MICROBIOLOGICAL METHODS

Checking the Validity of the Harvesting and Disaggregating Steps in Laboratory Tests of Surface Disinfectants

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A chemical disinfectant against surface-associated bacteria typically uses carriers (e.g., glass disks) that are purposely contaminated with bacteria prior to disinfection. After disinfection, the bacteria are harvested by mechanically separating them from the carrier surface to form a suspension of cells in a dilution tube. Bacterial clumps in the tube are disaggregated using mechanical or chemical techniques, thereby creating a well-mixed suspension of single cells suitable for enumeration. Efficacy is quantified by comparing the viable cell count for a disinfected carrier to the viable cell count for sham-disinfected (control) carrier. A test is said to be biased (invalid) if the observed efficacy measure is systematically higher or lower than the true efficacy. It is shown here for the first time that the bias attributable to the harvesting and disaggregating steps of a disinfectant test can be measured. For some conventional biofilm harvesting and disaggregating techniques, laboratory checks showed either negligible bias or important bias, depending on the disinfectant. Quantitative bias checks on the harvesting and disaggregating steps are prudent for each combination of carrier material, microorganism, and disinfectant. The quantitative results should be augmented by microscopic examination of harvested disinfected and control carriers and of the disaggregated suspensions.

Quantitative laboratory methods for testing a disinfectant against surface-associated bacteria typically use easily manipulated carriers (e.g., glass disks or slides). For a dried surface test, suspended bacteria are placed on each carrier and dried, a method that simulates the spill of a contaminated liquid. For a biofilm test, a bacterial biofilm is grown on the surface of each carrier using a growth protocol that emulates surface contamination in a specific moist, hydrated, or intermittently hydrated environment. Some of the carriers are disinfected and others serve as control carriers. At the end of the designated use time, the disinfectant is neutralized to stop its activity. Control carriers receive the same manipulations and neutralization as the disinfected carriers. For each carrier, the bacteria are harvested from the surface into a suspension, and any clumps of cells are disaggregated. Conventional dilution series and plating methods produce a count of CFUs, indicating the viable cells on the carrier. The counts conventionally are expressed as the density of viable cells, for which the units are either CFU/cm^2 (of carrier surface area) or CFU/carrier. The outcome of the test is the log reduction (*LR*) measure of disinfectant efficacy found by subtracting the mean of $\log_{10}$-transformed densities for the disinfected carriers from the mean of $\log_{10}$-transformed densities for the control carriers.

An acceptable disinfectant test must be relevant, practical, reproducible, and valid (unbiased). Of these criteria, absence of bias presents the greatest challenge. A quantitative test is unbiased if, over many independent tests of a specific disinfectant, each test following the standard operating procedure, the *LR* values are neither systematically too high nor too low. It is infeasible, however, to measure the bias for a disinfectant test, because the true *LR* value is unknown. Consequently, one cannot use a comparison to the truth for determining whether observed test outcomes are correct on average or are systematically incorrect. This makes it impossible to apply the definition of bias directly to disinfectant tests. For this reason, an indirect approach is required to provide assurance that a test is unbiased. Experts evaluate each step of the disinfectant test method and judge whether that step is valid. In this context, "experts" are those who review and approve methods in some official capacity, such as referees for a peer-review journal, committee members in a standard methods validation organization, or officials in a regulatory agency. If all steps are deemed unbiased, the disinfectant test, including the *LR* result, is assumed to be valid. This is the same strategy as used to provide QA for a production process, especially when the check of the finished product requires destroying the product,

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thereby making it economically impractical to use routine sampling inspection. Instead, the manufacturer will strive to maintain the quality of every step in the production process, thereby creating a finished product of acceptable quality.

For a disinfectant test, expert judgment may be the sole determinant of validity for some steps. For example, many of the laboratory manipulations during a disinfectant test are well-established microbiological techniques, and the validity of each such step is accepted without special check data. For other steps, laboratory data may be required to provide assurance that the steps are valid. For example, it has long been recognized that incomplete neutralization of a chemical disinfectant could invalidate disinfectant test results. In response to that concern, a neutralization check experiment (1) is often included in the standard operating procedure for a disinfectant test.

The premises of this paper are (*1*) if every step is valid, then the *LR* result is unbiased, (*2*) if a particular step is invalid, then it will cause the *LR* to be systematically higher or systematically lower (i.e., that step creates a bias), and (*3*) the bias due to a step can be measured. This paper focuses on two critical steps in a surface disinfectant test—harvesting and disaggregating.

Many techniques for accomplishing the harvesting step, the disaggregating step, or both steps simultaneously have been devised. The term harvesting indicates the laboratory manipulation that purposely separates surface-associated bacteria from the carrier, usually by mechanical means. Harvesting should not be confused with removal of bacteria from the carrier due to chemical effects during the disinfection step. The harvesting step has also been called removal or recovery. Harvesting techniques include scraping, mixing with a Vortex mixer (with or without beads), sonicating, stomaching, swabbing, and washing, where each technique can be applied with or without the addition of chemical agents such as surfactants or enzymes (2–7).

The disaggregating step has also been called destabilization (of the aggregates or the extracellular polymeric substance that binds biofilm bacteria), dispersion, resuspension, declumping, or disintegration. Commonly used disaggregating methods include mixing with a Vortex mixer (with or without beads), local agitation by repeated fill/expel pipetting when forming the dilution series, sonicating, and homogenizing, where each technique can be applied with or without the addition of chemical agents such as surfactants or enzymes (5, 6, 8–10).

Because the harvesting technique might not be able to safely remove every viable cell from a carrier surface, and the disaggregating technique might not completely and safely break up all clumps into single cells, it is prudent to consider the possibility that the harvesting technique or the disaggregating technique might bias the efficacy value. In fact, important bias due to the harvesting and disaggregating steps has been observed in practical circumstances. One case pertained to an antimicrobial-coated sewing cuff on an artificial heart valve (11). Laboratory antibacterial tests of the cuff showed good efficacy at resisting bacterial colonization. However, subsequent investigation utilizing confocal scanning laser microscopy showed that, in fact, the cuff was not effective at preventing bacterial colonization (11). Apparently the initial laboratory tests produced a biased *LR* because, unknown to the investigators, the harvesting efficiency for the antimicrobial coated cuff was lower than for the control cuff. Consequently, the viable cells on coated surfaces were undercounted, and the efficacy result was systematically too high.

In a different application, it was observed that a glutaraldehyde formulation had the fixative effect of increasing both attachment strength and microcolony cohesion in a biofilm (12). The investigators cautioned that conventional harvesting techniques were unlikely to remove the viable cells from disinfected carriers, thereby undercounting the viable cells.

In a study about the determination of bacterial numbers in the effluent of bioreactors by plate counts, it was found that the main problem was neither nonviable cells nor physiological specialists, but aggregates of bacteria (9). The investigators noted that many techniques for converting aggregates into isolated CFUs also killed part of the bacterial community. It is plausible that a viable, but injured, subpopulation within the disinfected cell suspension would be especially sensitive to disaggregation trauma. The result would be an artificially low viable cell count for disinfected carriers and an exaggerated efficacy measure.

As is illustrated in data below, a disinfectant might contain nonlethal dispersive ingredients, such as surfactants or enzymes. Those chemicals could promote more effective harvesting or disaggregating. The result would be relatively higher viable cell counts for disinfected carriers and a systematically too small *LR*.

Although the preceding examples point to an obvious need for laboratory methods for checking the validity of the harvesting and disaggregating steps, the development of check methods has lagged behind. A few methods have been devised for comparing the efficiencies of alternative harvesting or disaggregating techniques. Published evaluations show that the success of a harvesting or disaggregating method often depends on application-specific factors, such as surface material and bacterial species. Almost all published evaluations focused only on control carriers, disinfected carriers were not considered. The usual goal was to determine the technique that achieved the highest viable cell counts in the suspension. As will be shown below, existing check methods are potentially misdirected. A convincing check must consider disinfected carriers, as well as control carriers. Validity is not assured by high control carrier viable cell counts.

Among the published techniques for checking the validity of the harvesting step are methods for counting the cells on carriers (2, 5, 13, 14) and techniques for measuring the aggregate amount of biofilm on carriers (13, 15–18). The literature also contains methods for comparing the efficiencies of alternative disaggregating techniques. Among the published evaluations are viable cell counts before and after disaggregating (5, 9, 10) and quantitative microscopic

assessment of filtered samples of the suspension before and after disaggregating (5, 19).

The harvesting and disaggregating steps are not easily separated in some applications, e.g., disinfectant tests in which the bacteria are associated with surfaces that are soft, porous, granular, or fragile. For preparing food samples, soil samples, fabric samples, or samples of packing material (e.g., sand, peat, or granular activated carbon), various combinations of sonicating, stomaching, mixing on a Vortex mixer, and homogenizing—with and without the addition of glass beads or of chemicals such as enzymes, surfactants, or chelating agents—have proven effective at simultaneously harvesting and disaggregating surface-associated bacteria (8, 13, 20–24). These combination techniques are also used for carriers that are made of nonporous, hard materials, such as polycarbonate, steel, or glass (3, 25). By simultaneously harvesting and disaggregating, one minimizes sample manipulations, and thereby potentially reduces both the risk of contamination and the cost of experimentation.

Published laboratory evaluations of combined harvesting and disaggregating techniques include viable cell counts, total cell counts, active cell counts, and counts in electron microscope images (4, 13, 21, 23, 24). Because of the mechanical energy required to accomplish both harvesting and disaggregating, many studies checked whether the techniques caused cell injury or death. Some studies uncovered the lethal effects of a chemical additive, such as an enzyme or a chelating agent (23, 25). Because of the tradeoff between the beneficial effect of separating aggregates into single cells and the detrimental effect of damaging or inactivating cells, it is especially challenging to identify optimum specifications when applying a sonication method (8, 10, 13, 25, 26).

The main goal of this paper is to show how data from laboratory check experiments can be used to calculate a quantitative estimate of the bias due to harvesting or disaggregating. It is demonstrated that practical, reliable laboratory check experiments are feasible and that bias can be calculated by comparing the harvesting efficiency and disaggregating efficiency for disinfected carriers to the corresponding efficiencies for control carriers. This paper explains why it is not essential to achieve a high efficiency for control carriers. Rather, it is critical that the efficiency for the disinfected carriers is the same as for control carriers.

Calculating the Harvesting Bias and Disaggregating Bias

Let S_C and S_D denote the observed viable cell densities for control and disinfected carriers, respectively. The log reduction is:

$$LR = \log_{10}(S_C) - \log_{10}(S_D)$$

Here and elsewhere, the subscripts C and D denote control and disinfected carriers, respectively.

Harvesting Bias

Let X denote the numerical measurement of the bacteria on the surface of a carrier according to the chosen method; e.g., for a crystal violet (CRV) staining method (17), the quantity X denotes the corrected spectrophotometer measure of light absorbance, an indirect indication of the number of surface-associated bacteria. The symbols AH and BH are used as superscripts to denote "after harvesting" and "before harvesting," respectively. The measured bacterial load on the carrier is $X^{(BH)}$ before harvesting and is $X^{(AH)}$ after harvesting. Because of the manipulations required to conduct the measurement, it usually is not possible to measure both $X^{(AH)}$ and $X^{(BH)}$ on the same carrier. Let U denote the estimated harvesting efficiency fraction by a specified harvesting technique; then U is calculated as:

$$U = (X^{(BH)} - X^{(AH)}) / X^{(BH)}$$

The amount by which LR is biased due to harvesting is:

$$bias_h = \log_{10}(U_C) - \log_{10}(U_D)$$

If the harvesting technique removed all viable cells from the surface, then the conventional LR would be valid. For incomplete harvesting, the LR result is biased if and only if there is differential harvesting from the disinfected carrier compared to the control carrier, that is, if and only if $U_C \neq U_D$.

Disaggregating Bias

Consider a suspension of bacteria that had been harvested from the carrier and disaggregated according to the specific disaggregating protocol. One approach for checking the effectiveness of the disaggregating technique is to filter a sample of the suspension, examine the filter microscopically, and calculate the density of viability-stained cells for comparison to the density based on conventional CFU plate counts. Note that the microscopy and CFU counts are based on samples from the same disaggregated suspension.

Let M_C and M_D denote the microscopy-based viable cell density for the control and disinfected carriers. Let F_C and F_D denote the disaggregating efficiency fractions for the control and disinfected carriers:

$$F_C = S_C / M_C \text{ and } F_D = S_D / M_D$$

If the cells were completely disaggregated into individual cells, then each CFU corresponds to a single cell and F should be almost 1.0. On the other hand, if disaggregation was incomplete and many colonies were due to aggregates of bacteria, then S would be considerably less than M and F would be much less than 1.0. However, the main issue is bias, not attainment of a near-perfect disaggregating efficiency fraction. The formula for calculating the disaggregating bias of LR is:

$$bias_d = \log_{10}(F_C) - \log_{10}(F_D)$$

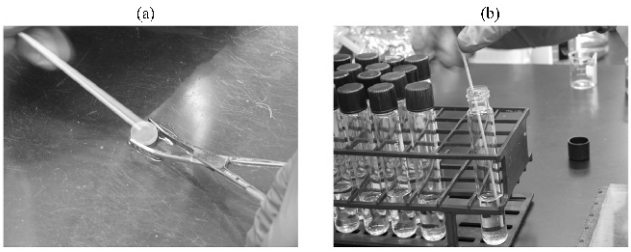

Figure 1. Harvesting by scraping the carrier face with a wooden applicator stick (a) and rinsing the stick in a dilution tube (b). The carrier face also was rinsed into the dilution tube (rinsing not shown).

The *LR* efficacy measure is biased if and only if there is differential disaggregation; consequently, there is no bias due to the disaggregating step if F_C and F_D are nearly equal, even if those disaggregating efficiency fractions are small.

Methods and Results for Demonstrating the Feasibility of Laboratory Bias Checks

To assess the feasibility of bias checks in the laboratory, a series of experiments was conducted in the Center for Biofilm Engineering at Montana State University. A few of the methods and results have been selected for presentation here. Although the experiments used biofilm bacteria, the check methods could be adapted to dried surface tests.

Harvesting Bias

A CRV stain technique was used to determine the fraction harvested. The carrier was placed for a specified time in a sterile 50 mL centrifuge tube that contained a specific concentration of CRV. To clear away the nonbound CRV, the carrier was rinsed three times by dipping in three separate 45 mL volumes of sterile dilution water in 50 mL centrifuge tubes. The bound CRV was eluted from the carrier with ethanol in a 50 mL centrifuge tube. The eluted CRV was poured into a cuvet and measured by a spectrophotometer (Genesys™ 20, Thermo Fisher Scientific, Pittsburgh, PA) absorbance reading at a wavelength of 540 nm.

To calculate the harvesting fraction, five types of carriers were required: control carriers and disinfected carriers that held unharvested biofilm; control carriers and disinfected carriers from which the biofilm was harvested; and "clean" carriers that were sterilized but not placed in the biofilm reactor. The harvesting efficiency fraction was calculated using $X^{(BH)}$, the mean spectrophotometer absorbance reading for the unharvested carriers minus the mean absorbance reading for the clean carriers and $X^{(AR)}$, the mean spectrophotometer absorbance reading for the harvested carriers minus the mean absorbance reading for the clean carriers. Note that the subtraction corrects the absorbance readings for potential background absorbance at 540 nm.

A *Pseudomonas aeruginosa* (ATCC 15442) biofilm was grown on glass carriers in either a Centers for Disease Control and Prevention (CDC) biofilm reactor (Biosurface Technologies, Bozeman, MT), according to the standard operating procedure (27), or a drip flow biofilm reactor (Biosurface Technologies), according to the standard operating procedure for the reactor (28). Each carrier from the CDC reactor was stained for 15 min in 5 mL 1% CRV, and the CRV was eluted from the carrier surface by immersion in 5 mL 95% ethanol for 10 min. The biofilm from the drip flow reactor was stained for 10 min in 45 mL 1% CRV, and the CRV was eluted from the carrier surface by 10 min immersion in 45 mL 95% ethanol. To check a harvesting technique, four carriers were removed from the same biofilm reactor and two were treated with the harvesting technique and two were unharvested. Some clean carriers received each CRV staining protocol to provide background readings.

Harvesting was accomplished by scraping with a wooden (white birch) applicator stick into a dilution tube (Figure 1) according to the standard protocol (7, 29). To assess the responsiveness of the CRV method on the control carriers, poor and intermediate harvesting efficiencies were created artificially by scraping only designated fractions of the carrier surface, e.g., scraping just in a narrow band across the middle of the carrier or scraping just half of the carrier surface. A good harvesting efficiency was created by scraping the whole surface. The results in Table 1 show that U_C was responsive to differing harvesting efficiencies, in that U_C increased when the carrier surface was completely scraped compared to partial scraping.

The CRV method was also applied to disinfected carriers so that $bias_h$ could be calculated. In each of four experiments, a *P. aeruginosa* (ATCC 15442) biofilm was grown in a CDC reactor on four glass disk carriers, two control carriers, and two disinfected carriers. In three experiments, the disinfected carriers were treated with 10 mL 1000 mg/L chlorine for 10 min and neutralized with sodium thiosulfate. In the fourth experiment, the disinfected carriers were treated with 1% pectinase for 10 min and neutralized by dilution with sterile dilution water. The biofilm was harvested from the carrier surface by scraping the whole face. Because these experiments did not include unharvested biofilm carriers, the calculation of *U* was based on the mean corrected absorbance readings over all unharvested biofilm carriers observed in the previous CDC reactor experiments. The harvesting efficiency fractions for the treated carriers were similar to the harvesting fractions for the control carriers. All harvesting bias values were negligibly small, ranging from –0.10 to 0.13 (Table 2). For harvesting by scraping and the materials, species, and disinfectants considered here, the CRV laboratory check indicated negligible harvesting bias.

The CRV check method was relatively easy and inexpensive. A potential disadvantage of the CRV stain is that it labels all biofilm components, not just the viable cells. During exploratory work on check methods, the investigators evaluated other stains, such as the monotetrazolium redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which stains actively respiring bacteria, and found promising results (data not shown). Although one can conclude from this work

Table 1. Results from the CRV check method for calculating the control carrier harvesting efficiency fraction (U_C). The geometric mean of U_C and the SD of $\log_{10}(U_C)$ across two carriers are shown for each harvesting method.

Harvesting method	Geometric mean of U_C	SD of $\log(U_C)$
Biofilm from the drip flow reactor		
Narrow band scraped	0.24	0.4942
Half scraped	0.54	0.1695
All scraped	0.87	0.0462
Biofilm from the CDC reactor		
Narrow band scraped	0.57	0.0439
Half scraped	0.22	0.1719
All scraped	0.69	0.1616

that a harvesting check method is feasible, further research is required to develop an optimized, reliable standard method.

Regardless of the quantitative harvesting check method, some disinfected and control carriers before and after harvesting should be inspected under a microscope. The pattern or location of bacteria remaining on carriers may provide guidance for improving the harvesting protocol. For example, glass disk carriers holding biofilm grown in a CDC reactor were observed under a Nikon Instruments Inc. (Melville, NY) SMZ1500 continuous zoom stereomicroscope with 7.5–110 magnification. Images of the stained biofilm showed that unscraped carriers contained extensive biofilm bacteria (Figure 2a), and the scraped carriers contained few bacteria (Figure 2b). The center portion of a scraped carrier face was fairly clean, and the few bacteria found on the surface were mainly within scratches. It appeared that a few clumps of biofilm remained on the carrier because they were pushed by the scraping stick to the edge of the carrier or were located on the beveled coupon edge where the final rinsing step missed them (Figure 2b). Images of the unstained biofilm showed an extensive biofilm on the unharvested biofilm carriers (Figure 3a). A high harvesting efficiency for 1 min of sonication ($U_C = 1.0$; data not shown) was visually confirmed (Figure 3b). The effectiveness of partial scraping is visually evident in Figure 3c for a half-scraped carrier and in Figure 3d for a carrier where only a narrow band across the middle of the carrier was scraped. Visual inspection of carrier surfaces required neither expensive microscope equipment nor highly specialized techniques, yet it provided important information about harvesting effectiveness.

Disaggregating Bias

The CTC stain was used to indicate viability for purposes of directly counting viable cells in the disaggregated suspension (30). A 1.0 mL sample of undiluted suspension was filtered through a 25 mm id, 0.22 m pore size, black, polycarbonate membrane filter (GE Water & Process Technologies, Trevose, PA). The filtering process caused any clumps to collapse into flattened aggregates that were one cell thick. The filter was rinsed three times with filter-sterilized ultrapure water and stained with 2 mL 0.04% (w/v) CTC (Polysciences, Inc., Warrington, PA) prepared in filter-sterilized ultrapure water for approximately 1 h in the dark. The stain was rinsed through the filter with filter-sterilized ultrapure water. The filter was mounted with nonfluorescing immersion oil on a glass microscope slide with a glass cover slip and examined via epifluorescence microscopy using a Nikon Eclipse E800 fluorescence microscope fitted with a CTC filter cube. With the objective set at 100 , 20 randomly chosen fields/membrane filter were each photographed using a fluorescence camera (Photometrics CoolSNAP fx, Roper Scientific Photometrics, Tucson, AZ). Each field covered a very small fraction (approximately 3 10^{-8}) of the effective filtration area. The fluorescent cells were counted in each field of view, and those direct counts were scaled up to provide a density for the carrier. Assuming that respiration is an accurate indication of viability, the CTC method provided a microscopy-based alternative to the CFU-based viable cell count method.

In each experiment, a *P. aeruginosa* (ATCC 15442) biofilm was grown on glass disk carriers in a CDC reactor. There were two control carriers and two disinfected carriers. For two experiments, disinfection was with 1000 mg/L chlorine for 10 min. For one experiment, disinfection was with 1% pectinase for 10 min. Because pectinase is known to break down the extracellular polymeric substance, it was expected to cause better disaggregation than for control carriers. For each carrier, biofilm was harvested by scraping. The suspension for each carrier was disaggregated by

Table 2. The $bias_h$ values and the geometric means of U_C and U_D for the CRV harvesting check method. The biofilm was harvested by scraping.

Experiment	Treatment (concentration and time)	Geometric mean of U_C	Geometric mean of U_D	$bias_h$
1	Chlorine (1000 mg/L for 10 min)	0.38	0.46	–0.10
2	Chlorine (1000 mg/L for 10 min)	0.52	0.65	–0.10
3	Chlorine (1000 mg/L for 10 min)	0.71	0.68	0.02
4	Pectinase (1% for 10 min)	0.71	0.53	0.13

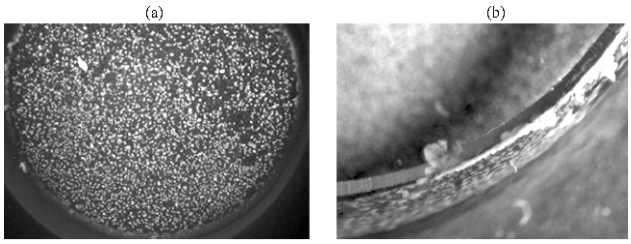

Figure 2. Microscope images of glass disk control carriers containing biofilm grown in the CDC reactor, viewed after staining with Syto 9. Panel (a) is the surface of an unscraped carrier, 7.5 magnification, and panel (b) shows an edge of a scraped carrier, 20 magnification.

homogenization at 20 500 rpm for 1 min. Samples were taken from each suspension for purposes of both CFU-based and CTC stain microscopy-based viable cell density counts, and the disaggregating efficiency fraction was calculated. When treating with chlorine, the two estimates of the disaggregating bias of *LR* were small: 0.05 and 0.28 (Table 3). When treating with pectinase, the disaggregating bias was –1.3, large enough to be of practical importance.

The pectinase data show that the CTC staining technique can detect differential disaggregating efficiency fractions. Although it is feasible to measure disaggregating bias, the CTC staining technique was time-consuming. Also, it depended on the assumption that CTC accurately identified viable cells. Other methods based on microscopy and image analysis also were successful, but the details are beyond the scope of this article. As a qualitative check on a disaggregating method, visual inspections of filtered samples are strongly recommended both before and after disaggregating.

Harvesting and Disaggregating Bias

Based on a couple of feasibility experiments, the investigators judged that, for checks utilizing only two disinfected carriers and two control carriers, it was not difficult to conduct both a harvesting check and a disaggregating check at the same time.

Discussion

This paper provides practical methods for addressing concerns about the harvesting or disaggregating steps in disinfectant tests against surface-associated bacteria. Most reported evaluations of the harvesting and disaggregating steps have ignored the fact that differential efficiency is the key issue. For example, even though a harvesting technique consistently removes just 0.1% of the bacteria from both disinfected and control carriers, it is satisfactory for disinfectant testing. The reason is that a 0.1% sample of the bacteria is counted on every carrier, control and disinfected, and the average calculated *LR* will be exactly the same numerical value as when 100% of the viable bacteria are counted on each carrier. On the other hand, a method that harvests almost all bacteria from the surface of a control carrier will produce a biased *LR* whenever the fraction harvested from disinfected carriers is consistently smaller.

Although many innovative methods have been published for harvesting and disaggregating bacteria, the extent to which those manipulations bias the outcome has not been calculated previously. Note, however, that a similar quantitative approach was used in a study conducted to estimate the bacterial population on leaves of living plants that was in attached biofilm (5). Those investigators showed how to calculate harvesting efficiency and use that efficiency for calculating the bias of the primary outcome, mean biofilm abundance.

The bias checks investigated here pertain only to the consistent misrepresentation of *LR* due to differential harvesting or disaggregating efficiencies, not to the overall bias that occurs when *LR* systematically departs from the "true" *LR* for a disinfectant. Work remains to be done on optimizing the most promising of the check methods. Although new ideas may be forthcoming because of technological advances, the harvesting check method based on staining the carriers with CRV was informative and practical. Labeling techniques have also proven useful, e.g., radiolabeling (31) or green fluorescent protein labeling (32). One should consider also laboratory checks based on nonbiological measurements, such as a dried weight method or total protein measurement, which are inexpensive and easy. Although the disaggregating check based on microscopic examination of the filtered suspension is feasible, the investigators found that there were many steps, and opportunities to make missteps, in the calculations required to express the microscopy-based density in exactly the same units as the conventional CFU-based viable cell density.

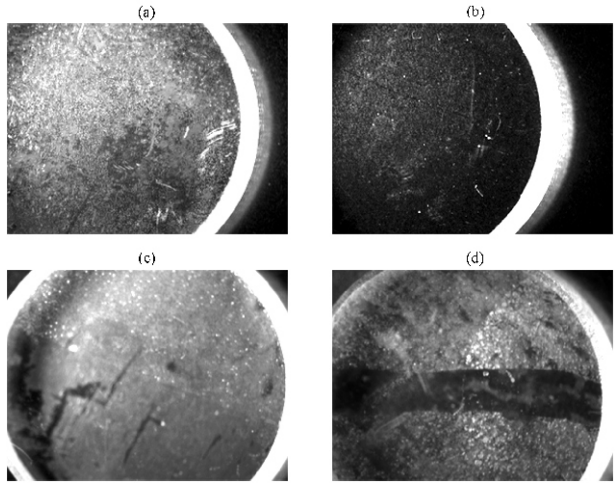

Figure 3. Microscope images of glass disk control carriers containing biofilm grown in the CDC reactor, viewed unstained. Panel (a) is the surface before bacteria were harvested; panel (b) is the surface of a carrier after harvesting by sonication for 1 min; panel (c) is the surface after the lower (and slightly left) half of the carrier face was scraped; and panel (d) is the surface after a narrow, horizontal band was scraped across the center of the carrier.

Table 3. The $bias_d$ values and the means of the log-transformed disaggregating efficiency fractions for the CTC stain disaggregating check method. The suspension was disaggregated by homogenization.

Experiment	Treatment (concentration and time)	Mean log F_C control	Mean log F_D disinfected	$bias_d$
1	Chlorine (1000 mg/L for 10 min)	–0.05965	–0.10843	0.05
2	Chlorine (1000 mg/L for 10 min)	–1.11453	–1.39245	0.28
3	Pectinase (1% for 10 min)	–1.41761	–0.07716	–1.34

Because of the potential for some bacteria species to reaggregate while in suspension, the time between disaggregation and plating may be an important factor (33).

Conclusions

A disinfectant test for surface-associated bacteria could produce a biased *LR* value due to either the harvesting step or the disaggregating step. The bias could be large enough to be of practical importance, in which case the observed efficacy is an invalid assessment of the disinfectant. Bias occurs if and only if the disinfected and control carriers produce different efficiency fractions. The results show that it is feasible to conduct laboratory check experiments to estimate the efficiency fractions and to calculate the associated bias of *LR*. It is recommended that disinfectant tests include harvesting and disaggregating checks for each important combination of carrier material, microbial species, and disinfectant. It is prudent to support the checks with qualitative, visual confirmation, e.g., microscopic examination of carrier surfaces.

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