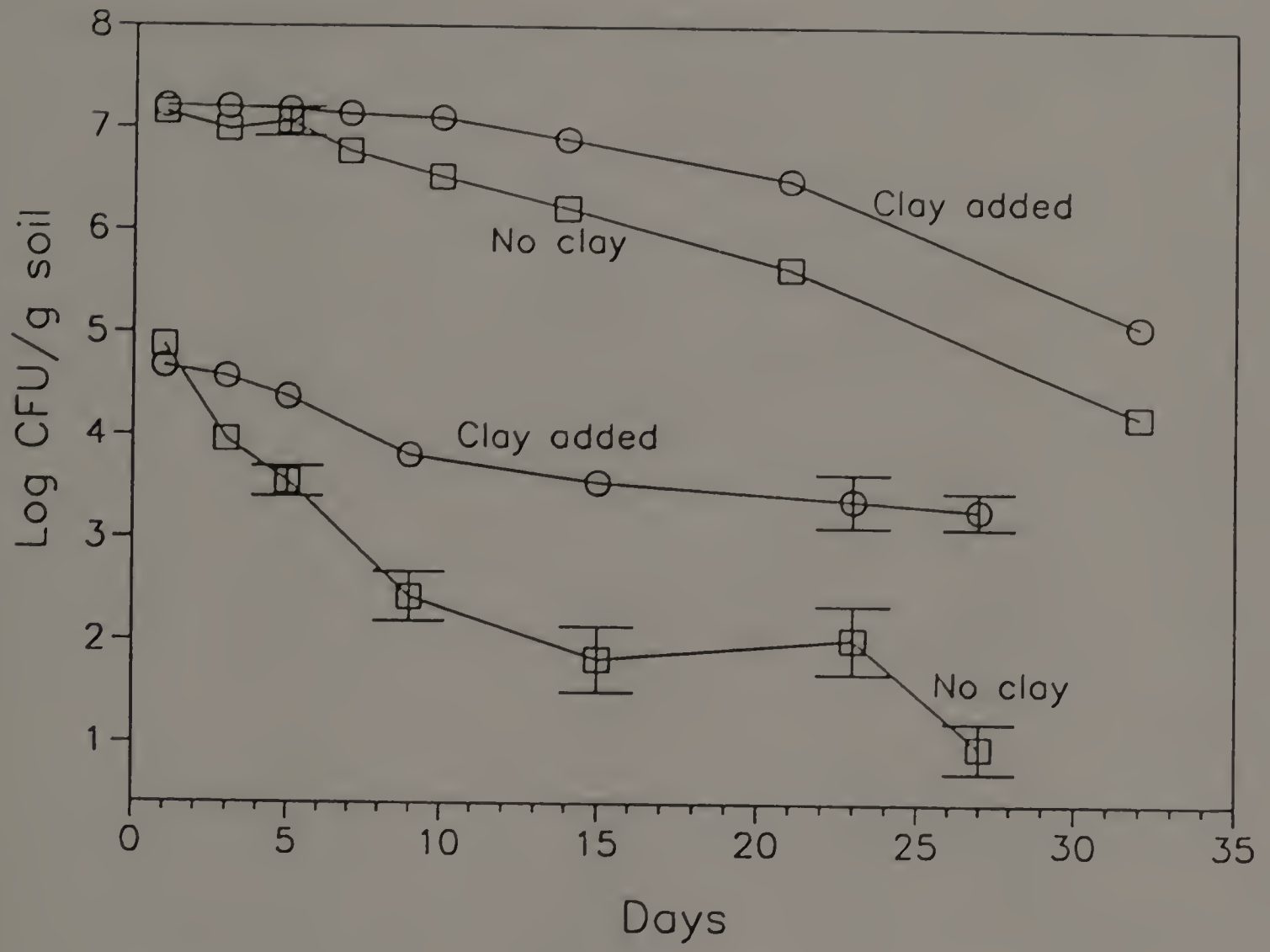

Fig. 2.3 *A. tumefaciens* A6 inoculated at two densities into field-moist soil with and without a 10% (w/w) bentonite clay amendment.

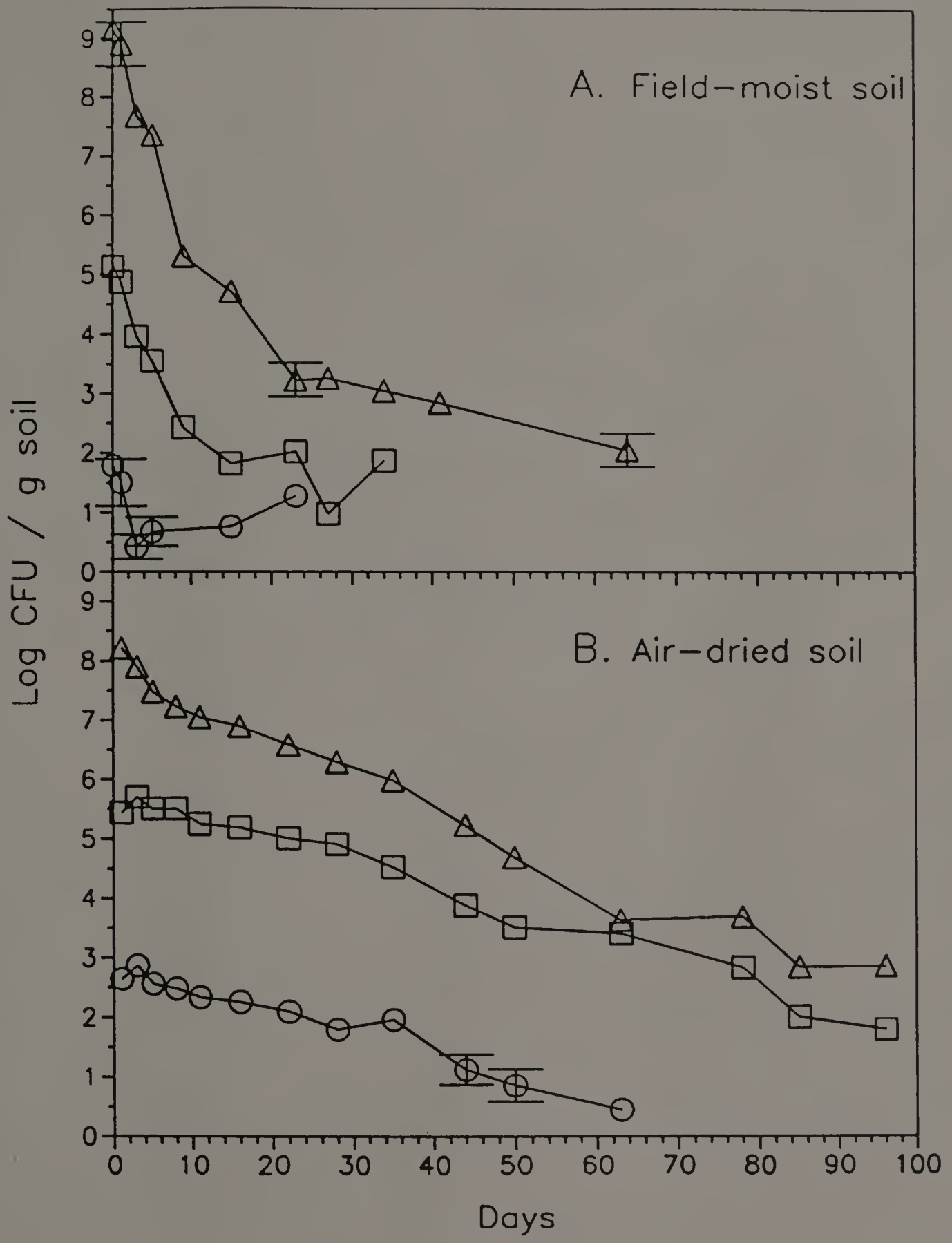

Fig. 2.4 *A. tumefaciens* A6, at three inoculum densities in "Field-moist" soil (0.1 ml liquid inoculum / 2 g moist soil) and "Air-dried" soil (0.5 ml liquid inoculum / 1.5 g dried soil).

CHAPTER III

CELL RECOVERY VARIABLES

Introduction

Monitoring the populations of organisms introduced into natural environments contributes information essential for evaluating the safety and efficacy of such introductions. Many factors complicate the accurate enumeration of organisms introduced into soil. Among these are the extensive background of indigenous organisms (Alexander, 1977; Danso *et al.*, 1973), the complexity of the soil matrix (Postma *et al.*, 1989; Postma *et al.*, 1990b; van Veen and van Elsas, 1986), and bacterial aggregation within and adhesion to soil particles (Balkwill and Casida, 1979; Hattori and Hattori, 1976; Marshall, 1980; Ozawa and Yamaguchi, 1986; Postma *et al.*, 1989 and 1991). Viable organisms can either be or become non-culturable (Colwell *et al.*, 1985; Roszak *et al.*, 1984), and the differences in physiology between free organisms and organisms attached to solid surfaces (Fletcher, 1991; Marshall, 1988; van Loosdrecht *et al.*, 1990) could effect enumeration methods that rely on culture in the laboratory.

Much work has been done to develop enumeration methods suitable for studying the survival kinetics of introduced microorganisms. The now classical selective plate count method that exploits the capacity of antibiotic-resistant mutants to grow on selective media (Danso *et al.*, 1973) remains a widely employed means to enumerate bacteria introduced into soil. Its advantages include practicality, inexpensiveness, and high

sensitivity. As few as 10-30 cells per gram of soil can be detected (Bushby, 1981; Danso and Alexander, 1974; Liang *et al.*, 1982). However, two important disadvantages detract from the value of this method. First, aggregated cells will cause underestimates in enumerations, and viable but non-culturable cells will not be counted at all. Second, antibiotic resistance may confer a competitive disadvantage, and the compromised ability of antibiotic-resistant mutant may discredit that organism as a suitable model for monitoring survival of the parent strain in the environment (Compeau *et al.*, 1988; Pettibone *et al.*, 1987; and Turco *et al.*, 1986). Recombinant bacteria, marked with genes that code for selectable characteristics other than antibiotic resistance such as the lac operon (Drahos *et al.*, 1986) or *xylE* (Macnaughton *et al.*, 1992; Winstanley *et al.*, 1991) also require dispersal of cells and culture in the laboratory.

Fluorescent-antibody and other immuno-coupling techniques have proven valuable (Bohlool and Schmidt, 1980; Crozat *et al.*, 1987; Dewey *et al.*, 1989; Dewey *et al.*, 1990; Page and Burns, 1991; Postma *et al.*, 1988) for many applications, and do circumvent some of the problems inherent in the selective plating method. Cells do not have to be culturable; in principle, all cells can be counted regardless of physiological state as long as cell walls are intact and presenting antigen. A competitively incompetant antibiotic-resistant mutant does not have to serve as a model organism. However, these techniques lack sensitivity, often do not distinguish living cells from dead cells, and may not distinguish an introduced organism from its indigenous relatives that present the same antigens. And, as with the selective plating method, cell association with particles affects accurate enumeration.

DNA probe methods offer great promise for detecting specific microorganisms in a complex background (Atlas and Sayler, 1988; Ford and Olson, 1988; Holben *et al.*, 1988) without the requirement for culturability. With the advent of PCR technology, detection of very low numbers of organisms in a natural environment has become possible (Bej *et al.*, 1991; Steffan and Atlas, 1988; Tsai and Olson, 1992). While these methods, perhaps coupled with a most probable number enumeration scheme, (Fredrickson *et al.*, 1988) potentially offer highly sensitive systems for monitoring microbial population dynamics, at this time such methods are technically impractical for many laboratories. In addition to technical inconvenience, problems remain with the maintenance of stable, unique gene segments, and definitive assessment of the importance of gene copy number has not yet been achieved (Fredrickson *et al.*, 1988).

In spite of limitations, selective plating with its practicality and sensitivity remains an important technique for enumerating introduced organisms. Our objective in this study was to analyze the impact of two variations on the details of this technique on the successful recovery of an introduced *Agrobacterium* and on its survival kinetics.

We examined the efficiency of cell recovery by means of the selective plating method in terms of two potentially influential factors: *i*, the strong association of introduced cells with large soil particles and aggregates; and, *ii*, the development, in the introduced bacteria, of dependence on extractable component(s) of soil for growth on selective agar medium.

Materials and Methods

Soil.

The soil used was the same Ninigret very fine sandy loam described in Chapter II. It was air-dried 24 h and sieved (< 2 mm). After sieving, the soil was air-dried for 24 h more till weight loss due to change in moisture content was 25%, and stored at 4°C.

Culturing and Enumerating Agrobacteria.

The same *Agrobacterium tumefaciens* A6, a natural isolate resistant to 100 μg ml^{-1} rifampicin described in Chapter II, was used in these experiments. The agrobacteria were cultured, harvested, washed, and resuspended in fresh buffer as described in Chapter II.

Recovered cells were enumerated by means of the pour plate method on the same medium reported in Chapter II for *A. tumefaciens* A6. In addition, one part of a soil extract, described below, was added to 9 parts of the modified T-Soy agar for some treatments.

Soil Extract Preparation.

The standard soil extract used throughout the experiment was prepared by autoclaving 1 part (by weight) of the 48 h air-dried soil in 4 parts of the phosphate buffer for 1 h. The autoclaved mixture was held for 24 h at room temperature, after which the

liquid portion was decanted and autoclaved for 20 min. The solids were discarded. Extract-amended media were prepared by aseptically adding 1 part, by volume, of sterilized soil extract to 9 parts of molten T-Soy agar after it had been autoclaved. The water-extraction used in one of the experiments was prepared in exactly the same way except that distilled water was substituted for the phosphate buffer.

Inoculation of Soil.

Soil was apportioned and inoculated in the same manner as described for "air-dried" soil in Chapter II.

Experimental Design.

On each sampling day the inoculated soil in each of duplicate tubes was suspended in 18.5 ml of sterile phosphate buffer (described in Chapter II) to achieve a 1:10 dilution. Each suspension was agitated for 45 s with a Vortex mixer, and then the tube was placed horizontally on a rotary shaker operating at 400 rpm for 10 min. This shaker arrangement produced a vigorous agitation that simulated a reciprocal shaking action. Upon removal from the shaker, suspensions were allowed to settle for 60 s. After the settling period, supernatant subsamples were withdrawn from approximately the top 3 cm of the suspension and transferred to milk dilution bottles of sterile phosphate buffer for serial 10-fold dilution. The soil suspension in the sample tube was then shaken by hand, and "freshly-agitated" subsamples were immediately withdrawn and transferred to a separate dilution series in the same manner as above. One set of replicate plates was poured containing aliquots from appropriate dilutions of the supernatant subsamples of

each of the duplicate tubes was plated on the rifampicin-T-Soy medium with the 10% soil extract amendment; another set was plated on the medium without soil extract. Similar sets of replicate plates were prepared from the freshly-agitated subsamples medium with and medium without soil extract amendment. From each appropriate dilution of each of the duplicate tubes, 3 to 20 replicate plates (determined according to the number of colonies per plate that was anticipated) were prepared using each of the two media (with or without soil extract). Extract-amended plates were incubated at 30°C for 3 days; non-amended plates were incubated at 30° for 5 days.

Statistical Analyses.

The methods used for the statistical analyses of the results of this investigation are provided in Appendix B.

Results

Survival Patterns.

Four different methods of enumerating *A. tumefaciens* A6 in soil recovered numbers of agrobacteria falling along four different curves (Fig. 3.1). The upper curve, repeated in each of the three subfigures (A, B, and C) of Fig. 3.1, shows the maximum number of *A. tumefaciens* A6 counts that could be recovered over a 7-month incubation following the organism's inoculation into non-sterile soil. The inclusion of a phosphate-buffer extract of soil in the plating medium and the inclusion of particles of all sizes from the initial soil suspensions prepared on any given sampling day were found to be necessary to achieve maximum cell recovery. The lower curve in each of the three subfigures shows the counts obtained when one or both of these factors were omitted. Inclusion or exclusion of large particles (and the CFU's associated with them) was accomplished by withdrawing the subsamples from a well-shaken slurry of inoculated soil either before or after the slurry had been allowed to settle 60 s. These two subsample types, distinguished only by the presence or absence of larger particles, were further tested for their response to a 10% by volume soil extract amendment in the plating medium. The same general pattern of survival is displayed by all four data sets for the first 100 days. (Fig. 3.1): an initially rapid rate of decline for the first two weeks was followed by a slower rate of decline through day 100. Counts on plates with soil extract (Fig. 3.1A, both curves) seemed to enter a third, very slow period of decline after about 129 days of incubation. On plates without soil extract (Fig. 3.1B and C, lower curves),

CFU numbers never entered this third phase of very slow decrease. Instead, a sudden acceleration was observed in the decline of the numbers of recovered cells, which ultimately fell below the limits of detection.

Fig. 3.1A illustrates the impact of excluding the larger silt and sand particles from a subsample. For the first few weeks of incubation, removal of large particles had little or no effect on the counts obtained from these subsamples. After about 45 days, however, excluding large particles by allowing 60 s of settling appeared to remove a progressively increasing fraction of the CFUs from the soil suspensions before plating. By the end of the 7-month incubation, more that 90% of the counts achieved by plating freshly-agitated total soil suspensions (with the complete range of particle sizes) were excluded by plating only the supernatant liquid that remained after 60 s of settling time. The difference between the upper and lower curves in Fig. 3.1A implies that an ever-increasing proportion of the total CFUs recovered by the optimized method (freshly-agitated suspensions, with extract) were associated with large particles.

Addition of soil extract amendment to the plating medium was shown to enhance the culturability of CFUs; the lower curve of Fig. 3.1B illustrates the effect of omitting the extract. Subsamples plated on media without soil extract exhibited reduced colony formation. The only difference between those two subsample sets was culturability, because at each sampling time both subsamples were withdrawn from the same freshly agitated soil slurry and, therefore, would have contained essentially the same number of CFUs. The enhancement of recovery by soil extract increased during the first three weeks and then remained nearly constant over the next four months. After day 129, the CFU's exhibited a precipitous decline in culturability on media without soil extract (Fig.

3.1B). Without the extract, no CFUs could be recovered after 5 months of incubation in soil, even though more than 10^3 *A. tumefaciens* A6 CFU's could be cultured per g of the same soil samples for the duration of the 7-month investigation when soil extract was included in the media.

When large-particle-associated CFUs and soil extract were both excluded, cell recovery was the lowest observed in this survival experiment (Fig. 3.1C). The combination of large particle removal and extract omission decreased counts by a greater factor than either effect achieved alone. Cells became unrecoverable by this protocol shortly after day 97, a full month before the agrobacteria fell below the limits that could be detected if large-particle-associated cells were included in the samples for plating (Fig. 3.1B).

Soil Extract Amendment: Phosphate Buffer Extraction Vs. Water Extraction.

On day 129, cell recovery with the standard soil extract (autoclaved for 1 h in phosphate buffer) was compared to recovery with an extract of soil prepared by autoclaving in distilled water and to recovery on plates without soil extract (Fig. 3.2). Omission of the buffer components from the extracting solution gave a product of different color and viscosity: the phosphate-buffer extract had a much darker brown color and greater viscosity than the water extract. In the case of subsamples withdrawn from the soil slurry immediately after agitation (Fig. 3.2A), the soil extract prepared with phosphate buffer increased recovery of cells by a factor of about seven in comparison to plates lacking soil extract. The enhancement brought about by the water-extract in the freshly agitated subsample set was only three-fold. When subsamples were withdrawn

from the supernatant of the soil slurry after 60 s settling time (Fig. 3.2B) no cells were recovered on unamended medium. Water-extract-amended medium supported minimal colony formation, and phosphate-buffer extract increased that recovery five-fold.

Standard Soil Extract and Colony Development Time.

Throughout the study, CFUs recovered from the inoculated soil on selective medium lacking the standard phosphate-buffer extract required two more days of incubation than those on extract-amended medium to develop into colonies of size large enough to be counted. In contrast, broth-cultured cells that were harvested at early stationary phase and plated directly, without incubation in soil, displayed no difference in time required for colony development between media with or without soil extract.

Changes in Colony-Localization Fraction.

Like those of most bacteria, the colonies of the *Agrobacterium* used in this study assumed three distinct morphologies in pour-plates depending on their location in or on the agar: surface colonies, interior colonies, and bottom colonies. A change in the quantitative distribution of these colony types was observed over time (Fig. 3.3). The fraction of total colonies that formed on either the surface or the interior of the agar rather than on plate bottoms was approximately 0.88 for all subsamples during the first 5 days of the experiment. Thereafter, the fraction of off-bottom colonies distinctly declined as a greater and greater proportion of the colonies developed on the bottoms of the plates. This decrease in the fraction of colonies forming off plate bottoms was evident with both freshly agitated suspensions (Fig. 3.3, filled circles) and supernatant

subsamples withdrawn after particle settling (Fig. 3.3, open circles) and with plates containing or lacking soil extract. Even during the first 5 days of incubation of the *Agrobacterium* in soil, the fraction of colonies forming above plate bottoms (0.88) was slightly although significantly ($P < 0.05$, by *F* test) lower than the off-bottom fraction (0.94, data not shown) of colonies formed when cells were pour-plated directly from broth-culture. The development of a significantly greater fraction (0.12) of bottom colonies than would be expected due to chance alone (0.06 as determined from plating the soil-free, broth-cultured cell suspension) suggests that these colonies developed from CFUs associated with particles too heavy to be readily suspended in molten agar.

Open circles in Fig. 3.3 represent the fraction of surface plus interior (*i.e.*, off-bottom) colonies to total colonies formed in plates of supernatant subsamples. The filled circles represent fraction of colonies formed off the bottoms of plates poured with freshly-agitated soil suspensions. This fraction appears to remain the same, at about 0.88 to 0.90, for approximately a week for all four cell-recovery protocols. Subsequently, the fraction of off-bottom colonies remained markedly greater for plates poured with the supernatant of soil suspensions that had been allowed to settle for 60 s. The off-bottom fraction of the supernatant samples (Fig. 3.3, open circles) showed a slow, apparently linear decline that contrasted with the rapid, exponential decline of the off-bottom fraction of the freshly agitated subsamples (Fig. 3.3, filled circles).

Addition of soil extract affected the rate and extent to which the fraction of off-bottom colonies declined (Fig. 3.3). The effect of soil extract on the decline of off-bottom fraction of colonies was more strikingly displayed on plates poured with freshly-agitated soil suspensions (Fig. 3.3, filled circles), so only these were subjected to further

statistical analysis (Appendix C). As described above, during the first 5 days of incubation, slightly less than 90% of the *Agrobacterium* colonies were formed off the bottoms of pour-plates. By the end of the experiment (Fig. 3.3A, filled circles) or by the time colonies ceased to be formed in agar plates (Fig. 3.3B, filled circles), only about one fifth of all colonies formed off the bottoms of the pour plates. Neither the initial nor final fractions of off-bottom colonies appeared to vary with the inclusion or omission of soil extract from the agar. The principal effect of the inclusion of soil extract in the plating media appeared to be to increase the time required for the off-bottom fraction of colonies to decline from 0.9 to 0.2. For example, the fraction of off-bottom colonies fell to 0.5 in plates with no soil extract after about 35 to 40 days of incubation of the *Agrobacterium* in soil (Fig. 3.3B, filled circles). Nearly 60 days of incubation were required for the off-bottom fraction to reach 0.5 in plates containing soil extract (Fig. 3.3A, filled circles).

Analysis to assess the statistical significance of the apparent effect of soil extract on the time required for the fraction of off-bottom colonies to reach 0.2 is provided in Appendix C. The nonlinear analysis of covariance (of the "freshly-agitated" soil suspension data) presented in Appendix C showed that the better fit of the data to nonlinear regression equation which included a term that accommodated different rates of decline resulting from the inclusion or exclusion of soil extract (Fig. 3.3A and B, solid lines) for the freshly-agitated samples (Fig. 3.3A and B, solid symbols) compared to the fit of a single regression curve for all the solid symbols pooled (Fig. 3.3A and B, dotted line) was highly significant ($P < 0.001$, by F test).

Comparison of Large Particle Exclusion Methods.

Fig. 3.4 offers a comparison of the numbers of off-bottom colonies in plates of freshly-agitated subsamples (open circles) to the numbers of all colonies recovered when supernatant subsamples were plated (open squares). Our methods offered two ways in which large-particle-associated CFUs could be distinguished from free and small-particle-associated CFUs in this study. The first way, depicted in Fig. 3.1A, separated the large-particle-associated CFUs from the others by allowing the large particles to settle out of the soil suspensions before subsamples were drawn for plating. The other method distinguished large-particle-associated CFUs as those that developed into colonies on the bottoms of pour plates. Fig. 3.4A shows a comparison of counts on media amended with soil extract; Fig. 3.4B compares counts on media without extract.

The high degree of overlap between the squares and circles in the two halves of Fig. 3.4 suggests that the survival kinetics of free or small-particle associated CFUs were approximately the same, regardless of the method used to exclude large particles. Moreover, the presence or absence of soil extract in the agar did not appear to affect the close agreement between the two methods for enumerating non-large-particle-associated cells.

A statistical test for differences in the numbers of cells recovered by the two different methods for excluding large particles is provided in Appendix D. The analysis presented in Appendix D, which accommodates the two-phase decline evident in Fig. 3.4A and B, determined that, in both Fig. 3.4A and Fig. 3.4B, the curve that best fits the squares (all colonies from supernatants of subsamples that had been allowed to settle 60 s) could not be demonstrated to differ significantly (even at the $P < 0.10$ level) from

the curve that best fits the circles (surface and interior colonies from freshly-agitated subsamples).

Interaction between the Effects of Soil Extract and Particle Association.

After a few weeks of incubation in soil, smaller numbers of *A. tumefaciens* A6 CFUs formed colonies on plates if soil extract was omitted from the agar (Fig. 3.1B, lower curve) or if cells bound to rapidly sedimenting particles were removed (by allowing 60 s of settling) from soil suspensions before they were plated (Fig. 3.1B, lower curve). When soil-extract-dependent cells and large-particle-associated cells were simultaneously prevented from forming colonies (Fig. 3.1C, lower curve), the lowest numbers of agrobacteria were recovered. When freshly-agitated (no settling) subsamples were plated without extract, counts did not fall below limits of detection until after day 142. Supernatant (60 s settling) subsamples, however, produced no detectable colonies on medium without extract after day 97. On day 112 either count-reducing effect alone, in comparison to optimal recovery (no settling, with extract), lowered recovery by about a factor of 10 (Fig. 3.1A or B); it might be expected that both effects acting together (60 s settling, no extract) would lower counts 100-fold. However, as can be seen in Fig. 3.1C, no CFUs at all were recovered on day 112 with 60 s settling and no extract: at least a 10,000-fold reduction in counts. Thus, a very great interaction between extract omission and large particle exclusion was obvious after day 100.

In order to determine whether some interaction occurred between the effects of soil-extract omission and large-particle exclusion before 100 days, the data from Fig. 3.1 were redrawn to show only the first 100 days of incubation (Fig. 3.5) and statistically

analyzed (Appendix E). One nonlinear regression model for these curves is depicted by the dotted lines in Fig. 3.5, and a second model, which is identical to the first except for the addition of a term accounting for soil-extract omission and large-particle exclusion interaction, is depicted by the solid lines (Fig 3.5) (model development is fully discussed in Appendix E). Fig. 3.5 shows two phases of decline for all four recovery methods. If the second phase, beginning sometime after 20 days, is examined, it can be discerned that the particle-exclusion effect (the difference between circles and squares) produces an ever-widening ∨ shape formed by the regression curves for counts from both extract-amended (Fig. 3.5, upper pair of curves) and non-extract-amended (Fig. 3.5, lower pair of curves) plates. The regression model that does not include the interaction term (Fig. 3.5, dotted lines) produces ∨'s that open the same amount: these curves imply that particle-exclusion reduces counts by a given, time-dependent function, regardless of the inclusion or omission of soil extract. The other regression model, with the interaction term (Fig. 3.5, solid lines), generates a wider ∨ formed by the curves of the counts on non-extract-amended plates (Fig. 3.5, open symbols) in comparison to the ∨ of the extract-amended plates (Fig. 3.5, solid symbols). The second model implies that supernatant subsamples, from which large particles were excluded, exhibited a greater loss of counts when soil extract was omitted from the plating medium than did the freshly-agitated subsamples (which included particles of all sizes on the plates).

An F ratio was calculated as described in Appendix B to discriminate between the two models and was found to be significant at the $P < 0.025$ level, thus statistically justifying the inclusion of the interaction term. The statistical analysis, therefore, determined that an interaction between the two count-reducing effects was operative

during the first half of the experimental period (up to 100 days) as well as during the second half (100-203 days).

Discussion

This investigation demonstrated the very significant differences in the quantitative recovery of the introduced *Agrobacterium* that resulted from seemingly minor changes in methodology. Not only did each factor alone (the settling of the soil suspension for only 60 s, or the 10% soil extract amendment to the T-Soy medium) prove significant, but so also did their interaction. Although the effects of particle-settling and soil extract amendment and their interaction were significant during the first 100 days of the study, none of these factors dramatically changed the overall pattern of survival of the introduced agrobacterium during that time period. There was a short initial burst of growth, then rapid decline followed by a slower decline. An initial burst is probably temporary reaction to the release of nutrients brought about by the wetting effect of the inoculum (Lynch, 1982). The two decline phases that follow are not unusual for an introduced organism; this pattern has been observed by other workers who have monitored survival for similar periods of time (Macnaughton *et al.*, 1992; Postma *et al.*, 1990a). It has been suggested that the early, rapid decline of dense populations, such as the 10^8 agrobacteria per g of soil used in this study may be caused by protozoan predation (Alexander, 1981; Postma *et al.*, 1990a). As bacterial populations are subsequently reduced, the slower decline that follows is often thought to result from starvation (Postma *et al.*, 1990a). Others have proposed or considered a distinction between short-lived and long-lived populations (Macnaughton *et al.*, 1992; Winstanley *et al.*, 1991).

It was during the second 3½ months of our study that our four variations of the recovery method resulted in marked differences in cell recovery and thereby generated very different survival kinetics curves. It bears emphasis here that the four different enumeration procedures were applied, on any given sampling day, to the same tubes of inoculated soil. Obviously, there was only one density of viable *A. tumefaciens* A6 cells in any given sample. Any differences among the counts produced by the four methods must reflect differential efficiencies of recovery and cannot be attributed to artifactual differences in numbers of agrobacteria in replicate soil samples. Only our optimal recovery method, which involved plating all particle sizes with their associated CFUs on extract-amended medium, produced data that hinted at the establishment of an equilibrium population. Without soil extract, extinction before 130 days would have been assumed. With soil extract but without cells associated with rapidly settling soil particles, the plate counts formed a pattern that could be extrapolated to predict continued decline well beyond the end of this study.

Increasing association of introduced organisms with soil particles over time is a well established phenomenon. Our work with *A. tumefaciens* A6 corroborates the findings of numerous studies (Balkwill and Casida, 1979; Ozawa and Yamaguchi, 1986; Postma *et al.*, 1990b; Postma *et al.*, 1989; and Postma *et al.*, 1991). We looked at particle association in two ways and got complementary results: 1.) by comparing, over time, cell counts obtained from a soil suspension immediately after a 10 min shaking period (while the slurry was still well-suspended) to counts obtained from the supernatant of that same suspension after it had been allowed to settle for 60 s, we determined that an ever-increasing proportion of the CFUs counted were associated with the particulate

matter that sedimented from suspension during that 60 s of settling time; and 2.) by monitoring colony localization in the agar plates, we observed that an ever-increasing fraction of the colonies formed on the bottoms of the petri plates, presumably because of their attachment to "heavy" particles. We consider it likely, considering the density of molten agar, that particles of sizes large enough to augment bottom-colony formation were at least as heavy as the particles that rapidly sedimented when settling of soil suspensions in buffer was allowed for 60 s. Therefore, we assume that the heavier particles that were present in the subsamples drawn from freshly agitated suspensions, but absent from the supernatant subsamples drawn after 60 s, corresponded approximately to the heavy particles that were associated with increased colony formation on the bottoms of plates. Surface and interior colonies were assumed to have developed, for the most part, from CFUs that were not associated with or easily dislodged from particles that sedimented in 60s in the sample tubes.

Hattori and Hattori (1976) extensively discussed the relative importance of soil particles and aggregates to bacterial survival, and indicated that the capillary pores within aggregates greater than about 20 μm in diameter are the most favorable habitats for bacterial life in soil. They also suggested that although bacteria do survive on the outside surfaces of particles, these are probably in a non-growing state. In contrast, bacteria in the capillary-pore environment may be able to metabolize substrates more readily than surface organisms and may even be able to multiply. Postma *et al.*, (1990b) demonstrated that introduced bacteria associated with particles or aggregates greater than 50 μm in diameter survived longer than cells that were free or associated with smaller particles. A consensus exists in the belief that capillary-pore microniches in larger

aggregates offer protective habitats (Heijnen and van Veen, 1991; Postma *et al.*, 1990b). Rough calculations with Stokes' Law suggest that permitting particles in suspension to settle for 60 s probably led to the loss of particles greater than about 30 μm from the supernatant, thereby removing most of the aggregates with optimal survival sites from the supernatant subsamples. Our study also involved only minimal effort to dislodge and disperse CFUs in the samples, and no special steps were taken to disrupt aggregates. Disruption and dispersal efforts have been shown to produce markedly higher counts from soil samples (Hopkins *et al.*, 1991; Macdonald, 1986;) Consequently, we suggest that the aggregate component of our freshly-agitated subsamples contributed more significantly to higher overall counts and increased bottom colony formation than did cells adhering to individual particles of silt or fine sand. Bottom colonies, having formed from particle- and aggregate-associated CFUs, were likely to have arisen from many cells comprising each CFU, whereas colonies growing on the surface or suspended within the agar were more likely to have developed from single free cells or very small clusters. We suspect that efficient dispersion of "heavy" aggregates prior to dilution and counting would have greatly reduced the apparent fraction of cells associated with rapidly sedimenting particles over the course of the entire experiment while increasing the overall counts.

Soil extract in agar medium clearly enhanced CFU recovery. Although this study did not attempt to determine what components of soil extract facilitated colony formation, the two- to four-fold increase in recovery achieved with the soil extract made with Na/K phosphate buffer compared to extract made with water suggests the possible involvement of humic substances. Cations of alkali metals, such as the Na^+ and K^+

of our buffer, interact with humic substances (Kononova, 1961). In addition, the phosphate in the buffer would be expected to complex polyvalent cations, such as Ca^{2+}, Fe^{3+}, or Al^{3+}, that act as flocculating agents joining humic acids into large, insoluble particles (Stevenson, 1982). Certainly, the buffer extract did display the characteristic brown color of humic acid. While soil-extract agar has long been considered an advantageous recovery medium for a broad range of soil organisms (Angle *et al.*, 1991; James, 1959), we had not expected substantial enhancement of recovery of an introduced organism that had been cultured and maintained in the laboratory on T-Soy broth and agar. Interestingly, after day 100, the soil-extract amendment abruptly became essential rather than merely beneficial. Both morphological and metabolic changes are known to occur in bacteria as they adjust to very low nutrient conditions (Fletcher, 1991; Marshall, 1988; Marshall, 1992; van Veen and van Elsas, 1986). The change in dependence on soil extract reported in this study may reflect similar kinds of changes in the *Agrobacterium*.

Subsamples from the supernatant of the suspensions after 60 s of settling, *i.e.*, subsamples missing the large particles and aggregates that settled out in that amount of time, benefitted more from the soil extract amendment than did the freshly agitated subsamples that contained all particle sizes. This interaction was detected by analysis of covariance applied to counts taken between 5 and 97 days of incubation. The same effect was obviously manifest after 100 days by the sudden absence of colonies on plates lacking both soil extract and large particles 1 month before failure of colony formation on the same medium was observed when large particles were included in the plated samples. Formation of colonies on the surfaces and suspended within agar plates was

also enhanced by soil extract, in that the plates poured with supernatant free of large particles had a much greater fraction of surface and interior colonies. The greater enhancement of cell recovery by soil extract for the supernatant subsamples might have been due to enhancement of formation of surface and interior colonies, *i.e.*, colonies that arose from CFUs likely not associated with large particles and aggregates. This phenomenon suggests that the non-particle-associated, or easily-dislodged CFUs had exogenous nutritional and/or other cultural needs for growth on solid medium that differed from those of CFUs that were distinctly associated with large, rapidly sedimenting particles. Apparently, these needs were met, at least to some degree, by soil extract. An extensive body of literature addresses the effects of attachment to solid surfaces on bacterial metabolism (Fletcher, 1991; Marshall, 1976; Marshall, 1982; Marshall, 1988; Stotzky, 1986; van Loosdrecht *et al.*, 1990; Zobell, 1943). To date, the only general theme to emerge from these studies is that attached cells frequently exhibit physiological activity that differs from that of free cells. Our results accord with this generalization.

This study addresses some of the difficulties inherent in population-monitoring methods that rely upon cell dispersion and culturability. As efforts to develop practical and accurate detection and enumeration methods continue, accommodations must be made to changes in the physiological status and degree of particle attachment that organisms may experience during prolonged incubations in soil.

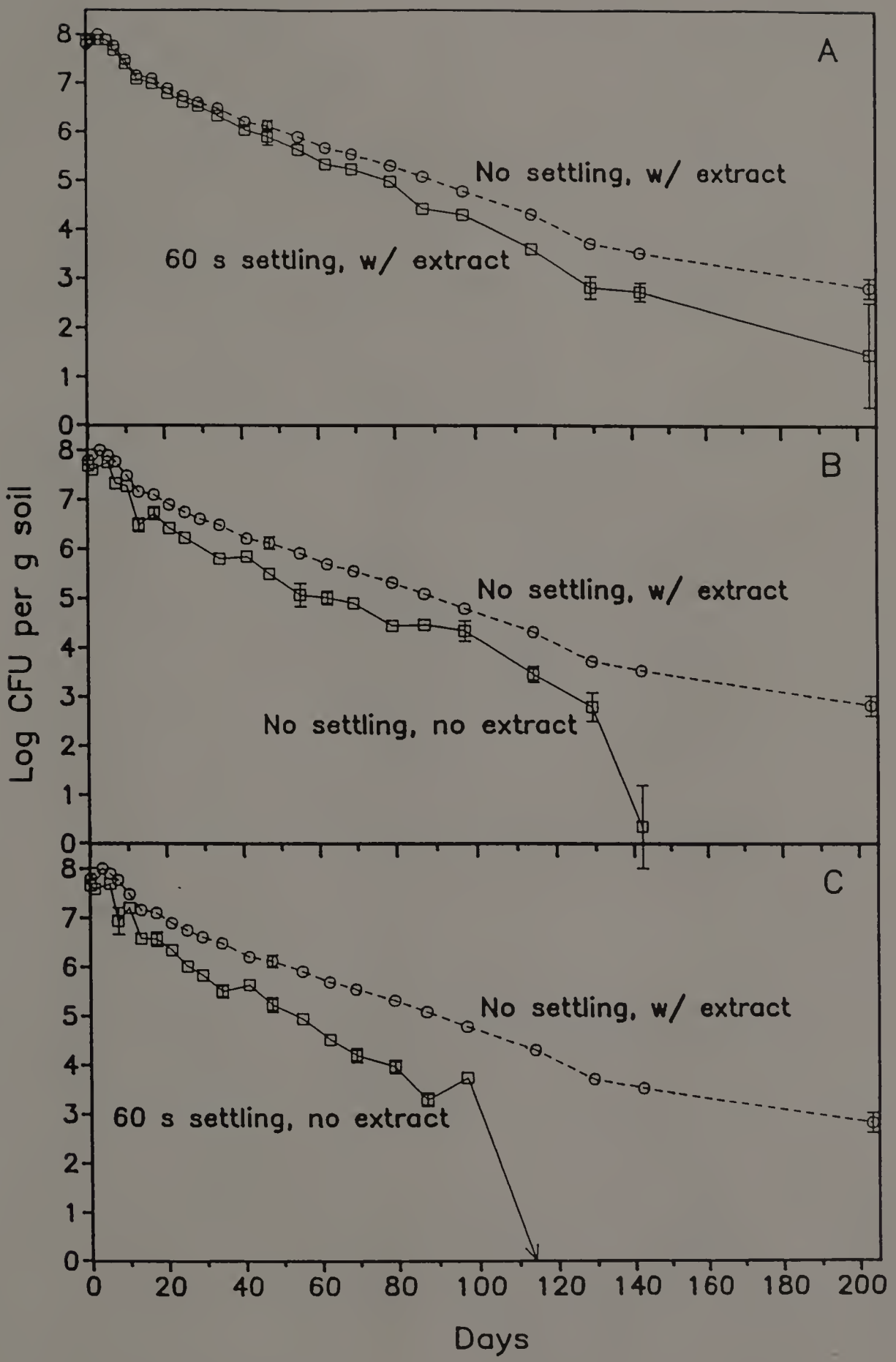

Fig. 3.1 Survival patterns of *A. tumefaciens* A6 inoculated into non-sterile soil as determined by four different recovery methods. The upper curve (repeated in A, B, and C) shows the numbers of cells recovered by an optimized method in which particles were not allowed to settle out of the suspensions before plating on medium that contained soil extract. The lower curves illustrate the effect of excluding particles (A), soil extract (B), or both (C).

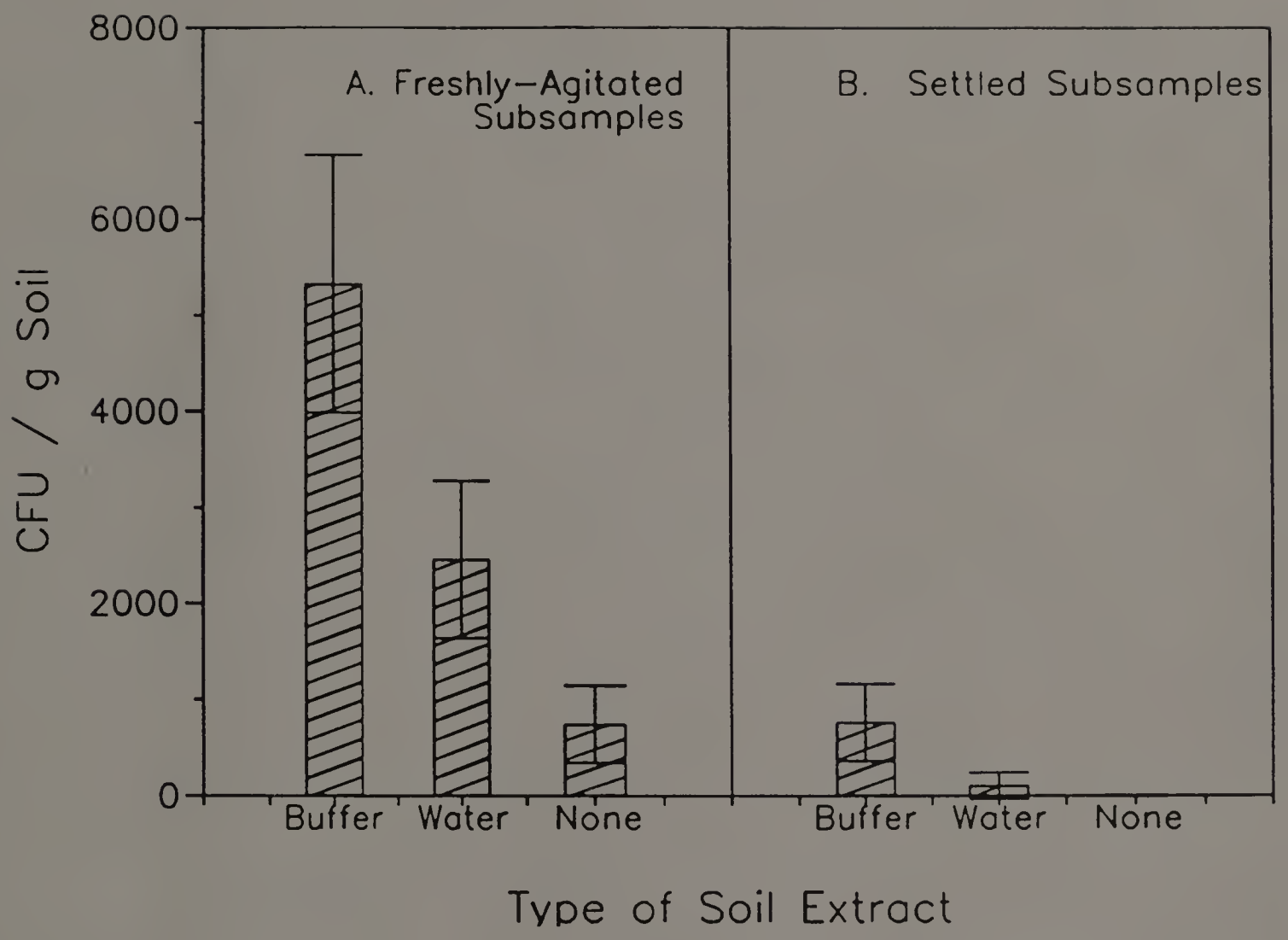

Fig. 3.2 Differential enhancement of recovery of *A. tumefaciens* A6 after 129 days of incubation in soil by the presence in the plating media of extracts of soil made with distilled water or phosphate buffer.

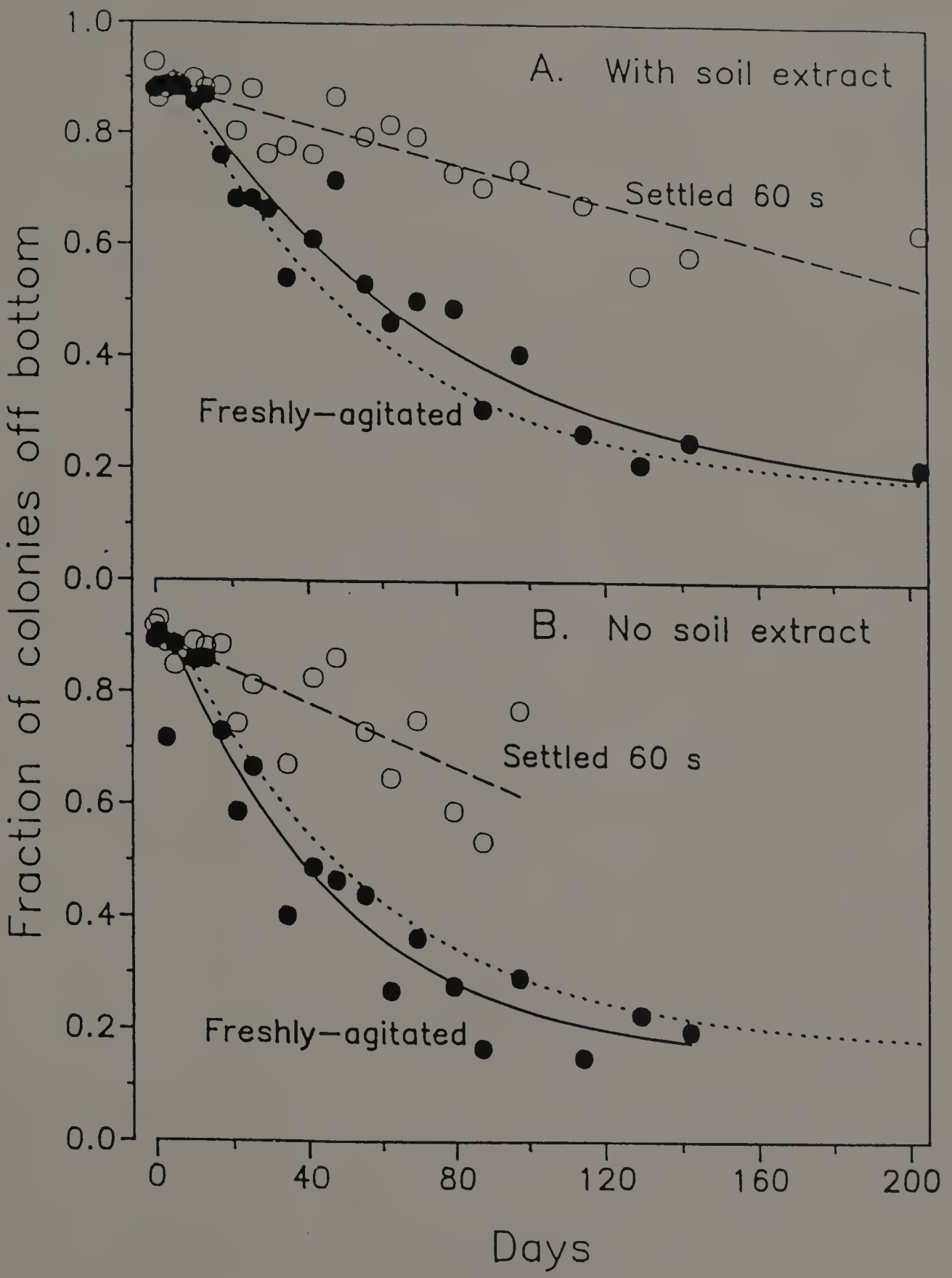

Fig. 3.3 Fractions of total colonies formed on surfaces or within interiors (but off bottoms) of pour plates. The dotted line (same in A and B) is a fit of equation (1)(Appendix C) to all data shown by filled circles (pooled). The solid lines are fits of equation (2)(Appendix C) which accommodates the different rates of decline resulting from the inclusion (A) or exclusion (B) of soil extract. The dashed lines were fit by linear regression.

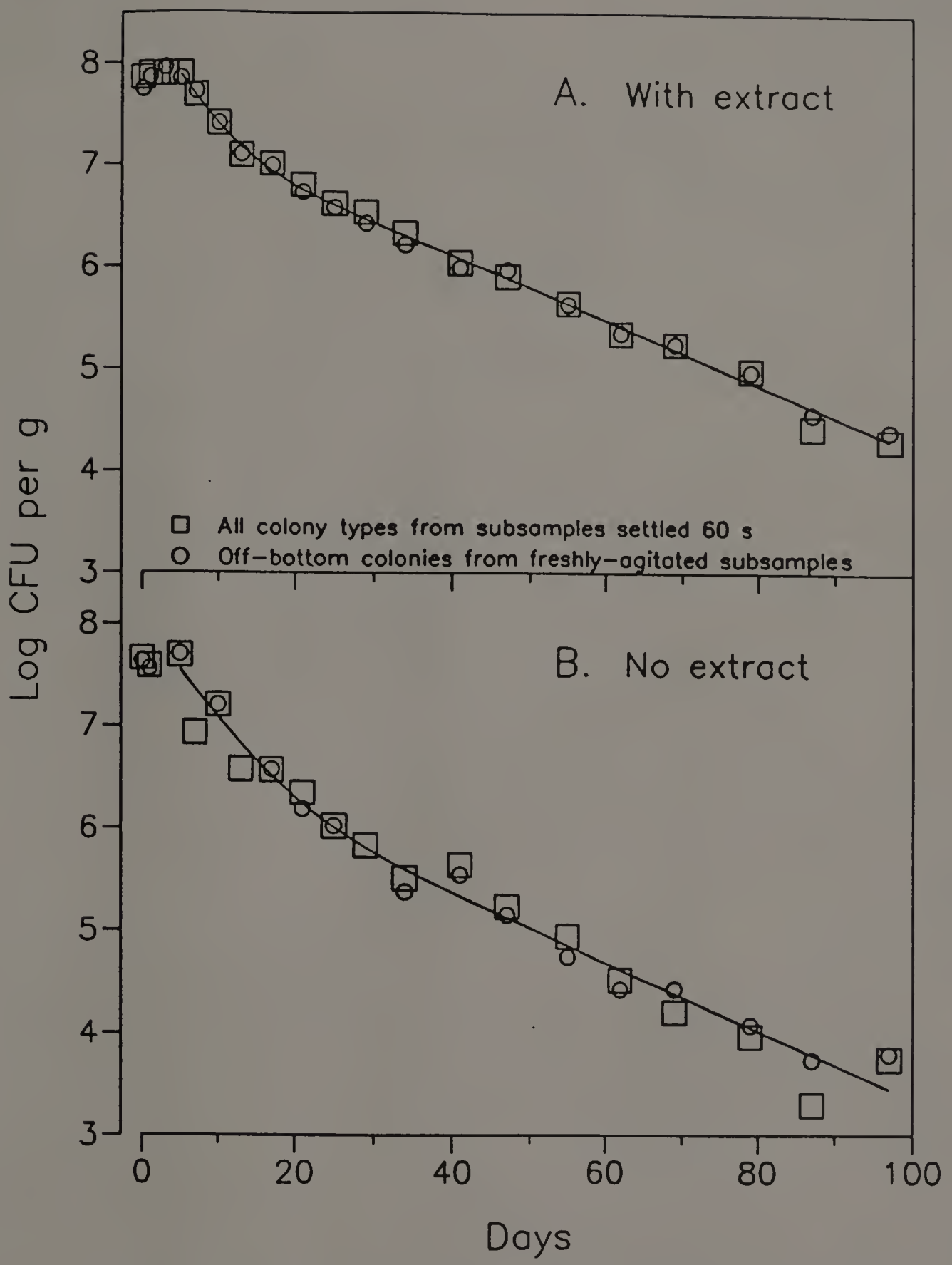

Fig. 3.4 Comparison of the effects of two methods for the exclusion of large particles on the recovery of *A. tumefaciens* A6. Open squares denote free and small-particle-associated CFUs recovered from the supernatants of soil suspensions after settling was allowed for 60 s. Open circles represent CFUs from freshly-agitated subsamples that formed colonies on the surfaces of in the interiors of pour-plates, *i.e.*, that were not associated with particles heavy enough to augment colony formation on plate bottoms.

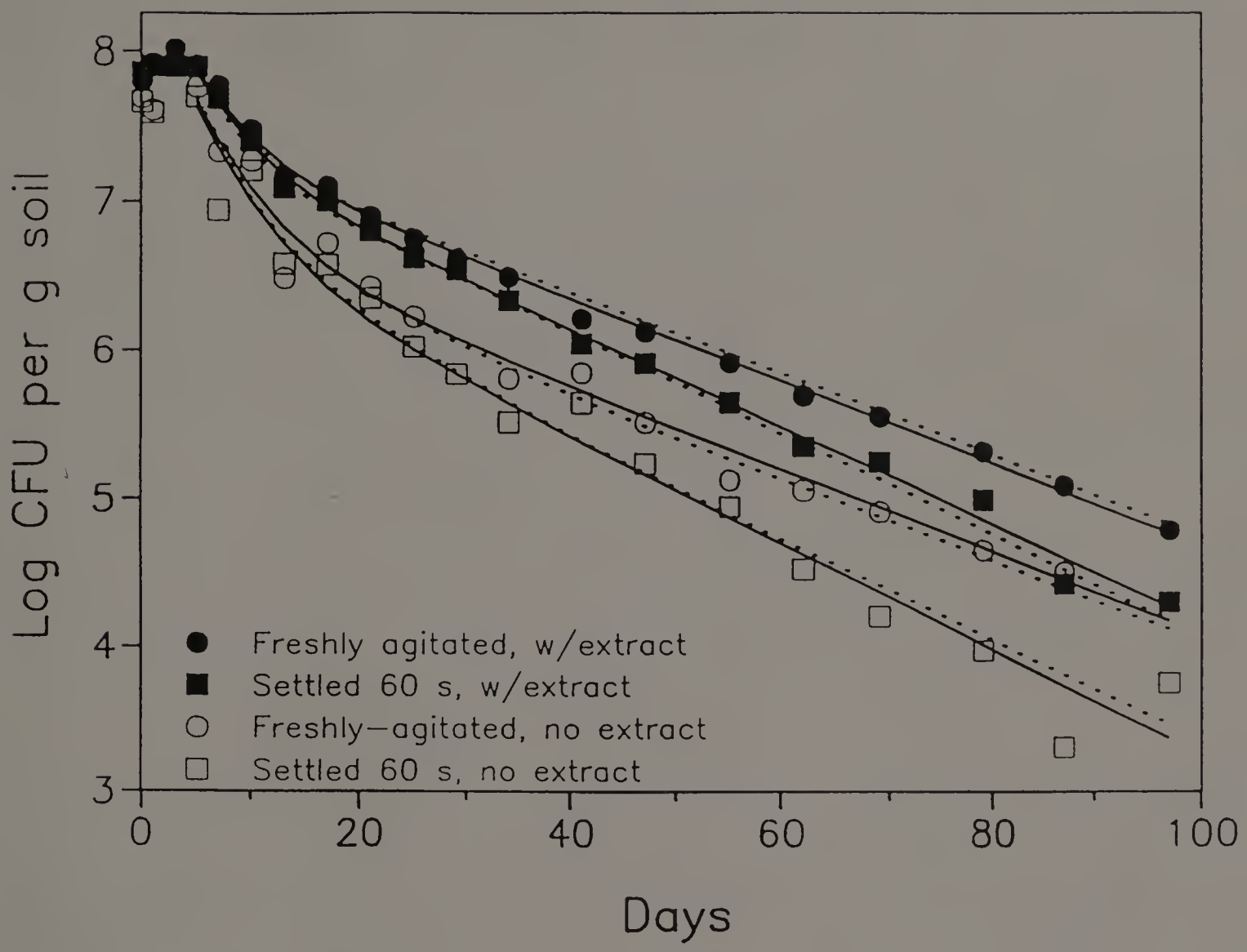

Fig. 3.5. Reduction of recovery of *A. tumefaciens* A6 from soil suspensions by omission of soil extract (open symbols) from the plating media or by removal of cells bound to large particles by permitting settling of soil suspensions for 60 s prior to plating. The curves show fits by nonlinear regression of two different models that include (solid lines) or lack (broken lines) a term for interaction between the effects of soil extract and particle association.

CHAPTER IV

THE INFLUENCE OF INOCULUM DENSITY

Introduction

Impending deliberate introductions of microorganisms into soil for enhancement of crop production, pest control, and biodegradation call for fundamental information about the ecology of introduced organisms. Both the success of an intentional introduction and its ultimate environmental impact depend on the ability of the introduced microorganism to survive, compete, and grow in a pre-established ecosystem. The survival of soil bacteria is influenced by a variety of biotic and abiotic factors such as predation, competition for nutrients, temperature, O_2 tension, moisture level, and the physical matrix of the soil. It is reasonable to assume that introduced bacteria will, at least initially, occur in relatively open spaces and be more vulnerable to stresses than are well-adapted indigenous bacteria (van Veen and van Elsas, 1986).

The population densities of introduced bacteria tend to decrease after inoculation into non-sterile soil, but little has been done toward developing mathematical models that describe and predict these survival kinetics. A few mathematical analyses have provided "rate constants of dying" (Peña-Cabriales and Alexander, 1979) or "half-life" values (Jensen and Sørensen, 1987). Crozat *et al.* (1987), Corman *et al.* (1987), and Postma *et al.* (1990a) have provided functions describing exponential damping to equilibrium population over logarithmically transformed data. Inoculum density is a fundamental

factor to consider in mathematical modeling of survival as well as in field applications. Several different survival patterns for bacteria introduced at varying inoculum densities can be postulated. If the general range of biotic and abiotic factors affecting microbial life in soil tend to impact the entire system in a relatively uniform manner, simulating, in a sense, a "well-mixed" system, these stresses could be expected to affect the introduced organisms in a rather homogeneous way. If all the introduced organisms were uniformly exposed to such influences, they would likely either fail to survive at any population density (non-equilibrated extinction) or survive and equilibrate at some "carrying capacity" (equilibrated persistence); die-out or survival patterns such as these imply that ultimate population density is independent of inoculum density. If, however, in the heterogeneous, discontinuous, and structured environment of the soil matrix, those stress factors impact organisms in a disconnected or non-uniform way, non-equilibrated or partially equilibrated persistence might result. In this situation, final population density is dependent upon inoculum density.

The proposed index of persistence (IP) described in the Appendix A is based on population density integrated over time. There are two conditions that a measure of persistence must meet in order to qualify as an IP as defined there:

> Condition 1.) Strains may have equal values for IP only if they are in some sense equally persistent or equally present over the period for which the IP is defined.
>
> Condition 2.) Values for IP must be independent of initial population density above any minimum threshold density required for establishment.

This particular investigation will be carried out in the interest of satisfying Condition 2. Equilibrated persistence or extinction (inoculum-density-independent phenomena) would establish the situation necessary to satisfy Condition 2 and facilitate the development of the IP described in the Appendix A.

In this study, we observed the influence of inoculum density on the survival of *Agrobacterium tumefaciens* A6 under three different inoculation and recovery regimens.

Materials and Methods

Soil.

The soil used was the same Ninigret very fine sandy loam described in Chapter II and it was prepared and stored as described in Chapter II.

Culturing and Enumerating Agrobacteria.

Rifampicin-resistant *A. tumefaciens* A6 was cultured and enumerated as described in Chapter II. Media was amended with soil extract for some treatments as described in Chapter III.

Soil Extract Preparation.

Soil extract was prepared as described in Chapter III. Only the phosphate buffer extraction was used in these experiments.

Inoculation of Soil.

The inoculation of field-moist and air-dried soil was done in the same manner as described in Chapter II.

Sampling Procedures.

For each experiment, duplicate tubes were sampled for each organism on each sampling day. The inoculated soil in each of duplicate tubes was suspended in 18.5 ml of sterile phosphate buffer to achieve a 1:10 dilution.

For the experiments in which the samples were settled for 60 s and plated without soil extract (Fig. 4.1 A and B), each suspension was vortexed for 60 s, then allowed to settle for 60 s. After the settling period, supernatant subsamples were withdrawn from approximately the top 3 cm of the suspension and transferred to milk dilution bottles of sterile phosphate buffer for serial 10-fold dilution. From each appropriate dilution of each of the duplicate tubes, 3 to 20 replicates (determined according to the number of colonies per plate that was anticipated) were plated in the rifampicin-T-Soy medium without soil extract as described in the section on enumeration of introduced bacteria. Plates were incubated at 30°C for 4 days.

For the "optimized" recovery method (Fig. 4.1 C) each suspension was vortexed for 45 s, and then the tube was placed horizontally on a rotary shaker operating at 400 rpm for 10 min as described in Chapter III. Upon removal from the shaker, suspensions were allowed to settle 60 s. After the settling period, the soil suspension in the sample tube was then re-agitated by hand-shaking and the "freshly-agitated" subsamples were immediately withdrawn and transferred to a dilution series and plated in the rifampicin-T-Soy medium with a 10% soil extract amendment (v/v). From each appropriate dilution of each of the duplicate tubes, 3 to 20 replicates (determined according to the number of colonies per plate that was anticipated) were plated. Extract-amended plates were incubated at 30°C for 3 days.

Results

The influence of inoculation and recovery factors (as discussed in Chapters II and III) can be seen in Fig. 4.1. All three subfigures, A, B, and C, present high, intermediate, and low inoculum densities of *A. tumefaciens* A6, but under different soil inoculation and cell recovery regimens. It is important to note that Figs. 4.1A and B show only the CFUs that were recovered from the supernatants of soil suspensions sampled (*i.e.*, were non-large-particle associated) and were culturable without soil extract (Chapter III addresses these recovery variables). These CFUs would presumably be a subset of the total viable population of introduced agrobacteria. The counts in Fig. 4.1C represent a larger portion of the total viable introduced cells in the soil: the most that could be recovered using methods studied in this investigation. The differences in survival patterns are greater between the inoculation variables (Fig. 4.1A, "field-moist" soil vs. Fig. 4.1B, "air-dried" soil) than between recovery variables (Fig. 4.1B vs. C).

Very rapid decline to below the limits of detection from all inoculum densities occurred when the agrobacteria were introduced into "field-moist" soil (Fig. 4.1A). Cells from the inoculum of 10^9 CFU per g of soil declined at the highest initial specific rate and the 10^2 inoculum declined the most slowly. The population densities in Fig. 4.1A appear to converge to some kind of equilibrium level before dropping below the detection limit. The percentage of change in population between days 0 and 23 was strongly related to inoculum density.

Fig. 4.1B illustrates the survival patterns of agrobacteria inoculated into "air-dried" soil and recovered using the same methods that produced the counts shown in Fig.

4.1A. Semi-logarithmic plots of the CFUs recovered from the low and intermediate-sized inocula were approximately linear with about the same slope. The high inoculum followed two distinct phases of decline: an initial phase of very rapid disappearance lasting for about two weeks was followed by a long period of slower decline. The second phase appears linear as was the decline of the other two inocula, but the rate of decline is faster.

The greatest persistence of cells, at all three inoculum densities, is illustrated by Fig. 4.1C, which shows counts of cells introduced into air-dried soil and recovered according to the most effective method used in this investigation. The overall pattern of the three inocula, relative to one another, is very similar to that of Fig. 4.1B: cells from the two lower inocula disappeared at about the same specific rate, while the high inoculum declined rapidly during the first two weeks (as in Fig. 4.1B) and then entered a phase of slower disappearance during which the specific rate of decline was still greater than that of the two lower inocula. However, the specific rates of decline were markedly lower than those shown in Fig. 4.1B, and CFUs from all three inoculum densities were recovered for a much longer period of time. Sufficient data are also presented in Fig. 4.1C to suggest that a third phase of very slow decline began around day 128 for cells from the 10^8 inoculum. No more soil samples inoculated with 10^5 cells g^{-1} were available for enumeration after day 142. Cells inoculated at 10^2 g^{-1} could not be recovered after day 128.

Discussion

The rapid decline of *A. tumefaciens* A6 introduced into field-moist soil (Fig. 4.1A) probably resulted from intense predation. Water in field-moist soil (approximately 25% in this case) would be held at fairly low water suction and a considerable portion of the capillary pore spaces would be filled with water before inoculation. Under these conditions, the liquid inoculum would remain primarily in the larger soil pores. Van Veen and van Elsas (1986) argued that bacteria introduced into large pore spaces are quite vulnerable to both predation and abiotic stresses. The slower rates of decline evident at the lower inoculum densities (Fig. 4.1A, B, and C) and the slowing rate over time at all three densities may be due to a density-dependent rate of predation, as described by Alexander (1981): the frequency of attack by the predator on its prey is a direct function of the densities of the feeders and the prey. As fewer and fewer agrobacteria remain in the large pore spaces, the rate of their elimination by predation slows. Inoculum density did influence the specific rate of decline of this organism: by day 23, the highest inoculum had declined by 6 log orders, the mid-range inoculum had declined by about 3 log orders, and the low inoculum had declined by a factor of only 30 (Fig. 4.1A) Because no effort was made in this experiment to recover cells that were associated with large particles or culturable only with the soil extract supplement, it is unknown whether a different pattern of survival would have been observed if cells protected within large, rapidly settling aggregates had been included in the counts.

In the experiments with "air-dried" soil (Fig. 4.1B and C) specific rates of decline were partially influenced by inoculum density over the duration of our investigation. In both cases (Fig. 4.1B and C) semi-logarithmic plots of cells introduced at densities of 10^5 and 10^2 cells per g fell along straight lines of similar slopes. However, the curve for the inoculum of 10^8 cells per g was shaped differently. The rate of decline was faster than that of the lower inocula, at least until population densities had decreased to about 10^4 CFU per g of soil, and two different phases of the decline were observed. The first phase was of very rapid decline from 10^8 to 10^7 CFU per gram, followed by a somewhat slower decline from 10^7 to approximately 10^4 CFU per gram. The main force influencing these rates of decline is likely to have been density-dependent predation, as it likely was for high inocula in field-moist soil. The intensity of predation was probably attenuated by a greater degree of enclosure of cells in protected microsites as a consequence of inoculation into air-dried soil.

We saw no evidence to suggest convergence of the three initial population densities in the air-dried soil (Fig. 4.1B and C) to some stable equilibrium density as reported by Crozat *et al.* (1982, 1987) and Corman *et al.* (1987). These investigators observed decline of higher inoculum densities (approximately 10^6 and 10^5 CFUs per gram of soil) and growth of a lower inoculum density (approximately 10^3) of a strain of *Bradyrhizobium japonicum* to reach an equilibrium density after about 45 days of a little more than 10^3 CFUs per g of soil. Numerous differences between our experimental design and those of Crozat *et al.* (1982, 1987) and Corman *et al.* (1987) could account for the differences between our results. The studies involved different organisms in different soils. Crozat *et al.* (1982, 1987) enumerated organisms using a fluorescent-

antibody technique and reported significant difficulty discerning cells from organomineral particles at cell densities of 10^3 or less per gram of soil, and used a bioassay based on soybean nodulation for their final estimation of population density. Crozat *et al.* (1982, 1987) subsampled from a soil inoculated and mixed in bulk, whereas we inoculated and sampled individual portions of soil. They did not indicate the initial soil moisture level at the time of inoculation.

Postma *et al.* (1990a), using an experimental design that more closely resembled ours (individual soil portions, inoculation into air-dried soil, and selective plating on antibiotic-amended medium), observed decline of rhizobia from densities of 10^8, 10^7, and 10^6 CFUs per gram of soil to equilibrium densities that differed significantly, with higher inocula producing higher final populations. They attributed rapid early decline to a rapidly acting factor such as predation, and the slower later decline to a slowly acting factor such as starvation.

A short-term experiment (21 days) by Dupler and Baker (1984) with different inoculum densities in soil showed a greater initial burst of growth from an inoculum of 10^5 cells per gram than from an inoculum of 10^7 cells per gram (growth of introduced cells immediately after inoculation is discussed in Chapter II). Winstanley *et al.* (1991) followed four inoculum densities of *Pseudomonas putida* strains in lake water for 28 days and concluded that, in some cases, their higher inocula were approaching different equilibria, thus indicating that final population densities were dependent upon inoculation density.

Of the limited number of investigations of the influence of inoculum density, some report final populations independent of initial inoculum size, while others report

dependence. Our results are inconclusive. The duration (203 days) of our longest incubation (Fig. 4.1C) was insufficient to permit us to characterize the ultimate fate of the *A. tumefaciens* A6 cells introduced at the highest and intermediate inocula. Convergence to a single equilibrium density, convergence to different equilibria, or extinction could have occurred. The failure of the cells from the 10^2 to persist may have occurred because this inoculum was below a threshold density necessary for establishment rather than because of an inherent inability of the organism in this system to persist at an equilibrium density. An inoculum of 10^3 cells per g might have grown and reached an equilibrium. More rigorous and exacting efforts to disperse cells associated with soil aggregates and to meet more fully the culture requirements developed by cells over long periods of incubation in soil might recover more CFUs than even the most efficient methods reported here. If better cell recovery could be achieved, even slower rates of decline than those exhibited in Fig. 4.1C might be detected. A recovery protocol improved by such efforts might also reveal whether the real viable populations of Agrobacteria would converge to an identical equilibrium density, or establish different equilibrium densities dependent upon inoculum densities, or continue to decline to non-equilibrated extinction.

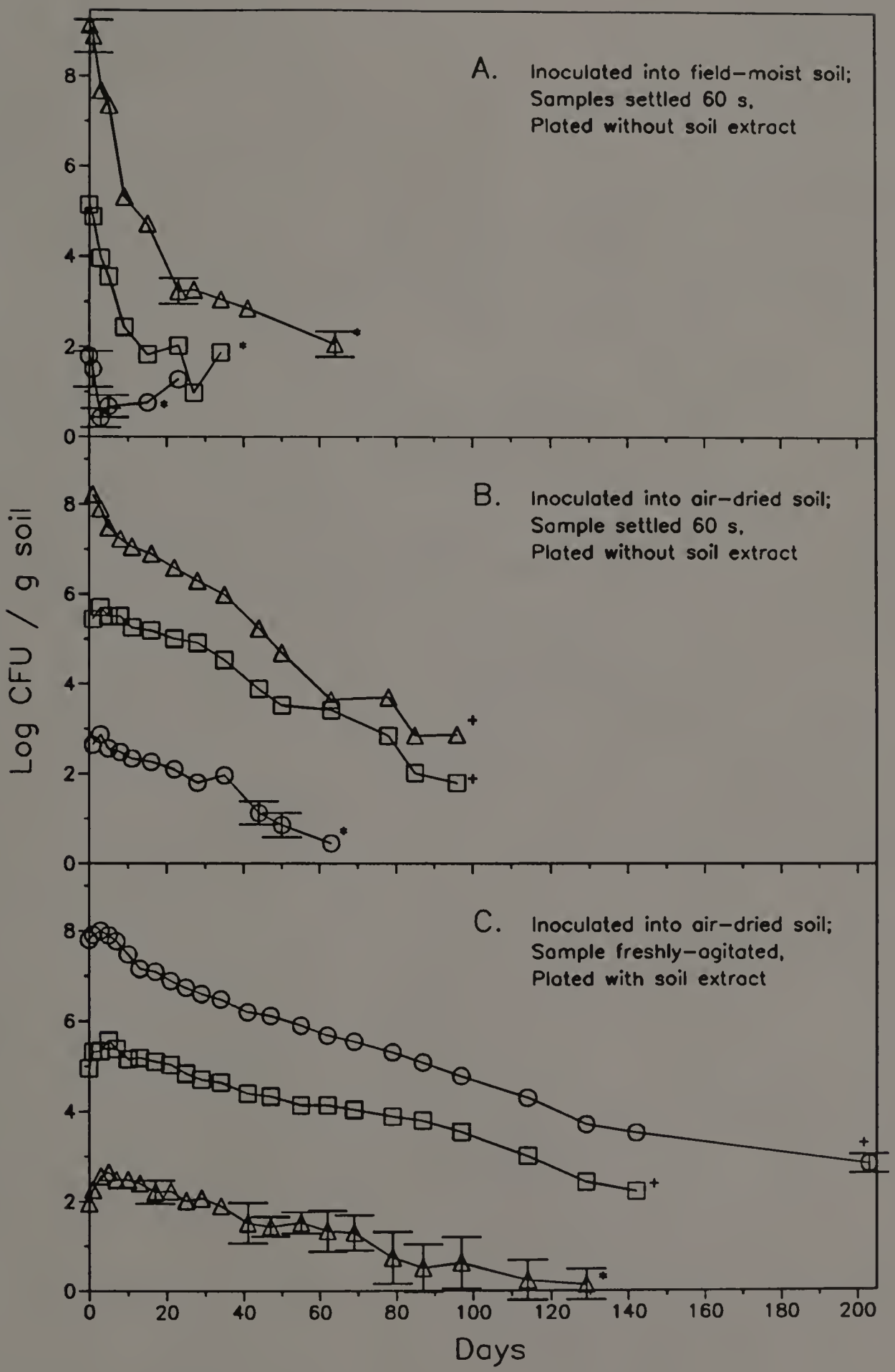

Fig. 4.1 Apparent survival at three inoculum densities of *A. tumefaciens* A6 introduced into soil using three different inoculation and recovery regimens. (*) CFUs not detectable after last sample day. (+) CFUs no longer enumerated after last sample day.

APPENDICES

Appendices A through E were written by Stephen Simkins, University of Massachusetts, Amherst, Massachusetts and are included with his permission.

APPENDIX A

A WORKING DEFINITION OF INDEX OF PERSISTENCE

The index of persistence we wish to develop would not be a universal property of a strain applicable to all soil types, but would apply only to a given, reproducible environment. In addition, the IP would quantitatively describe persistence over a specified period of time. We do not wish to place over optimistic restrictions on our development of an IP. However, there are two conditions that an IP must satisfy to be useful for our work under the other subobjectives of our collaborators at Michigan State University:

> Condition 1. Strains may have equal values for IP only if they are, in some sense, equally persistent or equally present over the period for which the IP is defined.
>
> Condition 2. Values for IP must be independent of initial population density.

Other properties that an IP must exhibit will be satisfied as a matter of course during its development, such as a requirement that a minimum of human and material resources should be consumed per IP measurement. Another desirable but, for our purposes, not essential property of an IP would be if it were possible to calculate values for IP_∞, i.e., a measure of the persistence of an introduced organism in the truly long run.

The first condition required of an IP is designed to prevent the assignment of identical values for IP to strains that persist to degrees that differ in any quantifiable manner. For example, it would be undesirable to assign an IP of 0 to any strain that falls below levels of detection by the end of the period specified for the IP, even if the strains die off at different rates. It would be equally undesirable to give an IP of 1 to any strain that appeared to reach an equilibrium population
density by the end of the period if different strains stabilized at different densities. Several candidates for an example of an IP were considered during the preparation of this proposal that failed to meet the first condition. In the following discussion, we will use the term "index of presence" to refer to indices that at least meet condition 1, irrespective of their ability to meet condition 2 for an index of persistence.

A simple yet defensible index of presence of a strain in an experimental habitat, would be to use something like the average number of cells present over all sampling times. A better index of presence might be obtained if the average were weighted to compensate for unequal intervals between sampling times. An even more satisfactory index of presence might be obtained by fitting a curve through a log cell number vs time plot and integrating the resulting expression (converted back to arithmetic axes) from $t = 0$ to $t = t_f$, the final sampling time. Thus, if we have an expression for population density as a function of time, B(t), then we have

$$\text{Index of presence} = \int_0^{t_f} B(t)dt$$

This expression is not ready for use as an index of persistence, because it does not necessarily meet condition 2, that an IP must be independent of initial population density. In fact, most mechanistic expressions for population density as a function of time with which the authors are familiar arise as solutions to a differential equations which depend intimately for their solutions upon the choice of B(0), and which cannot be solved unless that initial condition is specified.

We circumvent this problem by seeking a value for B(0) that is not an actual experimental inoculum density, but is instead some kind of "idealized" initial population density, Bi, that can be calculated from experimental data. Fig. A.1 shows three different patterns of population dynamics that might result following the introduction of a GEM strain into soil. Fig. A.1A shows the pattern we expect to observe most frequently: the introduced microorganisms die following population dynamics that can be approximated by exponential decay kinetics. An initial lag preceding this onset of exponential decline or the presence of an initial period of greater specific death rate that eventually stabilizes do not represent a essential differences from the pattern of kinetics shown in Fig. A.1A. The important property of this pattern of population dynamics is that the specific death rate is independent of initial population density. This type of microbial dynamics can be regarded as nonequilibrated extinction because the cells eventually die at a specific rate that is not density dependent

Fig. A.1B illustrates the population dynamics exhibited by a strain that stabilizes at an equilibrium density in the soil. The important property of this pattern of population dynamics it that the steady state population density is independent of inoculum density. In other words, the cells eventually persist at an equilibrated density. A pattern of population dynamics intermediate between the inoculum-size-independent extinction of Fig. A.1A and equilibrium in Fig. A.1B is shown in Fig. A.1C. Here, there appears to be an equilibrium trajectory followed by the population density. If low cell numbers are initially introduced, they may grow initially before entering exponential decline. A high inoculum density may rapidly decay before the decline slows. The characteristic feature of this type of decline kinetics is that high and low cell densities gives rise to log cell number vs. time plots that eventually overlap, i.e., the bacteria eventually die out, but their population levels on the way to extinction are equilibrated. If new inputs of organic carbon are not supplied to soil samples, all apparently stably persisting populations (cf. Fig. A.1B) must be slowly declining as the reserves of carbon in the soil are exhausted that maintain the soluble nutrient pool that they depend upon. Thus, in very long experiments, only the patterns shown in Figs. A.1A and A.1C would be expected to be observed, unless the introduced organism is a spore former.

For the two equilibrated patterns of population dynamics a useful choice for a Bi value with biological meaning is found by extrapolating the equilibrium pattern back to $t = 0$, as shown in Figs. A.1A and A.1B with dashed lines. Using this choice for an idealized value for initial population density, we obtain a function for B(t) that can be integrated over the time period of interest to obtain expressions for IP with units of cell hours per g. Their use may be justified because they are good approximations of integrated population density for dynamics following inoculation at levels in the vicinity of Bi.

For strains that follow nonequilibrated extinction kinetics, there is no such "natural" value for Bi. Non-zero values for Bi are needed to satisfy the first condition placed on an IP: that such a measure be equal for two strains only if those strains truly are equally persistent. If all strains with non-equilibrated extinction kinetics were assigned $Bi = 0$ and, hence, $IP = 0$, this condition would be violated for two strains with very different specific rates of decline. It would be better to assign $IP = 0$ to strains that can not be reisolated immediately following their inoculation into

soil. We should recognize that some strains may actually equilibrate at an equilibrium density or a low specific rate of decline at densities below the experimental limits of detection for those strains. These strains would possess a Bi but we would not be able to measure it. The possible existence of undetectable Bi values offers possible justification for basing a choice of Bi on the lowest values for Bi that could experimentally be detected. For example, all strains undergoing nonequilibrated extinction could be given Bi equal to the highest of all the limits of detection for the various strains used in a particular study.

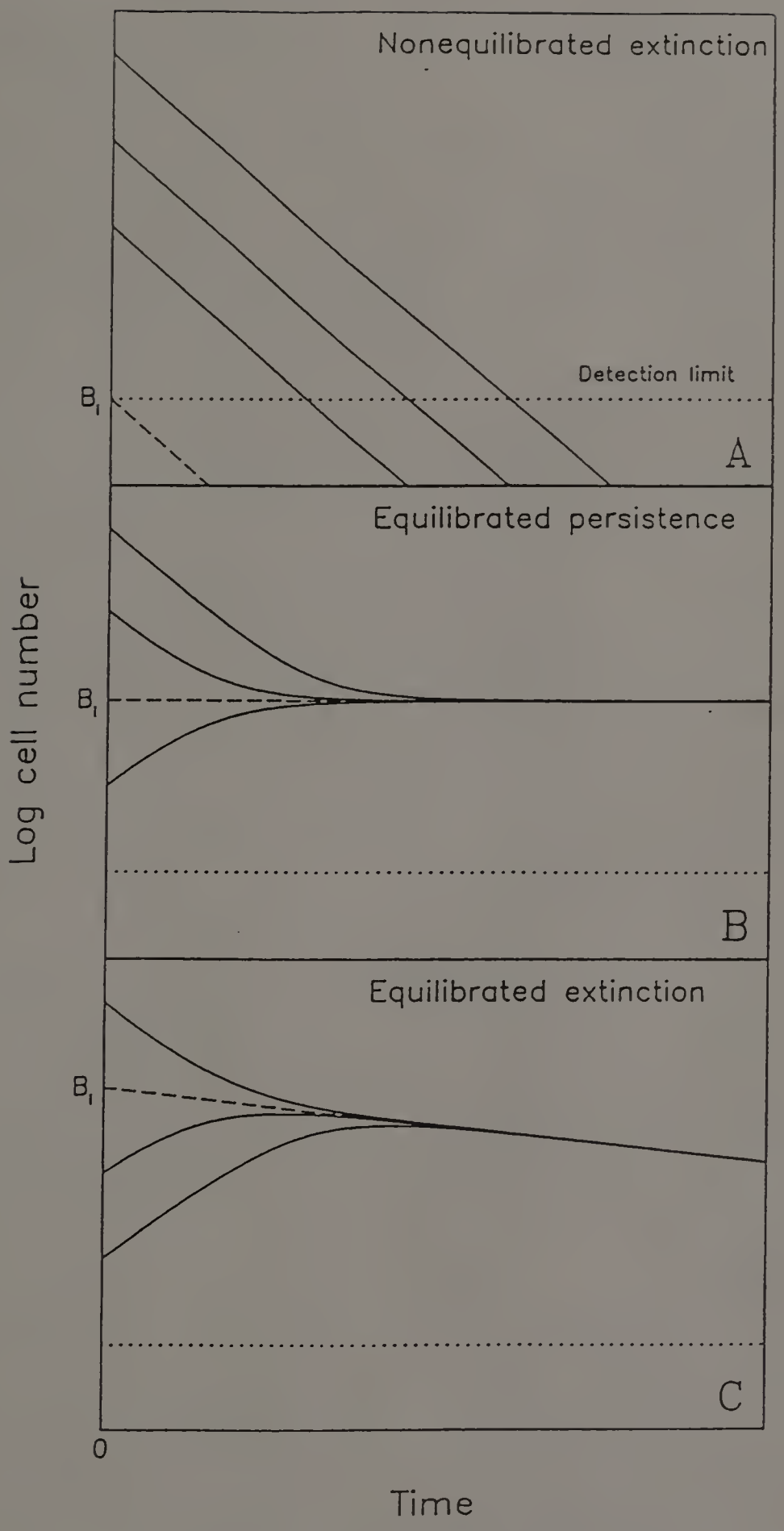

Fig. A.1. Possible patterns of survival kinetics.

APPENDIX B

METHODS FOR THE STATISTICAL ANALYSES

All statistical analyses were performed using a single-equation, multivariate nonlinear regression program (SINGLEQU) written and provided by Stephen Simkins, University of Massachusetts, Amherst, MA. The program uses the method of Marquardt (Bard, 1974) to estimate those values for the parameters of a nonlinear model that minimize the sum of squared residuals. The models fit by SINGLEQU may be multivariate in containing up to 12 independent variables (and 32 parameters). However, each model is only a single equation; the model must be a single-valued function that returns one predicted, dependent value for each unique set of values for the independent variables (and parameters) supplied to it.

The results of two or more regression analyses were often compared using an *F* test to discriminate between two potential models of best fit using a procedure described by Bard (1974). When two alternative nonlinear models containing different numbers of parameters (m_1 and m_2) leave different amounts of unexplained variation (expressed as residual sums of squares, RSS_1 and RSS_2), an *F* test can be applied to see whether the model with more parameters fits the data more closely than the less highly parameterized model to a degree that is more than would be expected by chance alone. If $RSS_1 < RSS_2$ and $m_1 > m_2$ (*i.e.*, if model 1 fits the data better than model 2 but has more parameters so a closer fit is expected of model 1), then the following *F* ratio can be constructed:

$$F = \frac{(RSS_2 - RSS_1)/(m_1 - m_2)}{RSS_1/m_1}$$

As the formula above suggests, the *F* ratio is associated with $m_1 - m_2$ degrees of freedom in the numerator and m_1 degrees of freedom in the denominator. A significant value of *F* calculated from the above formula supports the use of the model with more parameters and the closer fit to the data. If the value of F is not statistically significant, then the use of the more highly parameterized model cannot be defended even though it gives a tighter fit to the data. This use of the *F* test is closely analogous to the way that *F* tests are used in multiple linear regression to determine how many independent variables may justifiably be included in a model or how many polynomial terms may be included in a curvilinear model. Unlike its application to linear models, the use of this *F* test to discriminate between nonlinear models gives only numerically approximate results (Bard, 1974). To compensate for the uncertainty associated with this ratio, a value for *F* calculated to compare two nonlinear models will only be accepted as statistically significant in this work if it exceeds the value for *F* tabulated to be significant at the $P < 0.025$ level.

APPENDIX C

NONLINEAR ANALYSIS OF COVARIANCE TO TEST FOR DIFFERENCES IN RATES OF DECLINE

To assess the statistical significance of the apparent effect of soil extract on the time required for the fraction of off-bottom colonies to reach 0.2, the data shown by filled circles in Fig. 3.3 were fit to a model of the form:

$$\phi(t) = \phi_d e^{-\alpha t} + \phi_\infty \qquad (1)$$

where $\phi(t)$ is the fraction of *Agrobacterium* colonies forming off the bottoms of pour plates, ϕ_∞ is the final fraction of off-bottom colonies, ϕ_d is the amount by which the fraction of off-bottom colonies will decrease over the course of the experiment, α is a rate constant, and t is time. Equation (1) can fit data showing an exponential decline to an asymptotic value and is, thus, suitable for describing the counts (Fig. 3.3) from the portion of the incubation over which decreases in the off-bottom colony fraction occurred. However, equation (1) is incapable of fitting the lag of 5 days at the beginning of the experiment preceding the onset of discernable declines in off-bottom colony fraction. Because the availability of soil extract had no readily observable effect on the duration of this lag phase, no attempt to model the lag was made. Instead, equation (1) was fit only to plate counts obtained after 5 or more days of *Agrobacterium* incubation in soil. Separate fits (curves not shown) of equation (1) were made by nonlinear regression to the data (after day 5) in the two halves of Fig. 3.3. These fits gave values for α ($\pm$ one asymptotic standard deviation of estimation) equal to 0.013 ± 0.003 for the plates with soil extract and 0.026 ± 0.005 for the plates without extract. The tendency of soil extract to retard the decrease in off-bottom colony fraction was reflected in the lower value of the rate constant, α, estimated for the data obtained with soil extract.

A fit of equation (1) to all the data shown by filled circles (for days ≥ 5) in both halves of Fig. 3.3 is shown by the dotted curve in Fig. 3.3. The dotted curve is the same in both parts (A and B) of the figure. For this fit of equation (1), α was 0.019 ± 0.004. Most of the filled circles in Fig. 3.3A, (with soil extract) lie above this dotted curve, whereas most of the filled circle points in Fig. 3.3B lie below the curve. The variation left unexplained by this fit (the sum of squared residuals, RSS) was 0.243 (unitless). Equation (1) was modified to permit the data from the two halves of Fig. 3.3 to be fit using different values for α while sharing the same values for ϕ_d and ϕ_∞, as follows:

$$\phi(t) = \phi_d \exp[-(\alpha_1\delta_1 + \alpha_2\delta_2)t] + \phi_\infty \qquad (2)$$

where δ_1 and δ_2 are "dummy" independent variables that are set to 1 and 0, respectively, for results from plates with soil extract (Fig. 3.3A, filled circles) and are set to 0 and 1, respectively, for data from plates lacking extract (Fig. 3.3B, filled circles). When equation (2) was fit by nonlinear regression to the fractions of off-bottom colonies, the unbroken curves shown in Fig. 3.3 were obtained. The values estimated for α_1 and α_2 by this regression were 0.014 ± 0.002 and 0.022 ± 0.004, respectively. The sum of squared residuals left by this regression was 0.138 (unitless). which represented a significant ($P < 0.001$, by F test) reduction in unexplained variation compared to that left when data in the two halves of Fig. 3.3 had to be fit using a single value for α (RSS = 0.243). When equation (1) was independently fit to the data in the two halves of Fig. 3.3, the total unexplained variation (RSS) dropped only to 0.130, which did not represent a statistically significant improvement in fit over that achieved using equation (2). Thus, the two solid curves shown in parts A and B of Fig. 3.3 do not appear to differ significantly in either their initial values or their final asymptotes.

The analyses using equations (1) and (2) constitute a nonlinear analysis of covariance, customized to test for differences in α between the two halves of Fig. 3.3. These analyses show that the inclusion of soil extract in the plating medium resulted in a highly significant deceleration of the decline in the fraction of *Agrobacterium* colonies that formed off the bottom of the plates. Soil extract, therefore, appeared to enhance the culturability of CFUs that formed colonies on the surface or interior of the agar medium (CFUs that, presumably, were not associated with heavy, rapidly sedimenting particles) for a much longer period of time into the study than was observed when the amendment was omitted.

APPENDIX D

TEST OF DIFFERENCE BETWEEN NONLINEAR REGRESSIONS

A statistical test for differences in the numbers of cells recovered by the two different methods for excluding large particles was performed. The data shown in Fig. 3.4 were fit over the interval from 5 to 97 days using the following, double exponential model:

$$\log_{10}[\, B(t)\,] = \log_{10}[\, B_1 \exp(-\theta_1 t) + B_2 \exp(-\theta_2 t)\,] \qquad (3)$$

where B(t) is the number of cells as a function of time, B_1 and B_2 are the apparent initial numbers of two subpopulations of agrobacteria, one of which declines more rapidly than the other, and the two values of θ are constants describing the rates of declines of the two subpopulations. For example, the slope of the linear portion of the curves after about 50 days in the two halves of Fig. 3.4 is equal to $-\theta_2/\ln(10)$.

Equation (3) was fit by nonlinear regression once to the data shown by open circles in Fig. 3.4A and once to the data shown by the open squares, leaving unexplained residual sums of squares equal to 0.0706 and 0.0505, respectively. These values for residual sums of squares have no proper units, although they might be assigned the ill-defined, but illustrative units of $[\log_{10}(\text{ cells per g })]^2$. All data (both squares and circles) in Fig. 3.4A were pooled, and equation (3) was fit to this combined data set. The unexplained sum of squares (0.1450) left by the regression analysis of the pooled data did not exceed the sum (0.1211 = 0.0706 + 0.0505) of the unexplained sums of squares left by the two separate analyses by an amount that was statistically significant by F test even at the $P < 0.10$ level. This was interpreted to mean that there were no significant differences between the curves of best fit to the circles and squares in Fig. 3.4A. Accordingly, only one smooth curves is shown in Fig. 3.4A corresponding to the fit of equation (3) to the pooled data. The same analysis was repeated for the data presented in Fig. 3.4B with the same general result: the curves of best fit to the squares and circles in Fig. 3.4B could not be demonstrated to differ significantly from one another even at the $P < 0.10$ level. The two methods for enumerating cells not associated with large, rapidly sedimenting particles gave counts that were indistinguishable from one another with the statistical methods described here.

APPENDIX E

TEST FOR THE SIGNIFICANCE OF AN INTERACTION EFFECT

In mathematical terms, we wanted to approximate the numbers of agrobacteria recovered by our best method (using soil extract on plates poured with freshly agitated soil suspensions) with some smooth function, B(t), and to use two other functions to represent the effect of omitting soil extract, $f_s(t)$, and the effect of excluding large-particle-associated cells, $f_p(t)$. Thus, the numbers of cells recovered from freshly agitated slurries on plates without soil extract would be $B(t)f_s(t)$, and $B(t)f_p(t)$ cells would form colonies on plates with extract-containing agar made from soil slurries from which large-particles were removed by settling for 60 s. No great difficulty would be expected in finding functional expressions for B(t), $f_s(t)$, and $f_p(t)$ giving reasonable fits to the data shown in Figs. 3.1A and B. The question of interest would be whether those expressions could be used to predict the lower curve in Fig. 3.1C from the equation $B(t)f_s(t)f_p(t)$. This possibility would require testing against an alternative hypothesis: that some interaction between the effects of soil-extract omission and large-particle exclusion might occur. Model discrimination could be used, as described in the Methods section, to determine whether the numbers of agrobacteria recovered from the supernatants of soil slurries on plates without soil extract could be more defensibly modeled by the expression $f_s(t)f_p(t)B(t)$ or whether an interaction term was needed, for example, $B(t)f_s(t)f_p(t)f_*(t)$, where $f_*(t)$ is the interaction term.

Some note is probably worth taking of the fact that time is explicitly specified as an argument for $f_s(t)$ and $f_p(t)$ because the magnitudes of these effects change markedly during the experiment. Omission of neither soil extract nor large particles seems to affect cell recovery during the first 5 days of incubation (Fig. 3.1), but after 10 weeks of incubation omission of either extract or large particles reduces counts.

In testing for the significance of an interaction between the effects of soil extract and large particle removal, it was decided to use only data collected between days 5 and 97. Before 5 days of incubation, neither factor (soil extract nor large particles) appeared to affect cell recovery (Fig. 3.1). After 100 days, an enormous interaction between these two factors was so unmistakably present as not to be worth testing. On day 112, subtraction of counts associated with large particles reduced cell recovery (Fig. 3.1A) by a factor of about 10 as did omission of soil extract from the plating medium (Fig. 3.1B). One might expect that imposing both of these count-reducing effects simultaneously would reduce cell recovery by about a factor of 100. Instead, when soil-extract-free plates were poured on day 112 from soil suspensions lacking large particles, no colonies were recovered (Fig. 3.1C). To fall below our detection limits on day 112, the numbers of agrobacteria that could be recovered with this treatment must have been at least 10,000 times lower than the numbers of agrobacteria that could be recovered using soil extract and including large-particle-associated cells. Therefore, shortly after 100 days of incubation, strong interaction between the effects of soil extract and particle association appeared. Consequently, we restricted our study of the possible interaction between these two variables to periods of incubation of less than 100 days. Data from Fig. 3.1 on recovery of *Agrobacterium* are redrawn in Fig. 3.5 to show only the first 100 days of incubation. Counts prior to day 5 are shown on Fig. 3.5 but were not analyzed in any of the statistical procedures.

The semilogarithmic presentation of the data in Fig. 3.5 emphasizes the fact that error variance around these curves is stabilized by taking the logarithms of cell numbers per g of soil. Accordingly, logarithms of the models described above also need to be taken. For example, if

B(t) is a function known to describe the numbers of agrobacteria recovered (at any time t) using plates with soil extract poured with freshly agitated soil suspensions, then the log of the number of cells predicted to be recovered without soil extract from soil suspensions lacking large particles would be:

$$\log \text{CFU per g} = \log[B(t)] + \log(f_s) + \log(f_p) \quad (3)$$

If an interaction term, viz., $\log[f_*(t)]$, were required in the above expression it would also be added to the right side of the equation.

All the data in Fig. 3.5 can be simultaneously fit by a single nonlinear model. Each datum shown on Fig. 3.5 can be conceptualized as a single dependent variable ($\beta \equiv$ log CFU per g) that is a function of three independent variables, δ_1, δ_2, and t. The independent variables are time, t, and two "dummy variables", δ_1 and δ_2, that indicate experimental treatment. For counts measured according to a protocol in which large particles are removed (by settling) from soil suspensions before plating, δ_1 is set equal to 1, and $\delta_2 \equiv 1$ for counts on agar lacking soil extract. A value of 0 for either dummy variable denotes an enumeration procedure in which the corresponding count-reducing factor is not operative. Thus, any point in Fig. 3.5 may be symbolized as $\beta(\delta_1,\delta_2,t)$. For example $\beta(1,1,87) \simeq 3.3$ is the open square denoting the lowest cell numbers of any plotted in the figure.

The single model expression that was actually fit to the data by regression was developed from the following set of four equations:

$$\beta(0,0,t) = \log[B(t)] \quad (4)$$
$$\beta(1,0,t) = \log[B(t)] + \log[f_p(t)] \quad (5)$$
$$\beta(0,1,t) = \log[B(t)] + \log[f_s(t)] \quad (6)$$
$$\beta(1,1,t) = \log[B(t)] + \log[f_s(t)] + \log[f_p(t)] \quad (7)$$

Taken together, equations (4) through (7) represent the case where there is no interaction between the effects of soil-extract omission and the removal of large-particle-associated cells. To allow for the possible existence of such an interaction, equation (7) was replaced with

$$\beta(1,1,t) = \log[B(t)] + \log[f_s(t)] + \log[f_p(t)] + \log[f_*(t)] \quad (8)$$

Equations (4) through (7) show in diagrammatic form the structure of the functions used to represent the four broken curves in Fig. 3.5 in descending order, i.e., equation (4) would be fit to the top curve and (7) is the form for the bottom curve.

Equations (4) through (7) can be combined into a single expression by explicitly introducing the dummy variables on the right side of the equality, as follows:

$$\beta(\delta_1,\delta_2,t) = \log[B(t)] + \delta_2\log[f_s(t)] + \delta_1\log[f_p(t)] \quad (9)$$

with δ_1 and δ_2, which can only assume values of 1 or 0, serving to "turn on" those features of the general model that are desired to fit data from a particular treatment. Similarly, equations (4), (5), and (6) can be combined with equation (8) to yield:

$$\beta(\delta_1,\delta_2,t) = \log[B(t)] + \delta_2\log[f_s(t)] + \delta_1\log[f_p(t)] + \delta_1\delta_2\log[f_*(t)] \quad (10)$$

for the model that allows for possible interaction between soil extract and large particles.

Because the motivation for the present analysis was to test for interaction between the effects of soil extract and large particle removal, expressions for B, f_s, f_p, and f_* could be chosen arbitrarily, subject only to the condition that they provide a close fit to the observed data over the time interval (5 to 97 days) of interest. The following functions were found to provide satisfactory fits to the data:

$$B(t) = B_1\exp(-\lambda_1 t) + B_2\exp(-\lambda_2 t)$$
$$f_p(t) = \exp(-\mu_1 t)$$
$$f_s(t) = (1 - \nu)\exp(-\mu_2 t) + \nu$$
$$f_*(t) = \exp(-\mu_3 t)$$

where β is the common logarithm of the number of cells recovered per g of soil; B_1 and B_2 are the apparent initial densities (in cells g^{-1}) of rapidly and slowly disappearing cells; λ_i and μ_i are rate constants; and ν is the (asymptotically approached minimum) fraction of cells that will form

colonies without soil extract. Both f_p and f_* predict ever decreasing counts, but the expression for f_s is formulated to impose a limit, ν, on the fraction to which soil-extract omission can reduce counts. With these substitutions, equation (9) becomes

$$\beta = \log[\, B_1\exp(-\lambda_1 t) + B_2\exp(-\lambda_2 t)\,] - \delta_1\mu_1 t/\ln(10) + \delta_2\log[(1-\nu)\exp(-\mu_2 t) + \nu] \quad (11)$$

and equation (10) may be rewritten as follows:

$$\beta(\delta_1,\delta_2,t) = \log[\, B_1\exp(-\lambda_1 t) + B_2\exp(-\lambda_2 t)\,] - \delta_1\mu_1 t/\ln(10) + \delta_2\log[(1-\nu)\exp(-\mu_2 t) + \nu] - \delta_1\delta_2\mu_3 t/\ln(10) \quad (12)$$

Multivariate nonlinear regression was used to fit equation (11) simultaneously to all the data plotted in Fig. 3.5. The parameter values estimated by this regression were used to draw the curves shown with broken lines. The regression left unexplained variation (sum of squared residuals) equal to 1.270 $[\log(\text{CFU g}^{-1})]^2$. This regression explained 98.45% of the variation of all the log CFU values in Fig. 3.5 about their mean. Equation (12) was then fit simultaneously to all the data in Fig. 3.5 and left a value of 1.162 for the residual sum of squares. The values estimated by the two regressions for the parameters of these models are shown in Table 3.1. As expected, the model with the greater number (eight) of parameters, equation (12), left less unexplained variation than did equation (11), which had only seven parameters. An F ratio equal to 5.45 (with 1 degree of freedom in the numerator and 59 in the denominator) was calculated as described in the Methods section to discriminate between the two models and was found to be significant at the $P < 0.025$ level. Therefore, equation (12) was regarded as the more statistically defensible of the two models for the description of the data, and the corresponding curves are shown with solid lines in Fig. 3.5.

The only difference between equations (11) and (12) is that the latter includes a term allowing for interaction between the effects of soil-extract omission and removal of large-particle-associated cells. Thus, the results of two nonlinear regressions combined with a test for model discrimination provided statistical evidence that these two factors interact. The nature of the interaction can be deduced either from a careful comparison of the broken and solid curves in Fig. 3.5 or from a consideration of the sign for the value of μ_3 given in Table 3.1 for the parameter values estimated for equation (12). The broken curves depicting equation (11) that pass through filled symbols in Fig. 3.5 become almost perfectly parallel to the broken curves through the open symbols after about 50 days. Both pairs of curves form V-shapes opening down and to the right. The V-shaped pair of broken curves through the open symbols (corresponding to counts from plates lacking soil extract) lies below the corresponding pair of curves through the filled symbols. Otherwise, these two V-shapes are quite similar: the slopes of the lines involved are the same. When the possibility of interaction was introduced into the model by using equation (12) which produced the solid lines in Fig. 3.5, one of the two V-shapes became wider than the other: the V is wider for the open symbols than for the closed symbols. The width of opening of one of these V shapes is a reflection of the degree to which removing large particles reduces the number of colonies formed. Because the open symbols fit by the wider V shape correspond to counts on plates without soil extract, more counts appear to be lost from soil suspensions through settling of large particles when those suspensions are plated on media without soil extract than when plated on media with the extract. When both of these CFU-reducing effects are simultaneously at work, cell recovery is reduced by a significantly greater amount than would be expected in the absence of an interaction between them. The positive sign for the value of μ_3 in Table 3.1 conveys the same information. In equation (12), the final, interaction term, $-\delta_1\delta_2\mu_3 t/\ln(10)$, is added onto the rest of the model only when $\delta_1 = \delta_2 = 1$, i.e., when large-particle-associated cells are removed and the remaining suspensions are plated without soil extract. Since t and ln(10) are always positive, this term will be negative provided $\mu_3 > 0$, and counts are predicted to be reduced by the interaction of the effects of the absences of soil extract and large particles.

Table E.1. The values of parameters estimated by multivariate nonlinear regression for the fit of two models to data on the decline of populations of *Agrobacterium* A6 over a period of between 5 and 97 days after inoculation into soil. All values are shown plus or minus one asymptotic standard deviation of estimation.

Parameter (units)	Model	
	Equation (11)	Equation (12)
B_1 (10^6 CFU g^{-1})	2.91 ± 1.57	2.39 ± 1.03
B_2 (10^6 CFU g^{-1})	0.292 ± 0.041	0.272 ± 0.039
λ_1 ($days^{-1}$)	0.337 ± 0.084	0.292 ± 0.065
λ_2 ($days^{-1}$)	0.0625 ± 0.0024	0.0633 ± 0.0023
μ_1 ($days^{-1}$)	0.0155 ± 0.0016	0.0118 ± 0.0022
μ_2 ($days^{-1}$)	0.104 ± 0.022	0.135 ± 0.038
μ_3 ($days^{-1}$)	NA[a]	0.00736 ± 0.00301
ν (unitless)	0.192 ± 0.023	0.253 ± 0.036

[a]Not applicable to this model.

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