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General Interest

A Framework for Developing Research Protocols for Evaluation of Microbial Hazards and Controls during Production That Pertain to the Application of Untreated Soil Amendments of Animal Origin on Land Used To Grow Produce That May Be Consumed Raw

LINDA J. HARRIS,[1]*† ELAINE D. BERRY,[2] TYANN BLESSINGTON,[1] MARILYN ERICKSON,[3] MICHELE JAY-RUSSELL,[1] XIUPING JIANG,[4] KAREN KILLINGER,[5] FREDRICK C. MICHEL, JR.,[6] PAT MILLNER,[7] KEITH SCHNEIDER,[8] MANAN SHARMA,[7] TREVOR V. SUSLOW,[9] LUXIN WANG,[10] AND RANDY W. WOROBO[11]

[1]Western Center for Food Safety and [9]Department of Plant Sciences, University of California, One Shields Avenue, Davis, California 95616; [2]U.S. Department of Agriculture, Agricultural Research Service, U.S. Meat Animal Research Center, P.O. Box 166, Clay Center, Nebraska 68933; [3]Center for Food Safety, University of Georgia, 1109 Experiment Street, Griffin, Georgia 30223; [4]Department of Food, Nutrition, and Packaging Sciences, Clemson University, 217 P&A Building, Clemson, South Carolina 29634; [5]School of Food Science, Washington State University, P.O. Box 646376, Pullman, Washington 99164-6376; [6]Department of Food, Agricultural and Biological Engineering, Ohio Agriculture Research and Development Center, Ohio State University, 1680 Madison Avenue, Wooster, Ohio 44691; [7]U.S. Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Environmental Microbial and Food Safety Laboratory, 10300 Baltimore Avenue, Beltsville, Maryland 20705; [8]Department of Food Science and Human Nutrition, University of Florida, 359 FSHN Building, Newell Drive, Gainesville, Florida 32611; [10]Department of Animal Sciences, Auburn University, 240 Upchurch Hall, Auburn, Alabama 36849; and [11]Department of Food Science, Cornell University, 630 West North Street, Geneva, New York 14456, USA

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ABSTRACT

Application of manure or soil amendments of animal origin (untreated soil amendments; UTSAs) to agricultural land has been a long-standing practice to maintain or improve soil quality through addition of organic matter, nitrogen, and phosphorus. Much smaller quantities of these types of UTSAs are applied to land used for food crops than to land used for animal grain and forage. UTSAs can harbor zoonotic enteric pathogens that may survive for extended periods after application. Additional studies are needed to enhance our understanding of preharvest microbial food safety hazards and control measures pertaining to the application of UTSAs especially for land used to grow produce that may be consumed raw. This document is intended to provide an approach to study design and a framework for defining the scope and type of data required. This document also provides a tool for evaluating the strength of existing data and thus can aid the produce industry and regulatory authorities in identifying additional research needs. Ultimately, this framework provides a means by which researchers can increase consistency among and between studies and facilitates direct comparison of hazards and efficacy of controls applied to different regions, conditions, and practices.

The U.S. Congress, through the Food Safety Modernization Act (FSMA) *(81),* has directed the U.S. Food and Drug Administration (FDA) to establish science-based minimum standards for the safe production and harvesting of fruits and vegetables that may be or are typically consumed in a raw form, where such standards would minimize the risk for foodborne illnesses (proposed produce safety rule) *(82).* As stated previously in a companion document *(31),* FSMA includes specific directions to the FDA to address a number of subject areas including development of minimum standards related to biological soil amendments of animal origin and to agricultural water. FSMA also directs the FDA to establish a process to petition the FDA to request a variance from some or all provisions of the rule. Such a petition would be based on information that the variance is necessary and justified in light of local growing conditions. FSMA sets forth procedures, processes, and practices to be followed under the variance that collectively provide the same level of public health protection as the requirements of the produce safety rule to ensure that the unprocessed produce would not be considered adulterated.

The FDA has historically provided food processors with options that would allow them to take an alternative approach for some prescriptive provisions in regulations (e.g., juice hazard analysis critical control point plans: 21 CFR 120.24) *(80).* Alternative approaches must be supported by an assessment of the efficacy of the variant approach to reduce the risk of microbiological hazards for the given situation. For example, alternative standards associated with soil amendments and agricultural water would be appropriately applied to the commodities, conditions, and practices at an individual or group of operations and would be dependent on the data supporting the proposed alternative approach. The current situation is one in which

* Author for correspondence. Tel: 530-754-9485; Fax: 530-752-4759; E-mail: ljharris@ucdavis.edu.
† Except for the first author, all authors are listed in alphabetical order.

broadly applicable policy and standards must be drawn from studies based on disparate experimental conditions and methodologies that are generally difficult or impossible to compare on a study-to-study basis. In addition, even the most comprehensive studies reflect outcomes from only model systems or limited agroecoregions. Standards with prescriptive limits may be either overly restrictive or inadequately preventive of persistence and subsequent transference to crops. The purpose of this framework arises less from blanket deficiencies in perceived prior studies but as a mechanism to facilitate data gathering in a manner that significantly improves comparability and effective decision making relative to risk management.

Problem-solving research is critical to better understand produce safety hazards and to develop measures to minimize them. The primary goal of this document is to provide a framework for designing the type of research studies that would add to our understanding of preharvest microbial food safety hazards and control measures pertaining to soil amendments of animal origin (e.g., manure, urine, and bedding), excluding those of human origin (e.g., sewage sludge, human waste, and biosolids), that have not undergone a validated and verified zoonotic pathogen reduction treatment, i.e., untreated soil amendments (UTSAs). Validation of the efficacy of the treatments used to reduce pathogens before land application of soil amendment treatments or the use of foliar sprays containing watery extracts of UTSAs (e.g., compost or manure teas) were considered beyond the scope of this document, although some sections may pertain to the development of relevant research studies.

A similar document was prepared for designing research studies pertaining to the application of agricultural water to the edible portion of fresh produce *(31)*. Although the overall approach of the current document is similar to that of the agricultural water document, several factors herein apply solely to soil amendments. Numerous additional sources of information are available, including reviews by Franz and van Bruggen *(22),* Pachepsky et al. *(61),* van Elsas et al. *(83),* and Wei and Kniel *(89)*.

Studies that document the survival of foodborne pathogens in UTSAs after application to agricultural land provide an important basis for many elements in this document. The framework presented herein can be used by researchers to design studies and anticipate the scope and detail of data required. This framework also should be useful to the produce industry or competent authorities for their evaluation of the strength of existing data and thus should aid in identifying research needs. Use of the framework in this document could increase consistency among and between research studies and facilitate the direct comparison of hazards and efficacy of controls applied to different regions, conditions, and practices.

Developing appropriate research protocols involves many factors with multiple options, each of which may impact the experimental outcome and thus the applicability of the data. Under ideal circumstances, studies would incorporate generally accepted best practices for each experimental factor. Designing experiments that contain best or better choices is preferred; however, sometimes only good choices are feasible. This document provides an overview of the factors that need to be considered when developing a study that pertains to soil amendments. Suggestions for selecting the generally accepted good, better, and best practices for many of the experimental factors discussed in this document can be found in Table 1 (model system trials) and Table 2 (field trials). Categorization of practices as good, better, and best is highly subjective and may differ with the circumstance(s). Tables 1 and 2 and the discussions in this document are meant to highlight the range of factors that should be considered during the study design process and are not meant to advocate one approach over another. The authors also recognize that some flexibility in these approaches is needed to allow for modifications to study designs as new data emerge or because of site-specific circumstances. Attempts should be made to balance the range of choices for each relevant experimental factor. The study report must clearly articulate justifications for the choices made for design and implementation of the study. The research should be completed under the supervision of and interpreted by one or more experts with strong backgrounds in microbiology and agricultural systems who are fully aware of all applicable regulations. The research should employ appropriate methodologies and techniques.

This document aims to highlight the broad range of factors that may influence the survival of foodborne pathogens in UTSAs before or after application to agricultural land. The persistence of a specific foodborne pathogen is influenced by intrinsic properties of the target microorganism and the soil microbial community, the type and condition of the UTSA, and external factors derived from local agroecological conditions. The collective and specific impacts of these factors on pathogen survival are not precisely understood but should be considered when designing a study because they will influence the interpretation and applicability of the collected data. Experimental evaluation of a wide range of factors often is not possible and may not be necessary. Where feasible, practicable, and representative of commercial practice, researchers should evaluate the condition(s) that will introduce the greatest number of pathogens to the system and would most likely support pathogen survival after application of UTSAs to agricultural land. When the target organism is introduced under these conditions and then declines to a point where it cannot be detected, the study also should account for other conditions in which smaller amounts of pathogens are anticipated or the potential for survival is reduced. The sections that follow provide discussions and reference reports concerning some of the factors that can influence pathogen survival in UTSAs after application to agricultural land; these factors should be considered in the study design.

SOIL AMENDMENTS OF ANIMAL ORIGIN

Sources of soil amendments of animal origin. The animal source of the UTSA to be used in the study and the production or domestic husbandry system in which the

TABLE 1. *Generally accepted good, better, and best experimental designs for model system trials*[a]

Factor	Good design	Better design	Best design
Contained facility (e.g., growth chamber, greenhouse, microplots, lysimeters)	Replicated containers of at least 0.02 m^3 in greenhouse or programmable diurnal growth chamber with means to record or manage soil temp (i.e., temp-controlled pot immersion bath)	Contained microplots in greenhouse or microplots or lysimeters in controlled open-environment research facility with means to record or manage soil temp	Extracted soil core or constructed macroplot retaining soil aggregate and pore structure in controlled open environment with means to record soil temp and UV radiation and monitor microbial community composition
Soil	Soil is consistent with that in the region(s) of intended UTSA[b] application, e.g., texture (clay, sand, silt content), nutrients, moisture, and temp	Soil is from the region(s) of intended UTSA application and represents the expected range of soil texture, moisture, and temp consistent with growing area of interest	Soil is from the region(s) of intended UTSA application, has been recently used in appropriate crop rotation, and represents the expected range of soil texture, moisture, and temp consistent with growing area of interest
UTSA for inoculated studies	UTSA blend represents the composition diversity of intended application	UTSA represents the expected range of age, moisture, and temp exposure consistent with intended application	UTSA is acquired from the source(s) of interest; appropriate documentation of age and treatment is available; UTSA represents the expected range of moisture and temp exposure consistent with intended application
UTSA with naturally contaminated manure	UTSA contains a single or group of indicator organisms appropriate to the pathogen of concern, and those organisms are present in measurable quantities and can be distinguished from other microorganisms present in the UTSA and soil	UTSA contains the pathogen of concern, and those organisms can be distinguished from other microorganisms present	UTSA contains the pathogen of concern at levels comparable to those used for inoculated studies, and those organisms can be distinguished from other microorganisms present
Study organism (contained facility)[c]	Pathogen of concern, or nonpathogen or stably attenuated pathogen with some historical or laboratory-based data to support its use as a surrogate for the pathogen of concern in the UTSA(s) of interest	Pathogen of concern, or nonpathogen or stably attenuated pathogen with detailed historical or laboratory-based data to support its use as a surrogate for the pathogen of concern in the UTSA(s) of interest	Pathogen of concern, or nonpathogen or stably attenuated pathogen with detailed historical, laboratory- or field-based data to support its use as a surrogate for the pathogen of concern in the UTSA(s) of interest when compared with outbreak strains
Strain marker[c]	Selectable stable phenotype (e.g., marker for resistance to antibiotic not used in veterinary or human medicine or to detergents, metals, or pesticides) that is reasonably effective for suppressing the growth of the background microbiota for plate count and enrichment methods	Selectable stable phenotype (e.g., marker for resistance to antibiotic not used in veterinary or human medicine or to detergents, metals, or pesticides) that is effective for suppressing the growth of the background microbiota for plate count and enrichment methods and that allows for easy secondary confirmation of the inoculated strain	Selectable stable phenotype (e.g., marker for resistance to antibiotic not used in veterinary or human medicine or to detergents, metals, or pesticides) that is effective for suppressing the growth of the background microbiota for plate count and enrichment methods and a stable phenotype or genotype that allows for easy secondary confirmation of the inoculated strain
No. of replicates	Two fully replicated trials	Three replicated trials under the full range of conditions expected for intended UTSA application	More than three replicated trials under the full range of conditions expected for intended UTSA application

[a] Categorization of practices as good, better, and best is subjective and may vary with each experimental situation.
[b] UTSA, untreated soil amendment.
[c] In most cases, biosafety level 2 pathogens or organisms containing recombinant DNA will not be approved for use in model trials; however, there may be circumstances under which their use is approved (e.g., controlled research environments such as growth chambers and greenhouses, application of naturally contaminated UTSA). For lists of surrogate organisms, see Harris et al. *(31)*.

UTSA was generated should be specifically defined, because these factors will drive the methods of land preparation and UTSA application and possibly the volume of UTSA applied. Collectively, these factors also may, to some extent, define the related target pathogen risk(s) (see ''Identifying the pathogen(s) of concern in UTSAs'').

Considerations in type and form of UTSA selected for study. UTSAs have numerous sources, including commercial animal production (e.g., bovine, porcine, poultry, ovine, equine, rodent, and fish) and less traditional sources such as zoo animals and urban green waste (which may contain animal waste). The nutrient and microbiological contents of the UTSA depend on several factors, including source animal, animal diet, manure type (e.g., solid, slurry, or liquid), and amount and type of bedding material in the collected manure *(53)*. These and other factors, such as the manure collection and management system and the duration and condition of storage before land application, will affect the nutrient and microbiological contents of the UTSA applied and the subsequent survival of pathogens *(1, 86)*. The moisture content of the UTSA will impact the method of land application and the choice of application equipment (Table 3). Rate of application may be determined by the nutrient content of the UTSA and the soil test values for the current period of application *(53)*. The texture of the soil receiving the UTSA, the amount of organic material present, the method of postapplication incorporation, and local climatic conditions also may affect the persistence of residual pathogen populations *(19, 26, 34, 47, 48, 58, 69)*. For the reasons mentioned above, the study results will only pertain to the specific UTSA type(s), method(s) of application, and production system(s) evaluated and can only be extrapolated to other closely aligned system conditions. Thus, it is important to carefully select the UTSA for use in the study to ensure that it is representative of the actual commercial practice that the study is meant to reflect.

The UTSA may be applied to agricultural land as a liquid, slurry, high-water-content solid, or dried solid amendment *(53)* (Table 3). Solid UTSAs may be used alone or incorporated with other materials (e.g., bedding and green waste). Litter or solid manure may be stockpiled in the open environment or accumulated in large volumes (stacked piles) under cover and protected from weathering. Liquid that has been intentionally separated from the solids portions of manure is classified as a liquid UTSA. The composition and diversity of components of the UTSA may influence the outcome of a study. The study description must include the handling and management system from which the UTSA is sourced, a product analysis (chemical and microbial) of the UTSA, and the range of particulate sizes (solid UTSA) and degree of sediment present (liquid UTSA). When the UTSA being investigated is not homogenous, the range and extent of heterogeneity of chemical and microbial contents must be described. Variations in microenvironments can lead to spurious conclusions that are based only on calculated means.

Analysis of soils and UTSAs. A list of common UTSA and soil test methods is provided in Table 4. In many cases, methods used by commercial or institutional laboratories that specialize in soil or UTSA analysis are cited. The methods listed are current examples; other appropriate methods probably are available. Whatever methods are used, they should be recognized as applicable or appropriately validated for the subject soils or UTSAs. Chemical and microbiological analyses, when done, should be included in the study report.

As part of the nutrient management plan for crop production, a product analysis of both the soil and the UTSA is required in some localities. Chemical analyses for both soil and commercially marketed UTSAs will typically include the determination of nitrogen (nitrate nitrogen), phosphorus, potassium, and a variety of nutrients, including copper, sulfur, and zinc. The microbial analysis for the UTSA may include aerobic bacteria plate count and counts of *Enterobacteriaceae,* fecal coliforms, *Escherichia coli,* and enterococci (Table 4). The level of the target organism should be determined for studies that include naturally contaminated UTSAs. When the study design involves inoculation of the UTSA with a target organism, uninoculated control samples should be analyzed using the medium intended for enumeration or isolation of the inoculum. A general characterization of the indigenous microbial soil community *(84)* and/or indicators such as environmental or commensal *E. coli* and *Enterococcus* spp. *(45)* in the unamended soils may be beneficial to the understanding of subsequent microbiological responses of the amended soils in a study. Other useful measurements include moisture content, pH, soluble salts, total carbon, total solids, and volatile solids (Table 4).

Because of decomposition or exposure to the elements, the aging of UTSAs will influence the nutrient and microbial content and other characteristics that may influence the survival of the target organism(s) *(1, 34, 86)*. Background information on the age of the UTSA will provide important contextual information; to the extent possible, the age of the UTSA should be standardized among replicate trials. In some cases, approximate age will be discernible by the state of decomposition, but because of the variable methods of accumulation in place for UTSA handling and management at the source facilities, usually only a rough estimate of the mean age of mixed UTSAs will be possible. For this reason, use of an UTSA from a source facility with some type of record-keeping system that can provide a reasonably accurate age of the source (or range of ages for a heterogeneous UTSA) would be beneficial. Handling and management systems may incorporate features that constitute pretreatments, and these features should be documented as completely as possible because they may impact survival of inoculated microorganisms. For example, UTSAs subjected to intentional or unintentional aerobic or anaerobic digestion, including any heating before separation of solids and/or lagooning, may have reduced microbial levels, including those of indicator microorganisms such as *E. coli*. The population density of microbiological indicators in the UTSA at the time of application should be determined.

Rate and time of UTSA application. Under commercial practice, the amount of UTSA applied should ideally be

TABLE 2. *Generally accepted good, better, and best experimental designs for field trials*[a]

Factor	Good design	Better design	Best design
Site selection	Experimental field(s) shares some characteristics, moisture, and soil textures with the region(s) of intended UTSA[b] use	Field shares most of the dominant characteristics with the region(s) of intended UTSA use including climate and soil characteristics (e.g., texture, moisture), and commercial UTSA practices are consistent with intended UTSA use	Field is in the region(s) of intended UTSA use, represents the range or dominant expected soil textures, and replicates dominant commercial UTSA practices that are consistent with intended USTA use
Seasons or years	One season applicable to typical practice for more than 1 yr; 1 yr with more than one season	All seasons applicable to typical practice; more than 1 yr	All seasons applicable to typical practice; multiple years
Crop (when applicable)	Single crop or crop type and variety (with detailed justification for selection) applicable to intended UTSA application; crop type examples: almonds, citrus, table grapes	More than one crop type or variety applicable to intended UTSA application OR preliminary data providing scientific basis for selection of most vulnerable single crop type (greatest survival of pathogen of interest), time of year when postapplication survival is greatest, or links to outbreaks	All crop types are applicable to intended UTSA application OR detailed data providing scientific basis for selection of most vulnerable single crop or variety within a single crop (greatest survival of pathogen of interest) or time of year when postapplication survival is greatest
UTSA for inoculated studies	UTSA blend has the diverse composition of the intended UTSA application	UTSA represents the expected range of age, moisture, and temp exposure consistent with the intended UTSA of interest; C:N, constituent analysis, and ''microbiological stability'' are characterized	UTSA is from the source of interest and comes with detailed documentation of age, temp history, and handling treatment; represents the expected range of moisture and temp exposure consistent with the intended UTSA of interest; C:N, constituent analysis, and ''microbiological stability'' are characterized
Naturally contaminated UTSA	UTSA from a single source that contains the target pathogen or a single or group of surrogate organisms appropriate to the target pathogen, which are present at measurable levels after incorporation and can be distinguished from other microorganisms present in the UTSA with selective culture media	UTSA from more than one source that contains the target pathogen or a single or group of surrogate organisms appropriate to the target pathogen, which are present at measurable levels after incorporation and can be distinguished from other microorganisms present in the UTSA with selective culture media	UTSA from three or more sources that contain the pathogen of concern or a single or group of surrogate organisms appropriate to the target pathogen at levels comparable to those used for inoculated studies, and those organisms can be distinguished from other microorganisms present with selective culture media
UTSA application method	Typical of agricultural practices in region	Typical of agricultural practices in region of interest for crop type and crop cycle of interest	UTSA application practices that are typical for all crop types and crop cycles of interest in the region of interest are evaluated
Time of UTSA application and post-UTSA soil management practices	Conditions reflect reasonable air and soil temp and moisture conditions and time point in the crop cycle for the growing area and application of interest; site is managed using agricultural practices that are typical for the region and for crop type and crop cycle of interest	Conditions reflect reasonable air and soil type, temp and moisture conditions, and time point in the crop cycle; site is managed according to most local current post-UTSA cultural practices for the crop(s) of interest	Conditions reflect reasonable air and soil temp and moisture microclimates, soil types, and time point in the crop cycle that are expected in the growing area of interest; site is managed according to all local current cultural practices for the crop(s) of interest
Pathogen of concern	Pathogen is consistently isolated from the animal source of the UTSA(s) of interest (see Table 5)	Pathogen is consistently isolated from the animal source of the UTSA(s) of interest and has been linked to produce outbreaks in region or crop of interest	Pathogen is consistently isolated from the animal source of the UTSA(s) of interest, linked to produce outbreaks in region or crop of interest, and data provide evidence of greater survival in soils

TABLE 2. *Continued*

Factor	Good design	Better design	Best design
Study organism (for field trials involving inoculation)[c]	Nonpathogen or attenuated pathogen with some historical or laboratory-based data to support its use as a surrogate for the pathogen of concern in the UTSA(s) of interest	Nonpathogen or attenuated pathogen with detailed historical or laboratory-based data to support its use as a surrogate for the pathogen of concern in the UTSA(s) of interest	Nonpathogen or attenuated pathogen with detailed historical, laboratory- or field-based data to support its use as a surrogate for the pathogen of concern in the UTSA(s) of interest when compared with outbreak strains
Strain selection (for field trials involving inoculation)	Naturally occurring nonpathogenic strain isolated from fresh manure, OR single strain with some preliminary data on environmental fitness in the UTSA(s) of interest	Two strains with some preliminary data on environmental fitness in the UTSA(s) of interest, OR clear dominant isolate in series of environmental fitness studies in the UTSA(s) of interest	Mixture or cocktail of three or more strains with evidence of environmental fitness in the UTSA(s) of interest, OR clear dominant isolate in a series of environmental fitness studies in the UTSA(s) of interest
Strain marker[c]	Selectable stable phenotype from nonrecombinant laboratory manipulation that is reasonably effective for suppressing the growth of the background microbiota for plate count and enrichment methods	Selectable stable phenotype from nonrecombinant laboratory manipulation that is effective for suppressing the growth of the background microbiota for plate count and enrichment methods and that allows for easy secondary confirmation of the inoculated strain	Selectable stable phenotype from nonrecombinant laboratory manipulation that is effective for suppressing the growth of the background microbiota for plate count and enrichment methods plus a stable phenotype or genotype that allows for easy secondary confirmation of the inoculated strain
Inoculum preparation and incorporation into UTSA	Test strains are cultured to stationary phase in laboratory media, suspended in a neutral carrier, and uniformly distributed into the test UTSA and/or UTSA-amended soil	Test strains are cultured in laboratory media, suspended and held in UTSA subsample or UTSA extract, and uniformly distributing into test UTSA and/or UTSA-amended soil	Test strains are cultured in UTSA subsample or UTSA extract and uniformly distributing into test UTSA and/or UTSA-amended soil
Postapplication sampling plan and no. of replicates	Two fully repeated trials with at least two replicate blocks (that are not immediately adjacent) per factorial treatment	Three fully repeated trials with three or more replicate blocks (that are not immediately adjacent) per factorial treatment conducted at intervals spanning at least one seasonal cycle	More than three fully repeated trials with independent but matched sources of UTSA applied at intervals spanning a two-season cycle, three or more replicate blocks (that are not immediately adjacent) per factorial treatment

[a] Categorization of practices as good, better, and best is subjective and may vary with each experimental situation.
[b] UTSA, untreated soil amendment.
[c] In most cases, risk group 2 pathogens or organisms containing recombinant DNA will not be approved for use in field trials; however, there may be circumstances under which their use is approved (e.g., controlled research environments such as growth chambers and greenhouses, application of naturally contaminated UTSA). For lists of surrogate organisms, see Harris et al. *(31)*.

based on the nitrogen and/or phosphorus content of the material and the soil nutrient test values. Best commercial practice includes characterization of the UTSA and soil nutrients to ensure that the application rate meets but does not exceed the crop needs, given the soil test values for those nutrients at the time of application. When designing a survival study, the rate at which UTSA is applied to the field(s) is also an important consideration and should reflect the high end of the range used in commercial practice. Application of UTSAs is not a finely calibrated process, and higher rates of application are likely to occur at ''hot spots'' in the field. The rate(s) of application selected for the study should consider these potential hot spots, which may lead to higher levels of pathogens applied and/or longer pathogen persistence postapplication.

The study report should detail the equipment used for application, the condition of the soil surface (e.g., presence and relative amount of crop residue), and the method for and depth of incorporation (if applicable). Although it might be tempting in research settings to artificially homogenize the UTSA to reduce variability in aggregate size distribution and to facilitate uniform spreading of target mass per unit area, this action may influence microbial survival. The potential result is an accelerated rate of viability reduction, which should be avoided. The date of other soil and crop management events relative to manure application date (e.g., tillage, planting of cover crops, crop residue retained as cover, transplanting and planting, and type and rate of application for fertilizers, pesticides, and plant protectants) should be included. Photo documentation and georeference

TABLE 3. *Manure application techniques*[a]

Form of manure	Application technique
Solid: may include high-water-content solids, thermally treated and/or dried manure, or stockpiled manure, with or without incorporated bedding or litter (roughly 25% or more solids content)	Spreading onto cropland with a manure spreader; may be followed by incorporation or left unincorporated
Slurry: may include stored manure slurries or pumpable solid-liquid mixtures from storage tanks or lagoons (approximately 10–25% solids content)	Application onto cropland from enclosed tank truck or tank wagon (may be applied by injection directly into soil or by spray distribution onto soil with or without incorporation); pumping and distribution onto cropland by large-diameter sprinkler systems (gun or big gun sprinklers)
Liquid: may include liquids remaining after solids separation or lagoon storage or wastewater (typically less than 10% solids content; lagoon treatment systems typically are less than 1% solids content)	Pumping and distribution onto cropland by a variety of irrigation or other sprinkler systems

[a] Adapted from Miner et al. *(53)*.

coordinates of the application and incorporation may complement written documentation.

Weather event patterns and situations that may positively or negatively impact the survival of pathogens in the applied UTSA should be reported, including the time in days or weeks relative to application of the UTSA. These situations include major attraction of vectors such as flies or wildlife postapplication, major weed and volunteer crop development during the study, and if a crop is planted, major insect pest and disease pressures. Plant development may positively or negatively impact survival of pathogens in the applied UTSA by various mechanisms, including rhizosphere development and shading of soil surfaces that can affect total solar radiation, soil temperature, and soil moisture. Pest damage and disease development in the experimental crop may result in defoliation, frass deposits, significant root rot, or decaying vegetation and thus influence many experimental variables and potentially delay or accelerate inactivation of pathogens. Although limited data currently are available on the nature of these impacts, the trial-specific conditions encountered should be noted for future comparability between seasons and regions.

Time of application (date, time, and season). The date(s) and times of application of the UTSA should be provided in the study report. In some regions and for some crops, there may be a single relatively standard time of UTSA application. In other regions, the time of application may vary by year and current crop rotation. The persistence of enteric bacteria in manure-amended soils can differ according to the time of year, ambient temperature, or soil moisture *(6, 24, 32, 46, 54, 57)*. Some results suggest that survival of these bacteria in soils may be greater during cooler seasons *(6, 36, 46, 77)*. Thus, a study initiated in the summer months is unlikely to represent commercial application of UTSAs in the late fall or winter. The choice of application time should mimic the anticipated commercial practice. When commercial practice is variable, studies should include replicates in all the seasons in which UTSAs are applied under commercial practice or, when data are available, in the season(s) under commercial practice that is expected to result in the greatest pathogen persistence.

MODEL SYSTEMS

Model systems (e.g., laboratory, growth chamber, greenhouse, or microplot) can provide important information about the influence of some environmental variables on pathogen survival in agricultural environments. Such model systems may be needed because biocontainment and decontamination issues severely restrict the use of pathogenic microorganisms in open greenhouse and field-based research. The simulation of produce production environments in model systems to assess the survival of pathogens attributed to manure is extremely challenging, and minor changes in the protocol may have dramatic effects on pathogen survival.

The diversity of soil microbiota complicates the enumeration of foodborne pathogens; therefore, some pathogen survival studies have included use of pasteurized or sterilized soils *(43, 44)*. However, soil microbiota can have a significant effect on the survival of foodborne pathogens. Recently collected field soil is recommended for use in laboratory or greenhouse environments; soil that is pasteurized, sterilized, or frozen before use is usually not appropriate for these types of studies.

The characterization of important soil and UTSA properties (chemical and microbial analysis), as described above, should be included in the study report. Information on how the soil was collected, stored, and sieved (if applicable), the volume of UTSA applied to the soil, and the application method (surface or incorporated) also should be reported. Controlled environmental conditions and experimental factors of model systems do not always allow for the ''normal'' climatic fluctuations of environmental conditions, such as temperature, humidity, wind, rainfall, drying, and solar radiation, or other variables that occur with field studies, which impact the survival of various pathogens in unamended soil and manure-amended soil *(5, 59, 85)*.

The microbiota of water, soil, and plants and certain climate effects are impossible to fully replicate under laboratory conditions. However, a well-designed model

system that simulates natural conditions (e.g., temperature, humidity, soil type, and UTSA) can be used to identify a smaller set of variables to be evaluated in the field. Laboratory studies may be used to evaluate the survival of test microorganisms (see ''Microorganism Selection for Inoculation Studies'') under a range of scenarios and environmental conditions; those promoting the greatest survival are sometimes chosen for investigation in field studies as a way to limit the number of variables that need to be evaluated. The use of model systems alone or in conjunction with field studies should be clearly justified in a study report.

FIELD-BASED STUDIES

As with model systems, field studies of pathogen survival after application of UTSAs face distinct challenges. A review of the literature reveals wide variation in data regarding the persistence of manureborne pathogens in soils. In addition to the inherent differences among bacterial species and even among different strains within a species, this variation is governed to a large extent by the interaction of a number of factors specific to the production environment, including the region, its prevailing climate, and the geospatial characteristics and soil type of the field *(19, 26, 32, 47)*. The prevalence, growth, and/or persistence of pathogens in UTSA-amended soils are affected by seasonal conditions of temperature, rainfall, humidity, UV exposure, and solar radiation intensity *(5, 59, 85)*. To assist in interpretation of the data, when possible environmental factors that could have an impact on pathogen survival should be monitored and reported.

Field studies often incorporate attenuated pathogens or nonpathogenic indicator strains to limit the potential for dissemination of virulent strains into the environment. A list of surrogate organisms that have been used in field trials was provided by Harris et al. *(31)* and is discussed in detail below. Field studies also may use naturally contaminated UTSAs as an alternative to inoculation with laboratory-cultured microorganisms (see below).

Because of the larger scale of field studies, consistency and reproducibility of UTSAs and their application are more challenging, and higher data variability over laboratory-based model studies should be expected. Thus, sampling methods, numbers, and frequency of sample collection (discussed in detail below) should address the variability that is observed in field trials.

Site selection. Survival studies should be conducted in the same region(s) as those of commercial interest. Depending on the study objective, a commercial setting may be the only option; however, research facilities that are capable of replicating the representative commercial environment and management practices of interest also may be used. Some universities, colleges, governmental agencies, and private sector interests offer such field research facilities, often with restricted or limited access. Approval from the institution's biosafety committee is usually required to release certain (sometimes any) microorganism(s) (pathogen or surrogate) into a research field, and the approval process may take considerable time. Regardless of whether the site is a commercial or research field, the study should be compliant with local, state, and national biosafety regulations and legislation regarding the release of microorganisms to the agricultural environment.

The site selected for study should ideally have a uniform soil texture (within practical limits) because significant changes in texture may impact the study outcomes *(48)*. The site also should have topography representative of the targeted cropping region. In cases where a region has both flat and sloping or variably contoured terrain, selection of a flat or leveled site may give more consistent results. UTSA application to soil at sites with a minimum degree of sloping will have a lower potential for surface movement of the UTSA, nutrients, and microorganisms from the area of application under normal weather conditions. In cases where the commercial production in that region is primarily conducted on uneven terrain, replicated blocks of the field site should reflect the range of such variability in grade or terrain. When the UTSA is applied to a site with a high degree of slope (overall or in portions of the field plot), surface runoff of manure, nutrients, and microorganisms is more likely to occur *(2)*. This latter setup also may complicate the sampling regime necessary to measure the survival response of the target organism. Any site variation in drainage will increase the variability in soil moisture across the experimental site, which could have a major impact on the microbial persistence.

Geographic and land use characteristics of the field site and adjacent land that could affect soil conditions or soil microbiota should be noted in the study report. Some of the potential items to include would be (i) crops planted and soil amendments applied over the previous 2 to 3 years for the field site and adjacent land, (ii) areas of the field plot where flooding and/or erosion due to weather events are common, (iii) any occurrence of drainage to public or private surface waters (consult hydrological data in watershed maps), (iv) activities occurring adjacent to the field site that could potentially impact the study (e.g., livestock production facilities, manure storage, sewage treatment plants, human developments, and landfills), (v) whether the land has been fumigated in the past 3 years, (vi) types and levels of wild animal activities or insect pressure that occur on or near the field site at the time the study would be conducted, and (vii) level and duration of shading, such as from trees.

Site selection also should take into consideration the feasibility of protecting the site from introduction of variables that would affect the research outcome and preventing the release of microorganisms outside the study boundaries. Examples could include (i) installation of wind barriers to prevent erosion in some locales, (ii) diversion of runoff to grassland and vegetation barriers to minimize potential for drainage into surface waters, (iii) erection of fences to limit access by livestock, wildlife, and humans, and (iv) implementation of control measures to limit bird access to the site.

Climate. Weather data should be collected daily throughout the study period and compared with historical

TABLE 4. *Physicochemical and biological attributes of soil and soil amendments and methods commonly used to evaluate them*

Parameter	Example methods[a]	Reasoning and potential impact on the presence or survival of pathogens
Nitrate nitrogen	SMEWW 4500-NO_3^-: nitrogen (nitrate); TMECC 04.02-B: nitrate nitrogen determination[b]	Nitrogen content of soil and UTSA[c] may dictate amendment application rate, which will determine pathogen and fecal bacteria load applied to soil
Total nitrogen	EPA method 351.2: total Kjeldahl nitrogen by semiautomated colorimetry *(14)*; SMEWW 4500-N: nitrogen; SMEWW 4500-N_{org}: nitrogen (organic); TMECC 04.02-A: total Kjeldahl nitrogen, semimicro Kjeldahl technique; TMECC 04.02-D: total nitrogen by combustion; USGS method I-4650-03: alkaline persulfate digestion for total nitrogen and phosphorus in water	Nitrogen content of soil and UTSA may dictate amendment application rate, which will determine pathogen and fecal bacteria load applied to soil
Ammonium nitrogen	SMEWW 4500-NH_3: nitrogen (ammonia); TMECC 04.02-C: ammonium nitrogen determination	Nitrogen content of soil and UTSA may dictate amendment application rate, which will determine pathogen and fecal bacteria load applied to soil
Phosphorus	SMEWW 4500-P: phosphorus; TMECC 04.03-A: total phosphorus; TMECC 04.03-B: water-soluble phosphorus; USGS method I-4650-03: alkaline persulfate digestion for total nitrogen and phosphorus in water	Phosphorus content of soil and UTSA may dictate amendment application rate, which will determine pathogen and fecal bacteria load applied to soil
Potassium	SMEWW 4500-$KMnO_2$: potassium permanganate; TMECC 04.04-A: total potassium; TMECC 04.04-B: water-soluble potassium	Potassium content of soil and UTSA may dictate amendment application rate, which will determine pathogen and fecal bacteria load applied to soil
Total organic carbon, organic matter content	EPA method 415.1: total organic carbon *(13)*; TMECC 04.01-A: organic carbon, combustion with CO_2 detection; MSA-CM, chap. 34: total carbon, organic carbon, and organic matter	Measures of the organic content of soil; affects water retention and cation-exchange capacity especially in sandy soils; critical for plants and soil bacteria as a source of energy
Other mineral nutrients and trace elements	MSA-CM, chap. 18: aluminum; MSA-CM, chap. 19: sodium; MSA-CM, chap. 20: magnesium and calcium; MSA-CM, chap. 21: boron; MSA-CM, chap. 23: iron; MSA-CM, chap. 24: manganese; MSA-CM, chap. 26: copper and zinc; MSA-CM, chap. 33: sulfur	Typically determined for soils and manures to correct any deficiencies for plant requirements and/or to determine any accumulations to phytotoxic levels or pollution levels; may impact survival of microorganisms including soil bacteria or foodborne pathogens
Soluble salts (conductivity)	SMEWW 2510: conductivity; SMEWW 2520: salinity; TMECC 04.10-A: electrical conductivity for compost, 1:5 slurry method, mass basis; TMECC 04.15: soluble salts	High levels of soluble salts may negatively impact plant growth and require management as environmental pollutants; UTSAs are a source of soluble salts, which may impact amendment rate; salinity may impact survival of microorganisms in soil *(48)*
Moisture content	TMECC 03.09-A: total solids and moisture at 70 $\pm$ 5°C	Moisture content of soil and UTSA impacts the growth or survival of *E. coli* O157:H7 and other pathogens *(5, 88)*; moisture content of UTSA will determine the application method, which may influence pathogen persistence in amended soil *(69)*
Total solids	TMECC 03.09-A: total solids and moisture at 70 $\pm$ 5°C	Measure of the physical properties of a manure; total solids content of UTSA will determine the application method, which may influence pathogen persistence in amended soil *(69)*
Volatile solids	SMEWW 6200: volatile organic compounds; TMECC 05.01: biodegradable volatile solids; TMECC 05.10-A: volatile fatty acids in compost extract by gas chromatography; TMECC 06.07: volatile organic compounds	Measure of the organic content of manure or soil
pH	SMEWW 2310: acidity; SMEWW 2320: alkalinity; TMECC 04.11-A: 1:5 slurry pH	Important indicator of soil characteristics; soil pH dramatically impacts solubility and therefore bioavailability of all mineral nutrients; pH of UTSA and soils can impact the growth or survival of *E. coli* and foodborne pathogens *(66, 86)*
Soil temp	EPA field temp measurement (SESDPROC-102) *(16)*; SMEWW 2550: temp	Soil temp can impact the survival of foodborne pathogens *(32, 46)*

TABLE 4. *Continued*

Parameter	Example methods[a]	Reasoning and potential impact on the presence or survival of pathogens
Soil texture	Particle size analysis (clay, sand, and silt contents) *(20, 27)*	
Escherichia coli	CMMEF, chap. 8: *Enterobacteriaceae,* coliforms, and *Escherichia coli* as quality and safety indicators; SMEWW 9221: multiple-tube fermentation technique for coliforms; TMECC 07.01-C: *E. coli*	*E. coli* levels may be indicative of the age and/or handling of the UTSA and the likelihood that foodborne pathogens are present
Aerobic bacteria plate count	CMMEF, chap. 7: aerobic plate count; SMEWW 9215: heterotrophic plate count; SMEWW 9216: direct total microbial count	
Enterobacteriaceae	CMMEF, chap. 8: *Enterobacteriaceae,* coliforms, and *E. coli* as quality and safety indicators	*Enterobacteriaceae* population density may be indicative of the age and/or handling of the UTSA and the likelihood that pathogens are present
Fecal coliforms	CMMEF, chap. 8: *Enterobacteriaceae,* coliforms, and *E. coli* as quality and safety indicators; SMEWW 9221: multiple-tube fermentation technique for coliforms; SMEWW 9222: membrane filter technique for coliforms; SMEWW 9225: differentiation of coliforms; TMECC 07.01-B: fecal coliforms	Fecal coliform levels may be indicative of the age and/or handling of the UTSA and the likelihood that pathogens are present
Enterococci	CMMEF, chap. 9: enterococci; SMEWW 9230: fecal *Streptococcus* and *Enterococcus* groups; TMECC 07.03-A: *Enterococcus*	

[a] SMEWW, *Standard Methods for the Examination of Water and Wastewater (65)*; EPA, Environmental Protection Agency; USGS, U.S. Geological Survey (National Water Quality Laboratory, method I-4650-03 *(62)*); MSA-CM, *Methods of Soil Analysis*, part 3, *Chemical Methods (74)*; SESDPROC, Science Ecosystem Support Division operating procedure *(16)*; CMMEF, *Compendium of Methods for the Microbiological Examination of Foods (12)*.

[b] TMECC, *Test Methods for the Examination of Composting and Compost (76)*. Compost sampling and testing protocols included in TMECC were either provided by individual contributors or adapted for compost analysis from methods in the following reference sources: *Test Methods for Evaluating Solid Waste, Physical/Chemical Methods,* EPA publication SW-846, 3rd ed., 1990 (and as revised); *Official Methods of Analysis,* 15th ed., Association of Official Analytical Chemists, 1990; *Methods of Soil Analysis,* parts 1 through 3, Soil Science Society of America, 1996; *Recommended Chemical Soil Test Procedures for the North Central Region,* North Central Regional Research Publication 221 (revised), 1998; *Standard Methods for the Examination of Water and Wastewater,* 18th ed., American Public Health Association, 1992; and American Society for Testing & Materials (ASTM) standard test methods, 1988.

[c] UTSA, untreated soil amendment.

data, preferably over several years, for the region using one or more of the closest weather stations. Local historical weather data (e.g., mode and range of precipitation amount, relative humidity, air temperature, and solar radiation) are available through a number of public sources, including Internet sites hosted by the National Climatic Data Center of the National Oceanic and Atmospheric Administration and state and regional organizations *(31)*. Although public source weather data also may be used for readings of daily climatic conditions during the field trial, measurement of the actual conditions at the field sites would provide the greatest value for interpretation of variation in microbial data. Climatic conditions that should be monitored daily during the field trial include minimum, mean, and maximum (i) solar radiation (watts per square meter) and total UV radiation (joules per square meter), (ii) wind speed, (iii) relative humidity, (iv) air and soil temperatures, (v) evaporation rate, and (vi) total precipitation, with heavy precipitation events highlighted. These data, both mean values and any unusual spikes and dips, would be useful for interpretation of the persistence of the microorganisms in soils after application of an UTSA.

Soil management practices. The inherent chemical and physical properties of the soil used in an UTSA study and current and past management practices will influence the survival of microorganisms and thus are important elements to be characterized and documented in a survival study. Although a specific soil type may be predominant within a geographic region, variations in soil types and other soil characteristics are likely to occur that could potentially affect target organism survival. For example, pathogen survival is strongly affected by levels of assimilable organic carbon in sandy soils and positively affected by dissolved organic nitrogen *(21, 48, 49)*. As discussed in detail above and presented in Table 4, some of the basic soil properties to include in this characterization are soil texture (clay, sand, and silt contents), pH, electrical conductivity, rooting depth (a potential reflection of soil bulk density), moisture content, and nutrient content, which includes macronutrients and micronutrients such as those typically assessed when determining the need for crop fertilizer addition within a nutrient management framework. Organic matter content also is an essential element of the characterization because it can be a source of both rapidly metabolized and slowly

degraded carbon for the soil microbial community and can serve as protective microsite harborage for microbes.

Other important aspects of soil management include tillage practices; presence and functional status of drainage tiles; irrigation type, frequency, amount, and source; application of soil fumigants; and organic soil amendment(s) history. Documentation of historical data includes identification and date(s) for cover crops, crop residues, manure (solid and/or liquid), biosolids (sewage sludge), compost or compost extracts, or other types of organic fertilizers.

Other factors associated with prior soil-crop management that could influence microbial survival include the date(s) and amount of certain soil amendments used, such as poultry or horse manure that contain arsenicals or mercurials, or agrichemicals (e.g., copper sulfate, nitrapyrin [ammonia oxidation inhibitor], pharmaceutical fermentation residues, or similar materials). When used repeatedly over long periods, such amendments may accumulate to levels inhibitory to some microorganisms *(9, 10, 78)*. If readily known, their presence, range, and distribution should be documented as factors of potential influence on study outcomes.

UTSA application and incorporation. The application and/or incorporation of UTSAs should reflect the times during the year or cropping seasons when these materials typically would be applied or incorporated (as applicable). Rates of application should follow those normally used in full-scale soil nutrient and crop management systems. State agricultural extension publications that describe methods of application *(64)* also provide methods for calculating recommended application rates and calibrating the application equipment *(29)*. Documentation of the equipment used for UTSA application and/or incorporation and the calibration procedure used to determine the rate of application should be included in the report of tests involving inoculated UTSAs or naturally contaminated UTSAs. Cover crop presence or crop residue at the time of application and any incorporation or injection of amendments needs to be taken into account in the implementation of the study, and the types and amounts of cover crop or residue and the dates and times of these events should be provided in the report.

CROP

Potential contamination of the edible portion of the crop is the ultimate concern when applying UTSAs. Typically the application of UTSAs is defined as the number of days before some production-defined point, which includes time between application of UTSA and planting the crop, time between application and blossom or emergence of fruit, and time between application and harvest of the crop. In cases where the focus is on the time between application and planting or time between application and bloom or emergence, the study focus is on the survival of the target organism in UTSA-amended soils. However, it may be appropriate in some circumstances, e.g., when levels of target organisms fall below the experimental limit of detection in the soil, to include an assessment of transfer to the crop by planting, sampling, and examining the target crop(s) of interest through the point of harvest.

Horticultural food crops, including those typically consumed in a fresh uncooked state, are invariably part of a rotation program that can include other similar crops, agronomic crops, cover crops, and fallow periods of variable duration. In some regions, different crops may be planted on the same land several times within a single year. Preparation of the soil before planting may differ among crop types. Descriptions of the crop(s) and corresponding management practices are important because these factors may influence the survival of pathogens. Although it may not be necessary to include planting the crop as a component of the study, it may be important to prepare the land as if the crop of interest were going to be planted.

In contrast, for applications of UTSA to lands with an established perennial crop (e.g., tree fruits, tree nuts, and grape vines) the study, by necessity, must include the crop. Other variables likely to influence pathogen survival include the planting and type of cover crops, the type of irrigation system (e.g., sprinkler, flood irrigation, or subsurface drip), and the timing of tilling of the soil (if done). The age of the trees, vines, or plants will influence the density of the canopy that develops, which effectively alters the temperature, humidity, and UV exposure at the soil surface. Each of these factors should be considered in the study design. When the study aims to represent a wide range of practices within a crop type, the design should attempt to simulate those situations that would be predicted to favor greater survival of pathogens based on the latest available data.

USING NATURALLY CONTAMINATED UTSAs

The use of naturally contaminated UTSAs (containing appropriate indicator organisms or pathogens) may provide a viable alternative for locations for which inoculation of manure or soil with any organism is prohibited and conditions are suitable for this approach. When using naturally contaminated UTSAs that are suspected or known to be contaminated with foodborne pathogens, a job hazard analysis should be conducted before the study, and all personnel should be appropriately trained to limit personal exposure and ensure environmental containment. Microorganisms in naturally contaminated manure may exhibit environmental hardiness characteristics that may be encountered in actual practice. Evaluation of the persistence of the target pathogen in naturally contaminated UTSA-amended soils would be an ideal scenario; however, this situation may not exist for all UTSAs or pathogens. Measuring the survival and persistence of an appropriate indicator such as *E. coli* in naturally contaminated UTSAs may sometimes be the only option.

A number of issues related to naturally contaminated UTSAs should be addressed in the experimental design. It may be a challenge to source naturally contaminated UTSAs for the manure type or pathogen relevant to the study. Unlike inoculation experiments in which strain(s) and levels of the pathogen or surrogate can be standardized, the levels in naturally contaminated UTSAs will be dependent on the

level of shedding in the herd or flock. For some types of livestock, the relevant pathogen(s) of concern and/or the appropriate surrogates may be present in the fresh manure at levels adequate to quantitatively assess their survival in soils after application of the UTSA. However, a source with consistently high levels of the target organism may be difficult to find. Using naturally contaminated UTSAs with low levels of the target organism may preclude the use of enumeration techniques postapplication and thus require enrichment (measures of presence or absence) of larger or more numerous samples to demonstrate reduction of the target population in the soil. Detection of the target organisms may be complicated by the lack of selective markers, and the target organisms in naturally contaminated UTSAs are unlikely to be the same strains in all experimental replicates, thus increasing the potential for experimental variability. Some of these issues may be overcome with a mixture of naturally contaminated UTSAs from more than one source to allow for the evaluation of pathogen or indicator populations with potentially different survival characteristics. When manures from different sources are used, the probability of capturing isolates that are more environmentally hardy would be enhanced.

MICROORGANISM SELECTION FOR INOCULATION STUDIES

Biosafety. Where permitted, the addition of selected laboratory-cultured microorganisms to UTSAs may address some of the issues associated with use of naturally contaminated material. Selection of microorganisms for inoculation studies is often limited by the location of the study. Studies conducted in model systems or at qualifying environmental research facilities may be approved for a wider array of appropriate pathogens or surrogate organisms than is typically permissible for open-environment or field studies. Foodborne pathogens are usually classified as risk group (RG) 2 (e.g., *NIH Guidelines,* Appendix B) *(56)* and require the use of biosafety level (BSL) 2 handling procedures. Some foodborne pathogens in some countries may be classified as RG3 and must be contained with BSL-3 procedures. Use of RG2 and higher organisms in any research study requires prior approval from an appropriate institutional committee. Thus, for field studies, surrogates that are organisms classified as ''agents that are not associated with disease in healthy adult humans'' or RG1 or attenuated avirulent pathogens that have been categorized as RG1 are often used with appropriate site-specific permits or permission.

Introduction of recombinant DNA into RG1 organisms may restrict the use of these organisms in both model systems and field trials and may result in a change in required handling practice from BSL-1 to BSL-2 or require a separate approval process *(56)*; researchers should be aware of pertinent local regulations at the field site that may be different from or in addition to institutional requirements.

Airborne drift of naturally or artificially contaminated UTSAs during application to land. UTSAs in lagoons or ponds have sufficient moistened mass such that bioaerosols are not typically generated and transported beyond the lagoon perimeter. In contrast, UTSAs distributed through a center pivot irrigation system can generate aerosols that are transported beyond the field perimeter. UTSAs subject to conditions conducive for generation of very small particulates may be aerosolized during application. These conditions have the potential to contribute to transport and dissemination of UTSA particulates carrying the study organisms from field applications to areas beyond their property or designated application sites. Care should be taken to prevent or minimize these occurrences.

Identifying the pathogen(s) of concern in UTSAs. Foodborne pathogens commonly associated with manure from commercial animal production vary among animal species (Table 5) and differ widely in prevalence and level within and among animal species *(17)*. The survival of different pathogens (genus, species, and strains) in soil can vary considerably *(23)*. The appropriate microorganisms for challenge studies should be epidemiologically or ecologically relevant to the different types of UTSAs being studied, the physicochemical parameters during any storage intervals and conditions, and land application practices that pertain to the system of interest. The selection of the pathogen(s) of concern should be justified in the report. The discussions below on selection, propagation, and recovery of the test organism(s) are focused on bacteria. The same general principles could be applied if a parasite or virus were identified as the target organism.

Attenuated pathogens or other nonpathogenic surrogates. In many cases, the inoculation of UTSAs with the pathogen(s) of concern will not be feasible, and a surrogate will need to be selected. The choice of an appropriate surrogate to use in place of the pathogen of concern in the UTSA should be justified with supporting documentation in the study report. A list of surrogate organisms that have been used in field trials has been published *(31)*. Surrogates may survive better or worse than the pathogen of concern in UTSA-amended soils. Some data, either previously published or generated specifically for the study, should be presented to demonstrate that survival and persistence of the surrogate is comparable to and ideally not less than that of the pathogen of concern under conditions most likely to affect survival in the study. These data should be included in the study report or available to those reviewing the report.

There are advantages and disadvantages of using either nonpathogens or attenuated pathogens as surrogate organisms. Detection methods for nonpathogenic surrogates may be difficult, even when selective markers are used, because the indigenous microbiota may overwhelm the inoculated strain in laboratory tests. Attenuated pathogens are usually missing one or more genetic factors that contribute to pathogenesis. Attenuated strains used in field studies should lack structural virulence genes (i.e., genes encoding toxins or components of type 3 secretion systems) rather than contain mutations in regulatory genes or regulatory regions of virulence genes. Mutations in regulatory genes (e.g., *cya*

TABLE 5. *Pathogens found in manure*[a]

Pathogen	Presence in manure					
	Bovine	Avian	Porcine	Ovine	Equine	Caprine
Bacteria						
Campylobacter spp.	+	+	+	+		+
Clostridium perfringens	+	+	+			
Escherichia coli (Shiga toxigenic)	+		+			
E. coli O157:H7	+	+	+	+	+	
Listeria monocytogenes	+	+	+	+		
Salmonella	+	+	+	+	+	+
Yersinia enterocolitica			+		+	
Parasites						
Cryptosporidium spp.	+		+	+	+	
Giardia	+		+	+		

[a] Adapted from Millner *(52)*; for additional information on the presence of enteric human pathogens in manure, see Sobsey et al. *(73)* and Erickson *(17)*.

and *crp*) or within regulatory regions of virulence genes are easily compensated for by second-site point mutations that could result in reversion to virulence and heterogeneous populations of microorganisms. Inactivation of genetic factors often raises concerns about relative survival ability of the attenuated strain compared with the parent strain or other fully pathogenic organisms. To complicate issues further, there are currently no standardized means by which to determine when an attenuated RG2 organism is no longer capable of causing disease in healthy adult humans (i.e., becomes RG1).

When using attenuated strains, the potential for contamination of nearby commercial crops should be carefully evaluated. In almost all cases, commercial samples contaminated with these organisms would yield a positive test result (e.g., for *Salmonella* or *E. coli* O157:H7) regardless of their attenuated status.

Marker-assisted detection and enumeration. Process validation or environmental persistence and dispersal studies often can be more easily conducted with isolates genetically marked in some way to facilitate detection, recovery, and enumeration. Strains carrying markers, such as antibiotic resistance, xenobiotic degradation, luminescence, fluorescence, or other differential reporters, or capable of multiplying on a rare carbon or nitrogen source will aid in the selection and enumeration of the target pathogen or the surrogate from manure and amended soil samples containing high populations of background microorganisms. The use of such markers is also desirable in studies requiring enrichment at population levels below conventional levels of detection. Markers can also be utilized to conduct semiquantitative recovery with real-time PCR methodologies against a standard DNA or related reference (standard curve).

In several studies, antibiotic resistance has been used as a marker to detect the target microorganism in microbiologically complex environments such as manure and soils *(25, 60)*. Resistant variants of the target organism are typically isolated by stepwise exposure to increasing concentrations of the antibiotic. The genetic and physiological bases of these spontaneous mutations are nearly impossible to determine. One hypothesis is that these mutations result in the modification of targets of the antibiotics or a selection of a strain with a generally altered stress resistance. Therefore, before use of such antibiotic-resistant strains, at least preliminary laboratory data should be collected to demonstrate the fitness of the mutant with respect to that of the parental wild type.

A more laborious alternative to using antibiotic resistance markers would be to select individual colonies and screen for unique pulsed-field gel electrophoresis or other patterns or to use PCR assays to identify specific genes unique to the target organism. Some studies have employed green fluorescent protein introduced chromosomally or in a plasmid as a marker *(18, 37–41, 44, 49, 69)*. Ma et al. *(50)* outlined useful approaches for testing the stability and burden of plasmids containing the green fluorescent protein gene and found that some strains of *E. coli* O157:H7, *Salmonella,* and *Listeria* containing the green fluorescent protein plasmid can be stable for many generations without adversely affected growth rates. Although the markers work well, these organisms carry recombinant DNA, which will likely restrict their use as described above. A discussion of key elements of selection and precautions in application of surrogates was provided by Beuchat et al. *(7)* and Sinclair et al. *(72)*.

For optimal evaluation of data, newly developed strains with markers that can be selected or screened must be characterized for fidelity or minimal variation in physiological characteristics from the parent strains and for the stability of these markers in the absence of selection and under conditions simulating environmental stressors *(51)*. Regardless of the nature of the genes, marker stability is affected by the location of the genetic modification and the degree of gene expression *(11)*. The stability of the markers and their effect on growth, survival, and any desired or critical phenotypic traits should be determined for each strain before its use in UTSA challenge studies.

Inoculation with a single organism or a cocktail of strains. Either a single isolate or a mixture of strains can be used to inoculate UTSAs. Use of a mixture of three to five strains with different genotypic and phenotypic stress tolerances is typically preferred over the use of a single isolate *(7)* but may not always be feasible. The selection of representative strains that have been isolated from the species-specific UTSA, the relevant commodity, or the related production environment may be appropriate. When a cocktail of strains is used, all strains should be individually screened to confirm that there is no antagonism among them due to the production of bacteriocins or other antimicrobial factors.

MAINTENANCE, CULTIVATION, AND PREPARATION OF INOCULUM

Many choices must be made when establishing procedures for inoculum preparation for challenge studies. Although each of these choices has the potential to influence the environmental fitness or survival of the inoculum, it is not clear that they always have an impact *(75)*. However, carefully considered standard operating procedures should be established for culture preparation with the goal of maximizing environmental survival and minimizing the potential for the culture preparation to be a variable among replicated trials. The discussions below are meant to highlight factors that should be considered when developing protocols for inoculum preparation. The methods used for the study and the rationale for these choices (where appropriate) should be included in the study report.

Inoculum preparation. At the initiation of the study, isolates should be screened to confirm their identity and important phenotypic and genotypic traits (e.g., no change in extrapolysaccharide production or absence of specific virulence genes). Bacterial strains selected for UTSA inoculation studies should be isolated from freeze-dried cultures or cultures stored in a freezer rather than on slants, plates, or under oil at refrigeration or room temperatures. Even though growth and metabolism of microorganisms is reduced at temperatures below optimum but above freezing, these cultures are still subject to genetic changes that could alter the fitness of the organism. Typically, a single well-isolated colony will be inoculated into or onto a medium with the appropriate additives (e.g., antibiotics) to maintain identifiable markers (if needed) and incubated at the appropriate temperature until the culture has reached an appropriate growth phase (exponential, early, or late stationary phase) (Table 2). Organisms grown to stationary phase are more likely to survive acute stress. It may be possible to further culture or incubate the inoculum in the UTSA when the form of the UTSA (liquid or slurry) is amenable to direct inoculation. This approach would allow the inoculum to adapt to the conditions of the UTSA, thereby providing an approximation of the physiological condition of cells in naturally contaminated UTSAs *(58, 70)*. This culture step also may reduce the potential for physiological shock to cells that are transferred directly from laboratory medium or carrier to UTSAs or soil. As a general rule, multiple transfers of cells to new growth media should be minimized to reduce the potential for genetic drift and any subsequent changes in phenotype *(55, 67)*.

Carrier medium. Once cells have been cultured, they are usually transferred to a carrier medium to form the inoculum. The prepared inoculum may be further incubated or held for a period before inoculation of the target UTSA or soil. A suitable carrier medium may be a commercial preparation (e.g., sterile deionized water, phosphate-buffered saline, or 0.1% peptone) or a manure-based preparation such as autoclaved bovine feces slurry *(70),* UTSA, or soil. The carrier medium should stabilize the inoculum preparation but not directly influence the response of the organism after addition to the target UTSA *(35, 68)*. If the study microorganism(s) has been prepared in a commercial laboratory medium, the cells are often collected by centrifugation and may be washed one or more times to remove residual medium. These cells are suspended in the selected carrier and then applied to the target UTSA. If the inoculum has been cultured in the UTSA or similar preparation, the inoculum may be applied directly into a solid or liquid UTSA, which could then be applied to soil *(70)*. The volume of the inoculum should be taken into account; the volume of the inoculum should be small enough so as not to significantly affect the moisture content or other intrinsic properties of the target UTSA or soil.

Sampling inoculum before application. Before the inoculum is transferred to either the carrier or UTSA, the microbial population density should be determined at various stages using an appropriate selective medium specific for the target microorganism(s), e.g., (i) after the initial growth of bacteria in broth or UTSA, (ii) immediately before and after blending with the UTSA, and (iii) after application to the environmental matrix. These data can be used to determine whether the population density has been affected by suspension of the target organism in the carrier medium or after incorporation into the UTSA. If a multistrain inoculum is being prepared, the population density should be separately determined for each strain and for the strain mixture.

Amount of inoculum applied. The level of an enteric pathogen shed by animals can vary widely *(3, 17, 33)*. Final levels of the target organism in the inoculated UTSA should reflect the upper end of the expected range even when the frequency of occurrence of these levels is not high. Use of lower inoculum levels should be adequately justified.

Method of inoculation. Regardless of the consistency (liquid or solid) of the UTSA matrix, the inoculum should be evenly distributed. The methods used to distribute the inoculum should be adequately described. Inoculum can be incorporated into liquid UTSAs that have a flowable consistency by stirring (e.g., in a sterile vessel with a sterile magnetic stir bar), by use of a commercial mixer, or by shaking with sufficient agitation to achieve even distribution. Solid UTSAs and inoculum should be

thoroughly mixed using manual or mechanical means to ensure that all the material comes into contact with the inoculum. Replicate solid UTSA samples from different locations in the container (or stack) or liquid UTSA samples should be taken for population density determinations to ensure that the inoculum has been uniformly distributed.

Personal protective equipment. Personal protective equipment as appropriate (e.g., gloves, laboratory coats, body suits, goggles, face masks, or respirators) should be provided to individuals involved in the collection, handling, and transportation of UTSAs to the laboratory and experimental site and during the application, incorporation, manipulation, and sampling of either naturally contaminated or artificially inoculated UTSAs. Personnel should be aware of specific facility requirements when the UTSA is collected directly from an animal production or domestic husbandry operation. At such facilities and during transportation of the UTSA, personnel should be aware of the microbiological hazards of the UTSA, the physical dangers of the environment, and other hazards (e.g., asphyxiation, poisoning, and explosion) when manure is stored in confined spaces. Personnel should be aware of any federal or state occupational safety and health standards that exist for work in and around manure facilities and may want to contact state agricultural extension programs to identify practices that would minimize working hazards.

The use of additional and specialized protective equipment (full face respirator with specific filters) may be necessary during certain process steps when the potential for aerosolization of infectious agents exists. All personnel, including farm labor and maintenance workers, should be aware of the hazards and should be provided with appropriate personal protective equipment upon entering the experimental site. All equipment (e.g., manure applicator, tank truck, spray system, tractor, disk, and other tillage implements) used at the experimental site should be cleaned and then sanitized (or sterilized when practical) after use to prevent cross-contamination. The safe disposal of waste generated in the laboratory and at the experimental site will depend on the study organism and the specific requirements of the Institutional Biosafety Review Committee; specific requirements and procedures may vary among these committees and among state and regional offices.

RECOVERY OF TEST ORGANISMS FROM UTSAs, SOIL, AND UTSA-AMENDED SOIL

Sample collection of both soil and UTSAs for nutrient and microbial analysis should be carried out on the day of the experimental application of the naturally contaminated or inoculated UTSA (either laboratory- or field-based studies) both before and after application. Steps should be taken to ensure that the UTSA or UTSA-amended soil samples analyzed are representative of the bulk matrix being assayed. Although pooling samples either in the field or before enumeration or enrichment will reduce costs and labor associated with analysis *(42, 55, 63),* the decision to use composite samples should be balanced against the ability to determine the level and degree of heterogeneity among samples. In addition, the temperature of the soil (and depth of measurement) and the air on the day and at the time of application should be recorded.

Sample collection tools. Sterile or sanitized nonreactive tools and containers should be used to protect UTSA and soil samples collected for microbial analysis from cross-contamination. Presterilized sampling devices (e.g., soil core sampler, blades, scoops, containers, and bags) may be purchased or prepared in the laboratory. Appropriate personal protective gear should be used to protect both the workers and the integrity of the samples.

Sampling UTSAs. For solid UTSAs, special attention should be paid to visible variations in moisture and composition or structure. When considerable variations exist, multiple samples should be taken and analyzed to ensure that the test results reflect both the range and the mean populations of target and background microbiota for the entire mass of material being applied. Additional challenges may exist when larger volumes of UTSAs are incorporated into the study design. Uneven exposure to the environment and/or pile heating may lead to differences in composition between the surfaces of the manure stack or stockpile and the entire mass of material stored. Surface material (0- to 5-cm depth) will differ from material at a depth of 5 to 100 cm *(71),* and the sampling method should take this difference into consideration. Multiple samples should be collected from each cross section of a solid UTSA pile *(76, 87).*

Ideally, for larger volumes, stacked and stored material would be mixed before arrival at the land application site, and then subsamples of a defined size (based on material uniformity) would be collected during the course of the land application at periodic intervals (i.e., at the beginning, middle, and end of application). One way to sample during spreading of the UTSA is by laying pieces of clean tarp or plastic sheet on top of the soil at several locations in the field *(87).* UTSA landing on the tarp from one pass of the equipment can be subsequently sampled. This sampling approach also could be used to estimate variability in application rate, which would help to inform sampling needs.

Liquid samples may be obtained from constructed lagoons and lagoon sludge and from manure slurry pits or ponds. When obtaining samples from a storage unit, the material should be thoroughly agitated before sampling; analysis of several samples will help assess the adequacy of mixing. Consideration should be given to obtaining samples immediately before and at periodic intervals during field application to ensure samples are representative of material that is actually applied. Liquid UTSAs that are surface applied can be sampled by periodically collecting material from the tank truck, liquid cannon, or irrigation pivot or from containers preplaced in transects across the field within the spray deposition range but out of range of the transiting vehicle. For subsurface injection of liquid UTSAs, samples must be obtained from the tank containing the UTSA.

Sampling soil. There are several methods for collecting soil samples, and factors related to relevant agricultural

practices used in the study should be considered when selecting collection methods to ensure the best chance of target organism recovery. A method should be selected that involves parameters that accurately reflect the applied UTSA, affected layers of soil, and presence of crops or crop residues that may influence the survival and spatial distribution of the target organism in a treated area. Sample collection should take into consideration the type of crop (root or above ground) and cultivation methods (tillage, bed preparation, or side dressing). For soil core samples, factors to be considered are width, depth, and amount. The width of the core sample should be documented because it influences sample size, and wider samples may reflect a greater array of microenvironments. The depth of the sample will depend on the factors of importance to the study objectives and should reflect typical agricultural practices of interest.

In some areas, such as fields in which the edible portion of the crop does not directly contact the soil (e.g., orchards) or where the UTSA is not incorporated after application, drag swabs of the soil surface or a core from the top layer of soil involved in UTSA application may be acceptable. Where the UTSA is incorporated into the soil or used for fields of root crops, drag swabs would not be an accurate representation of the risk associated with crop contamination. The potential influence of root crop or cover crop may necessitate examination of soil at a greater depth. Spatial heterogeneity in pathogen survival can occur with soil depth and soil location (rhizosphere versus bulk soil). When a cover crop is grown on the field following application of the UTSA, samples should be obtained near the plants because root exudates can provide readily available carbon and an environment that is more conducive to pathogen survival compared with the bulk soil *(4, 60)*. For root crop fields, the depth of the soil samples taken should be at least the depth of the soil at the maximum root depth for that particular crop.

Size of the individual analyzed units. Sample size is often chosen subjectively, with a wide range of values reported for similar experimental conditions. The mass of individual samples should be sufficient to perform all analyses and large enough to represent soil heterogeneity. As a systematic approach to identifying the best sample mass, a resampling-based preliminary trial can be conducted before the start of the experimental trial. With this approach, samples of different amounts would be obtained systematically from subplots, and the range, mean, and standard deviation would be calculated for each of these sets. By using a sampling software program, such as Shortcut in Sample Size Identification (SISSI; available free of charge for noncommercial purposes; www.robertoconfalonieri.it/software_download.htm), the sample amount could be targeted to the size for which the rate of change of means becomes negligible. Because samples will be heterogeneous, collecting a representative subsample for individual analyses is important *(63)*. Laboratory personnel should collect representative subsamples from randomly selected locations. Homogenization (such as mixing or grinding) of samples taken from a larger batch for analysis is critical, and the effectiveness of such homogenization should be verified.

Methods for recovery. Methods used for sample analysis must allow for the accurate and reproducible recovery of the target microorganism. A full discussion of the methods used for recovery of pathogens from manure-amended soil is beyond the scope of this document, but sources such as the FDA *Bacteriological Analytical Manual* (BAM) *(79),* Environmental Protection Agency methods *(15),* the U.S. Composting Council's *Test Methods for the Examination of Composting and Compost (76), Standard Methods for the Examination of Water and Wastewater (65),* or other validated published studies should provide detailed methodologies for specific pathogen analysis.

The purpose of sample preparation for microbiological analysis is to retrieve all or most microbial cells or spores of the target pathogen from the sample matrix. Common practice is to suspend fresh samples in some type of diluent (e.g., Butterfield's phosphate buffer, saline, or buffered peptone water) at a ratio of 1:9 initially and then to mix thoroughly by stirring, shaking, or stomaching the sample for a defined time. The mixing method selected should adequately break up clumps or aggregates present in the mixture to efficiently release microbial cells from the sample matrix. For pathogen enumeration, aliquots of this mixture or serial dilutions may be directly plated on selective agar. Appropriate resuscitation techniques that allow for recovery of viable but stressed or injured cells may be required.

When a standard method is not used for recovery, some data to demonstrate the efficacy of the method used for recovering the target organism should be provided. The specific method used for recovery will influence the reporting of the results. Results may be reported on a per-gram basis for both enumeration and presence-absence testing (e.g., log CFU per gram, log most probable number [MPN] per gram, or 2% positive of 300 100-g samples).

Methods for enumeration and endpoint determination. Conventional culture methods for pathogen detection from UTSA-amended soil include direct plating, MPN methods, and enrichment (presence-absence) testing. In some cases, the sensitivity of direct plating methods may be improved by either filtering the sample first or concentrating the cells by centrifugation. The presence of high levels of background microbiota in UTSA-amended soil often necessitates the use of an appropriate selective agar for enumeration of the target organism or use of a step to separate target cells from the matrix (e.g., immunomagnetic bead separation). When the pathogen population is or is expected to be below the experimental detection limit, an enrichment approach using larger sample sizes should be applied. Often, enrichment procedures include a preenrichment step to repair injured cells and a selective enrichment to selectively grow the target organism.

Enrichment-based approaches do not permit direct quantification but indicate only presence or absence of the target organism in a certain documented sample size. MPN-based methods are an exception because they use a series of dilutions that are enriched (in appropriate medium containing the appropriate selective marker agent) and then scored

TABLE 6. *Study report checklist*

Information type	Specifics	Description
Specific to the study objectives		
Untreated soil amendment (UTSA)	Source	Describe all sources of the UTSA subject to this study; include information on the animal(s), domestic husbandry production practices, and feed (if known)
	Soil amendment characteristics	Document physicochemical characteristics of the soil amendment (see Table 3), amt and type of bedding, manure collection and management, and duration and condition of storage
	Rate of application	Describe the range of application rates following the commercial practice and based on the UTSA nutrient content
	Application method	List all application methods and equipment used and the corresponding soil test results and UTSA characteristics
	Time of application details	List historical time(s) of day, season, age of crop, and time to planting or harvest (as appropriate)
	Postapplication incorporation	Describe the method of postapplication incorporation (if applicable)
Region	Geospatial characteristics of field(s)	Include elevation, level, slope, and direction of slope
	Geographic location	Provide information on the location, including latitude and longitude, of the field(s) under this application
Crop	Crop description	List crop type(s) and varieties covered under this application
	Crop management practices	Provide information on typical crop rotation, pest management, and other practices
Soil	Soil management practices	Describe field and bed prepn, fumigation, conditioning, tilling, nutrient management history, and other practices
	Soil type and texture	Describe critical factors (e.g., pH; salinity; soil survey description; clay, sand, and silt content; organic matter content; major nutrients (nitrogen, phosphorus, potassium); minor nutrients (aluminum, boron, calcium, copper, iron, magnesium, manganese, sodium, sulfur, zinc) (see Table 4)
	Microbiological analysis	When this analysis is done, include a general characterization of the indigenous microbial community and/or indicator organisms
Climate	Climate history	Describe avg weather during the relevant period and prevailing conditions
Justifications	Commercial vs this study	List all the justifications and modifications made between commercial practices and this study (a table is recommended)
Include in the study report		
Introduction		
Purpose of the study		List study objectives
Justification of the study design		Provide any preliminary or previously published data in support of the exptl design; include historical data on the use of UTSA and time to harvest for crop
Materials and Methods		
System selection	Model system	Describe system (e.g., growth chamber) and environmental conditions used; provide rationale when study is based solely or primarily on data from a model system; justify model used
	Field-based study	Describe geographic location, use, and geospatial characteristics of field(s) used in the study and adjacent land; provide rationale for site selection; include data (minimum, mean, and maximum) for solar and total UV radiation, wind speed and direction, relative humidity, air and soil temp, evaporation rate, and total precipitation (with heavy precipitation events highlighted) immediately before and during the study
UTSA	Source	Describe animal source of UTSA used in the study; include information on animal production practices and feed (if known)
	Soil amendment characteristics	Nutrient and microbial data, moisture, amt and type of bedding, and duration and condition of storage
	Application details	Indicate the rate(s), date(s), and time(s) of application used in the study; indicate presence of cover crop or crop residue at time of application
	Postapplication incorporation	Describe the mechanism of postapplication incorporation
Crop	Crop description	List type(s) and varieties of crops and rotations included in the study design (if relevant)
	Crop management practices	Describe production practices that were used before and during trials (e.g., irrigation dates, times, and duration; nutrient management history; pesticide application) if relevant
Soil	Soil management practices	Describe practices used during the study
	Soil type	Describe critical factors, including texture

TABLE 6. *Continued*

Information type	Specifics	Description
Microorganism(s)	Pathogen(s) of concern	Identify pathogen(s) relevant to manure source(s)
	Surrogate organism	Data to demonstrate that survival and persistence of the surrogate is comparable to those of the pathogen of concern under conditions most likely to affect survival during the study
	Selected microorganisms	Justify selected microorganism(s), including strain, cocktail vs single strain, and marker selection
	Inoculum prepn	Describe maintenance, cultivation, and prepn of inoculum, including media and incubation time and temp, and provide rationale for choices
	Inoculum carrier	Describe the inoculum carrier medium
	Inoculum enumeration	Describe procedures used for enumeration of microorganism(s) in the inoculum
Inoculation	Application protocol	Provide inoculation levels and rationale for level used, frequency and method of inoculation, and environmental conditions at time of inoculation
	Time of inoculation	Provide time of day, date, plant age, and no. of days before typical harvest (also describe personal protective equipment used when pathogenic strains are used)
Recovery and detection	Sample prepn	Provide sample size and weight, diluent type, volume or ratio, and recovery method (e.g., rinse, swab, or homogenize); record soil and air temperatures at sampling; provide times and temp conditions between sampling and sample processing
	Time of sampling	Provide date, time of day, and plant age at time of sampling
	Enumeration	Describe methods used for enumeration (time, temp, medium), and justify choices
	Enrichment	Describe procedures used for enrichment (time, temp, medium), and justify choices
	Other detection methods	Describe any other procedures that were used for detection
Sampling plan	Sampling strategy	Provide rationale for no. of sample units, no. of samples analyzed, and sampling intervals
	Replicates	Justify no. of replicates and no. of field locations (if applicable)
	Study duration	Provide rationale for duration of study
Controls	Control type	Describe each control and provide rationale for no. of controls used
Statistical analysis	Statistical test(s)	Describe statistical approach and rationale; report confidence level
Results		
Data	Raw concn data	Provide unadjusted concn data (e.g., plate counts, dilution, and sample size) and measure of variability (e.g., SE and SD)
	Raw prevalence data	Provide unadjusted prevalence data (e.g., no. of positive samples, sample size, and total no. of samples processed)
	Adjusted data	Provide calculated concn and prevalence data, measure of variability, and exact *P* values
Summarized data	Summary	Provide detailed summary of study results
	Figures or tables	Provide graphical or tabular summary of study results
Discussion	Interpretation of results	Provide interpretation of results within the context of this application
	Limitations	Discuss limitations of the applicability of the data and explain potential reasons when discrepancy is seen between the data and expected results
	Conclusions	Include key findings and recommendations; indicate what factors might warrant new challenge study

in a semiquantitative manner to estimate the number of organisms in a sample. A useful MPN calculator for calculating values for unconventional sample sizes and numbers can be found in the BAM, Appendix 2 *(8)*; this Web site has a link at the bottom of the page for downloading an Excel spreadsheet.

The choice of quantification or enrichment method(s) and pooling strategy (if employed) should be provided in the report. Background microbiota may be able to grow on selective media especially when the level of the inoculated organism reaches the lower limit of detection. For both enumeration and enrichment samples, a subset of suspect colonies should be selected to confirm by biochemical, serological, or DNA-based tests that the organism recovered is the inoculated strain (where inoculum has been applied) or the target organism (for naturally contaminated UTSAs). The number of isolates selected will depend on the experimental design and the results of initial confirmation tests, which should provide some information on the likelihood that the background microbiota are capable of multiplying in the recovery medium.

DNA-based quantification, detection, and identification methodologies are continuing to improve as is their potential to characterize the microbiomes of soils and their interactions with pathogen survival kinetics. These methods may be useful for identification of organisms recovered from the samples. However, these methods are not typically useful for enumerating or detecting organisms that are in

very low abundance and nonuniformly distributed. DNA purification from soils can be problematic, especially when the target DNA is in low abundance.

Control samples. In addition to sampling the inoculated UTSA and UTSA-amended soil, adequate numbers of uninoculated control samples (UTSA and soil) should be analyzed to determine the background microbiota (e.g., aerobic plate count and thermophilic coliforms) and to verify the absence of the target microorganism.

Storing and shipping samples. The time between collection and processing of samples should be minimized. Samples should be handled in a controlled manner during transport to the laboratory, recognizing the potentially biohazardous nature of the samples and the need to protect the target microorganism(s) from further inactivation or growth beyond what occurred in the field. When samples contain BSL-2 organisms, appropriate and approved shipping methods must be used. Samples should be cooled and held on ice (avoiding direct contact with the ice) or chilled; high and low temperature extremes should be avoided. Care should be taken to avoid freezing the samples because freezing and thawing may result in changes to microbial populations. The sample temperature during the time of transport and preprocessing storage should be documented.

EXPERIMENTAL DESIGN, SAMPLING STRATEGY, AND STATISTICAL ANALYSIS

Numerous factors need to be considered when developing a study's experimental design and sampling strategy. Background knowledge of the variability in the composition of the target UTSA, field characteristics, and soil types in the region of interest is necessary for study design development. Project investigators should consider and discuss the identified potential factors influencing survival of the target organism and reduce variability in the key factors as much as possible. Consultation with a statistician is useful for guidance in development of the study's experimental design, sampling strategy, and data analysis.

Size and number of field locations. Each plot should be large enough to establish subplots for repeated sampling over time. Sufficient space is needed within the plot to ensure that undisturbed areas are available for sample collection over the course of the experiment. A minimum of two and preferably three or more blocks that are not located adjacent to each other should be evaluated, as described in Table 2. Hence, soil variability between and within single plots should be documented and compared with that in the region for which the study results may be applied (Table 2).

Sample size (number of samples per time point) and statistical power. A statistically valid sampling scheme is an important component of the overall study design. The required number of samples analyzed at each point is governed by the variability of a sampling unit within the plot and the degree of precision desired. Historical knowledge of the variability of the factors under consideration in the region of the study, such as characteristics of the UTSA and soil types, would assist in calculating the necessary number of samples to achieve a given statistical power. When this information is unavailable, preliminary data may need to be generated before initiating the study *(28)*. A discussion of the ability of the adopted sampling scheme and soil sampling method to capture the presence of the target organism should be provided in the study report.

Field soil sampling is often a major source of uncertainty in pathogen detection studies. As analytical techniques improve, the control of sampling error frequently becomes the limiting factor in documenting pathogen inactivation. Because of the variability in the properties of untreated manure and soil, the distribution of a target organism within a research plot can range over many orders of magnitude *(9, 21, 30, 77)*. The sampling plan adopted should include sufficient sample numbers to account for this variability. Factors to consider include the number of samples, sample size (width, depth, and amount), location of the sample within a subplot or field, and whether, how, and when composite samples are used.

An initial rapid decrease in pathogen level after UTSA application is often followed by the survival of low levels of a subpopulation of the pathogen for an extended period of time *(6)*. As target organism levels approach the lower limits of detection, it is best practice to increase the number of samples analyzed at each sample time to enhance the probability of detection of the target organism. More samples (in some cases hundreds of samples) and/or larger samples sizes as one approaches levels of the target organism that are below the point of quantification but within the probability of presence-absence detection is a reasonable expectation.

Duration of the study and sampling intervals. The length of the study will be specifically related to the research objectives. A primary focus of many studies probably will be to document the initial levels or presence of target organisms immediately after UTSA application and to target levels at some later point (e.g., planting, blossom, or harvest). Sampling should be initiated immediately after UTSA application and at appropriate intervals thereafter. To capture the typical initial rapid die-off of foodborne pathogens in UTSA-amended soil, samples may need to be collected more frequently immediately after application.

When the focus is measuring pathogen inactivation, the study is typically concluded when the pathogen is no longer recovered from the soil in noninhibitory enrichment medium at one or more time points. Recovery of the target organism by enrichment is dependent upon sample size, and designating the ''absence'' of an organism from a production area is dependent on a combination of sample size and sample numbers (e.g., not detected by enrichment of 150 100-g samples). Unfortunately, no standardized approach exists to establish an appropriate number of samples or sample size. Initial data can be used to determine when an increase in sampling size will be necessary. Two

consecutive observations of ''no detection'' in an increased sampling regime is the often-accepted sensible endpoint for field trials. The number of samples needed depends on the accuracy and sensitivity required and the heterogeneity of the UTSA and soil being examined. The report should include a discussion of the considerations taken when determining the end of the trial.

Number of replicate experiments and system variability. Studies should be replicated over time (e.g., repeated in more than 1 year). Because of the many sources of variation discussed above, some of which cannot be directly controlled, experimental trials conducted in more than 1 year will strengthen the overall findings of a given study. Although a 1-year study may be acceptable under some circumstances, multiple seasons and years are likely needed to capture the breadth of applicable environmental conditions (Table 2). Data from the initial year or the first 2 years of a study can be used to guide decisions on the need to generate additional data.

LIMITATION OF STUDY

For the reasons discussed in this document, the study results will pertain to only the specific UTSA type(s), method(s) of application, and production system(s) evaluated and can be extrapolated only to other closely aligned system conditions. Results of either model or field studies conducted on a specific soil type with specific crop, soil, and amendment management practices may not reflect precisely the same pathogen reduction rates that might be achieved in a produce production locale that differs in one or more of the factors described in the foregoing discussion (e.g., soil and crop type and management practices; manure type, source, age, and rate and method of application; and geographical and climatic conditions). Until more results of carefully conducted and documented studies become available, it will be difficult to extrapolate results from different sites even within regions. Presently, the database of information available on pathogen survival in UTSA-amended soils is insufficient to support broad comparisons and generalizations across soil types, crops, and soil and amendment management practices. As such information becomes available from the types of studies suggested here, meta-analyses and other statistical analyses may prove useful for developing methods to adjust for locality differences.

INFORMATION TO INCLUDE IN A STUDY REPORT

The study should be completed by or under the supervision of one or more experts in microbiology or a related field who are fully aware of all applicable regulations. Involvement of experts in agricultural production (e.g., soil science, horticulture, or plant pathology) should also be considered, especially for field trials. The report should provide sufficient information to allow an assessment of the adequacy of the study (Table 6). The introduction should clearly outline the specific objectives of the study and include a review of pertinent literature and a description of relevant commercial practices pertaining to the UTSA and crop(s) of interest. The experimental design should be adequately described, and justification for the choices made should be included in the report (Table 6). Differences between commercial practice and the final experimental design should be considered and discussed in the context of the research results. Methods should be outlined in sufficient detail to allow for evaluation and interpretation of the results.

In some cases, it may be appropriate to include both (i) the raw unadjusted concentration data (e.g., actual plate counts, dilutions, and sample size), final summarized data, and measures of variability (e.g., standard error and standard deviation) and (ii) the raw prevalence data (e.g., number of positive samples, sample size, and total number of samples processed). The discussion should provide an interpretation of the results and any limitations associated with the applicability of the data. Potential sources of variability, such as environmental conditions, should be documented, and their potential influence on study results should be discussed. The conclusions should contain key findings and any notable recommendations.

Specific information (as applicable) should be collected for each study and provided in the report. Table 6 provides checklists for (i) information specific to the study objectives and (ii) information to include in the study report.

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