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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 Quality of Agricultural Water Contacting Fresh Produce That May Be Consumed Raw

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ABSTRACT

Agricultural water may contact fresh produce during irrigation and/or when crop protection sprays (e.g., cooling to prevent sunburn, frost protection, and agrochemical mixtures) are applied. This document provides a framework for designing research studies that would add to our understanding of preharvest microbial food safety hazards and control measures pertaining to agricultural water. Researchers will be able to use this document to design studies, to anticipate the scope and detail of data required, and to evaluate previously published work. This document should also be useful for evaluating the strength of existing data and thus should aid in identifying future research needs. Use of this document by the research community may lead to greater consistency or comparability than currently exists among research studies, which may ultimately facilitate direct comparison of hazards and efficacy of controls among different commodities, conditions, and practices.

The U.S. Congress, through the Food Safety Modernization Act (FSMA), 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 consumed in a raw form where such standards would minimize the risk for foodborne illnesses (produce safety rule). FSMA includes specific directions to the FDA to address a number of subject areas including development of minimum standards related to agricultural water and biological soil amendments of animal origin (soil amendments). FSMA also directs the FDA to establish a process for states and foreign governments 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 in light of local growing conditions, and procedures, processes, and practices to be followed under the variance are reasonably likely to ensure that the produce is not adulterated and provide the same level of public health protection as the requirements of the produce safety rule.

The FDA has indicated that it may provide an option to growers that would allow them to take an alternative approach for some prescriptive provisions (e.g., standards associated with soil amendments and agricultural water), similar to past regulations (e.g., juice hazard analysis critical control point plans: 21 CFR 120.24) *(78)*. Any alternative approach should be supported by an assessment of its efficacy for reducing microbiological hazards for the given situation. It is anticipated that the rule may provide information that would assist growers in determining when a particular alternative might be appropriately applied to the commodities, conditions, and practices at an individual operation and what types of data would be needed to support an alternative approach.

Additional research is critical to enhance our understanding of produce safety hazards and to develop measures

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needed to minimize them. The primary objective 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 agricultural water. Validation of agricultural water treatment methods or postharvest uses of water were not considered although some aspects of this document may pertain to the development of relevant study designs. It is anticipated that researchers will be able to use this document as an approach to design studies and to anticipate the scope and detail of data required; the produce industry or competent authorities should also find this document useful for evaluating the strength of existing data and thus as an aid in identifying future research needs. Use of this document may lead to greater consistency or comparability than currently exists among research studies, which may ultimately facilitate direct comparison of hazards and the efficacy of controls among different commodities, regions, conditions, and practices.

Developing appropriate research protocols involves many factors with multiple options, each of which may impact the experimental outcome. 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, it is understood that sometimes only good choices are feasible. Table 1 offers suggestions for selecting the generally accepted good, better, and best practices for many of the experimental factors discussed in this document. Attempts should be made to balance these choices, and the study report should clearly articulate justifications for the choices made. The research should be completed under the supervision of and interpreted by an expert(s) with a strong background in microbiology who is fully aware of all applicable regulations pertaining to the research. The research should employ appropriate, validated methodologies and techniques. Factors that need to be considered in developing a study that pertains to agricultural water are provided in the following sections.

AGRICULTURAL WATER

Agricultural water may contact fresh produce during irrigation and/or when crop protection sprays (e.g., for cooling to prevent sunburn, for frost protection, and for application of agrochemical mixtures) are applied. A detailed list of published surveys that have evaluated the prevalence of foodborne pathogens in environmental water has been compiled by Erickson *(25)* at the University of Georgia Center for Food Safety. Traditionally, two agricultural water microbial monitoring approaches have been taken by the produce industry to ensure the adequacy of agricultural water when it is applied directly to fresh produce surfaces *(68)*: (i) qualitative testing for the presence or absence of human pathogens in a given volume of water or (ii) quantitative testing of indicator microorganisms (e.g., *Escherichia coli*). Both approaches have advantages and disadvantages for ensuring the adequacy of agricultural water when applied directly to fresh produce during production.

Human pathogens can survive for variable lengths of time in water and on various portions of fresh produce; see the detailed bibliography compiled by the Center for Food Safety *(12)* and recent reviews by Levantesi et al. *(54),* Suslow *(68),* and van Elsas et al. *(79)*. The persistence of a specific human foodborne pathogen in either environment is determined by the intrinsic properties of the microorganism, the properties and surface structure of the plant, and external factors derived from local agroecological conditions and the production practices used in cultivation of the crop.

Water from diverse sources (e.g., subsurface, surface, reclaimed, and municipal) has been used in the production of fruit and vegetable crops. Considerable seasonal or climatic variations in water quality are possible, notably where supplies are drawn from surface or subterranean sources. The specific water source(s), the distribution system(s), and the intended use(s) of the water pertaining to the study should be adequately described as outlined below.

Water source. Each water source applicable to the study should be described in detail including, as appropriate, information on the conveyance system and its condition and, if used, impoundments or reservoirs, the intended use, and the application method(s). Water quality may impact microbial survival; characteristics that are relevant regionally, such as nitrogen, pH, turbidity, conductivity, total dissolved solids, and carbon, should be provided in the study report (Table 2). Unless the water is from a consistent source (e.g., deep well), each of these water quality measurements are reasonably likely to change from sample to sample and day to day. In some cases these measurements may be available from public sources such as regional irrigation districts and state departments of natural resources or environmental protection. Ranges and average measurements with standard deviations or standard errors should be provided.

Preapplication treatment of water. Treatments are occasionally used to improve the microbiological quality of agricultural water when applied directly to the fresh produce during production. Mechanical (e.g., sand filters and settling ponds) or chemical (e.g., flocculants and antifoaming agents) treatments may be used to remove particulate matter or suspended solids. Other chemicals (e.g., chlorine and copper) that would directly affect microbial populations may be added to irrigation water. Water-soluble fertilizers may be added to irrigation water and applied through an irrigation system (i.e., fertigation). If any of these treatments are applicable, they should be noted, and relevant maintenance records and repair policies for equipment used in their application should be described in detail because these factors may influence the ability of pathogens to survive on the surface of a fruit or vegetable. When the study pertains to water use for application of agrochemicals, the specific agrochemical(s), chemical concentration(s), water temperature(s), and standard mixing methods should be described along with the maximum and minimum times

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

Factor	Good	Better	Best
Location	Growth chamber	Greenhouse	Field
Seasons or years (field trial)	One season, more than one location	More than one season, single location	Multiple seasons, multiple locations[a]
Site selection (field trial)	Site shares some characteristics of commercial growing region for crop of interest; conditions reflect reasonable air and soil temps and relative humidity for targeted region and crop	Site shares most characteristics of commercial growing region for crop of interest; conditions reflect reasonable air and soil temps and relative humidity for targeted region and crop	In the growing area of interest and replicates range of microclimates expected for the targeted region, season(s), and crop
Water source (used for experimental crop cultural management)	Water matches most quality ranges or extreme parameters of water source of interest	Water matches expected or characterized quality ranges or extreme parameters of water source of interest	Water source consistent with range of sources of interest
Crop[b] (study applicable to single crop)	Current commercial variety	Current commercial variety commonly grown in region of interest	Current commercial variety(ies) commonly grown in region of interest and preliminary or historical data suggest this variety is most likely to support survival of pathogen of concern
Crop (study applicable to more than one crop)	Single crop or crop type (with detailed justification for selection); crop type examples: citrus, leafy greens, tomato	All crop types in variance application are evaluated, OR preliminary data provide scientific basis for selection of most vulnerable crop type or time of year the crop is most vulnerable	All crops in variance application are evaluated, OR preliminary data provide scientific basis for selection of most vulnerable single crop (greatest survival of pathogen of interest) or time of year the crop is most vulnerable
Organism[c]	Nonpathogen or attenuated pathogen with some historical or laboratory-based data to support its use as a surrogate for the pathogen of concern	Nonpathogen or attenuated pathogen with detailed historical or laboratory-based data to support its use as a surrogate for the pathogen of concern when compared with outbreak strains	Nonpathogen or attenuated pathogen with detailed historical or laboratory- or field-based data to support its use as a surrogate for the pathogen of concern when compared with outbreak strains
Strain selection	Single strain with some data on environmental fitness	Two strains with some data on environmental fitness	Mixture or cocktail of three or more strains OR one clear dominant isolate in a series of environmental fitness studies
Strain marker[c]	Selectable stable phenotype (e.g., antibiotic resistance marker) that is reasonably effective for suppressing the growth of the background microbiota for plate count and enrichment methods	Selectable stable phenotype (e.g., antibiotic resistance marker) that is very effective for suppressing the growth of the background microbiota for plate count and enrichment methods	Selectable stable phenotype (e.g., antibiotic resistance marker) that is very 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
Management practices (field trial)	Site managed according to some local, current cultural practices including plant surface wetness regimes for targeted region and crop	Site managed according to most local, current cultural practices of interest including plant surface wetness regimes for targeted region and crop	Site management replicates local, current cultural practices of interest
No. of replicates	Two replicate trials	Three replicate trials in >1 yr	More than three replicate trials in >1 yr

[a] May vary with each experimental situation.
[b] Crop should be at relevant maturity or developmental stage for purpose of the study.
[c] In most cases, biosafety level 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). Lists of surrogate organisms are provided in Tables 5 and 6.

TABLE 2. *Physicochemical attributes of water that may influence the survival of pathogens*

Parameter	Example methods	Instrument utilized	Reasoning	Attribute
Water temp at sampling	EPA[a] field temp measurement (SESDPROC-102)[b] *(23)*	Thermometer	Temp correlated positively with *Salmonella* MPNs[c] *(37)*; inverse relationship observed between bathing water temp and percentage of *Salmonella*-positive samples *(65)*	Temp/decay rate
pH	EPA field pH measurement (SESDPROC-100) *(21)*	pH meter	Easy and familiar to measure	Acid/base
Oxidation-reduction potential (ORP)	EPA field measurement of ORP (SESDPROC-113) *(22)*	ORP meter	*E. coli* level varied inversely with ORP *(37)*	Acid/base balance
Turbidity	EPA field turbidity measurement (SESDPROC-103) *(20)*	Portable colorimeter	Higher percentage of *Salmonella*-positive samples observed during intense turbidity *(65)*	Protectant
Conductance	EPA field specific conductance measurement (SESDPROC-101) *(24)*	Conductivity meter	Enterococci correlated positively with conductivity *(37)*; could be related to salinity	Osmotic stress
Total dissolved solids	EPA method 160.1: residue, filterable *(17)*	Conductivity meter or drying oven and analytical balance		Protectant, nutrient source
Total organic carbon	EPA method 415.1: organic carbon, total *(18)*	Apparatus for total and dissolved organic carbon		Protectant, nutrient source
Nitrogen	EPA method 351.2: determination of total Kjeldahl nitrogen by semiautomated colorimetry *(19)*	Chemical hood, heating unit, balance, glassware, sulfuric acid, block digestor with tubes, continuous flow analysis equipment, pump, colorimetric detector		Protectant, nutrient source

[a] EPA, Environmental Protection Agency.
[b] SESDPROC, Science Ecosystem Support Division operating procedure.
[c] MPN, most probable number.

that would occur between mixing and application to the crop (including holding over multiple days or incorporation of new with old mixtures). The water used in the research study should be treated as it would be commercially for the particular application being studied.

Application method and quantity. The methods used for the application of irrigation water and crop protection sprays (e.g., furrow, surface drip, microsprinkler, and overhead sprinkler for irrigation; spray for agrochemical application) and the range of application rates (volumes of water) should be defined. If possible an estimate of the amount of water that is used on the crop for each water application of relevance to the study should be provided (e.g., acre-feet of water for irrigation and gallons per acre for agrochemical sprays).

Time of application (day, season, and harvest). Food safety risks from application of contaminated water are more likely when the water is applied closer to harvest. Time of application should be described to include, if relevant, time of day, season, age of the crop, and minimum projected number of days to harvest.

LABORATORY-BASED MODEL SYSTEMS

Laboratory-based model systems can provide important information about the influence of some environmental variables on pathogen survival in agricultural environments. Biocontainment and decontamination issues severely restrict the use of microorganisms in open greenhouse and field-based research. Consequently, available scientific literature on the survival of foodborne pathogens in water or on fresh produce crops has been largely confined to studies carried out in laboratory-based model systems (e.g., benchtop, growth chamber, and controlled greenhouse).

It is extremely challenging to simulate produce production environments or to assess the survival of pathogens attributed to preharvest water contamination of produce. Even minor changes in experimental protocols may affect pathogen survival. The diversity and types of water sources used for production agriculture complicate water studies,

both in the field and when designing a model system. Water sources across the United States do not have a standard temperature, flow rate, turbidity, total dissolved solids, specific conductance (electrical conductivity), pH, or microbial content. In addition, weather can influence foodborne pathogen levels in water and the survival of these microorganisms in water and on crops. Protective niches or biospheres that promote survival of foodborne pathogens may be present on the plant surface and may be influenced by plant surface topography and properties (e.g., numbers and locations of stomata) *(6, 51, 52)*. Protection can also be provided by shading (e.g., top versus bottom of leaves and higher versus lower regions of the plant) or by specific production practices *(27, 36)*.

The microbiota of water, soil, and plants and certain climate effects, such as rainfall and wind, are impossible to replicate under laboratory conditions. However, a well-designed model system that simulates natural conditions (e.g., temperature, humidity, and soil type) can be used to identify a smaller set of variables to be evaluated in the field. Whole plants should be used in model studies, and the soil and water used in laboratory-based systems should not be sterilized. Laboratory studies can evaluate the survival of test microorganisms under a range of scenarios and environmental conditions; those promoting the greatest survival can then be chosen for investigation in field studies as a way to limit the number of variables that need to be evaluated.

FIELD-BASED STUDIES

Laboratory-based studies on the fate of foodborne pathogens in water or on produce plants are generally carried out in controlled experimental systems in which as many variables as possible are normalized and controlled or are absent; these variables can include temperature, humidity, UV intensity, and water and soil chemistries. In contrast, field-based studies are subject to disruption from unpredictable natural events including extremes of weather, variations in soil and water quality, and damage caused by vermin, arthropods (insects and mites), or plant diseases. Consequently, strategies are needed to account for these sources of experimental variation, and experimental designs must include an assessment and identification of environmental variables that could influence the fate of test microorganisms. Parallel laboratory-based and field-level studies may be appropriate. Despite careful planning, a field trial may fail to yield useful results because of factors out of the researcher's control. Consequently, a greater number of replicate trials may be needed.

Production practices often differ significantly from one region to another and sometimes from year to year within a single region. These practices may also differ with the size or scale of the operation. It may be beneficial to engage commercial grower(s) or experienced field biologists to review the research protocol to ensure that, when possible, the experimental design is representative of the appropriate current commercial practice(s).

Site selection. Planning experiments that will introduce microorganisms to the agricultural environment must be done in strict compliance with local and national biosafety regulations and legislation. Detailed information on the geographic characteristics of the study site should be provided in the report. Water application to sites with low slopes will have a low potential for surface movement of microbes from areas of application under normal weather conditions. When water is applied to sites with high slopes (overall or in portions), surface runoff of inoculated microorganisms may occur. The latter situation could lead to localized areas of increased microbial pooling, which increases the potential for survival or growth.

The following geographic characteristics should be noted if present: (i) areas where weather events leading to flooding and/or erosion are common, (ii) animal activities that could disseminate the pathogens, and (iii) uneven terrain. A history of land use (e.g., the crops planted and soil amendments applied) on the study site within the previous 2 to 3 years should be provided. Sites where there is risk of drainage carrying the inoculated organism(s) to surface waters or public or private water supplies (consult watershed maps and hydrological data) should be avoided, or appropriate mitigation practices should be adopted.

Studies can be carried out in a commercial setting, and depending on the study objective, this may be the only option in some cases. Many universities, colleges, governmental agencies, and private sector interests can offer field research facilities with restricted or limited access. If such facilities are used they should be located close to and capable of replicating the representative commercial environment and management practices of interest. Release of any microorganism into a research field will usually require prior approval from a biosafety committee (even when nonpathogens or surrogates are used); such approval may take considerable time.

Protection of site. When possible, the site should be protected from variables that may influence experimental outcomes or that would lead to distribution of inoculated organisms outside of the study boundaries. Fencing to reduce wind erosion may be necessary in some locales. Standard bird control measures should be employed where needed. Likewise, the surrounding environment should be protected from possible cross-contamination with the microorganisms introduced as part of the study. Standard animal control measures should be considered to limit access by livestock or wildlife. It may be appropriate to clearly identify the test site with signage to discourage trespassing.

Climate. Accurate weather data are essential for the interpretation of yearly or seasonal variation in microbial data. Weather data (e.g., range and mode of precipitation amount, relative humidity, and air temperature) should be available from a local public source (e.g., National Climatic Data Center of the National Oceanic and Atmospheric Administration or state equivalent) (Tables 3 and 4). If using public source data, consider using readings for the past 5 years from one or more of the closest locations; the location(s) should be included in the study report. The same

TABLE 3. *Comparison of the climatic and environmental measurements provided by national and California agencies*

Climatic and environmental measurements	Stated in water variance document	NOAA, NCDC, and NESDIS[a]	CIMIS[b]
Solar radiation (W/m^2)	✓		✓ (daily)
UVB radiation (J/m^2)	✓		
Precipitation rain gauge amt	✓	✓ (daily) ✓ (hourly)	✓ (daily)
Precipitation time	✓	✓ (hourly)	✓ (hourly)
Precipitation duration	✓	Calculate from data	Calculate from data
Mean relative humidity (%)	✓	✓ (hourly)	✓ (daily) ✓ (hourly)
Evaporation (mm)	✓		✓ (daily: evapotranspiration)
Air temp		✓ (daily: max, min, avg, departure from normal, avg dew point, avg wet bulb) ✓ (hourly: dry bulb, wet bulb, dew point)	✓ (daily: max, min, avg, dew point)
Soil temp	✓		✓ (daily)
Sunrise and sunset time		✓ (daily)	
Significant weather or weather type		✓ (daily) ✓ (hourly)	
Snow or ice on ground (in.)		✓ (daily)	
Pressure (in. of Hg)		✓ (daily) ✓ (hourly: station pressure, pressure tendency, net 3-h change, sea level pressure, altimeter)	✓ (daily: avg vapor pressure [kPa])
Wind		✓ (daily: resultant speed, direction, avg speed, max 5 s, max 2 min) ✓ (hourly: speed, direction, gusts)	✓ (daily: avg wind speed, wind run [km]) ✓ (hourly: wind speed, wind direction)
Sky conditions		✓ (hourly)	
Visibility		✓ (hourly)	
Degree days			
Soil moisture			

[a] NOAA, National Oceanic and Atmospheric Administration; NCDC, National Climatic Data Center; NESDIS, National Environmental Satellite, Data, and Information Services. Data are available through a paid monthly or yearly subscription. More information on packages is available at http://www.ncdc.noaa.gov/oa/mpp/#MR. International data can be collected from the following sites: http://www7.ncdc.noaa.gov/CDO/georegion and http://cdo.ncdc.noaa.gov/pls/plclimprod/cdomain.abbrev2id.

[b] CIMIS, California Irrigation Management Information System. Data received free of charge. A sample of the data is shown on the following site: http://wwwcimis.water.ca.gov/cimis/frontSampDailyReport.do. Stations are not the same as those in the NCDC.

data should be reported for the time period during the field trial.

A wide variety of devices are available to measure climatic conditions at the site of the field trial. Although these measurements do not usually apply to all circumstances, they may provide information that would be useful for interpretation of results: (i) solar radiation (W/m^2) and UVB radiation (J/m^2), (ii) precipitation (rain gauge amount, time, and duration), (iii) mean relative humidity (%), (iv) evaporation (mm), (v) leaf wetness period, (vi) air and soil temperatures, and (vii) wind speed and direction.

CROP

Selecting crop variety. The specific crop type evaluated will depend on the objectives of the study. This document was developed specifically for fruits and vegetables that may be consumed raw. This includes crops with an outer rind or skin that is typically not consumed, such as melons and citrus, because contamination on the outer surface of these types of produce can be transferred to the edible portion of the fruit during preparation *(59)*.

Study results for one crop variety may not apply to other varieties (e.g., data for apples may not apply broadly to all pome fruit, and data for romaine lettuce may not apply to all leafy greens). Thus it may be necessary to consider individual varieties within a single crop or to evaluate combinations of crops that are commonly grown together. When the data are available, varieties demonstrating the greater potential for pathogen survival should be chosen.

Some of the considerations for crop selection include varieties that are common to the region in question, relative numbers of acres planted or volume produced, previous association with outbreaks of foodborne illness, and association with product recalls as a result of isolation of foodborne pathogens.

Age of the crop to which the test water is applied. The water use pertaining to the study (e.g., water for irrigation, water for pesticide application, or both) and the

TABLE 4. *Comparison of the climatic and environmental measurements provided by regional climate centers*

Climatic and environmental measurements	Regional Climate Centers					
	High Plains[a,b]	Midwestern[c]	Northeast[a,d]	Southeast[a,e]	Southern[a,f]	Western[g]
Solar radiation (W/m^2)		✓ (coming soon)				✓ various time frames, limited data[h]
UVB radiation (J/m^2)						
Precipitation rain gauge amt	✓ (daily: amt, year-to-date amt, 30-yr normals) ✓ (monthly)	✓ (daily)	✓ (daily: amt, year-to-date amt, 30-yr normals) ✓ (monthly)	✓ (daily: amt, year-to-date amt, 30-yr normals) ✓ (monthly)	✓ (daily: amt, year-to-date amt, 30-yr normals) ✓ (monthly)	✓ (daily data based on multiple years) ✓ (monthly data based on multiple years)
Precipitation time						
Precipitation duration						
Mean relative humidity (%)		✓ (hourly)				
Evaporation (mm)	✓ (monthly)	✓ (coming soon)	✓ (monthly)	✓ (monthly)	✓ (monthly)	
Air temp	✓ (daily: max, min, avg, 30-yr normals) ✓ (monthly: max, min, avg, departure from normal)	✓ (daily: max, min, avg) ✓ (hourly: temp, dew point, wet bulb)	✓ (daily: max, min, avg, 30-yr normals) ✓ (monthly: max, min, avg, departure from normal)	✓ (daily: max, min, avg, 30-yr normals) ✓ (monthly: max, min, avg, departure from normal)	✓ (daily: max, min, avg, 30-yr normals) ✓ (monthly: max, min, avg, departure from normal)	✓ (daily data based on multiple years: max, min) ✓ (monthly data based on multiple years: max, min)
Soil temp						
Sunrise and sunset time						
Significant weather or weather type						✓ (monthly data based on multiple years)
Snow or ice on ground (in.)	✓ (daily: amt) ✓ (monthly: new snowfall, depth on ground)	✓ (daily)	✓ (daily: amt) ✓ (monthly: new snowfall, depth on ground)	✓ (daily: amt) ✓ (monthly: new snowfall, depth on ground)	✓ (daily: amt) ✓ (monthly: new snowfall, depth on ground)	
Pressure (in. of Hg)		✓ (hourly)				✓ (monthly data based on multiple years)
Wind		✓ (hourly: speed, direction)				✓ (monthly data based on multiple years: speed, direction)
Sky conditions						✓ (monthly data based on multiple years: speed, direction)
Visibility						✓ (monthly data based on multiple years)
Degree days	✓ (monthly: heating, cooling, growing)	✓ (daily)	✓ (monthly: heating, cooling, growing)	✓ (monthly: heating, cooling, growing)	✓ (monthly: heating, cooling, growing)	✓ (monthly data based on multiple years: heating, cooling)
Soil moisture		✓ (weekly)				

[a] Data available are derived from the Climate Information for Management and Operational Decisions system (http://www.rcc-acis.org/index.php). [b] http://www.hprcc.unl.edu/. [c] http://mrcc.isws.illinois.edu/. [d] http://www.nrcc.cornell.edu/. [e] http://www.sercc.com/. [f] http://www.srcc.lsu.edu/. [g] http://www.wrcc.dri.edu/. [h] Data are available for certain locations (Oregon, Washington, Idaho, Montana, Wyoming, and Utah) in this region via the University of Oregon Solar Radiation Monitoring Laboratory (http://solardat.uoregon.edu/), the Washington State University Agricultural Weather Network (http://www.weather.wsu.edu/), and the national weather data site (http://www.pnwpest.org/wea/).

timing of the application of the water inoculum (e.g., horticultural maturity of the edible portion of the crop or time to harvest) should be considered when formulating an experimental plan.

To assist in experimental design and evaluation, an overview of the historical use of the water and time to harvest should be provided. Historical data can provide information about the normal use and timing of water applications to ensure the experimental design is based on normal practices or to provide guidance about alternative uses or timing. For example, when the study focus is water used in agrochemical application to the edible portion of the crop, the grower(s) can document past application dates for the target chemical and corresponding harvest dates after application. The greatest risk for microbial survival is with the shortest times between water application and harvest because of the limited time for pathogen reduction. In addition, the larger surface area of plants near harvest allows application of higher inoculum densities per unit and thus greater potential for survival per unit at harvest. The data generated from the study will usually apply only to the time period evaluated in the study (e.g., water applied 3 weeks before harvest) and not to shorter time intervals (in this example, less than 3 weeks before harvest).

Soil and crop management practices. Management of the experimental site should closely simulate typical or predominant agricultural conditions and practices. Factors to consider include (i) soil type; (ii) field preparation such as plowing, ripping, stubble disking, leveling, disking, and listing of beds; (iii) soil fumigation; (iv) soil conditioning (addition of compost, pellets, emulsions, or other amendments) and tilling; (v) pest management practices to control weeds, insects, and diseases; (vi) crop rotation schedules; (vii) cultivation for weed control; (viii) preplant and postemergence fertilization; (ix) management of the previous crop's residues; and (x) other relevant factors. Records should be maintained for the duration of the experiment, and a summary of this information should be archived including references to any production manuals used for guidance in establishing experimental protocols.

Harvest practices. Typical harvest practices should be described for the crop pertinent to the study because regional and crop-specific differences could impact research results. Some crops are harvested in a single pass, some are harvested in two or more passes without further irrigation, and some crops are harvested and reirrigated for a second or multiple harvests. In other situations, adjacent fields may be at the state of preplant or preemergence irrigation, under preharvest irrigation, or being harvested. The potential for contamination from sources that are separate from the crop being studied, but integral to on-farm water management, and the potential for unintended contamination of the study area should be noted. The potential for transference from adjacent areas by irrigation-management aerosols, other foliar treatments, equipment, human activities, and specific harvest practices related to data development should be recorded.

MICROORGANISM SELECTION

Biosafety. Foodborne pathogens must be handled in biosafety level (BSL) 2 facilities. Outside of a containment facility, the use of microorganisms containing recombinant DNA requires special permits. Thus a wider array of experimental microorganisms can be selected for studies conducted in model systems or in qualifying research facilities than would be permissible for open-environment testing. Even when pathogen surrogates or nonpathogenic organisms are used in field trials, appropriate permissions or permits may be required. Separate permits may be required for any organism carrying recombinant DNA. Some local regulations may completely prohibit the use of genetically modified organisms outside of containment facilities.

Both attenuated pathogens and nonpathogenic bacteria and viruses have been used as surrogates for foodborne pathogens in field trials (see ''Attenuated pathogens or other nonpathogenic surrogates'' and Tables 5 and 6, respectively). Although, by definition, attenuated pathogens are not pathogenic, most carry genetic factors that would be detected in commercial pathogen tests and in surveillance testing programs. The potential for the surrogate organism to contaminate commercial production should be carefully considered.

As an alternative to inoculation of field plots with microorganisms cultured in a laboratory setting, noncomposted manure or manure teas have sometimes been used. In these cases, the microbiota present in the manure become the inoculum. However, it is difficult to standardize microbial levels and types, thus introducing an additional variable to the study design.

Identifying the pathogen(s) of concern. If possible, the microorganisms for inoculation studies should be epidemiologically or ecologically relevant to the water sources being studied or the crop to which the water is applied. For those commodities for which no specific data exist, outbreak and recall data for fresh produce in general or an evaluation of the literature on relative environmental fitness of foodborne pathogens could be used to select a target pathogen. Although the pathogens of concern may be bacteria, viruses, or parasites, this document has focused on procedures that would be applicable to handling bacteria. These methods would need to be modified for field studies that involve viruses or parasites (e.g., different culture and detection methods), but the basic principles would still apply.

Cocktails versus single strains. Typically, a mixture of strains (pathogen or surrogate) with variable genotypic and phenotypic stress tolerances or presumptive enhanced host adaptations is preferred over the use of a single isolate. Consideration should be given to selecting one or more strains that were isolated in the test region or from the crop(s) being studied. When there are potential variations among different strains of the tested microorganism in terms of growth and survival, using three or five strains individually or in combination as the inoculum should be considered. When a cocktail of strains is used, the strains

within the cocktail should be screened (e.g., by cross-streaking) to demonstrate that there is no antagonism among them. Ultimately, for field trials, the choice and number of strains used may be dictated by the permits obtained for the study.

Attenuated pathogens or other nonpathogenic surrogates. In most cases, surrogate organisms will need to be used for field trials. Surrogates (either attenuated pathogens or nonpathogenic microorganisms) to be used in studies not conducted within a BSL-2 or BSL-3 containment facility (which may include specifically approved research farm locations) should be thoughtfully selected and validated in comparison with pathogenic forms. Attenuated (reduced virulence or avirulent) pathogens, sometimes used as surrogates, are usually missing one or more genetic elements that have been linked to infectivity or virulence of the organism (e.g., stx_1 and stx_2 in *E. coli* O157:H7 and *rpoS* in *Salmonella*). Demonstrating that an organism is missing genetic elements is relatively easy, but providing definitive evidence that a strain is not capable of causing illness (is a nonpathogen) is considerably more difficult. There is no formal mechanism to apply for BSL-1 classification for an organism that is attenuated. Attenuated pathogenic strains that have been used as surrogates in a wide range of trials have rarely been subject to animal feeding trials *(38),* and classification of an attenuated strain as categorically nonpathogenic or BSL-1 is subjective at best. The risk of applying an attenuated pathogen that is weakened but not incapable of causing disease should be carefully evaluated, and appropriate precautions should be taken to protect the inoculation site and neighboring areas. For sites that are adjacent to commercial production, application of an attenuated pathogen carries additional risk if the organism were to survive or move beyond the test site. In the latter case the isolation of the organism in a commercial crop may trigger a positive outcome in routine product testing, which would be unacceptable, even if one were able to prove that it is a well-characterized attenuated strain.

Selection of a nonpathogenic microorganism that was, for example, isolated from the region and crop of interest is a viable alternative. However, comparison of the environmental fitness of the nonpathogenic surrogate and the target pathogen should be well documented.

Typically, a surrogate will have the following attributes *(7)*: (i) similar characteristics to those of the pathogen of concern such as growth, inactivation kinetics, attachment capacity, susceptibility to sublethal stress injury, and resuscitation; (ii) inducible stress tolerance resistance traits (pH, heat, desiccation, osmotic pressure, etc.); (iii) ease of detection; and (iv) differential or unique phenotypic and/or genotypic traits from background isolates.

In addition, for field trials it is important that survival of the selected surrogate mimics that of the pathogen on growing plant materials and under environmental stress. There are relatively few well-characterized surrogates for use in field trials. Therefore, it may not always be possible to use more than one surrogate or a cocktail of strains. Table 5 provides a list of attenuated pathogens, and Table 6 provides a list of nonpathogenic strains (including some strains of *E. coli*) that have been used as surrogates for foodborne pathogens in field trials. Results among various surrogate microorganisms can differ significantly, and a surrogate used in one environment may not be suitable for another; justification for the choice of the surrogate should be provided. If no directly relevant published comparison data are available (e.g., comparison with the pathogen of interest), studies may need to be conducted to establish that the surrogate strain is suitable for use based on an evaluation of the criteria above; these data should be included in the study report.

Marker-assisted detection and enumeration. Environmental persistence and dispersal studies are preferably conducted with isolates genetically marked in some way to facilitate detection, recovery, and enumeration. Strains carrying markers, such as antibiotic resistance, xenobiotic degradation (complex or unique chemical degradation), lux, green fluorescent protein (GFP), or other differential reporters, will aid in the selection, enrichment, or enumeration of the target pathogen or the surrogate from produce samples containing high populations of background microorganisms.

The selection of a marker should be carefully considered. Numerous studies have used either antibiotic resistance genes or GFP (both plasmid and chromosomal) as the marker for tracing the target microorganism in microbiologically complex environments such as manure and soil (Tables 5 and 6). When the genetic marker is derived by recombinant DNA methods its use in field trials may be restricted or prohibited.

Strains with selectable markers will need to be characterized to ensure that there is minimal variation of physiological characteristics from parent strains and that the marker is stable in the absence of selection and under conditions simulating environmental stressors *(58)*. Regardless of the nature of the genes, marker stability was shown to be affected by the location of the genetic modification and the degree of gene expression *(13)*. The stability of the markers and the effect of the promoter:-marker pairing on the growth, survival, and any desired or critical phenotypic traits (e.g., cell size or serological markers) of bacteria should be determined for each strain before use. Some strains of *E. coli* O157:H7, *Salmonella,* and *Listeria* labeled with the plasmidborne GFP gene can be stable for many generations without adversely affecting growth rates *(55)*.

MAINTENANCE, CULTIVATION, AND PREPARATION OF INOCULUM

There are many ways to prepare microbial cultures for inoculation. Methods used to prepare the inoculum should be adequately described. A discussion of key elements of culture methods for inoculation of postharvest fruits and vegetables was presented by Beuchat et al. *(3)*; many of the key points apply here.

TABLE 5. *Attenuated foodborne bacterial pathogens that have been used to assess survival in model studies or field-based research*

Organism	Designation(s)[a]	Original source	Genotype (strain modifications)	Detection methods	Available data on infectivity or comparison with infectious strains	Biosafety level (source)[b]	Comments	References
Bacteria								
E. coli O157:H7	B6-914	CDC[c]	stx_1^-, stx_2^- (Amp^R, pGFP, Rif^R)	Fluorescence (302–366 nm); ampicillin- or rifampin-supplemented media	No	Unknown	Construct, GFP[d] labeled	*31–33, 43, 44*
E. coli O157:H7	ATCC 700728, NCTC 12900, PTVS 154	Naturally occurring isolate from humans (Public Health Laboratory, Austria)	stx_1^-, stx_2^- (Rif^R or Nal^R)	Rifampin- or nalidixic acid–supplemented media	Yes; comparative studies with diverse pathogens, ATCC 43888 and *E. coli* in lab and growth chamber	BSL-1 (ATCC)	Sold as a quality control standard for *E. coli* O157:H7 tests	*2, 4, 11, 36, 50, 61*
E. coli O157:H7	DM3	A derivative of strain NCTC 12900 (ATCC 700728)	stx_1^-, stx_2^-, eae^- (strain *DM3n:* Nal^R)	DM3n: MacConkey with nalidixic acid	Yes; reduced adherence and invasion of HEp-2 cells	BSL-1 (parent strain)	Insertional inactivation of *eae* in DM3 (strain contains recombinant DNA)	*84*
E. coli O157:H7	ATCC 43888, CDC B6914-MS1, PTVS 155	Isolated from human feces (CDC)	stx_1^-, stx_2^- (pGFPuv/Amp^R, Kan^R, Rif^R)	Fluorescence (365 nm); ampicillin- or rifampin-supplemented media	Yes; comparative studies with diverse pathogens in lab and growth chamber	BSL-2 (ATCC)		*11, 26, 27, 36, 50, 67, 69, 70, 73*
E. coli O157:H7	CV267	Cattle isolate	stx_1^-, stx_2^- (pGFPuv/Amp^R)	Fluorescence (365 nm); ampicillin-supplemented media	No	Unknown		*26, 27*
E. coli O157:H7	6980-2, 6982-2	IEH Consulting Group (Lake Forest Park, WA)	stx_1^-, stx_2^- (pGFPuv/Amp^R)	Fluorescence (365 nm); ampicillin-supplemented media		Unknown		*26, 27*
E. coli O157	20001383	Cattle waste	Non–verotoxin producing	Tryptic soy broth supplemented with novobiocin followed by plating onto CHROMagar O157, modified sorbitol MacConkey agar	No	Unknown		*40, 41*

TABLE 5. *Continued*

Organism	Designation(s)[a]	Original source	Genotype (strain modifications)	Detection methods	Available data on infectivity or comparison with infectious strains	Biosafety level (source)[b]	Comments	References
E. coli O157:H7	MD46, MD47	MD46 is a knockout mutant of F5456, associated with 1997 alfalfa sprout outbreak; MD47 is a knockout mutant of K4992, associated with 2006 lettuce outbreak	stx_1^-, stx_2^-, eae^- (pGPF, Amp^R)	Ampicillin-supplemented media	No	Unknown		*82*, unpublished
Salmonella enterica serovar Typhimurium	χ3985, PTVS 177	Unknown	Deletion mutant: Δcrp-11 Δ[zhc-1431::Tn*10*], Δcya-12 Δ[zid-62::Tn*10*] lacking adenylate cyclase and cyclic AMP receptor protein (Rif^R)	MacConkey agar + 1% maltose and colony confirmation; PTVS 177: rifampin up to 80 μg/ml	Yes; slower growth rate than the wild-type strain, but ability to attach to, invade, and persist in gut-associated lymphoid tissue of chicks is retained	Unknown	Nonpathogenic derivative (LD_{50}: $>4 \times 10^9$ CFU for 1-day-old chicks) of χ3761, a highly virulent *S. enterica* serovar Typhimurium strain (oral LD_{50} of 3×10^3 CFU for 1-day-old chicks); strain developed as vaccine in chickens. PTVS 177: robust environmental survival in soil and on plants	*38, 45, 46, 57, 69, 70*
S. enterica serovar Typhimurium	SL1344	Unknown	Chromosomal GFP pUC18T-mini-Tn*7T*-Gm-gfpmut3; M913 (fliGHI::Tn*10*) deficient in motility, M935 (cheY::Tn*10*) deficient in chemotaxis	Scanning electron microscopy, illumination intensity	No	Unknown	GFP labeled	*35, 51, 52*

TABLE 5. *Continued*

Organism	Designation(s)[a]	Original source	Genotype (strain modifications)	Detection methods	Available data on infectivity or comparison with infectious strains	Biosafety level (source)[b]	Comments	References
S. enterica serovar Typhimurium	ATCC 14028	Animal tissue	MAE 110 and MAE 119 are cellulose-negative or aggregate-negative derivatives of ATCC 14028 (GFP, Gen^R, Kan^R)	Fluorescence (365 nm); kanamycin-supplemented media	No	Unknown		*31, 85*
Viruses								
Poliovirus 1	LSc2ab	Sabin Type I oral poliomyelitis vaccine	57 base substitutions	Plaque assay	Yes	Unknown	Attenuated vaccine strain	*53, 64, 72*

[a] Culture collections: ATCC, American Type Culture Collection (www.atcc.org); CECT, Colección Española de Cultivos Tipo, Spain; CIP, Collection de L'Institut Pasteur, France; NCTC, National Collection of Type Cultures, UK. Specific information on these and other international culture collections can be obtained from the World Federation for Culture Collections (www.wfcc.info). Microorganisms and viruses also may be given designations consisting of letters and serial numbers, often including the researcher's initials or a descriptive symbol of locale or laboratory in the designation (e.g., TVS 353, 354, etc., named for T. V. Suslow).

[b] Biosafety level (BSL) designation is indicated where known. Regardless of BSL designation, researchers should consult with their institutional biosafety committee.

[c] CDC, Centers for Disease Control and Prevention, Atlanta, GA.

[d] GFP, green fluorescent protein.

Strain preparation. Selected bacterial strains should be isolated directly from frozen stock cultures onto nonselective agar medium and incubated at an appropriate growth temperature. If required, antibiotics to maintain fluorescence or other selective markers should be included in the medium. A single well-isolated colony should be removed with a sterile loop, inoculated into a liquid broth medium (which may also contain the selective marker), and incubated at the appropriate temperature to stationary phase. Strains can be grown with or without constant agitation, but conditions should be consistent for each inoculation and transfer. The strain identity should be confirmed using an appropriate method.

Inoculum preparation. As a general principle, the inoculum should be prepared in a manner that maximizes the strain characteristics that are being tested (e.g., resistance to desiccation). The conditions pertinent to the likely source of contamination should be considered when developing inoculum preparation procedures. In most cases, it would be rare that contamination of the water supply would occur immediately before application to the crop. Thus a hold time (e.g., 18 to 24 h) between inoculum preparation and inoculation of plants may be valid. In addition, the growth medium composition (e.g., nutrient rich or poor, or neutral, high, or low pH), plate or broth culture, and temperature of incubation (cool or optimum) may influence survival. It is generally accepted that stationary-phase cells are appropriate; cells that have been nutrient deprived or adapted to acidic or alkaline conditions may also be appropriate depending on the characteristics of the water.

Few systematic studies on inoculum preparation are available. In some cases, the way in which microorganisms are handled prior to inoculation had little practical influence on survival (e.g., on lettuce surfaces *(71)*). In other cases, the preparation and handling of the test microorganism significantly impacted the survival of the organism after inoculation (e.g., on nut kernels *(75)*).

A description of the culture conditions should be provided in the study report along with the rationale for the selected methods. The inoculum should be grown in the presence of the selective agent (e.g., marker antibiotic) that will be used in the recovery medium, and the stability of the marker in the absence of selection during growth and recovery should be reported.

(i) Broth inoculum preparation. Overnight broth cultures grown in laboratory media should be washed by centrifugation to remove nutrients or inhibitors. The pellet should be suspended in the carrier medium and can be held, until use, as appropriate to the objectives of the study (e.g., refrigerated, on ice, mixed with agrochemicals, and held at ambient temperature).

(ii) Plate inoculum preparation. An overnight broth culture should be spread on nonselective agar plates (containing antibiotics if appropriate) to produce a bacterial lawn after incubation. Large (150 by 15 mm) petri dishes are useful for this purpose. Following an appropriate incubation time at an appropriate temperature, cells can be collected, suspended in the carrier medium, and held refrigerated or on ice until use. Bacterial lawn cultures should be prepared carefully to avoid introducing contaminants from the medium (e.g., nutrients or inhibitors). Washing the cells by centrifugation and suspension in a carrier broth is sometimes employed for these cultures *(71)*.

Carrier medium. The carrier medium used to suspend the inoculum in the test water should be described. Common carriers include, but are not limited to, 0.1% peptone, Butterfield's phosphate buffer, and sterile water. In many cases it may be appropriate to suspend the inoculum directly into the water source pertinent to the study.

Water used as a carrier and during cultivation. Water will typically be used both before and after inoculation in the cultivation of the test crop. Although it is preferable to use the agricultural water source that is the subject of the study, site selection may dictate that the target water source is not the same water source used in the experimental studies. Thus the specific water source(s) used in the study (both carrier and during cultivation) and its physicochemical attributes (Table 2) should be described.

The potential influence of water quality variables should be considered in the design of studies and the analysis of experimental data and should be discussed in the study report. The systematic examination of the effect of each water quality parameter on microbial survival is not necessary. However, a prudent experimental design should consider the potential range or extremes in water quality for a specific application. When information is available, water quality parameters that have the potential to enhance the environmental fitness of the inoculated organisms should be chosen.

Sampling inoculated water prior to application. Factors such as pH, oxygen reduction potential, turbidity, and conductivity may influence microbial survival and recovery both in the water and on the crop. This means that the water used for the experiment should approximate the water that is actually being applied to the crop in question. The relevant water factors noted above should be measured for each experiment to control for variability and ensure reproducibility. Likewise, it may be of value to sample the inoculated water to determine the level of indigenous microbiota (e.g., heterotrophic plate count) and perhaps any relevant indicators (e.g., thermotolerant coliforms).

Preapplication treatment of water. Specific agrochemicals mixed into the water prior to application of the water to the crop may have a microbiological impact that must be considered. When there is an impact (increase or decrease in populations of foodborne pathogens), then the time (from addition to the water to application to the crop, including storage time) and the water temperature should be factored into the experimental design. The time incorporated into the design should be the time, under normal practice, that would result in the greatest number of pathogens prior to application to the crop.

TABLE 6. *Nonpathogenic surrogates that have been used to assess survival in model studies or field-based research*

Organism	Designation(s)[a]	Source	Detection methods	Pathogen comparison data available	Comments	References
Bacteria						
E. coli	TVS 353, 354, 355	Surface irrigation water (TVS 353), romaine lettuce (TVS 354), and sandy loam soil (TVS 355) samples from the central California coast near Salinas	Rifampin-supplemented media	Yes; direct comparative data with attenuated and pathogenic isolates in lab and growth chamber	Individual isolates differentiated by PFGE and REP-PCR[b]; comparative survival studies for soil, water, mixed greens, and spinach	*36, 50, 69, 73*
E. coli	NAR	Water	Nalidixic acid–supplemented media	Compared with DM3n (attenuated *E. coli* O157:H7)		*84*
E. coli	ATCC 25922	Clinical isolate	Colilert quanti-tray system (IDEXX, Westbrook, MA)	Yes; hydrophobicity, attachment, and recovery similar to that of *E. coli* O157:H7	Irrigation water studies; transfer to crop examined	*16, 49, 66*
E. coli	P36	Isolated from pork slaughterhouse	Bioluminescent (*lux* CDABG); kanamycin-supplemented media	No		*80, 81*
E. coli K-12	ATCC 35695, MC 4100, LMM1010	*E. coli* K-12 derivative	Nalidixic acid– or erythromycin-supplemented media	Yes; lower attachment and recovery than *E. coli* O157:H7	Common bacteriophage host	*29, 49*
E. coli K-12	ATCC 25253	Clinical isolate	Streptomycin-supplemented media	Yes (for food processing applications)	Widely used laboratory control strain	*16, 29*
E. coli K-12	EQ1	*E. coli* K-12 derivative	Nalidixic acid–supplemented media	No		*40*
Clostridium sporogenes	CIP 79.3, ATCC 19404NA, NCTC 532	Gas gangrene	Differential reduced clostridial medium incubated anaerobically	No	Media testing quality control strain	*34*
Listeria innocua (serotype 6b)	CIP 80.12, NCTC 11289	Human feces	Modified Oxford medium, MPN[c] in Fraser broth	Yes; *L. innocua* survived similarly or slightly better on parsley leaves than did *L. monocytogenes*	Direct inoculation of parsley leaves in the field	*15, 34*
L. innocua (serotype 6a)	CECT 910, ATCC 33090, NCTC 11288	Cow brain	Palcam agar	Yes; growth rate similar to that of *L. monocytogenes* ATCC 19114 in shredded lettuce		*30, 63*
L. innocua	LiP60	Isolated from organic amendments	MPN in Fraser broth	Yes; *L. innocua* survived similarly or slightly better on parsley leaves than did *L. monocytogenes*		*15, 34*

TABLE 6. *Continued*

Organism	Designation(s)[a]	Source	Detection methods	Pathogen comparison data available	Comments	References
Pseudomonas fluorescens	TVS 074	Derivative of A506 (S. Lindow, Univ. of California, Berkeley), the EPA[d] labeled biocontrol agent in BlightBan, originally registered by T. Suslow	Rifampin up to 80 μg/ml and UV fluorescence on Kings medium B	Yes; direct comparative data with attenuated and pathogenic isolates in lab and growth chamber; plant attachment and environmental fitness traits span range observed in pathogens and attenuated strains; less resistant to desiccation than *Salmonella* PTVS 177	Tier 1 toxicology studies available; registration and EPA exemption from residue tolerance on all crops	*74*
Viruses[e]						
Coliphage	PRD1, PRD-1, MS2, ATCC 15597-B1, ATCC 16696-B1	*Enterobacteriaceae*	Plaque assay (PFU method), RT-PCR[f]	Yes (for some strains); recovery rates after chlorine treatment similar to those for hepatitis A virus on strawberries, lettuce, amd cherry tomatoes	Irrigation water studies; transfer to crop examined	*1, 10, 66*
Murine norovirus 1	MNV, MNV-1.CW1, MNV P3, ATCC PTA-5395	Mouse	Confocal microscopy, plaque assays, qRT-PCR	Yes; comparisons indicate that MNV has potential to be a useful surrogate for human norovirus	Selection of norovirus surrogate for surface or groundwater BSL-2	*1, 9, 83*
Canine calicivirus	CaCV strain 48	Domestic dog	RT-PCR	Yes; no apparent difference in virus internalization when compared with human norovirus		*76*
Porcine sapovirus (SaV) Cowden strain, cell culture adapted	TC-Po/SaV/GIII/Cowden/1980/US	Pig	Cell culture to detect infectious virus; real-time RT-PCR to detect viral RNA	Yes; stable at low pH (3.0), similar to human norovirus by infectivity assay; SaV and norovirus share similar resistance to chlorine and heat (56°C) treatments by real-time RT-PCR	BSL-2	Unpublished data: Dr. Q.-H. Wang, 2011, Ohio State University, Food Animal Health Research Program, OARDC, Wooster, OH (used in greenhouse)

[a] Culture collections: ATCC, American Type Culture Collection (www.atcc.org); CECT, Colección Española de Cultivos Tipo, Spain; CIP, Collection de L'Institut Pasteur, France; NCTC, National Collection of Type Cultures, UK. Specific information on these and other international culture collections can be obtained from the World Federation for Culture Collections (www.wfcc.info). Microorganisms and viruses also may be given designations consisting of letters and serial numbers, often including the researcher's initials or a descriptive symbol of locale or laboratory in the designation (e.g., TVS 353, 354, etc. named for T. V. Suslow).

[b] PFGE, pulsed-field gel electrophoresis; REP-PCR, repetitive element PCR.

[c] MPN, most probable number.

[d] EPA, Environmental Protection Agency.

[e] Some viruses that are not human pathogens may nevertheless be classified as BSL-2 and thus are unsuitable as a surrogate in many situations.

[f] RT-PCR, reverse transcription PCR.

Inoculum enumeration. Inoculum suspended in a carrier medium should be enumerated by an appropriate method to determine the initial bacterial population after suspension in the carrier medium and immediately before application to the crop. Appropriate methods may include serial dilutions and plating onto nonselective media (containing antibiotics when appropriate) and selective media, membrane filtration, or most-probable-number (MPN) methods. Selective media should not be used alone because of the potential for the presence of injured organisms. When a multistrain cocktail is being used, initial levels can be standardized to a consistent optical density; all individual strains should be enumerated separately before being combined (using equal volumes of each individual strain), and inoculum levels in the final inoculum preparation should also be determined.

Level of inoculum applied to test plants. Determining the level of inoculum to use in an experiment is difficult because of the variability and uncertainty of contamination in surface water sources. Postoutbreak investigations and ongoing monitoring programs suggest that irrigation waters typically contain very low levels of human pathogens *(37, 48)*. Water quality in the agricultural environment is variable, and occasional increases or ''spikes'' in contamination are known to occur, but predicting the frequency, magnitude, and causes of both point source and non–point source spikes in water contamination remains largely unachievable. Furthermore, the relationships between pathogen level, timing of application relative to harvest (under varying environmental conditions and crop traits), and survival on the crop are unclear. The available and practical enumeration and recovery methods for different food and water matrices limit the level of quantitative detection that can be achieved for specific human pathogens. Practical restrictions in sampling regime and the same issues of recovery efficiencies also limit enrichment-based detection. Inoculum levels can sometimes have a significant impact on survival of pathogens on produce *(28)* and resulting cross-contamination *(60)*. In other cases, inoculum levels play a minor role in survival *(71, 75)*. Survival may be enhanced with higher inoculum levels or preinduction of stress-tolerance traits in the pathogen to reflect environmental survival expectations. Rapid and significant declines in bacterial populations may be observed within a short time of inoculation; decline is often not linear, and distinct and prolonged tailing may be observed *(4, 61)*. Low levels of inoculated organisms may persist for long periods of time, and the magnitude or rate of initial reductions cannot be used to predict persistence *(4, 36, 61)*.

Given these uncertainties and limitations, it is not possible to recommend a level of inoculation that will suit the diversity of experimental scenarios implied by this document. Consequently the objective(s) of the specific studies should guide the level of inoculum applied in the experiments, and justification should be provided in the study report. In general, lower numbers of stress-adapted or stabilized cells in the inoculum is preferred over unrealistically high numbers not known to be present in any reasonable water source. The inoculum level is often calculated from an estimate of maximum levels of the target foodborne pathogen or indicator organism (e.g., *E. coli*) that likely would be present in the water of interest (determined by historical data) multiplied by a fixed factor (e.g., 100) to account for uncertainties in existing data.

Uniform application of inoculum on the test plants. Liquid inoculum should be evenly distributed throughout the water sample (e.g., by stirring with a magnetic stir bar or by shaking or vortexing with sufficient agitation, depending on the volume being inoculated).

Method and time of application. The delivery system for the microbial inoculum should be considered. When possible, the inoculum should be applied in a way that would mimic normal agricultural practice. The time between application of the inoculum and harvest of the crop should be carefully considered in the experimental design. When practical for the purposes of the study, the highest volume application that would be used on the crop should be considered (highest potential for contamination and highest potential for survival). Application by spray in the presence of high winds should be avoided. The experimental design should consider water application and time to the earliest point of harvest. Survival of pathogens will be influenced by the age of the crop, and a greater survival potential would be expected closer to harvest.

Personal protective equipment. Because large numbers of microorganisms are typically used during application, appropriate personal protective equipment should be provided to those directly involved in application (e.g., gloves, laboratory coats or body suits, goggles, and respirators). Appropriate personal protective equipment should also be considered during sample collection and, as applicable, for farm labor personnel who may need to enter the experimental site after inoculation. Equipment used for application of the inoculum (e.g., tubing and spray containers) should be disposed of or sanitized as appropriate. Be aware that these procedures are typically required by an institutional biosafety review committee and requirements may vary among different organizations.

RECOVERY OF TEST MICROORGANISMS FROM THE CROP

This section addresses some of the issues that should be considered for recovering the test microbe from the edible portion of the crop. Pathogens or surrogates applied to a crop in a field can vary in their ability to survive. Factors affecting microbial survival that should be considered include direct sunlight and shade, rain and drainage patterns, and prevailing winds. A discussion of the statistical considerations regarding numbers of samples to test is included below (see ''Experimental Design, Sampling Strategy, and Statistical Analysis''). All sampling, subsampling, compositing, and microbial protocols should be evaluated to determine the sensitivity of the protocol.

Sample collection tools. As in any well-controlled microbiological experiment, tools and equipment should be

cleaned with soap and water to remove dirt and debris and then sanitized and rinsed thoroughly with potable water. Alternatively, presterilized sampling devices (e.g., blades, scoops, containers, and bags) may be purchased or prepared in the laboratory. Personal protective equipment, such as gloves, should be used and care should be taken to ensure that cross-contamination among samples does not occur.

Size of the individual analyzed units. The size of the individual sample is often chosen subjectively or for convenience, with a wide range of values reported in the literature for similar experimental conditions. However, it is more appropriate that individual sample sizes be chosen on the basis of a number of factors including weight and surface area of the edible portion of the crop (or serving size for the food in question) and the lower limit of detection. Whole or partial plants or one or more pieces of fruit or vegetable may be considered a sample (e.g., head of lettuce or individual lettuce leaves, one melon, or 10 cherries).

It is reasonable to expect that survival of the target microorganism will vary depending on its location on the fruit or vegetable architecture (e.g., surfaces that are primarily in shadow and able to retain water longer will likely support survival better than surfaces that are dry and exposed to UV radiation). Unless whole edible portions are used as the sample, areas or sections most likely to support survival should be sampled or prior studies should be considered to guide sampling strategies.

Samples should be handled in a controlled manner during transport to the laboratory, recognizing the potentially biohazardous nature of the samples as well as the need to protect the target microorganism from further inactivation or growth beyond what occurred in the field. High and low temperature extremes should be avoided, and testing should be timely. The sample temperature during the time of transport and preprocessing storage should be recorded and documented. Care should be taken to avoid freezing the samples, because freezing and thawing may result in changes to microbial populations.

Methods for recovery. A full discussion of the methods used for recovery of pathogens from fresh produce is beyond the scope of this document, but sources such as the FDA *Bacteriological Analytical Manual (77),* the U.S. Department of Agriculture Microbiological Data Program (www.ams.usda.gov/mdp), and the *Compendium of Methods for the Microbiological Examination of Foods (14)* provide detailed methodologies for specific analytical purposes. The optimum method may differ depending on the organism and the crop. Sample preparation may include washing the whole sample or swabbing a defined portion of the outer surface, homogenizing in buffer, or a variety of other treatments. The organisms that were inoculated onto the produce may be viable but stressed or injured, so appropriate resuscitation techniques that acknowledge this possibility may be required. For some products, homogenization or blending should be avoided; in some cases these procedures result in release of antimicrobials that may kill the target organism in the diluent or restrict its growth on agar media or in an enrichment broth. When a standard or validated method is not used for recovery, data validating the efficacy of the method used in the study should be provided. The specific method for recovery will influence the reporting of the results. Results may be reported on a per-gram, per-unit, or per-surface-area basis for both enumeration and presence-absence testing (e.g., log CFU/g, log CFU/cm^2, or log CFU per piece of fruit; 2% positive results from 300 100-g samples, 10% positive from 50 500-cm^2 areas, or 0% positive from 400 pieces of fruit).

Methods for enumeration and end-point determination. Methods commonly used to determine the presence of a microbe in a food or water sample include direct enumeration, filtration and plating, MPN-based methods, and enrichment (presence-absence) testing. In direct enumeration, the level of the organism in question is determined by dilution of the sample in buffer, and colonies arising from cells in the sample are enumerated on agar plates containing the appropriate selective marker agent (e.g., antibiotic). Enrichment-based approaches do not permit direct quantification but indicate only presence or absence of the target organism in a certain sample size. MPN-based methods use a series of dilutions that are enriched (in appropriate medium containing the appropriate selective marker agent) and then scored 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 *Bacteriological Analytical Manual*, Appendix 2 *(5)*. (At the bottom of the Web page is a link to download an Excel spreadsheet.)

To reduce the costs and labor associated with analysis of individual site samples, samples may, in some cases, be composited prior to enrichment *(47, 62)*. Although it would be desirable to analyze (enrich) the entire composite sample, the volume of medium required may be excessive. For presence-absence testing, a two-class attribute sampling plan *(42)* may be used to determine the number of subsamples that should be analyzed from each composite sample.

A justification for the choice of quantification or enrichment method(s) and compositing strategy (if used) should be provided. For both plate counts and enrichment samples a subset of colonies should be selected to confirm that the organism is the inoculated strain. Even with the use of antibiotic resistance markers, background microbiota may be able to grow on selective media especially when the level of the inoculated organism reaches the limit of detection. There is also a possibility that background microbiota will grow in some enrichment media even when antibiotics are added. Confirming the identity of a number of isolates either from plate counts or positive enrichment broths is prudent. The number of isolates to process 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 is capable of multiplying in the recovery medium.

Control samples. In addition to sampling the inoculated product it may be useful to include uninoculated controls to determine the background microbiota (e.g., aerobic plate count and thermophilic coliforms).

EXPERIMENTAL DESIGN, SAMPLING STRATEGY, AND STATISTICAL ANALYSIS

This section addresses some of the design criteria and expectations that should be considered during development of sampling protocols and sample process parameters.

Sample size (number of samples per time point) and statistical power. Sample size should be based on the research objectives and should consider the limits of detection for the methods used. It may be useful for the researcher to meet with a statistician or other expert to determine the statistical power of the study design. All sampling, subsampling, compositing, and related protocols should be evaluated to determine the sensitivity and the resulting confidence level, such that for any given sampling scheme one could state the degree of confidence in detecting the microorganism in question if it were present at a given mean log level. It is important to take into account that the levels of microorganisms on inoculated plants can change nonuniformly on treated surfaces and develop nonuniformly on newly formed tissues postinoculation. Sampling plans should anticipate such changes in microbial distribution. These effects are typically increased at time points further away from the inoculation and should be taken into account when evaluating the validity of the sample size and tissues included.

Number of replicate experiments and system variability. Biological systems are inherently variable. In addition to the wealth of published information describing this variability on plant surfaces, both above and below ground, there is abundant information and experience that demonstrates the lack of uniformity of irrigation source water. This is particularly true and relevant for the diverse sources of untreated surface water. Some water applications or production systems may be inherently more variable because of, for example, the presence of microclimates within the test area or where water cannot be uniformly applied; irrigation patterns are seldom uniform across a field or orchard. These factors may lead to increased variability in the data collected, which may lead to a need for more replicates to separate treatment effects. Each sampling survey and experimental study of controlled treatment effects should be replicated. The involvement of an agricultural engineer with experience in measuring system variability may be useful for designing experiments that involve application of water in agricultural settings.

The execution of a single trial is rarely considered rigorous; however, when the study data complement those from previous similar studies of directly comparable design then a single replicate may suffice. Acute and seasonal environmental (i.e., weather) conditions may affect pathogen survival in soil, on crops, and on noncrop vegetation; therefore, field trials intended to support broad conclusions regarding the environmental biology of pathogens in an agricultural setting are usually conducted in more than one season and typically in more than 1 year, for perhaps as many as 3 or more years. Exceptions to this generalization are possible on a case-by-case basis. Dependent upon the specific objectives and associated design elements, a minimum of three replications per treatment or variable within an experiment and two repeated experiments of essentially identical design are the accepted standard for field trials.

Number of field locations. Although specific water and weather conditions may predominate within a geographic region, variations may occur that could potentially affect pathogen inactivation. Therefore, collection of data from more than one field location and from field locations that are not located adjacent to each other should be considered (Table 1). Rationale for site selection should be provided. For example, highly uniform fields (e.g., uniform slope, uniform soil texture, and similar distance from regional landscape features) across a broad area may support the selection of a single central location. More variable field or regional profiles may require comparative studies that encompass the key known sources of potential influence on water source or crop environments (e.g., a regional valley with fields of varying soil texture bands, variable distance from a large water body, marine influence on the duration of leaf wetness, variable slope, and microclimate). These data can be found in regional agricultural databases (e.g., SoilWeb *(8)* for California).

Heterogeneity in field environments. It is often assumed that microorganisms introduced onto crops via water would uniformly contaminate the plot. However, various factors contribute to differential microorganism inactivation (i.e., pH; organic material concentrations; leaf, blossom, fruit, and root surface variability; spatial heterogeneity in microbiota; and edge effects in a field) and lead, over time, to micro and spatial heterogeneity *(36, 39, 56)*. The measured population is, at best, an estimate of the combined survival, growth, death, and dispersal on any plant surface. The sampling plan adopted to quantify the presence of contamination at a given time should include sufficient sample numbers and adequate sample size to account for this variability. Populations of individual introduced organisms such as an applied surrogate will invariably be more heterogeneous among different plants than populations of a composite group such as total mesophiles. Sample collection should be randomized across a unit survey area, preferably according to a random-site generator program, or across all replicated plots for each treatment in an experimental trial. It may be appropriate to conduct a preliminary background population assessment under the relevant conditions to provide data to a consulting biometric statistician to determine appropriate sampling protocols. The rationale for the sampling plan used should be discussed.

Duration of study and sampling intervals. Although it was designed for a different purpose, the recommendation from the National Advisory Committee on Microbiological Criteria for Foods challenge study document *(62)* was adapted for use here.

Challenge studies should be conducted for at least the intended period of interest, from point of water application to point of harvest. The sampling interval should be determined based on prior experience with similar crops and in consideration of the likely duration of survival or rate of growth or inactivation. Depending on situation and expected outcomes, it may be appropriate to test on a more frequent basis early in the study (e.g., hourly or daily) and at longer intervals (e.g., weekly) later in the study. For estimation of kinetic parameters, it may be necessary to have more than five sampling points.

When measuring pathogen inactivation, the study is typically concluded when the pathogen is no longer recovered from the product in noninhibitory enrichment media at more than one time point. In field trials the end of the trial may also be defined by maturity of the crop or typical harvest time. Pathogen recovery by enrichment is dependent upon sample size, and ''absence'' from a production area is dependent on sample numbers. Sample numbers are not anticipated to be constant. Greater sample numbers (in some cases hundreds of samples) as one approaches ''statistical zero'' below the point of quantification but within the probability of presence-absence detection is a reasonable expectation. Two consecutive observations of no detection in an increased sampling regime is the often accepted sensible endpoint for field trials. The trial may also end when the crop is considered ready for harvest. The report should include a discussion of the considerations taken when determining the end of the trial.

When quantitative data are obtained using direct enumeration or MPN-based methods, trends in the data can be used to predict microbial survival. In some challenge studies, a majority of the introduced microbes are inactivated in a log-linear manner. This initial inactivation can be followed by the survival and persistence of low levels of pathogens for an extended period of time, depending upon the characteristics of the organism and the matrix. Hence, it may not be appropriate to use the initial log inactivation rates (e.g., *D*-values) as a basis for determining pathogen or indicator levels. A variety of mathematical models can be used to describe non–log-linear microbial inactivation over time. The use of such models to describe microbial survival on crops in field trials has not been extensively validated, so they should be used with care. When models are not used, microbiologists often default to a direct approach in which they analyze samples by enrichment and after two successive negative sample observations conclude the pathogen has been ''eliminated'' and the experiment has been successful. When this approach is used, care must be taken to measure and report starting levels and detection limits and to consider the statistical limits of any claim regarding ''complete elimination'' of pathogens or indicators.

LIMITATION OF STUDY

Data collected in a single production system may not be sufficient for the validation of risk mitigation strategies meant to be applied by growers in disparate agroecological zones or applied to more than one crop. The report should include a description of the specific growing parameters (crop, region, and season) to which the study would apply. Extension of the data for crops, regions, or seasons other than the one in which the study was conducted should be carefully considered and well justified. The study will apply only to the timing interval between application of the water and time of no detection and longer.

SUGGESTIONS FOR INCLUSION IN A STUDY REPORT

The study report should be completed under the supervision of an expert with a strong background in microbiology who is fully aware of all applicable regulations. The study report must provide appropriate information, including an interpretation of the results, so that others can assess the adequacy of the study. The report should begin with an introduction that includes the objectives of the study and reviews the data supporting the experimental design. The report should include information characterizing the crop, production practices, and source(s) and application of water. The materials and methods should be described as they would be in a scientific publication. It may be appropriate to include both raw and summarized data, both of which should be clearly presented. A discussion should provide an interpretation of the results and any limitations on the applicability of the data. The conclusions should contain key findings and any recommendations and should indicate the types of changes in product formulation or processing that could warrant a new inoculation study.

Specific information (as applicable) should be collected for each study and provided in the report. Table 7 provides checklists for (i) information specific to the study objectives and (ii) information to include in the study report.

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TABLE 7. *Study report checklist*

Information type	Specifics	Description
	Specific to the study objectives	
Water	Water source(s)	Describe all water sources subject to this study
	Water characteristics	Document physicochemical characteristics of the water source(s) (Table 2)
	Intended use(s)	Describe all intended uses of water
	Preapplication treatment(s)	Describe water treatment(s) prior to application (if relevant); includes agrochemical mixing details
	Application method(s)	List all application methods and provide rates (volumes)
	Time of application details	List historical time(s) of day, season, age of crop, and time to harvest at the time of water application
Region	Geographic location	Provide information on the location including latitude and longitude of the field(s) under this application
	Geospatial characteristics of field(s)	Include elevation, level, sloping, and direction of slope
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 preparation, fumigation, conditioning, tilling, nutrient management history, and other practices
	Soil type	Describe critical factors, e.g., pH; salinity; soil survey description; clay, sand, and silt content; organic matter content; major nutrients: nitrogen (N), phosphorus (P), and potassium (K); minor nutrients: iron, manganese, copper, zinc, boron, molybdenum, and aluminum
Climate	Climate history	Describe average weather during the relevant period and prevailing conditions
	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 experimental design; include historical data on water use and time to harvest for crop
Materials and Methods		
Laboratory-based model system	Model description	Describe system (e.g., growth chamber) and environmental conditions used
	Rationale and justification	Provide rationale when study is based solely or primarily on data from a model system and justification of model used
Field-based study	Study site	Describe geographic location and geospatial characteristics of field(s) used in the study and provide rationale for site selection
	Climate conditions at site	Include data for temperature, rainfall, humidity, UV exposure, solar radiation, and wind at field trial site immediately prior to and during the study
Crop	Crop description	List type(s) and varieties of crops included in the study design
	Crop management practices	Describe production practices that were used before and during trials (e.g., irrigation dates, times, and duration; nutrient management history; and pesticide application)
Soil	Soil management practices	Describe practices used during the study
	Soil type	Describe critical factors
Microorganism(s)	Pathogen(s) of concern	Identify pathogen(s) relevant to water source(s) or crop(s)
	Selected microorganisms	Justify selected microorganism(s) including strain, cocktail vs single strain, and marker selection
	Inoculum preparation	Describe maintenance, cultivation, and preparation of inoculum including media, incubation time, and temp
	Inoculum carrier	Describe the inoculum carrier medium; include physicochemical attributes if medium is water
	Inoculum enumeration	Describe procedures used for enumeration of microorganism(s) in the inoculum
Inoculation	Application protocol	Provide level, frequency, and method of inoculation and environmental conditions
	Time of inoculation	Provide time of day, date, plant age, and number of days before typical harvest
Recovery and detection	Sample preparation	Provide sample size and weight, diluent type, volume or ratio, and recovery method (e.g., rinse, swab, or homogenize); 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

TABLE 7. *Continued*

Information type	Specifics	Description
	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 number of sample units, samples analyzed, and sampling intervals
	Replicates	Justify number of replicates
	Study duration	Provide rationale for duration of study
Controls	Control type	Describe each control and provide rationale for number
Statistical analysis	Statistical test(s)	Describe statistical approach and rationale
Results		
Data	Raw data on organism levels	Provide unadjusted level data (e.g., plate counts, dilution, and sample size) and measure of variability (e.g., standard error or standard deviation)
	Raw prevalence data	Provide unadjusted prevalence data (e.g., number of positive samples, sample size, and total number of samples processed)
	Adjusted data	Provide calculated level 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 the study objectives
	Limitations	Discuss limitations of the applicability of the data
	Conclusions	Include any recommendations; indicate what factors might warrant new challenge study

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