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Airborne Endotoxin from Indoor and Outdoor Environments: Effect of Sample Dilution on the Kinetic *Limulus* Amebocyte Lysate (LAL) Assay

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Airborne endotoxins in occupational environments are a potential respiratory hazard to individuals. In this study, airborne endotoxins were collected using open-face and button aerosol samplers from inside animal housing units and downwind from agricultural production sites and a wastewater treatment plant. Filter extracts were then diluted to examine the effect of interfering substances on the kinetic Limulus *amebocyte lysate (LAL) assay. In most cases, the overall endotoxin concentration was shown to decrease with increasing dilution up to 1000-fold, suggesting the presence of enhancing substances in the filter extracts. This dilution-dependent effect was most prominent in the open-face endotoxin samples, while button samples displayed little effect. Using a joinpoint regression model, it was determined that a dilution factor of 50 to 100 was generally sufficient to eliminate the presence of enhancing substances. After screening the data for dilution dependent effects, the airborne endotoxin concentrations were determined. The highest endotoxin concentrations, ranging from 2841 to 49,066 endotoxin units m^{-3} of air, were found inside swine farrowing and finishing barns. Airborne endotoxin concentrations were 10- to 100-fold lower inside a dairy barn and downwind of other agricultural production sites and the wastewater treatment plant. Examination of dilution-dependent effects should be considered essential when utilizing the LAL assay, especially if values are to be used for regulatory purposes.*

Keywords agriculture, airborne, dairy, endotoxin, *Limulus* amebocyte lysate assay, swine

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INTRODUCTION

Endotoxins are derived from the outer membrane of gram-negative bacteria and are a potential respiratory health risk when aerosolized.[1] Acute exposures to high levels of airborne endotoxin may cause influenza-like symptoms, while low-level exposures can cause airway inflammation accompanied by decreased lung function, cough, and chest tightness.[2–4] Chronic exposure to endotoxin-containing organic dusts from industrial and agricultural settings can lead to byssinosis and chronic bronchitis in workers.[5–7] Some reports suggest, however, that environmental and occupational exposures to endotoxin may protect against atopic sensitization, asthma, and cancer.[3,8–10]

While endotoxins are ubiquitous in the environment, elevated airborne concentrations are found in agricultural (e.g., livestock production, harvesting operations) and industrial (e.g., textile) settings.[11–13] Because of the negative health implications associated with airborne endotoxin in occupational settings, the Dutch Expert Committee on Occupational Standards has proposed a health-based 8-hr exposure limit of 50 endotoxin units (EU) m^{-3}.[14] If implemented, a 10- to 1,000-fold reduction of airborne endotoxin would be required to reduce related health risks in agricultural settings.[11] To ensure the highest degree of quality assurance and control with respect to endotoxin sampling, transportation, storage, and analysis, the European Committee for Standardization developed a guidance document for the assessment of workplace exposures to airborne bacterial endotoxins.[15] In an effort to improve on the European standard, researchers have investigated the effects of filter type, transport conditions, extraction solutions, and storage conditions on the analysis of airborne endotoxin.[16–18]

One issue that has received limited attention deals with the dilution of endotoxin samples prior to their analysis via the *Limulus* amebocyte lysate (LAL) assay.[19,20] Although the LAL assay is a commonly used procedure to quantify endotoxins in agricultural dusts,[21,22] the assay is also sensitive to interference by a variety of chemical substances (e.g., β-glucans, Tween, proteins) and possibly by certain filter collection media.[17, 23–25] Diluting the samples also dilutes out the interfering substances, reducing inhibition or enhancement of the LAL assay.

In some cases though, sample dilution may cause the endotoxin to be below detection limits. Hollander and co-workers[19] found that inhibition and enhancement of the LAL assay

occurred in some samples from occupational environments (e.g., agricultural, textile), with up to a 3-fold difference between diluted and nondiluted samples. Understanding the effect of interfering substances on the LAL assay is particularly important if the values are to be used for regulatory purposes. Analysis of nondiluted samples could provide misleading information about endotoxin exposure levels and potential health risks.

In this study, airborne endotoxin was collected from several agricultural settings and at a wastewater treatment plant using both open-face and button aerosol samplers. The main objective was to examine the effect of sample dilution on the kinetic LAL assay to verify the presence of interfering substances. After screening the data for dilution-dependent effects using linear, nonlinear, and joinpoint regression models, airborne endotoxin concentrations were determined for the various indoor and outdoor environments. Information presented in this study may be useful to individuals who currently use or intend on using the LAL assay to quantify endotoxin in airborne environmental samples.

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MATERIALS AND METHODS

Collection of Airborne Endotoxin

Airborne endotoxins were collected from within a swine finishing and farrowing barn and freestall dairy barn, while outdoor samples were collected downwind from a wastewater treatment plant, open-lot dairy, and open-freestall dairy and during soil tillage and dry bean threshing events. An outdoor background sample was collected immediately south of the USDA-ARS laboratory in Kimberly, Idaho.

The endotoxins were collected on 25-mm, 1.0 μm pore-size polycarbonate track-etch filters (Whatman, Florham Park, N.J.) that were housed in 25-mm, open-face Delrin filter holders (Pall Corporation, East Hills, N.Y.) or button aerosol samplers (SKC Inc., Eighty Four, Pa.). While the particle size collection characteristics of the open-face samplers are unknown, the button samplers were designed to improve the collection characteristics of dust with an aerodynamic diameter of $<$ 100 μm (i.e., inhalable particles). The samplers were mounted on surveying tripods at a height of 1.5 m, and vacuum was applied at about 2 L min^{-1} for 2 hr using a Vac-U-Go sampling pump (SKC Inc.). According to the manufacturer of the button sampler, sampling efficiency is maintained within $\pm$ 30% at flow rates ranging from 2 to 5 L min^{-1}. However, the button sampler only approximates the ACGIH®/ISO sampling criteria for inhalable particle mass when operated at 4 L min^{-1}, thus, for the purposes of this article we cannot claim that the airborne endotoxins were associated with inhalable particles.

Four open-face and four button samplers were utilized at each location and only one sampling session was conducted at each of the nine locations, resulting in a total of 36 open-face and 36 button samples being collected during the entire campaign. Exposed filters were stored in pyrogen-free tins and transported to the laboratory in a cooler with ice packs. The filters were then transferred to 2-mL, pyrogen-free polypropylene tubes and stored dry at -20°C until processed. Trip blanks were used during each sampling event to ensure that the filters were not contaminated during preparation, transport, and storage. Prior to sampling, the open-face and button samplers were depyrogenated by soaking in 70% ethanol, rinsing with pyrogen-free water, then autoclaving for 30 min. All other materials were either purchased pyrogen-free or depyrogenated by heating at 250°C for 30 min.

Endotoxin Extraction and Analysis

Extraction and analysis of the airborne endotoxins were conducted according to the method described by Dungan and Leytem.[18] In brief, 1.5 mL of pyrogen-free water (PFW) containing 0.05% Tween 20 (v/v) was added to the 2-mL polypropylene tubes, which were sonicated at room temperature for 20 min. Depending on the sample, the extracts were then diluted up to 2000-fold in PFW-Tween and then 100 μL aliquots were dispensed into a pyrogen-free 96-well microplate (Corning Inc., Corning, N.Y.). The microplate was then incubated at 37°C for 15 min.

Afterward, 100 μL aliquots of Kinetic-QCL reagent (Lonza, Walkersville, Md.) were added to each well, and then the microplate was immediately placed into a PowerWave spectrophotometer (BioTek Instruments Inc., Winooski, Vt.). An 8-point standard curve ranging from 0.005 to 50 EU mL^{-1} was prepared using lyophilized *Escherichia coli* O55:B5 (Lonza). Linear regression coefficients of the standard curves were $\geq$ 0.97. Quality control operations included analysis of trip blanks and method blanks and a duplicate sample for every eight samples.

Statistical Analyses

Data were fit to liner and nonlinear regression models using SigmaPlot 11.0 (Systat Software Inc., Chicago, Ill.). Joinpoint regression models were developed using Joinpoint 3.4.2 (National Cancer Institute, Bethesda, Md.). In all statistical models, endotoxin concentration was the dependent variable and dilution factor was the independent variable. The two-sample *t*-tests for the means were performed using SigmaPlot 11.0. Statements of statistical significance were based on $P <$ 0.05 unless stated otherwise.

RESULTS AND DISCUSSION

While a variety of buffers and detergents have been used to extract endotoxin from filters,[20,26–28] Tween 20 in PFW is generally preferred as extraction efficiencies have shown to be up to seven-fold higher than with PFW only.[16,29] The presence of Tween in the LAL assay, however, has also been shown to shift the endotoxin calibration curve toward a higher analytical response.[17,18,26] It was believed that the Tween was either reducing the activity of the lipopolysaccharide molecule (i.e., endotoxin) or proenzyme in the LAL assay. To avoid calibration errors, it is essential that endotoxin standards

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TABLE I. Examination of Dilution-Dependent Effects in Endotoxin Extracts Using Linear, Nonlinear, and Joinpoint Regression Models

		Linear Model			Nonlinear Model[A]		Joinpoint Model			
Endotoxin Source	**Sample**	r^2	**Slope**	***P*-value**	r^2	***P*-value**	**Joinpoint[B]**	**Slope 1**	**Slope 2**	***P*-value**
Background	Open-face	0.19	–0.0191	0.09	—	—	—	—	—	—
	Button[C]	0.62	–0.0269	< 0.001	0.65	0.001	—	—	—	—
Wastewater treatment plant	Open-face	0.12	–0.0068	0.04	0.46	< 0.001	50	–0.0049	0.0011	0.04
	Button	0.23	–0.0074	< 0.01	0.48	< 0.001	50	–0.0201	–0.000135	< 0.001
Soil tillage	Open-face	0.20	–0.0131	< 0.01	0.87	< 0.001	50	–0.0473	0.0001	0.02
	Button	0.17	–0.0080	0.01	0.75	< 0.001	50	–0.0219	0.0000	0.03
Bean threshing	Open-face	0.10	–0.0131	0.08	—	—	–	–	–	–
	Button	0.42	–0.1710	< 0.001	0.84	< 0.001	50	–0.0270	0.0014	< 0.01
Freestall dairy	Open-face	0.01	–0.0040	0.55	—	—	—	—	—	—
	Button	0.10	–0.0087	0.06	—	—	—		—	—
Open-lot dairy	Open-face	0.01	0.0011	0.67	—	—	—	—	—	—
	Button	0.14	–0.0066	0.03	0.74	< 0.001	50	–0.0291	0.0011	< 0.01
Freestall dairy barn	Open-face	0.00	0.0021	0.75	—	—	—	—	—	—
	Button	0.44	–0.0709	< 0.001	0.85	< 0.001	100	–0.0119	–0.000293	< 0.01
Swine farrowing barn	Open-face	0.04	–0.0993	0.27	—	—	—	—	—	—
	Button	0.14	–0.3011	0.04	0.01	0.88	250	0.0013	–0.000415	0.15
Swine finishing barn	Open-face	0.00	–0.0097	0.80	—	—	—	—	—	—
	Button	0.22	1.7400	< 0.01	0.49	< 0.001	1500	0.0000	0.0001	< 0.01

[A]All dilution curve data was fit to a three-parameter single exponential decay curve, except the swine finishing barn sample, which was fit to a three-parameter single exponential growth curve.
[B]Dilution factor at which the slope of two regression lines converge.
[C]Too few data points available to run joinpoint model.

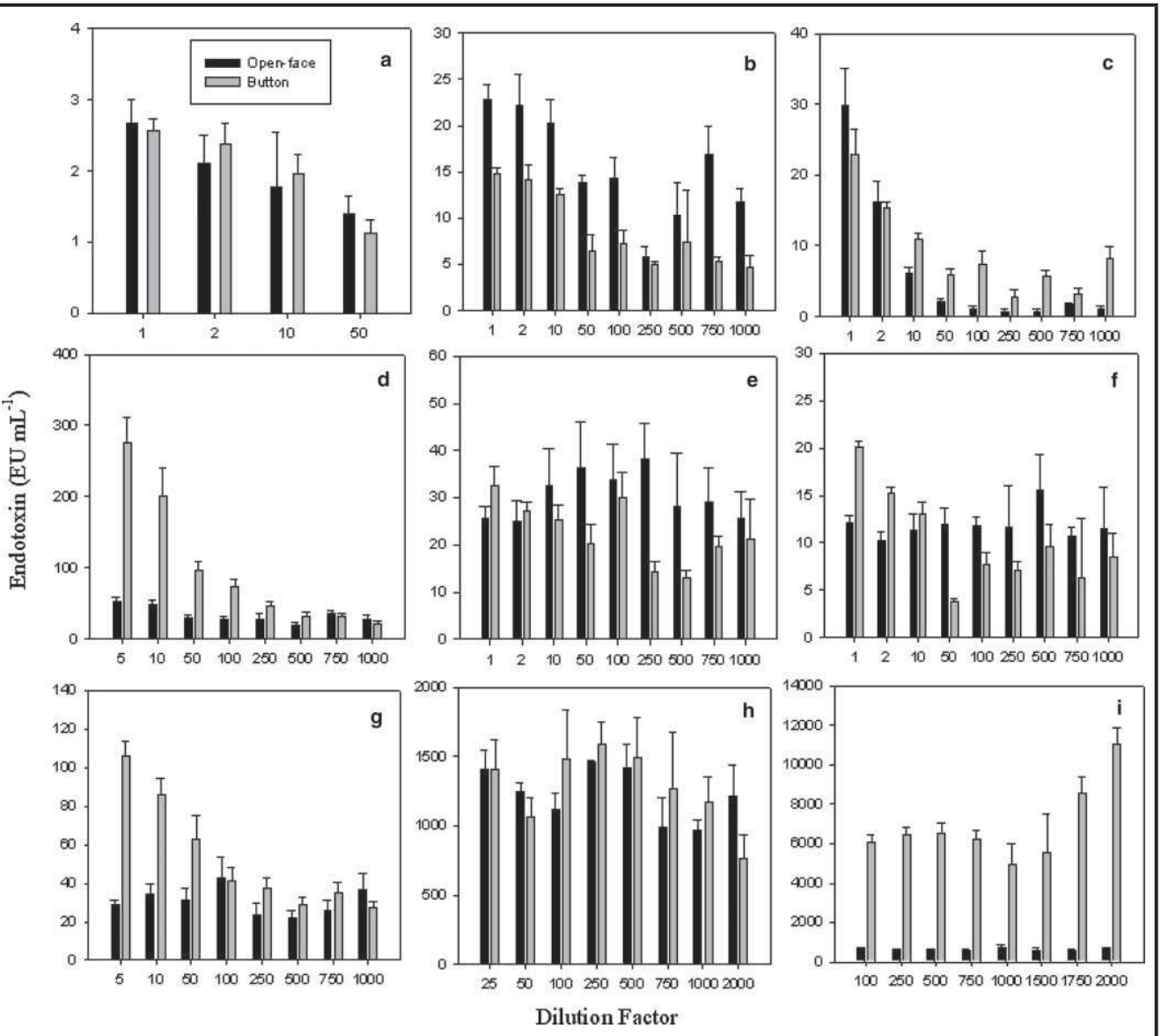

FIGURE 1. Dilution curves of airborne endotoxin collected using open-face and button aerosol samplers from (a) background, (b) wastewater treatment plant, (c) soil tillage, (d) bean threshing, (e) freestall dairy, (f) open-lot dairy, (g) freestall dairy barn, (h) swine farrowing barn, and (i) swine finishing barn. All data were corrected for dilution by multiplying the sample concentration by the dilution factor. Error bars indicate the standard error of the mean ($n = 4$).

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also be prepared with Tween if the filter extractant contains Tween.[18]

In this study, filter extracts from indoor and outdoor samples (most collected from agricultural settings) were diluted with PFW-Tween and analyzed using the kinetic LAL assay (Figure 1). The effect of dilution was most dramatic on the button samples collected downwind from the soil tillage and bean threshing events and open-lot dairy and from inside the freestall dairy and swine finishing barns (Figures 1c,d,f,g, and i, respectively). Except for the dilution curve from the swine finishing barn, the data suggest that enhancing substances were present in the filter extracts, as endotoxin concentrations were shown to decrease with increasing dilution (i.e., when corrected for dilution factor). A similar trend also occurred with the open-face endotoxin samples from the wastewater treatment plant and soil tillage event but not with any of the other open-face samples. In the button filter extract from the swine finishing barn, the endotoxin concentration was shown to gradually increase with increasing dilution up to 2000-fold, suggesting the presence of inhibitory substances (Figure 1i).

To more closely examine trends among the diluted extracts, the data were fit to linear and nonlinear regression models (Table I). In several cases, the P-values were > 0.05 after fitting the data to the linear regression models; thus, the null hypothesis was accepted, providing evidence that the slope of the regression lines did not differ from zero. Dilution curve

TABLE II. Airborne Endotoxin Concentrations at Various Indoor and Outdoor Environments After Being Screened for Dilution-Dependent Effects

Endotoxin Source	Endotoxin (EU m^{-3} of air)		*n*		*P*-value[B]
	Open-Face	Button	Open-Face	Button	
Background	10.9 ± 1.3[A]	11.8 ± 1.0	4	4	0.72
Wastewater treatment plant	69.4 ± 6.3	34.6 ± 5.5	6	6	<0.01
Soil tillage	7.3 ± 1.2	34.3 ± 3.8	6	6	<0.001
Bean threshing	150.4 ± 11.8	220.3 ± 28.0	8	6	0.18
Freestall dairy	176.9 ± 13.3	129.3 ± 10.2	9	9	<0.01
Open-lot dairy	69.2 ± 5.0	43.1 ± 4.5	9	6	<0.001
Freestall dairy barn	153.1 ± 11.9	194.6 ± 13.8	8	5	0.10
Swine farrowing barn	6506 ± 294	6911 ± 670	8	5	0.61
Swine finishing barn	2841 ± 301	49066 ± 5670	8	3	<0.001

[A]Average endotoxin concentration ± standard error of the mean.
[B]Statistical differences determined using a two-sample *t*-test. *P*-values < 0.05 indicate a significant difference between the open-face and button endotoxin concentrations for that row only.

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data with a slope statistically similar to zero were the open-face filter samples from the background, bean threshing, freestall dairy (button sample as well), open-lot dairy, freestall dairy barn, swine farrowing barn, and swine finishing barn. The linear regression data suggest that dilution of the filter extracts had no effect on the final concentration, and therefore, enhancing and inhibitory substances were absent or at very low concentrations.

To examine nonlinear trends, dilution curves with linear regression *P*-values < 0.05 were then fit to a three-parameter single exponential decay or growth model (Table I). The decay model fit the dilution curve data reasonably well (r^2 values ranging from 0.46 to 0.87), suggesting that enhancing substances were likely present in the filter extracts. In one case, though, the decay model did not fit the data well, resulting in a low r^2 value of 0.01 (i.e., button sample from swine farrowing barn). The only occurrence where inhibitory substances appeared to be present was in the swine finishing barn filter extract, where the dilution curve was fit with an exponential growth model ($r^2 = 0.49$).

The dilution curve data were further analyzed by fitting it to a joinpoint model to determine the point (i.e., dilution factor) at which the slope of two regression lines converge. In all cases, except the dilution curve of the button sample from the swine farrowing barn, the *P*-values were $<$ 0.05, indicating that the two slopes were statistically different (Table I). For the wastewater treatment plant, soil tillage (both open-face and button), bean threshing, and open-lot dairy (button sample only), the joinpoint was determined at a dilution factor of 50, while a dilution factor of 100 and 1500 was calculated for the button samples from the freestall dairy barn and swine finishing barn, respectively. The joinpoint of 250, determined for the swine farrowing barn dilution curve, was not statistically significant ($P = 0.15$). Interestingly, in most cases the dilution curves for the button samples followed an exponential decay trend, while the open-face endotoxin curves had linear regression slopes near zero. This may be related to the type or size of particulate matter the endotoxins are associated with, as the button samplers are designed to collect particles with an aerodynamic diameter <100 μm. Because of this size restriction, the button sampler could be capturing more fine particulate matter that interferes with the LAL assay than is captured using the open-face sampler.

Using the results from the linear and joinpoint regression models, data were screened for dilution dependent effects on the LAL assay. If the slope of the line was determined to be statistically similar to zero, then all dilution curve data points were averaged. When a joinpoint was determined, the data from that point to the highest dilution were averaged. Because the slope of the second regression line approached zero, it was assumed that dilution had little effect on the overall endotoxin concentration from the joinpoint to the highest dilution factor.

Table II presents the airborne endotoxin concentrations (EU m^{-3} of air) at each of the sampling sites after being screened for dilution-dependent effects. These values fall within ranges obtained by other researchers in similar environments.[11,30–33] For these 2-hr sampling events, the highest concentrations were found inside both swine barns and were 10- to 100-fold greater than in the freestall dairy barn. Similar results were obtained by Seedorf and co-workers,[34] who found that inhalable and respirable endotoxin concentrations were up to 100-fold greater in swine and poultry houses than in cattle houses. Compared with outdoor environments, indoor animal production systems generally contain elevated airborne concentrations of dust, bacteria, fungi, endotoxin, and other microbial by-products, since they accumulate within the housing structure.[35–37] The source of endotoxin-containing dusts within animal housing units are animal feces and bacteria-contaminated plant materials.[38]

Both swine barns contained mechanical ventilation systems that were in operation during endotoxin sampling. The freestall dairy barn was naturally ventilated with roof vents and side curtains (down at the time of sampling), which may explain the presence of much lower airborne endotoxin concentrations. Immediately downwind from the freestall dairy barn the endotoxin concentrations were similar to those inside the barn, but were about two-fold higher than downwind from the open-lot dairy. The downwind endotoxin concentrations from the open-lot dairy were markedly similar to those obtained at the wastewater treatment plant. The lowest airborne concentrations were obtained from the background site and during the soil tillage event. Endotoxin concentrations that were well above the proposed Dutch threshold of 50 EU m^{-3} occurred when downwind of the freestall dairy and bean threshing event and inside the dairy and swine barns.

CONCLUSIONS AND RECOMMENDATIONS

Animal production facilities, crop production sites, and municipal wastewater treatment plants are a source of endotoxin and produce airborne concentrations greater than found in background environments. When quantifying airborne endotoxin via the LAL assay, a dilution series of the filter extracts should be analyzed to verify the presence of inhibitory or enhancing substances. Failure to properly evaluate dilution-dependent effects can result in a severe over- or underestimation of airborne endotoxin concentrations. While about one-half the samples collected in our study did not display dilution-dependent effects (most open-face samples), a dilution factor of 50 to 100 was generally sufficient to eliminate the presence of enhancing substances. In the case of button samples from the swine finishing barn, the presence of inhibitory substances was not evident until a dilution factor of 1500 or higher was applied.

Because the scale of the dilution effect was quite large, one should consider diluting samples to as high a dilution factor as possible to detect inhibition and enhancement. If airborne endotoxin values from similar studies are to be used eventually for regulatory purposes, then testing dilution-dependent effects should be required before data are accepted. In addition, while not performed in this study, an assessment of inhibition and enhancement can also be performed by spiking the dilutions with equal concentrations of endotoxin standard.[19] However, one must be made aware that testing all samples during complex studies for dilution effects may not be possible, as the LAL assay is quite expensive. It therefore might be more cost-effective to test dilution-dependent effects in select samples from the same ambient environments, then select a dilution factor for use in all similar samples.

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