Persistence of *Bacillus thuringiensis* subsp. *kurstaki* in Urban Environments following Spraying^{▽†‡}

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Bacillus thuringiensis subsp. *kurstaki* is applied extensively in North America to control the gypsy moth, *Lymantria dispar*. Since *B. thuringiensis* subsp. *kurstaki* shares many physical and biological properties with *Bacillus anthracis*, it is a reasonable surrogate for biodefense studies. A key question in biodefense is how long a biothreat agent will persist in the environment. There is some information in the literature on the persistence of *Bacillus anthracis* in laboratories and historical testing areas and for *Bacillus thuringiensis* in agricultural settings, but there is no information on the persistence of *Bacillus* spp. in the type of environment that would be encountered in a city or on a military installation. Since it is not feasible to release *B. anthracis* in a developed area, the controlled release of *B. thuringiensis* subsp. *kurstaki* for pest control was used to gain insight into the potential persistence of *Bacillus* spp. in outdoor urban environments. Persistence was evaluated in two locations: Fairfax County, VA, and Seattle, WA. Environmental samples were collected from multiple matrices and evaluated for the presence of viable *B. thuringiensis* subsp. *kurstaki* at times ranging from less than 1 day to 4 years after spraying. Real-time PCR and culture were used for analysis. *B. thuringiensis* subsp. *kurstaki* was found to persist in urban environments for at least 4 years. It was most frequently detected in soils and less frequently detected in wipes, grass, foliage, and water. The collective results indicate that certain species of *Bacillus* may persist for years following their dispersal in urban environments.

Bacillus thuringiensis subsp. *kurstaki* is a common organic pesticide used to control defoliating pests including the gypsy moth, *Lymantria dispar*. The gypsy moth is a major forest pest that is especially predominant along the eastern seaboard and in the Midwestern United States. Over the last 20 years, thousands of acres have been treated with *B. thuringiensis* subsp. *kurstaki* to suppress or eradicate gypsy moth populations. The bacterium is applied to foliage as a water-based slurry. *B. thuringiensis* subsp. *kurstaki* is not typically harmful to mammals; but its toxin, when ingested, is lethal to the *Lymantria dispar* caterpillar (19, 22, 28).

In a recent review, Greenberg et al. (7) determined that *B. thuringiensis* provides the best overall fit as a nonpathogenic surrogate for *Bacillus anthracis* for spore fate and transport based on pathogenicity, phylogenetic relationship, morphology, and comparative survivability to biocides.

B. thuringiensis and *B. anthracis* are both Gram positive and aerobic, and they both form metabolically inactive endospores in response to environmental conditions. *B. anthracis*, the etiological agent of anthrax, is a bacterium of considerable concern in the biodefense community, but it is difficult to study its behavior in the environment, particularly for the wide-area releases often postulated in terrorist scenarios. Outdoor pesticide releases of *B. thuringiensis* subsp. *kurstaki* can therefore

provide insight into the environmental fate and transport of *B. anthracis* following a deliberate release.

The literature on the persistence of *Bacillus* spp. in the environment is incomplete and contradictory; however, previous studies have demonstrated that viable *Bacillus* spp. spores may persist and remain dormant in laboratories or rural environments for years to decades. Early research on *B. anthracis* indicated that spores could survive indefinitely in a dry environment, such as in dust, or on laboratory swabs or blood spots on clothing (32). Manchee et al. detected *B. anthracis* in soil where the agent had been dispersed 40 years previously (14). Hendriksen and Hansen found that *B. thuringiensis* subsp. *kurstaki* was persistent in bulk soil in cabbage plots for 7 years (9).

Studies on *Bacillus* spp. survival on leaves contain a wide range of results, with *B. thuringiensis* detected for days to years (6, 8–10, 19–22). In 1998, Smith and Barry recovered *B. thuringiensis* from leaf samples for 12 months and more postapplication in sprayed, previously sprayed, and nonsprayed areas (26).

The literature on *Bacillus* spp. persistence in water is predominantly focused on *B. anthracis* for biodefense. Although these studies may not be representative of general environmental conditions (33), they indicate that *B. anthracis* can remain viable in chlorinated or pond water for 2 years (3, 12).

It is difficult to use data from the existing literature, which was largely collected in laboratories and undeveloped areas, to assess the implications for an urban area after a biological attack. However, understanding persistence in the urban environment will be critical to formulating effective response, restoration, and recovery plans. To that end, under the auspices of the joint Defense Threat Reduction Agency–Department of

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FIG. 1. Location of Fairfax County 2008 spray areas. Spray area 35 is circled in red. Numbered yellow polygons represent all 2008 spray blocks.

Homeland Security Interagency Biological Restoration Demonstration, soil, surface, water, and vegetation samples were collected from two urban areas (Seattle, WA, and Fairfax County, VA), up to 4 years after spraying, and analyzed for the presence of viable *B. thuringiensis* subsp. *kurstaki*.

Sampling of historic spray areas (1 to 4 years after spraying) occurred in the Seattle, WA, area. However, in 2008 (the year of this experiment), the Washington State Department of Agriculture did not identify a need to spray for gypsy moth. For that reason, Fairfax County, VA, was sampled immediately after spraying and then at intervals for up to 1 year. The results are reported below.

MATERIALS AND METHODS

***B. thuringiensis* subsp. *kurstaki* application.** Fairfax County, VA, delivered one application of a commercial formulation of *B. thuringiensis* subsp. *kurstaki* (Foray 76B; Valent Biosciences, Libertyville, IL) via helicopter at a rate of 470 liters per km². Since the manufacturer optimizes their product for toxin activity and does not measure the *B. thuringiensis* subsp. *kurstaki* concentration in the final formulation, a sample of the spray suspension was obtained from Fairfax County authorities for laboratory analysis. In Seattle, Foray 48B or Foray XG (Valent Biosciences, Libertyville, IL) were applied aerially or by ground as a 2% suspension on at least three successive occasions. According to the records, application rates ranged from 10 to 1,000 liters per km². Since no archived spray material was retained, samples of Foray 48B and Foray XG were obtained from the manufacturer for laboratory analysis.

Sample locations and design. Sample collection occurred in 2008. One spray area in Fairfax County, VA, was sampled immediately before (background) and after *B. thuringiensis* subsp. *kurstaki* spraying and then at 6, 12, 24, and 48 weeks after spraying. Fairfax County's 2008 spray areas are shown in Fig. 1; the spray area sampled was designated block 35. Block 35 covers 0.737 km² and is largely residential. During each sampling interval, including background, a total of 245 samples were collected from this spray block. In Seattle, WA, samples were collected in locations that the Washington State Department of Agriculture sprayed between 2007 and 2004, Fig. 2. The Seattle spray blocks ranged from 0.02 to 0.4 km² and were predominantly commercial or mixed commercial/residential areas with tree canopy cover <15%. Detailed information on Seattle spray block and *B. thuringiensis* subsp. *kurstaki* application parameters is available from the Washington State Department of Agriculture (<http://agr.wa.gov/PlantsInsects/InsectPests/GypsyMoth/ControlEfforts/PastControlEfforts/PastControlEfforts.aspx>). A control area that had not been sprayed by the Washington State Department of Agriculture was also sampled to determine the background presence of *B. thuringiensis* subsp. *kurstaki* in the Seattle area (Fig. 2). Tables 1 and 2 show the spray block size and the timeline

FIG. 2. Locations of spray areas sampled in the Seattle, WA, area.

for sample collection in each location. Between 218 and 240 samples were collected from each spray block, with a total of 242 samples collected from the control block.

Three sampling schemes were used to characterize each spray area: probabilistic, close, and targeted (15, 30). Initial sample numbers were chosen to give 99% confidence that at least 95% of the area was without detectable spores provided all samples were negative. A grid or transect technique was used to define the probabilistic sample locations; a 9.1-by-9.1-m grid was used to define sample locations in relatively homogeneous areas, such as parking lots, while a smaller, 2.7-by-2.7-m grid was used to define sample locations in heterogeneous areas such as residential neighborhoods. Probabilistic sampling implies uniform distribution of the agent interrogated; to test this assumption, a set of "close" (or secondary) samples were collected for a randomly chosen 10% of the probabilistic samples at each site. Close samples were of the same type as their associ-

TABLE 1. Estimated amounts of *B. thuringiensis* subsp. *kurstaki* applied in Fairfax County, VA, and remaining at the time of sample collection

Wk sampled	Size (km ²)	<i>B. thuringiensis</i> subsp. <i>kurstaki</i> ^a		
		Estimated amt (g) applied	Estimated amt (g) remaining	Estimated no. of spores remaining
0 (after spraying)	0.737	5,460	5,460	10 ¹⁴
6			4,000–5,000	10 ¹⁴
12			3,000–4,000	10 ¹⁴
24			1,000–2,000	10 ¹⁴
48			500–1,000	10 ¹³

^a The amount applied assumes 7.4 kg of viable *B. thuringiensis* subsp. *kurstaki* per km², a one-time application. For the amounts remaining, the lower number assumes a 100-day half-life and the higher number assumes a 200-day half-life. The number of spores remaining assumes 10¹¹ spores/g.

TABLE 2. Estimated amounts of *B. thuringiensis* subsp. *kurstaki* applied in Seattle spray areas and remaining at the time of sample collection

Yr	Spray area	Size (km ²)	<i>B. thuringiensis</i> subsp. <i>kurstaki</i> ^a		
			Estimated amt (g) applied	Estimated amt (g) remaining	Estimated no. of spores remaining
2007	Kent	0.1	71	6–20	10 ¹¹ –10 ¹²
2006	Madison	0.24	10,000	60–800	10 ¹² –10 ¹³
2006	Rosemont	0.022	16	<1–1	10 ¹⁰ –10 ¹¹
2005	Eastlake	0.049	2,000	1–50	10 ¹¹ –10 ¹²
2004	Bellevue	0.044	2,000	<1–20	10 ⁹ –10 ¹²

^a The amount applied assumes multiple applications at rates of 740 liters km⁻² for Kent, Madison, and Rosemont, 620 liters km⁻² for Eastlake, and 200 and 250 liters km⁻² for Bellevue. For the amount remaining and the number of spores remaining, the lower number assumes a 100-day half-life and the higher number assumes a 200-day half-life. The number of spores remaining assumes 10¹¹ spores/g.

ated sample and were collected one foot to the north of the original sample whenever possible (the results of the “close” samples are not analyzed explicitly in this article; as predicted, the assumption of uniform distribution was incorrect). Finally, a set of targeted samples were collected in locations where *B. thuringiensis* subsp. *kurstaki* was likely to persist based on literature information (e.g., shady or damp locations; standing water). An additional 10% of the total number of samples was collected as field blanks at each site.

Sample collection. Sample collection was performed using the protocols contained in the BioWatch Outdoor Program Guidance (29). Samples from Fairfax County, VA, included soil, wipe, water, grass, and leaf samples. Samples from Seattle, WA, included soil, wipe, and water samples. Vegetation was not collected in Seattle; due to the longer duration between spraying and sample collection, it was assumed the bacteria would have been washed off the foliage.

In brief, the collection of soil samples involved scraping any organic material off the soil surface with a sterile spatula and collecting enough solid soil from the top 5 cm to fill a 50-ml conical tube up to the 30- to 50-ml level. Using a sterile pipette, 10 ml of standing outdoor water (e.g., puddle or lake) was collected into a 50-ml conical tube. Grass was cut using sterile scissors, with enough material collected to fill a 50-ml conical tube up to the 30- to 50-ml level, and leaves were cut from trees using sterile scissors with enough material collected to fill a 50-ml conical tube up to the 30- to 50-ml level (leaves that had fallen on the ground were not collected). Wipe samples were collected using sterile rayon gauze (7.6 by 7.6 cm; Dynarex Corp.) premoistened with 3 ml of phosphate-buffered saline (Fisher Scientific, Pittsburgh, PA). An area of ~1 m² was wiped using vertical then horizontal S strokes; the wipe was then placed in a 50-ml conical tube. All conical tubes were capped and sealed with Parafilm and placed in a self-sealing bag. The bag was wiped with a hospital grade bleach wipe (Dispatch; Caltech Industries, Midland, MI), allowed to air dry, and then placed on ice in a cooler for transport to the analytical laboratory.

Sample preparation and DNA extraction. For soil samples, 0.4 g of the sample was used for DNA extraction. For vegetation samples, a quarter of the sample was washed with 10 ml of PET buffer (100 mM sodium phosphate buffer with 10 mM EDTA and 0.01% Tween 20; Fisher Scientific), and an aliquot of the wash was used for DNA extraction. For wipes, the entire sample was washed with 2 to 3 ml of PET buffer, and an aliquot of the wash was used for DNA extraction.

DNA was extracted from soil and aliquots of vegetation and wipe washes using the commercial FastDNA spin kit for soil (MP Biomedicals LLC, Solon, OH). Extracts of the same type (e.g., soil) were pooled together by location (i.e., samples collected in close proximity) to minimize analysis time and cost. Each pool combined a maximum of three extracts. Extracts were then subjected to a molecular screen using *B. thuringiensis* subsp. *kurstaki*-specific real-time PCR.

Screening analysis (molecular screen). PCR inhibitors such as humic acid and cellulose are common in environmental samples. To test for inhibition following extraction, each batch of samples was tested for the presence of inhibitors by performing real-time PCR amplification of 16S ribosomal DNA (rDNA). Regardless of the source, environmental matrices such as soils typically contain large amounts of 16S rDNA. If no amplification was observed from the soils, which were expected to contain large amounts of target material, it was determined that inhibitors were likely present. When amplification of the 16S rDNA was unsuccessful, the batch of samples was diluted to 1:10 or 1:100 with DNA- and DNase-free water and tested again to ensure amplification prior to running *B. thuringiensis* subsp. *kurstaki*-specific real-time PCR.

DNA extract pools were tested for the presence of *B. thuringiensis* subsp. *kurstaki* DNA using real-time PCR with two *B. thuringiensis* subsp. *kurstaki*-specific primers designed as LANL. The sensitivity, specificity, and the ability of

these primers to detect *B. thuringiensis* subsp. *kurstaki* DNA in a wide matrix of samples were tested (see the supplemental material). Both 16S and *B. thuringiensis* subsp. *kurstaki* real-time PCR assays used the Bio-Rad iQ SYBR green Supermix (Bio-Rad, Hercules, CA) and LANL-designed 16S and *B. thuringiensis* subsp. *kurstaki* PCR primers (Invitrogen, Carlsbad, CA) in 25- μ l reactions that contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 0.2 mM concentrations of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), iTaq DNA polymerase at 25 U/ml, 3 mM MgCl₂, SYBR green I, 10 nM fluorescein, stabilizers, 7.5 nM forward and reverse primers (*B. thuringiensis* subsp. *kurstaki* or 16S), and 5 μ l of DNA extract (diluted or undiluted). The PCR cycling conditions were as follows: a melting temperature of 95°C for 2 min, followed by 45 cycles of melting at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 70°C for 10 s. Real-time PCR was conducted in a Bio-Rad iCycler iQ real-time PCR detection system.

Results were reported as threshold cycle (*C_T*) values. The *C_T* values were converted to an estimate of target genome copy number based on a standard curve generated for each plate of samples using serial dilutions of commercially available *B. thuringiensis* subsp. *kurstaki* DNA (American Type Culture Collection [ATCC], Manassas, VA). The threshold for selection of a sample for viability analysis was >1,000 target genome copies for either primer. Each plate subjected to real-time PCR contained the following controls: (i) a no-template control to ensure there was no background contamination; (ii) a panel of controls with known concentrations of *B. thuringiensis* subsp. *kurstaki* DNA (obtained from ATCC, Manassas, VA) in the form of genome copy number (10⁰ to 10⁶) to enable conversion of *C_T* values to estimated genome copy number for each reaction; and (iii) a set of reactions comprising a diversity panel that included DNA from *B. thuringiensis* subsp. *kurstaki* near neighbors and unrelated organisms to ensure the specificity of the assay.

Viability analysis. Pooled or individual samples that had an estimated *B. thuringiensis* subsp. *kurstaki* genome copy number of >1,000 target copies for either of the two primer pairs were processed for viability. In addition, at least 10% of samples that were negative by the molecular screen were assessed by plate culture to determine the reliability of the screen. To do this, fresh aliquots of the corresponding unpooled soil, water, vegetation, or wipe samples were washed with Trypticase soy broth (TSB; Becton Dickinson/Fisher Scientific). The supernatant was then removed (1.5 ml for soil samples and 200 μ l for water, vegetation, and wipe samples) and repooled using the same pooling scheme used for PCR. Pooled samples were heat treated (80°C for 10 min) to kill all vegetative cells. Then, 100 μ l of the pooled sample was added to Trypticase soy broth (broth culture), or three serial dilutions (10⁰ to 10⁻²) of each pooled sample were plated on Trypticase soy agar medium (Becton Dickinson/Fisher Scientific) containing cycloheximide (50 mg/liter; Sigma-Aldrich, St. Louis, MO) to inhibit fungal growth. Incubation occurred at 36°C for 16 h for broth culture and 24 h for plate culture (11). Broth cultures were observed for turbidity, and plates were observed for colonies with *B. thuringiensis* morphology. A culture result of growth or no growth was reported.

To confirm that broth cultures contained viable *B. thuringiensis* subsp. *kurstaki*, 50- μ l aliquots of the broth were taken at *t* = 0, before incubation and then at *t* = 16 h. The aliquots were subjected to heat lysis to make DNA available for *B. thuringiensis* subsp. *kurstaki*-specific real-time PCR using each of the two primers used in the molecular screen. Viable *B. thuringiensis* subsp. *kurstaki* was confirmed to be present if the ratio of the estimated target genome copy number at 16 h compared to zero hours was greater than 10.

To confirm that colonies on the plates were from viable *B. thuringiensis* subsp. *kurstaki*, a loopful of material was scraped from the 10⁰ dilution plate of each positive sample and suspended in 50 μ l of distilled water. This material was

TABLE 3. Time elapsed between *B. thuringiensis* subsp. *kurstaki* application and sample collection, number of sample pools for all matrices, pooled field blanks, and pooled lab duplicates analyzed for each area

Sampling location	Time elapsed	Total sample pools (n)	Field blank pools (n)
Fairfax County, VA, block 35			
Background	NA ^a , prespray	137	6
	1 day	140	6
	6 wk	138	6
	12 wk	147	7
	24 wk	146	7
	48 wk	128	6
Seattle, WA			
Seattle, control	NA (not sprayed)	89	8
Kent, 2007	10 mo	90	6
Madison, 2006	22 mo	94	8
Rosemont, 2006	22 mo	89	8
Eastlake, 2005	38 mo	128	7
Bellevue, 2004	50 mo	111	6

^a NA, not applicable.

subjected to heat lysis and subjected to *B. thuringiensis* subsp. *kurstaki*-specific real-time PCR using the same two primers used in the molecular screen. The results were reported in the form of C_T values.

Analysis of spray suspensions of *B. thuringiensis* subsp. *kurstaki* (Foray76B, 48B, and XG) was also conducted by plate culture as described above. An aliquot of the spray suspension was diluted 1,000-fold in phosphate-buffered saline (PBS) and heat treated. Serial dilutions of this preparation (100- μ l aliquots) in PBS were plated, and colony counts were obtained after overnight incubation. Triplicate measurements were made, yielding average values.

Quality assurance and control. Quality assurance was implemented for both sample collection and analysis to ensure high confidence in the data.

Sample collection. All samples were collected by personnel wearing clean, disposable nitrile gloves and booties. Gloves were changed after collection of each sample. Each sample was individually bar-coded and chain of custody was electronically tracked from collection through analysis using custom field software and a laboratory information management system (LIMS). Field blanks, analyzed to ensure no contamination occurred during sample collection, comprised 10% of the samples collected.

Sample analysis. During laboratory analysis, samples and extracts were contained and manipulated in closed conical, centrifuge, and microcentrifuge tubes and/or sealed deep well blocks to minimize aerosolization and cross-contamination. A positive control consisting of a soil sample spiked with a known concentration of *B. thuringiensis* subsp. *kurstaki* spores was included in each batch of samples processed to ensure performance of the assays. A total of 10% of all samples were processed as duplicates in the laboratory to assess internal consistency. All samples that had an estimated *B. thuringiensis* subsp. *kurstaki* genome copy number of >1,000 copies were processed for viability and, in order to test the effectiveness of the PCR screen, >10% of all samples that failed the PCR screen were also processed for viability.

The quality of DNA extracted is dependent on the matrix and the extraction process used. The DNA extraction kit used in this project was specific for DNA extraction from soils. Other matrices sampled in the present study presented less of a challenge for extraction. To ensure that the DNA extraction process was successful, soil extracts were tested for the presence of DNA by PicoGreen with the Quant-iT PicoGreen dsDNA assay kit from Molecular Probes (Invitrogen, Carlsbad, CA). Soil extracts were used since they were expected to contain the most DNA and would therefore show high levels of fluorescence when stained with PicoGreen. Two reference soil samples that represented different soil types, sand and clay, were spiked with a known concentration of *B. thuringiensis* subsp. *kurstaki* spores and processed with each batch. If the reference extracts showed negative results, then the DNA extraction process was repeated for that batch of samples.

RESULTS

Spray suspension analysis. Samples of each commercial *B. thuringiensis* subsp. *kurstaki* spray formulation were obtained, and viable spore concentrations were determined. A concentration of $1.3 \times 10^9 \pm 5 \times 10^8$ CFU of *B. thuringiensis* subsp. *kurstaki* per ml of suspension was measured from samples provided by the Virginia Department of Agriculture. A concentration of $8.3 \times 10^8 \pm 1 \times 10^7$ was measured from samples of Foray 48B and a concentration of $6.0 \times 10^9 \pm 3 \times 10^8$ was measured from samples of Foray XG (provided by Valent Biosciences, since the Washington State Department of Agriculture does not maintain samples of historical spray material).

Based on the results of culture analysis and the application rate provided by Fairfax County, ~5 kg of *B. thuringiensis* subsp. *kurstaki* was applied to spray block 35 (Table 1). Table 1 also includes an estimate of the amount of *B. thuringiensis* subsp. *kurstaki* remaining at the time of sampling, based on literature half-life values of 100 and 200 days (19).

Using the results of analysis of *B. thuringiensis* subsp. *kurstaki* spray material representative of the spray formulation the application rate provided by Washington State, the estimated amount of *B. thuringiensis* subsp. *kurstaki* applied in each spray block sampled is given in Table 2. Approximately 2 kg of *B. thuringiensis* subsp. *kurstaki* was applied to Bellevue and Eastlake, approximately 10 kg of *B. thuringiensis* subsp. *kurstaki* was applied to Madison, and less than 100 g was applied to Rosemont and Kent. Table 2 also includes an estimate of the amount of *B. thuringiensis* subsp. *kurstaki* remaining at the time of sampling, based on literature half-life values (19).

Environmental sample analysis. The numbers of sample pools and field blanks analyzed for each spray area are presented in Table 3, with the time elapsed between *B. thuringiensis* subsp. *kurstaki* application and sample collection.

Urban soils were found to be the most reliable reservoir for viable *B. thuringiensis* subsp. *kurstaki*. Soil results from Fairfax County are presented graphically in Fig. 3; soil results from Seattle, WA, are presented in Fig. 4. The largest percentages of soil samples containing detectable DNA and viable cultures were obtained immediately after spraying in Fairfax County ($t = 0$ in Fig. 3). Viable *B. thuringiensis* subsp. *kurstaki* was

FIG. 3. Soil analysis results as a percentage of total samples passing the *B. thuringiensis* subsp. *kurstaki* PCR screen (■) and *B. thuringiensis* subsp. *kurstaki* culture (□) for Fairfax County, VA, plotted according to weeks after *B. thuringiensis* subsp. *kurstaki* spraying. N, number of sample pools analyzed.

FIG. 4. Soil analysis results as a percentage of total samples passing the *B. thuringiensis* subsp. *kurstaki* PCR screen (■) and *B. thuringiensis* subsp. *kurstaki* culture (□) for Seattle, WA, plotted according to the *B. thuringiensis* subsp. *kurstaki* spray date. N, number of sample pools analyzed.

detected in Fairfax County soils throughout the 48-week duration of that experiment. In Seattle, WA, the largest percentages of samples containing detectable DNA and viable cultures were obtained in the most recently sprayed block (Kent 2007, sampled 1 year after spraying), and the smallest percentage of samples with detectable *B. thuringiensis* subsp. *kurstaki* was obtained from soils collected 4 years after spraying (Bellevue 2004 data, Fig. 4). A downward trend in the percentage of samples containing detectable DNA and viable cultures was observed with increasing time after spraying; however, viable *B. thuringiensis* subsp. *kurstaki* was still detected 4 years after spraying. A total of two background soil samples (6.7%) collected in Fairfax County, VA, were positive by PCR; however, both were determined to be negative by culture. A total of three of soil samples (8.1%) collected from the control area in

Seattle, WA, were positive by PCR; however, all were determined to be negative by culture.

Other sample types were less reliable reservoirs of *B. thuringiensis* subsp. *kurstaki* compared to soil and exhibited more variability. Table 4 presents wipe, water, grass, and leaf results. Wipes were collected from all locations and from a number of urban surfaces, including, but not limited to, concrete, asphalt, and metal (e.g., manhole covers). No wipe, water, grass, or leaf samples collected in the Fairfax County background samples or the Seattle control area were found to be positive by PCR or culture.

Viable *B. thuringiensis* subsp. *kurstaki* was obtained from wipe samples in Fairfax County at all time points except at $t = 0$. The highest percentage of culturable wipes was collected at in Fairfax County at $t = 48$ weeks, although no wipes from that sample set passed the PCR screen. Similar variability was observed for the Seattle wipes. PCR-positive samples were only observed from the Madison 2006 and Kent 2007 spray blocks; wipes from Eastlake 2005 and Kent 2007 contained viable *B. thuringiensis* subsp. *kurstaki*, but the wipes from Madison 2006 did not.

Water samples were collected from all locations except Madison 2006, Rosemont 2006, and Bellevue 2004. No water pools from Fairfax County passed the PCR screens; however, some pools collected from Fairfax County (at $t = 0$, $t = 6$, and $t = 48$ weeks) contained viable *B. thuringiensis* subsp. *kurstaki*. At $t = 48$ weeks, 50% of the water pools from Fairfax County were culturable. Seattle water samples contained no detectable *B. thuringiensis* subsp. *kurstaki* by PCR or culture.

Grass and leaf samples were only collected in Fairfax County, and the results were similarly inconsistent. Few grass and leaf samples from Fairfax County obtained after $t = 0$ passed the PCR screen. However, viable *B. thuringiensis* subsp. *kurstaki* was detected in grass and leaves at $t = 0$, 6, and 12 weeks (Table 4).

Laboratory duplicate sample analysis showed ca. 90% agree-

TABLE 4. Percentage of pooled samples passing the *B. thuringiensis* subsp. *kurstaki* PCR screen and *B. thuringiensis* subsp. *kurstaki* culture for wipe, water, grass, and leaves for each location^a

Location and time period	% Samples											
	Wipe			Water			Grass			Leaves		
	n	PCR	Culture	n	PCR	Culture	n	PCR	Culture	n	PCR	Culture
Fairfax County, VA												
Background	51	0	0	6	0	0	21	0	0	23	0	0
0 wks	42	7	0	7	0	14	15	80	7	21	48	10
6 wks	40	10	20	6	0	17	14	0	7	16	0	6
12 wks	40	0	13	3	0	0	17	0	0	20	0	5
24 wks	41	2	5	5	0	0	15	0	0	18	6	0
48 wks	39	0	26	6	0	50	13	0	0	19	0	0
Seattle, WA												
Seattle, control	39	0	0	3	0	0	0	NA	NA	0	NA	NA
Kent, 2007	40	23	3	1	0	0	0	NA	NA	0	NA	NA
Madison, 2006	40	5	0	0	NA	NA	0	NA	NA	0	NA	NA
Rosemont, 2006	39	0	0	0	NA	NA	0	NA	NA	0	NA	NA
Eastlake, 2005	43	0	16	1	0	0	0	NA	NA	0	NA	NA
Bellevue, 2004	44	0	0	0	NA	NA	0	NA	NA	0	NA	NA

^a The percentages of total pooled samples (n), samples passing the *B. thuringiensis* subsp. *kurstaki* PCR screen (PCR), and *B. thuringiensis* subsp. *kurstaki* culture samples (Culture) for wipe, water, grass, and leaves were determined for each location. NA, not applicable for samples not collected.

ment in duplicate samples, irrespective of the location or sample type. The Seattle, WA, samples had 92.4% agreement laboratory duplicates, with 7.6% of duplicates not in agreement. The Fairfax County, VA, samples had 86.8% agreement, with 13.2% of duplicates not in agreement.

DISCUSSION

The purpose of this study was to determine how long *B. thuringiensis* subsp. *kurstaki* persists in urban environments at detectable levels. *B. thuringiensis* subsp. *kurstaki* was found to persist for at least 4 years; data for the past 4 years were not collected. Sampling of historic spray areas (1 to 4 years after spraying) occurred in relatively small, well-separated locations in the Seattle, WA, area. The year sampling occurred in Seattle, the Washington State Department of Agriculture did not perform any new *B. thuringiensis* subsp. *kurstaki* spraying. For this reason, Fairfax County, VA, was sampled immediately after spraying and then at various intervals for up to 48 weeks.

Using the reported half-life of *B. thuringiensis* subsp. *kurstaki* in cabbage patches (100 to 200 days) (19), estimates of the amount of *B. thuringiensis* subsp. *kurstaki* remaining in the spray areas at the time of sample collection were made (Tables 1 and 2). There are many assumptions in these estimates, the most implausible of which is that all *B. thuringiensis* subsp. *kurstaki* was applied to and remained inside the spray area boundary (1, 28). However, the estimates likely give a conservative upper bound of the amount of *B. thuringiensis* subsp. *kurstaki* remaining at the time of sampling. The results of this experiment are consistent with the overall trend indicated in Tables 1 and 2: positive samples were collected in decreasing numbers as the time after spraying increased, but positives were still observed 4 years after spraying.

The major difference between the estimates in Tables 1 and 2 and the results lies in the different trends observed in Fairfax County and Seattle. Fairfax County shows a more marked and less regular decline in the percentage of samples containing viable material over 1 year. Seattle shows a steadier but less steep decline. This may be due to several factors. First, significant flooding occurred within 1 week after spraying in Fairfax County. This may have transported part of the initial spray material out of the sampled area. Second, it may be due to climatic or environmental differences between the urban areas. Many factors have been postulated to contribute to *Bacillus* spp. persistence or decay, including regional climatic conditions, soil alkalinity, the presence of shale, sandstone, or limestone in the soil, and potential interactions with the rhizosphere or earthworms (5, 9, 24, 32). These factors were not characterized in this experiment. Finally, it is also possible these and other anomalies in the data were an artifact produced by inherent uncertainties in the sample collection process. Environmental sample collection techniques are difficult to standardize between sample collection personnel, and individual variability may have contributed to the overall results. Because the present study investigated viability trends only and the results are qualitative (presence/absence) rather than quantitative, it is difficult to draw a conclusion on the significance of the decay trends.

Soil was the best matrix for recovery of viable *B. thuringiensis* subsp. *kurstaki*. It is difficult to determine whether this was

because *B. thuringiensis* subsp. *kurstaki* was predominantly present in soils, compared to other matrices, because *B. thuringiensis* subsp. *kurstaki* was most efficiently collected in soils (e.g., soil volumes sampled were higher than wipe volumes), or because *B. thuringiensis* subsp. *kurstaki* was most efficiently extracted from soil samples or whether this was a combination of multiple factors. The results, however, are not inconsistent with previous studies on the persistence of *Bacillus* spp. in rural soils. Manchee et al. found that 13% of soil samples collected 40 years after release at a largely rural biological warfare testing plot contained viable *B. anthracis* (14). In our study, 15% of soil samples collected at an urban, highly trafficked Seattle location contained viable *B. thuringiensis* subsp. *kurstaki* after 4 years (Fig. 4). Several other studies have detected *Bacillus* spp. in soil following its deliberate application (12–14, 23); the results indicate persistence up to 6 months in a Russian field (13) and up to 7 years in a cabbage plot in Denmark (26). Differences among these results may be explained by more or less favorable climatic and environmental conditions (5); however, the data in aggregate clearly indicate that soil is a favorable reservoir for viable spores, whether the soil is in a rural or urban environment.

Other sample matrices were less reliable reservoirs of viable *B. thuringiensis* subsp. *kurstaki*. Wipes produced more consistent results than water and vegetation, but no more than 26% of wipes in Fairfax County and 16% in Seattle were viable at any time point (Table 4). Although literature on the persistence of *Bacillus* on surfaces and in water is sparse, it does indicate a strong dependence upon surface type and climatic and environmental conditions (e.g., sunlight and moisture) (10, 16). *B. thuringiensis* has been found to persist on vegetation for days in direct sunlight (10) to a year on spruce needles (22). One lab study recovered viable *B. anthracis* from canvas after 41 years (25), and another evaluated persistence on wood, laminate, aluminum, polyvinyl chloride, and other surfaces, and recovered viable *B. anthracis* for up to 987 days (4). Comparisons of these data to laboratory studies are not wholly appropriate since the transient nature of water and vegetation likely contributed to the inability to recover viable *B. thuringiensis* subsp. *kurstaki* from these matrices over longer periods of time. However, the results of the present study are consistent with literature reports from other experiments (10, 22).

Secondary to demonstrating persistence in urban environments, the present study illustrates the differences between PCR screening and culture for environmental sample analysis. In some cases, culture analysis was more sensitive than the PCR screen, as demonstrated in samples from Fairfax County at $t = 12$ and $t = 48$ weeks (Fig. 3) and in Seattle from Eastlake 2005 and Bellevue 2004 (Fig. 4). In all cases, these samples contained small amounts of material ($<1,000$ genome copies by PCR), and the corresponding culture results were likely due to significant differences in the processing of samples for culture versus PCR: (i) larger aliquots of sample were used for culture than for PCR; (ii) DNA extraction efficiency for PCR is not 100%; and (iii) residual PCR inhibitors can affect the quantity of genome copies estimated.

In this experiment, both assays provided independent information on the presence of *B. thuringiensis* subsp. *kurstaki*, although viable cultures were also confirmed by PCR. While knowledge of viable agent by laboratory culture is important

for assessing public health impacts in biological restoration efforts, it may not always be the best indication of presence of an agent in the environment. *Bacillus* spp. may be sensitive to sample collection methods, specific media, and laboratory conditions, all of which can render it viable but nonculturable in a laboratory setting (2, 17). PCR allows rapid detection with specificity and sensitivity (18).

On the other hand, poor extraction efficiency and the presence of inhibitors (e.g., humic matter) can reduce PCR's effectiveness (27, 31). In this experiment, sample aliquots for viability testing were plated along with the sample matrix, allowing higher sensitivity of the viability assay in some cases; the results of soil samples from $t = 12$ weeks at Fairfax County are an example (Fig. 3). This outcome was especially pronounced when PCR concentrations were near the predetermined cutoff threshold of 1,000 genome copies. It should also be noted that PCR detects free DNA and nonviable agent. When time and cost are not a constraint, ideally, both techniques should be used for analysis of environmental samples.

The goal of the present study was to provide information on the persistence of a near neighbor of *B. anthracis* to inform efforts to determine how to restore an urban environment following a biological attack. Analysis of *B. thuringiensis* subsp. *kurstaki* persistence from two urban areas, one on the east coast and one on the west, demonstrated *B. thuringiensis* subsp. *kurstaki* can be expected to persist in urban environments for prolonged periods of time. At 48 weeks after *B. thuringiensis* subsp. *kurstaki* was applied, 85% of soils, 26% of wipe samples, and 50% of water samples contained viable *B. thuringiensis* subsp. *kurstaki* in Fairfax County, VA. In Seattle, WA, 77, 53, 25, 23, and 15% of soil samples contained viable agent at 1, 2, 3, and 4 years, respectively, after the *B. thuringiensis* subsp. *kurstaki* spray events. Consistent with the literature, soil was a reliable reservoir for *Bacillus* spp. at both locations. The results obtained from other environmental matrices (surface wipes, water, and vegetation) were less easily interpreted, but viable samples at various time points provided additional evidence of persistence in these different matrices in urban areas. Since *B. thuringiensis* subsp. *kurstaki* is a reasonable surrogate for fate and transport studies (7), it can be inferred that *B. anthracis* may persist for several years if released in an urban environment.

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